UNDERSTANDING THE GENETIC INTERACTIONS THAT REGULATE HEAT AND DROUGHT TOLERANCE IN RELATION TO WAX DEPOSITION AND YIELD STABILITY IN WHEAT (*TRITICUM AESTIVUM* L.)

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2014

Major Subject: Molecular and Environmental Plant Sciences

ABSTRACT

Wheat (*Triticum aestivum* L.) has been a major food crop for nearly 8000 years. Breeders continue to face an ongoing battle to produce stress tolerant cultivars that are able to feed a rapidly increasing global population. The ability of varieties to perform similarly in grain yield across various environments is an important trait that is critical to successfully keep up with food demands with decreasingly available arable lands. The work described in this dissertation focused on defining and understanding the genetic interactions of epicuticular wax and high temperature and drought tolerance and its association with yield stability, to better aid breeders in stress tolerance selection. The effect of high temperature on epicuticular wax, yield attributes and yield stability were investigated in a recombinant inbred line population of 180 individuals from a Halberd x Len cross by physiological and molecular techniques.

Epicuticular wax offers advantages in protecting the plant from both biotic and abiotic stresses. Under HT conditions, EWL can reduce chlorophyll fluorescence by reflecting excess irradiation and also reduce stomatal conductance, helping to regulate the rate of transpiration. QTL for epicuticular wax with large effects were detected on chromosomes 2A, 2B, 3A, 6B, and 7A. A large effect QTL for epicuticular wax was detected in three field environments on chromosome 2B (*QWax*.tam-2B.1) with the favorable alleles contributed by Halberd. QTL for yield stability and yield components stability indices with large effects were detected on chromosomes 1A, 1B, 2A, 2B, 3B, 6B, and 7A. A large effect QTL for yield stability was detected by five stability statistics

over diverse field environments on chromosome 1B (*Qyieldss*.tam-*1B*) with Halberd contributing the favorable alleles. High EWL may promote stable yields but its sensitivity to environmental conditions makes it challenging to definitively point to it as a source of improved stability. Although there were mixed relationships with yield performance and environments, the stability statistics QTL provide strong evidence that genetic variation may be heritable and could have implications for breeding programs targeting a set of environments rather than a single environment.

DEDICATION

This dissertation is dedicated to the great people in my life:

Claudette, my mother, you are an incredible, tremendous and inspiring person. Thank you for painstakingly instilled qualities of leadership, perseverance, and determination.

Mayiah and Ma'Keyana, you are and have been my inspiration, I gratefully express my appreciation and gratitude. Thank you for your unwavering support and encouragement.

The Clarke family of St.Kitts, some roads are not easily traveled or great things effortlessly attained. Nothing is beyond reach but we have to dare to.

I lack words to express my utmost gratitude.

To all I say a heartfelt and gracious

THANK YOU

ACKNOWLEDGEMENTS

I would like to extend a heartfelt and gracious thank you my graduate advisor and mentor, Dr. Dirk B Hays, and Drs. Amir Ibrahim, Russell Jessup and Alan Pepper for serving on my committee. Drs. Hays and Ibrahim allowed me to take advantage of their vast knowledge base, with regular office visits to voice any and all inquiries and suggestions. Words do not exist that can truly express my gratitude for the years of teaching and guidance.

Thanks you to my friends and colleagues and the staff and faculty of the Soil and Crop Sciences Department for their assistance and for making my time at Texas A&M University a wonderful experience.

NOMENCLATURE

CTP	Canopy temperature
ChFl	Chlorophyll fluorescence
EW	Leaf epicuticular wax
KNS	Kernel number per spike
QTL	Quantitative trait loci
MSHW	Mean single head weight
SRI	Spectral reflectance indices
Spm2	Spike per meter squared
SC	Stomatal conductance
THKW	Thousand kernel weight
Yield	Plot grain yield

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Drought and high temperature continue to limit the grain yield of wheat (*Triticum aestivum* L.) globally (Hays et al., 2007; Fischer, 2011; Gourdji et al., 2013). Environmental influences of drought and excess heat are the main culprits responsible for millions of tons of losses in crop yield and production (Mohammadian et al., 2007; Islam et al., 2009). High soil temperature affects mortality of seedlings and crop establishment; however, high temperature during floral development is particularly devastating. Over the past decade heat and drought have become an ever-increasing problem that has crippled agriculture and livelihoods of countries, thus affecting the world's agricultural production and food availability. Wheat production has fluctuated over the past decade as temperatures have increased, while the demand for wheat is expected to increase dramatically to feed a population of 9 billion by 2050, requiring an additional 3,339 million tons of cereal (FAO, 2012). That will require a 0.8 percent annual increase in total production by 2050 to meet the demand. In the summer months of 2010, the major wheat-producing nation of Russia experienced a heat wave that resulted in loss of 4.5 million tons of wheat, prompting a halt on exportation for the remainder of the year (USDA, 2010). Similarly in India, a heat wave engulfed the country during the maturation months of March and April resulting in 20% loss for wheat farmers in 2010. As the world population has grown at a rapid rate, there is increasing demand on plant breeders to develop varieties that are able to produce

consistent high yields and quality. The development of wheat plants with increased tolerance to drought and high temperatures will be of high significance. As such, enhanced ability to select lines with resistance to abiotic stress will play an important role in improving grain yields.

Plants have developed a variety of adaptive mechanisms to combat high temperature stress that include a thick cuticle, increased transpiration, leaf trichomes, leaf rolling, heat shock proteins, and osmoprotectants. Leaf epicuticular waxes coat leaves and stems offering protection by acting as a barrier between the plant and the environment, limiting water loss and increasing excess light reflectance in photosynthetic and non-photosynthetic, near-infrared and infrared wavelengths. Water deficit in peanuts (*Arachis hypogaea*) and jojoba (salt-sensitive) resulted in increased leaf epicuticular wax deposition (Rao et al., 1981; Mills et al., 2001). The amount of light a plant deflects is dependent on the thickness of the cuticular wax layer and this phenomenon was observed when carnations (Reed and Tukey, 1982) and barley (Giese, 1975) were exposed to increasing levels of irradiation resulting in a thicker leaf wax layer. Excess light to leaf regions can result in irreversible damage to photosystem II, especially the very sensitive D1 protein, due to the excess heat (Lombardini, 2006). Reduced light absorption can help reduce transpiration by lowering the vapor pressure between air and plant tissue (Shepherd and Griffiths, 2006). The association between increased epicuticular wax, decreased canopy temperatures and improved yields has previously been identified (Richards et al., 1986; González and Ayerbe, 2009), but its role with increased yield stability has yet to be elucidated. In addition to understanding

these interactions, it is also imperative to identify QTLs associated with regulating increased leaf epicuticular wax and increased yield stability. A better understanding of this molecular and physiological characteristics of leaf epicuticular waxes that confer improved adaptation to high temperature stress will be essential in breeding tolerant cultivars.

1.1. Effects of high temperature on plant physiology

High temperatures and water deficit stress are two factors that limit food production worldwide (Barnabás et al., 2008). In plants, response to high temperature and water deficit stress takes place at the physiological, molecular and cellular levels and has been associated with effectiveness in suppressing reactive oxygen species (Sairam and Saxena, 2000; Barnabás et al., 2008). It is often quite difficult to separate the effects of water deficit and high temperature stress on plant growth and development due to their relatively close association (Lombardini, 2006). In wheat, high temperature is destructive to photosynthetic processes, causing a rate reduction at a much earlier stage than other processes (Al-Khatib and Paulsen, 1999). High temperature stress during the sensitive reproductive stages of wheat contribute to many physiological and metabolic changes, as well as disruption in hormone homeostasis (Barnabás et al., 2008). One of the main physiological changes that occurs is modification of cell membranes causing membrane fluidity to be disrupted (Barnabás et al., 2008).

Plants utilize a number of mechanisms to overcome abiotic and biotic stress. The ability to survive in such adverse conditions is based a plants' ability to adapt or

acclimate to its surroundings (Lombardini, 2006). Adaptations in plants occur at a genetic level and also as a result of selection for tolerance over many generations, while acclimation is based on a response due to exposure from prior stress conditions (Lombardini, 2006). The response to high temperature stress in cereals at the cellular level has been measured, and observations suggested that responses in protoplasts, chloroplasts and thylakoids involve common photochemical components (Al-Khatib and Paulsen, 1999). Heat shock proteins (HSP) play a role in thermotolerance by acting as chaperones for proteins, preventing their denaturation. The effects of high temperature stress can be measured by monitoring the activity of heat shock proteins (HSP), reactive oxygen species (ROS), hormones and sugars (Frank et al., 2009). According to the study, HSP were highly up-regulated and ascorbate peroxidase (APO) genes were constitutively expressed, suggesting a role in high temperature stress tolerance (Frank et al., 2009). High temperature stress is usually accompanied by oxidative stress, inducing production of the ROS hydrogen peroxide and the subsequent increase of its' scavengers, such as APO. High temperature stress also resulted in increased 1-aminocyclopropane-1-carboxylic acid (ACC) and ethylene levels as well as the increased expression of sucrose phosphate synthase genes (Hays et al., 2007; Frank et al., 2009). Sucrose phosphate synthase production is thought to stimulate osmoregulation during periods of osmotic stress (Huber and Huber, 1996). Bennett et al. (2012a) reported a significant positive correlation between high temperature stress and grain yield under irrigation, but no significant correlation was observed between drought treatment and yield. It is imperative that plant breeders develop crops that are efficient

users of water and to high temperatures as water resources decline (Barnabás et al., 2008).

1.2. Effects of water deficit on plant physiology

Grain yield is a complex trait that is highly environmentally influenced and is a product of intricate physiological processes (Graybosch, 2001; Quarrie et al., 2005; Barnabás et al., 2008; González and Ayerbe, 2009; Bennett et al., 2012a). A number of physiological traits have been reported to correlate with high temperature and water deficit, including maturity, height, awns, grain yield and canopy temperature (Trethowan et al., 2002; Quarrie et al., 2005; Olivares-Villegas et al., 2007; Bennett et al., 2012a). Water deficit response is extremely variable and highly dependent on the genetic constitution of a species (Kim et al., 2007; Barnabás et al., 2008; González and Ayerbe, 2009). Water deficit also often results in production of ROS in mitochondria, chloroplasts and cytoplasm, resulting in increased ascorbate peroxidase (APO), lipid peroxidation (LPO) and glutathione reductase (GR) activity (Sairam and Saxena, 2000). In fact, GR activity under water deficit conditions increased by more than 50% relative to irrigated conditions (Sairam and Saxena, 2000). GR and APO are known ROS scavengers that have the ability to reduce damage to cellular components including proteins, DNA and organelles during periods of environmental stress. These mechanisms are in place to prevent the negative impact that abiotic and biotic stress can have on seed production. The effects of water deficit on yield have been investigated extensively across a number of agriculturally important crops to gain deeper insight into the

underlying mechanisms involved in tolerance and to develop better tolerant cultivars. In one such study, the devastating effect of water deficit stress, especially during the reproductive stage, resulted in a 37% loss of sesame seed yield after plants were subject to post-flowering water deficit stress (Kim et al., 2007). Similarly, in a RIL population of wheat (Seri/Babax), water deficit stress mostly affected the time from emergence to anthesis and grain filling, reducing grain yield by 35% to 82% (Olivares-Villegas et al., 2007).

1.3. Molecular mapping and QTL identification

A quantitative trait loci (QTL) is described as a genomic region associated with variation at allelic and phenotypic levels related to a trait of interest (Doerge, 2002). A QTL can be identified through a process known as mapping which is effective for analyzing complicated traits such as yield and yield components (Jaccoud et al., 2001; Paillard et al., 2003; Li et al., 2007). Molecular markers, a fragment of DNA that is associated with a specific region of the genome, is ran across the population of interest to identify regions associated wit the traits of interest. The past decade has seen rapid progress in integrating statistical analysis software with molecular markers created a path to develop genetic maps based on QTL effects (Cuthbert et al., 2008). QTL mapping has shifted to efficient methods that are low in cost, namely simple sequence repeats (SSR), single nucleotide polymorphism (SNP) and diversity array technology (DArT). SSR marker development has enabled substantial gain in the resolution of creating genetic maps and the ability to reveal higher polymorphism than previous

methods such as RFLP and AFLP (Röder et al., 1995; Paillard et al., 2003). SSR markers has become the molecular marker of choice due to robustness and requirement for a small amount of DNA, exhibiting linkage disequilibrium with economic traits at high probability (Donini et al., 1998; Gupta et al., 2003). SNP markers on the other hand detect changes in a single base in the genomic segment of two individuals and although initial development is costly, data analysis is simple and cheap making them ideal markers for QTL identification (Koebner and Summers, 2003; Landjeva et al., 2007). SNP markers produce a binary output with a potential to have a high density of markers (Koebner and Summers, 2003). DArT marker technology utilizes an array of genetic clones hybridized to genomic DNA from the population of individuals and not gel resolution (Jaccoud et al., 2001). Most of the genetic maps created for wheat (*T. aestivum* L.) are based on inbred populations, mainly recombinant inbred line (RIL) populations (Francki et al., 2008).

Approximately 2 to 10% of phenotypic variation in crops accounted for by QTLs was associated with yield and yield related traits (Quarrie et al., 2005). One of the most critical factors when breeding for wide adaptation and yield stability is the stable expression of a QTL (Quarrie et al., 2005). Epistatic interactions between numerous QTLs frequently complicate grain yield with other traits such as early flowering, grains per spike, harvest index and grain number associated with higher yields (Kusterer et al., 2007; Li et al., 2007; McIntyre et al., 2010). Recent advances in marker technology have afforded significant advances in detection and analysis of QTLs associated with specific traits or responses and has resulted in detection of major and minor QTLs that are

closely associated with grain yield and yield components. Five major QTLs are associated with grain yield were identified in a wheat RIL population on chromosomes 2A, 2D, 3B and 6A (Li et al., 2007). In this same study, twenty-seven QTLs associated with yield components were also identified, 9 for thousand kernel weight, 7 for kernel number per spike, and 11 for spike number (Li et al., 2007). Yet in another study, 99 putative QTLs associated with grain yield and yield components were identified in Chinese wheat RIL population (Wang et al., 2008). The QTLs were detected on all chromosomes except for 6A, 7A and 7B with a large number showing small effects and a small amount showing major effects. Furthermore, not surprisingly, pleiotropic effects were detected across 13 loci located on chromosomes 1A, 1B, 1D, 2A, 2B, 2D, 3A, 3B, 4B, 4D, 5B, 6D and 7D (Wang et al., 2008). Variation in environmental conditions between heading and maturity affect grain yield specifically, especially if an environmental stress occurs just before heading (Maccaferri et al., 2008). Water deficit treatment of durum wheat revealed 32 distinct major QTLs, 16 QTLs associated with grain yield, 15 for heading date and 11 associated with plant height (Maccaferri et al., 2008). In yet another study, seventeen yield component QTLs were mapped to chromosomes 2D, 3A, 4A, 5A, and 6B from an identified 56 QTL with environmental association (Börner et al., 2002).

1.4. Leaf epicuticular wax

Water deficit and high temperature are two of these major environmental stresses that have devastating effects on many agricultural crops. High irradiance can be

problematic to most plants especially since photosynthesis only requires about half of the light that a plant receives to carry out the necessary reactions required (Shepherd and Griffiths, 2006). To produce one gram of organic matter a plant must utilize approximately 500 grams of water through the soil-plant continuum. This delicate balance must be maintained in order for the plant to maintain its' homeostasis in normal conditions. The regulatory systems of plants are negatively affected by abiotic and biotic stresses often resulting in decreased seed yields. Some plants have developed mechanisms that allow for reduced water loss by transpiration, thus effectively utilizing the limited amount of water available to them. These plants have developed physiological responses to counteract water deficit and high temperature stress, one of which is a waxy coating covering leaves and stems, offering protection against the environment and limiting water loss by acting as a hydrophobic barrier (Bird et al., 2007). The plant cuticle possesses two distinct layers, cutin, a polyester polymer and cutan, a hydrocarbon polymer. Cuticular wax is a hydrophobic layer consisting of long chained aliphatic carbons resting on the outer most layer of the cuticle embedded in a polymer cutin (Shepherd and Griffiths, 2006). A polymer matrix of embedded wax and surface deposits comprise the cutin layer, the outermost layer of a leaf (Shepherd and Griffiths, 2006). The composition of cuticular wax has been characterized mainly in *Arabidopsis thaliana*, where a co-regulated relationship between synthesis and secretion of polyesters and wax was identified (Shepherd and Griffiths, 2006). Waxes are secreted and synthesize by epidermal cells and are composed of a mixture of complex acyl lipids (Taiz and Zeiger, 2002). Long chained fatty esters, free fatty acids and free fatty alcohols

are the primary constituents of cuticular waxes (Kunst and Samuels, 2003). An analysis of wheat epicuticular wax composition found primary alcohols as the main components, followed by esters, alkanes, fatty acids, and beta-amyrines. Octocosan-1-ol was the most common primary alcohol and the most common constituent of leaf epicuticular wax in wheat (Koch et al., 2005). The exact functions of the cuticle wax layer are still being evaluated and are believed to play important roles in other areas of the plant. For example, the wax layer is theorized to aid in reflection of excess UV light that plants are exposed to during daytime periods (Kolattukudy et al., 1987; Jenks et al., 1992; Shepherd and Griffiths, 2006).

Epicuticular wax characteristics have been influenced through selective breeding for glaucousness, the visible blue-white waxiness on wheat leaves and glooms. Glaucousness was reported to positively correlate with increased epicuticular wax load and reflectance (Koch et al., 2005; Shepherd and Griffiths, 2006). The composition of epicuticular wax of common bread wheat at developmental stages has been investigated laying the foundation for specified characterization of wheat epicuticular wax composition under certain environmental stresses (Bianchi and Corbellini, 1977; Koch et al., 2005). There is evidence suggesting the amount of light reflected by plants is based on the thickness of the cuticle wax layer (Shepherd and Griffiths, 2006). Excess light to leaf regions can result in irreversible damage to photosystem II, especially the very sensitive D1 protein, due to the excess heat (Lombardini, 2006). The loss of proper function results in decreased photosynthesis, thereby limiting grain yield. A variety of abiotic stresses appear to affect numerous genes (‘OsGL1’, *Glossy* 1-like gene family in

rice) involved in stress response, thus causing a diverse response to protect sensitive mechanisms (Islam et al., 2009). Several families of genes that play a role in wax biosynthesis and transportation have been identified. One such gene family is ATP binding cassette transporters (ABC), a sub-family of the White-Brown Complex (WBC) family in *Arabidopsis* (Sánchez-Fernández et al., 2001). Enzymes in the *eceriferum* family seems to play vital roles in was deposition, production and exportation (Hooker, 2002; Kunst and Samuels, 2003; Pighin et al., 2004). CER6 and CER10, two well-studied enzymes of the *eceriferum* family, are essential for production of cuticular waxes (Kunst and Samuels, 2003). CER5, an ATP binding cassette (ABC) transporter has also been reported to be required for wax exportation to the cuticle layer in *Arabidopsis* (Pighin et al., 2004). CER4, a fatty acyl-CoA reductase, localized in the endoplasmic reticulum, is required for cuticular wax production (Rowland et al., 2006). In *Arabidopsis*, overexpression of an epidermis specific promoter, CER6, resulting in increased wax deposition to the stems (Hooker, 2002). In another study, overexpression of the wax production gene (WXP1) conveyed increase water deficit tolerance in alfalfa (Zhang et al., 2005). However, few studies have sought to identify QTLs associated with leaf epicuticular wax. In a recent study of coconut (*Cocos nucifera* L.), a total of forty-six QTLs related to epicuticular wax synthesis were identified (Riedel et al., 2008). In rice, only two QTLs associated with epicuticular wax in response to water deficit stress were identified in the population (Srinivasan et al., 2008).

Richards et al. 1986 reported a difference in epicuticular wax between glaucous and non-glaucous lines after the onset of stem elongation. Reports suggest that WBC11

gene is required for wax transport to the leaf surface and a possible interaction with CER5 is required for the process (Bird et al., 2007). The identification of the genes that play a major role in epicuticular wax variation and biosynthesis increased high temperature and water deficit stress tolerance will provide needed insight into gene family functionality in stressed environments. Understanding the mechanism involved in these responses will provide clarity and direction in the development and breeding of high quality high temperature and drought tolerant cultivars.

1.5. Transpiration

Transpiration is the evaporation of water from plants mainly through the leaves and is directly related to carbon dioxide diffusion through stomates for photosynthesis. The stomata allow plants to adjust the rate of transpiration in their leaves opening in response to low intercellular carbon dioxide concentrations and light intensity. The relationship between carbon dioxide and water evaporation is exploited to measure stomatal conductance indirectly as a function of the rate of transpiration and photosynthesis. Transpiration measures the rate at which carbon dioxide and water vapor is expelled from and taken in by leaves in millimole per meter squared per second (mmol m^{-2} s^{-1}). Reduced transpiration is accompanied by a decrease in carbon dioxide availability and a reduction in the rate of photosynthesis. Plants adapted to arid and semiarid climates typically have a thick waxy layer (Oliveira et al., 2003). The ability of a plant to limit water loss through the leaf epidermis is essential to survive severe water deficit conditions (Araus et al., 1991). Tolerant genotypes use effective strategies such as

increased epicuticular wax deposition to adjust levels of tolerance to water deficit and may be advantageous in limiting transpiration by partial plugging of stomata (Araus et al., 1991; Elham et al., 2012). Therefore, epicuticular waxes have the potential to aid in allowing a plant to survive water deficit and high temperature conditions by improving water-use efficiency and reducing transpiration (Richards et al., 1986; Clarke et al., 1991). As plants age, stomata in older leaves become less active than their younger counterparts and develop increased stomatal resistance.

Water deficit conditions affect not only transpiration rate but also epicuticular wax deposition (Clarke and Richards, 1988; Clarke et al., 1991; Samdur et al., 2003; Kim et al., 2007; England and Attiwill, 2011; Elham et al., 2012). To minimize the loss of water in water deficit conditions, the stomata close, limiting the loss of water through transpiration (Samdur et al., 2003). Epicuticular wax provide a viable mechanism to help alleviate the effects of water deficit and high temperature environments by reducing the need for leaves to transpire continually (Clarke and Richards, 1988; Sánchez et al., 2001; Samdur et al., 2003; England and Attiwill, 2011). In peanuts (Samdur et al., 2003) pea plants (Sánchez et al., 2001), and wheat (Elham et al., 2012) epicuticular wax was observed to increase as an adaptation to water deficit conditions. In fact, the pea plants under water deficit conditions not only increased epicuticular wax on their leaves, but simultaneously lowered canopy temperature (Sánchez et al., 2001). In a study of isogenic durum wheat lines, glaucous isogenic lines were observed to have reduced photosynthesis and transpiration under water deficit conditions than their non-glaucous counterpart (Richards et al., 1986). This suggests that leaf glaucousness, through its

effect on conductance, was able to reduce transpiration directly, but had less of an effect on photosynthesis (Richards et al., 1986). In a study of water stressed wheat, increased epicuticular wax was highly associated with reduced transpiration but epicuticular wax and glaucousness together produced a larger effect on transpiration (Clarke and Richards, 1988). In *Eucalyptus regnans,* cuticle thickness and leaf waxiness reduced cuticular transpiration, and was negatively correlated with stomatal conductance in young developing leaves (England and Attiwill, 2011). It is important to note that epicuticular wax levels increase as leaves age but eventually plateau with a subsequent decrease as age increases (Samdur et al., 2003). Similarly, in young leaves, transpiration rate is low but gradually increases during leaf expansion, and subsequently declines at the onset of senesce, coinciding with vigorous stomatal conductance in developing leaves and decline in older leaves (England and Attiwill, 2011). However, Clarke and Richards (1988) suggested that the increase in transpiration rate as leaves mature might be a result of the gradual decline in epicuticular wax levels as the natural aging process of leaves takes effect.

Leaf temperature affects leaf vapor pressure. As air and leaf temperature increases, transpiration also increases due to the increasing vapor pressure gradient. Epicuticular wax effectively reflects incident light and excess irradiation during high irradiation events, mostly in the 750-800 nm region (Reicosky and Hanover, 1978; Mauseth, 1988). The reduction in irradiation and light by epicuticular wax reduces canopy temperature, which may be advantageous in high temperature environments by helping plant to reduce transpiration effectively (Mohammadian et al., 2007). Reduced leaf temperature

due to high epicuticular wax may lower respiration rate, allowing for efficient photosynthesis (Reicosky and Hanover, 1978). Canopy temperature was effectively reduced in pea plants subjected high temperature stress as a result of increased epicuticular wax deposition on their leaves (Sánchez et al., 2001). In high temperature environments, leaves stripped of epicuticular wax were unable to reduce their rates of transpiration and photosynthesis, and the rates were significantly higher than leaves with an intact epicuticular wax layer (Mohammadian et al., 2007). In a Brazilian native *J. mollissima*, removal of epicuticular wax resulted in a lower transpiration rate when compared to leaves with an intact epicuticular wax layer, further demonstrating its effectiveness in managing transpiration (Figueiredo et al., 2012). Furthermore, an increase in leaf epicuticular wax significantly reduced photoinhibition, thereby limiting damage to the PSII reaction center in the photosynthetic pathway (Mohammadian et al., 2007). However, Figueiredo et al. (2012) reported that the presence of epicuticular wax did not reduce leaf temperature under water deficit conditions.

1.6. Chlorophyll fluorescence

The effect of environmental factors on photosynthesis and photosynthetic capacity mechanisms has been studied through chlorophyll fluorescence. Fluorescence is the re-emittance of light energy, red and far-red light, by the photosynthetic machinery. Changes in fluorescence emission are complementary to changes in heat dissipation and photosynthetic rate. Photosynthetic metabolism and gas exchange of the leaf is closely related to chlorophyll fluorescence, providing a way to study the effects of

environmental stresses on photosynthesis (Smillie and Nott, 1982; Delieu and Walker, 1983; Ireland et al., 1984). The rapid and non-destructive measurement of chlorophyll fluorescence provides information on the status of photosystem II (PSII) and photoinhibition. Chlorophyll fluorescence is typically measured by the ratio F_v / F_{max} (F_m), where F_v is the difference between F_{max} and F_0 (minimum level of fluorescence) and F_{max} is the maximum level of light fluoresced in the induction curve. When exposed to environmental stresses, a decrease in the ratio F_v / F_{max} indicates damage to the photosynthetic machinery resulting from the onset of photoinhibition (Keck and Boyer, 1974; Baker et al., 1983; Strand and Öquist, 1985).

Photoinhibition is influenced by other environmental stresses other than light intensity, such as high temperature and water deficit (Souza et al., 2004; Ribeiro et al., 2008). In *L. lanigerum*, an evergreen shrub of the family Proteaceae, leaves with an epicuticular wax layer subjected to high levels of sunlight resulted in limited photoinhibition, but leaves without epicuticular wax showed significantly higher rates of photoinhibition (Mohammadian et al., 2007). Epicuticular wax appear to be a mechanism for reducing photoinhibition in plants by offering protection from high solar irradiation events (Barker et al., 1997; Roháček and Barták, 1999; Mohammadian et al., 2007; Yang et al., 2009). In leguminous trees, *Piptadenia moniliformes* and *Trischidium molle*, water deficit conditions caused reduced chlorophyll content, apparently as a preventive mechanism against photoinhibition (Souza et al., 2010). In a study of varying water deficit conditions, chlorophyll content had a rate of decrease that was correlated with water deficit (Araus et al., 1998). Chlorophyll content of flag leaves in a durum

wheat population naturally decreased as grain filling progressed but it was noted that the environment as well as genotype significantly impacted grain filling and fluorescence (Araus et al., 1998). Araus et al. (1998) reported that less than 4% and 10% of grain yield variability was explained by F_v / F_{max} from rainfed and irrigation treatments, respectively. Durum wheat genotypes with the lowest adaptation of flag leaves to sunny-dry conditions are more productive since they are better to maintain there water status (Araus et al., 1998).

1.7. Spectral reflective indices

Spectral reflectance is the characteristic of plant leaves that are quantified by measuring the portion of the incident reflected energy. Chlorophyll, carotenoids and chromophores respond to photosynthetically active radiation. These pigments absorb energy between wavelengths of 400-700 nm, the visible light spectrum (Araus et al., 2001; Prasad et al., 2007a). Chlorophyll is composed of four classes chlorophyll *a*, chlorophyll *b*, chlorophyll *c* and chlorophyll *d*. Chlorophyll *a* and *b* are present in abundance in green plants, whereas chlorophyll *c* and *d* are found in cyanobacteria and protists. The carotenoid group is large, but beta-carotene is the main carotenoid pigment that absorbs energy in plant leaves. Chlorophyll *a* absorbs light mainly in the violet-blue and orange-red wavelengths, between 400-450nm and 640-680nm with maximum absorbance peaks occurring at wavelengths of 430nm and 662nm (Araus et al., 2001). Chlorophyll *a* absorbs little energy in the green-yellow-orange area and consequently give leaves their characteristic green appearance (Lichtenthaler et al., 1998). Chlorophyll

b absorbs energy between 430-480nm and 630-660nm with maximum absorbance occurring at wavelengths of 453nm and 642nm. Chlorophyll *b* is able to transfer the energy to chlorophyll *a* for ultimate conversion to chemical energy (Araus et al., 2001).

Carotenoids are accessory pigments that extend the light absorbance of leaves, mainly in the region that chlorophyll is unable to absorb, the blue region (Frank and Cogdell, 1996). Beta-carotene is the main carotenoid pigment involved in energy absorbance and reflectance within this group. Absorbance occurs throughout the UV region but mainly in the visible region between 400-500 nm, the blue region, with a maximum absorbance peak at 470nm. The chromophores, phycoerythrin and phycocyanin are also accessory pigments that engage in energy absorption in the region of the spectrum that chlorophyll is not equipped to exploit. The chromophores operate mainly in the visible light region of the spectrum. Phycoerythrin absorbs energy between 500-600nm, with a maximum absorbance peak at 565 nm, whereas phycocyanin absorbs energy between 525-650nm, with a maximum absorbance peak at 620 nm. The energy in the near-infrared region, 750-1150 nm, is at too high an energy state, prohibiting energy absorption by chlorophyll and carotenoids (Araus et al., 2001; Prasad et al., 2007a). Instead, multiple tissues, such as parenchyma cells in the mesophyll, reflect the light energy to reduce the damaging effect on the photosynthetic machinery.

Spectral reflection exploits the association of specific wavebands of the spectrum with specific plant traits. These associations or spectral reflectance indices (SRI) are used to determine different morphophysiological properties of crop plants (Reynolds et al., 1994; Araus et al., 2001). The SRI is based on mathematical equations incorporating

various wavelength and their sum or ratios or differences. The most widely used SRI is normalized difference vegetative index (NDVI), which is an indirect assessment of canopy biomass and photosynthetic area and simple ratio (SR), which indicates the photosynthetic area (Aparicio et al., 2000; Raun et al., 2001). NDVI is the normalized difference between near-infrared and visible bands. Red-normalized difference vegetative index (RNDVI) and green-normalized difference vegetative index (GNDVI) predicts grain yield and biomass and estimates the photosynthetic area of the canopy. The photochemical reflective index (PRI) is used as an estimator of radiation-use efficiency and the water index (WI) indicates the leaf and canopy water status of the plant. The water indices, normalized water index-1 (NWI-1), normalized water index-2 (NWI-2), normalized water index-3 (NWI-3) and normalized water index-4 (NWI-4), assess relative water content, canopy temperature and leaf water potential (Babar et al., 2006a; Prasad et al., 2007a; Prasad et al., 2007b).

The vegetative indices can assess the health of crop plants by serving as indicators of abiotic and biotic stress. A high reflectance of the near-infrared waveband indicates the presence of enough green tissue to scatter light. A high vegetative index indicates that crop plants photosynthetically active regions are operating at normal levels (Penuelas et al., 1995a). Chlorophylls and carotenoids absorb the maximum or close to the maximum amount of energy that is available to them. The parenchyma as well as other tissue in the mesophyll is actively and efficiently reflecting the energy of the harmful near-infrared radiation from the leaves of the canopy indicating that the photosynthetic machinery is operating at levels that are efficient and productive

(Lichtenthaler et al., 1998). Low vegetative indices indicate reduced activity of photosynthesis, reflection and stomatal conductance. The loss of green tissue, hence, the decrease in chlorophyll content results in a decrease in the vegetative indices due to maturity or abiotic stresses. The decline in activity can be due to a number of stresses that includes mineral, high temperature, water deficit, salinity, water and radiation (Lichtenthaler, 1996; Kumar et al., 2002).

The water indices indicate the water health of the canopy. When the water indices are low, it indicates a healthy plant with normal water movement throughout the plant (Peñuelas et al., 1997; Babar et al., 2006b). This is the case in situations where the plant is receiving sufficient water to perform its' required functions. High water indices indicate that sufficient water is not available for the plants to perform their normal functions (Peñuelas et al., 1997). The plants are water stressed and the higher the minor absorption band at 970 nm, the greater water stressed the plants. This has been demonstrated in winter wheat, where during good water status the water indices were low but high during periods of water deficit at certain growth stages (Peñuelas et al., 1997). Furthermore, the water indices was able to effectively measure the change in canopy water status in response to salinity stress in 10 barley genotypes (Peñuelas et al., 1997).

Spectral reflectance indices have been used as an indirect tool for selecting high yielding genotypes under various environmental conditions. The SRI were able to assess the effects of elevated levels of carbon dioxide and ozone on canopy function and structure of soybean using the leaf area index (LAI) and photochemical reflective index

(PRI) (Gray et al., 2010). The association of spectral reflectance indices with grain yield and biomass have been well documented (Araus et al., 2001; Babar et al., 2006a; Prasad et al., 2007a; Prasad et al., 2007b; Gutierrez et al., 2010). Spectral reflectance indices were able to differentiate genotypes based on grain yield in a trial of 15, 25 and 36 spring wheat genotypes (Babar et al., 2006a). These results were supported in trials of winter wheat (Prasad et al., 2007a; Prasad et al., 2007b; Prasad et al., 2009) and bread wheat (Gutierrez et al., 2010). The spectral reflectance indices were able to identify environmental stressed plants including water-stress (Jiang and Carrow, 2007; Gutierrez et al., 2010; de Jong et al., 2012), high temperature stress (Gutierrez et al., 2010; de Jong et al., 2012) and salinity (Peñuelas et al., 1997). Identifying the ideal growth stage for collecting spectral reflectance data can depend on numerous factors, but various reports suggest that heading and grain filling are best to distinguish genotypes for grain yield in wheat (Royo et al., 2003; Babar et al., 2006a; Prasad et al., 2007b; Gutierrez et al., 2010).

Delayed senescence or 'stay-green' is an important trait that has been identified in sorghum and wheat (Harris et al., 2006; Tian et al., 2012; Tian et al., 2013). The trait is categorized into functional and non-functional stay-green types. The functional stay-green possess chlorophyll and continue to undergo photosynthetic activity, whereas, the non-functional, although still possessing chlorophyll, have no photosynthetic activity (Thomas and Howarth, 2000). The longer duration of photosynthesis has often led to greater yields in the stay-green types than the wild types (Lopes and Reynolds, 2012; Kumari et al., 2013) . In wheat, it was observed that a stay-green mutant, *tasg* 1, had a

larger flag leaf green area than wild type under water deficit and high temperature stress at grain filling (Tian et al., 2012). However, there was no difference in chlorophyll *a* content at the initiation of senescence between the wild type and *tasg* 1 mutant, but subsequently decreased at a faster rate in wild type compared to *tasg* 1 (Tian et al., 2012; Tian et al., 2013). Therefore, when measuring spectral reflectance it is imperative to carefully assess the stages that spectral reflectance data will be collected.

Strong genetic and phenotypic correlations must exist between spectral reflectance indices and grain yield and biomass in order for it to be used as breeding tool. Spectral reflectance indices have been a strong genetic correlation with biomass and grain yield when assessed in 25 winter wheat genotypes (Prasad et al., 2007a; Prasad et al., 2009). An interesting result of various studies identified the water based indices as having stronger associations with biomass and grain yield than the vegetative indices, also exhibit higher heritability with grain yield than vegetative indices (Prasad et al., 2007a; Prasad et al., 2009; Gutierrez et al., 2010). The water based indices can distinctly separate low yielding and high yielding genotypes much more effectively than vegetative or pigment based indices in high temperature and water deficit environments (Prasad et al., 2009; Gutierrez et al., 2010). According to previous studies, spectral reflectance indices exhibit a linear relationship with grain yield. The vegetative indices, RNDVI, GNDVI and SR positively associate with grain yield while the water indices, WI, NWI-1, NWI-2, NWI-3 and NWI-4 are negatively associated (Prasad et al., 2007a; Prasad et al., 2007b; Gutierrez et al., 2010).

1.8. Stability statistic

The idea of stability is not unambiguous and depends on how it is perceived by plant breeders (Lin and Binns, 1988). Defining and measuring adaptability has hindered plant breeders from exploiting genetic differences in adaptability (Finlay and Wilkinson, 1963). The inability to measure complex environments qualitatively is the main obstacle impeding breeding programs from identifying genotypes that perform consistently across diverse environments (Finlay and Wilkinson, 1963). The specific description of what constitutes stability and hence a stable genotype still remains blurred. A stable genotype is one that performs consistently, in terms of yield, in diverse environments (Finlay and Wilkinson, 1963; Eberhart and Russell, 1966). A well adapted and stable genotype is found in the top one-third of stability statistics in diverse environments (Fox et al., 1990). Finlay and Wilkinson et al. (1963) developed a linear regression model that is able to measure phenotypic stability quantitatively across a range of environments. A regression coefficient, b, equal to or close to one is considered to have average stability or adaptability. A regression coefficient sufficiently above one is considered to be specifically adaptable only in high yielding environments and values close to zero is considered to be specifically adaptable only in low yielding environments (Finlay and Wilkinson, 1963). A variety with maximum yield potential and maximum phenotypic stability in the most favorable environment is considered to be ideal. Genotype by environment (GxE) interactions of crop plants can be reduced by integrating a mixture of genetics into varieties (Eberhart and Russell, 1966). The GxE interactions make it difficult to compare varieties across environments since there ranking in each

environment will differ (Eberhart and Russell, 1966). Stratification of macro-environments is an effective method to reduce GxE interactions and the regression model, b_i, partitions GxE interactions into variation response of the variety to environmental indexes and unexplained deviations from the regression (Eberhart and Russell, 1966). This model considers a variety to be stable if b_i=1 and the deviation from the regression, s_d^2, equals to zero. Shukla et al. (1972) developed an alternative to the regression approach of stability analysis, designated as stability variance (σ_i^2). The stability variance is based on separation of GxE interaction into two components, thus providing a better measure for genotype stability. It defines stability as the lack of interaction between GxE but combines additive genotypic effect, additive environmental effect and random error to determine the mean performance of a genotype (Shukla, 1972). A simple method for determining the stability of a genotype is the use of the coefficient of variation (Francis and Kannenberg, 1978). The coefficient of variation (CV_i) of each genotype is calculated as a percentage and represents the variation across environments (Francis and Kannenberg, 1978). It considers a genotype as stable if it produced high yields and its performance was consistent, meaning it had a small amount of variation across environments (Francis and Kannenberg, 1978).

The stability statistics or measures are divided into two distinct groups, parametric and non-parametric statistics. The parametric stability statistics take a few assumptions into consideration, mainly that the data normally distributed. Non-parametric stability statistics makes no assumptions, are not sensitive to errors in measurements and observations can be deleted without causing estimates to vary greatly unlike the

parametric counterparts (Nassar and Huehn, 1987). Non-parametric statistics are based on the ability of genotypes to stabilize in diverse environments and are ranked accordingly (Nassar and Huehn, 1987). There are four main non-parametric stability statistics that is based on rank, $S_i^{(1)}$, $S_i^{(2)}$, $S_i^{(3)}$, and $S_i^{(6)}$ (Nassar and Huehn, 1987; Hühn and Nassar, 1989; Huehn, 1990a). The $S_i^{(1)}$ statistic measure a genotypes' mean absolute rank difference over the number of environments, $S_i^{(2)}$ measures the common variance or between-ranks variance across the number of environments, $S_i^{(3)}$ measures the absolute deviations of rank squares for each genotype and $S_i^{(6)}$ measures the rank squares for each genotype relative to mean ranks (Becker, 1981; Nassar and Huehn, 1987; Hühn and Nassar, 1989; Huehn, 1990b). $S_i^{(1)}$,and $S_i^{(2)}$ estimates only the stability of a genotype, whereas $S_i^{(3)}$, and $S_i^{(6)}$ measures performance and stability of a genotype (Becker, 1981; Hühn and Nassar, 1989). The phenotypic stability of $S_i^{(1)}$ and $S_i^{(2)}$ values are tested for differences among genotypes using the goodness of fit test, chi-squared with one degree of freedom (Nassar and Huehn, 1987). The $Z_i^{(1)}$ and $Z_i^{(2)}$ statistics tests the phenotypic stability of a specific genotype within the trial against the alternative hypothesis that it is not stable (Nassar and Huehn, 1987). When $Z_i^{(1)}$ and $Z_i^{(2)}$ are lower than the critical chi-squared value there are no significant differences among genotypes. $S_i^{(1)}$ and $S_i^{(2)}$ are strongly correlated with each other and provide clear and relevant interpretation of data (Nassar and Huehn, 1987).

The common parametric stability statistics were classified into four groups, A, B, C and D, based on their method of calculation (Lin and Binns, 1988). Coefficient of variance (CV) and genotype mean square (S_i^2) make up group A. Wricke's ecovalence

(W_i^2), Shukla's stability variance (σ_i^2), Plaisted and Peterson's mean variance component (θ_i) and Plaisted's variance component for GE interaction ($\theta_{(i)}$) comprise group B. Group C and D comprise of Finlay and Wilkinson's regression coefficient (b_i), Perkins and Jinks' regression coefficient (β_i), Eberhart and Russell's deviation parameter (δ_i^2) and Perkins and Jinks' deviation (δ_i^2), respectively. Later, the concept of stability was further classified into three types, type I, type II and type III. In type I, a genotype with a small among environmental variance is considered stable. The type II idea considers a genotype that has a mean response of all genotypes in a trial parallel to the environmental response as stability. Finally, in type III a small residual mean squares from the regression model on the environmental index is ruled as stable (Lin et al., 1986).

Stability statistics have been used to identify stable genotypes in bread wheat (Akcura and Kaya, 2008; Mohammadi et al., 2012), sunflower (Tabrizi, 2012), palm oil (Rafii et al., 2012), and lentil (Asghar et al., 2006). In bread wheat, grain yield was observed to have a positive significant correlation with the non-parametric statistics $S_i^{(1)}$, $S_i^{(2)}$, $S_i^{(3)}$, and $S_i^{(6)}$ (Flores et al., 1998; Akcura and Kaya, 2008; Farshadfar et al., 2012). In a malting barley study, two genotypes were identified as stable with good yields across environments (Bantayehu, 2009). In a study of six sunflower cultivars, positive and significant correlation were identified between environmental variance, regression coefficient (b_i), genotypic coefficient of variation, and coefficient of determination (Lin et al., 1986; Tabrizi, 2012). Evaluation of eighteen bread wheat cultivars across diverse environments, the dynamic and static concept of stability was observed and it was noted

that linear regression models were more useful in detecting phenotypic stability (Mohammadi et al., 2012). Stability and yield must be considered simultaneously to develop a successful breeding program, since a genotype's performance must follow the environment (Mohammadi et al., 2012; Rafii et al., 2012).

1.9. Rationale and objectives

The earth's surface temperature has increased by about 2 ^{0}C over the past decade and according to scientists this trend is expected to continue (Ortiz et al., 2008; Gourdji et al., 2013; USEPA, 2013). As the largest food crop in the world and a major staple in many countries especially, developing and undeveloped countries, it is important that new drought and heat tolerant wheat cultivars be developed that produce high grain yields during hotter and dryer conditions that will prevail in the future. Food shortages and high food prices will create worldwide chaos and instability, especially in developing countries, as has been seen over the past decade, in the event termed the Arab Spring.

The **long-term goal** of this project is to use marker-assisted selection of known tolerance QTLs, to develop new wheat cultivars that produce ideal yields during heat stress. *The aim of this proposal* is identify the link between heat tolerance and increased epicuticular wax and identify QTLs associated with both heat tolerance, increased leaf epicuticular wax content and yield stability. The *central hypothesis* of this proposal is that leaf epicuticular wax helps to confer heat tolerance and increased yield stability by reducing transpiration and internal high temperatures by reflecting excess radiation.

Studies have shown an association between cooler leaves, heat tolerance and grain yield (Ayeneh et al., 2002; Kirigwi et al., 2007; Mason et al., 2011). It has been shown that high epicuticular wax also decreases canopy temperatures during abiotic stress. An understanding of the role that epicuticular wax plays in tolerance for abiotic stress and identification of associated QTLs will aid in our future development of improved varieties.

The specific objectives this dissertation were to: 1) define the role of increased leaf and spike wax on increased spectral reflectance, and its effect on reduced canopy and spike temperatures and transpirational cooling, 2) define the role of increased epicuticular leaf wax on reducing canopy temperatures, and its influence on increasing yield stability during heat stress and, 3) define the genetic association and overlap between QTLs regulating increased epicuticular leaf wax and increased yield stability during reproductive stage heat stress.

1.10. Location of field trials

The state of Texas, located in the southwest of the United States, is one of the states that comprise the Great Plains. The state displays a variety of environmental niche that makes it ideal for conducting field studies. The state is divided into twelve environmental niches ranging from semi-arid and arid in the west to humid subtropical in the east. Chillicothe, a member of the rolling plains niche, specifically, the low rolling plains experience hot dry summers with annual average rainfall of approximately 23 inches (Fig. 1). The east central environment encompasses the city of College Station

(Fig. 1). The climate is described as subtropical and temperate with hot summers and experience average annual rainfall of approximately 39 inches. Uvalde is a unique environmental niche located in the south of Texas (Fig. 1). Its' climate is described as continental, semi-arid and subtropical-sub humid with hot and humid summers experiencing annual average rainfall of approximately 23.22 inches. Ciudad de Obregon is located in northern Mexico in the coastal state of Sonora (Fig. 1). It experience a desert like climate that is hot and dry with little rainfall averaging less than 15 inches annually.

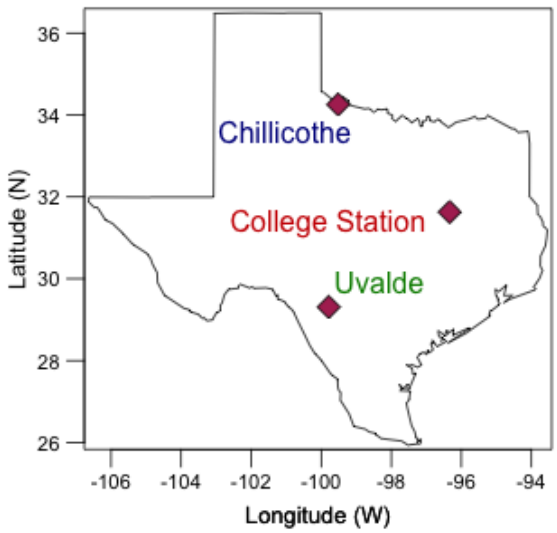

Fig. 1 Representative maps of the field trial locations in Texas and Mexico.

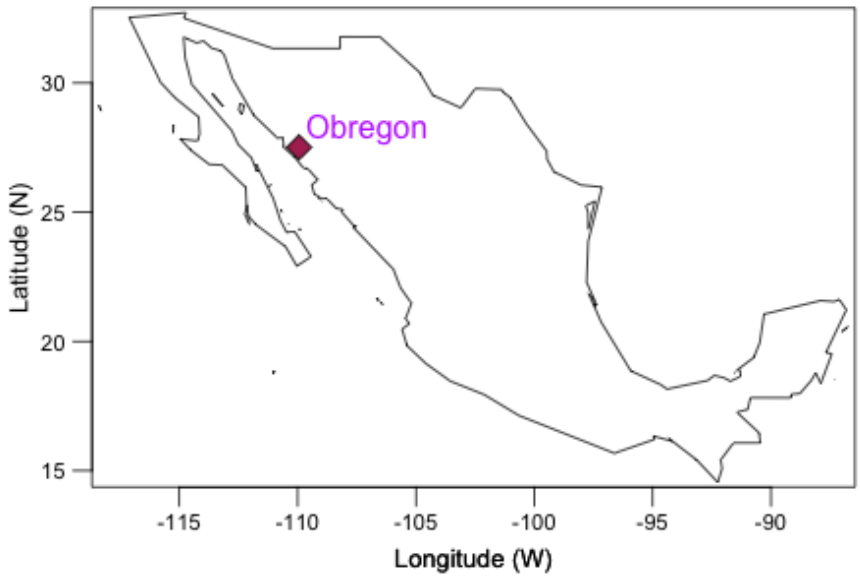

Fig. 1 Continued

CHAPTER II

CHANGES IN LEAF EPICUTICULAR WAX LOAD AND ITS EFFECT ON CANOPY TEMPERATURE, TRANSPIRATION, CHLOROPHYLL FLUORESCENCE AND SPECTRAL REFLECTANCE IN ELITE WHEAT CULTIVARS (*TRITICUM AESTIVUM L.*) EXPOSED TO HIGH TEMPERATURES DURING REPRODUCTIVE STAGES

2.1. Introduction

Wheat (*Triticum aestivum* L.) breeding strategies to develop high yielding and abiotic stress tolerant wheat are based on the development of a large segregating population with ensuing selection among the population. Current popular methods tend to be time consuming and expensive due in part to the large populations that are created and the need for field evaluations for several years at multiple locations. Empirical selection, the primary method of selection for yield, may not fully account for the environment by genotype interactions (Prasad et al., 2007b; Gutierrez et al., 2010). The past decade has seen advancement in methods specific for yield selection, incorporating physiology and morphology into the yield selection criteria (Reynolds et al., 1998). Canopy temperature depression, stomatal conductance, chlorophyll fluorescence and physiologically diagnostic indices are some of the physiological criteria currently in use (Reynolds et al., 1998; Aparicio et al., 2000; Babar et al., 2006a).

The ability of a plant to survive high temperature (HT) conditions depends in part on its ability to reduce the amount of radiation entering the leaf and to water loss through

both stomatal and epidermal transpiration. Epicuticular wax (EW) is an important adaptation trait in this function covering aerial surfaces in a manner that forms a barrier between the environment and the plant, offering protection against both abiotic, such as excess irradiation, water loss, high temperatures, and biotic stresses (Jenks et al., 1992; Kunst and Samuels, 2003; Shepherd and Griffiths, 2006). In previous studies, it has been observed that high temperature stress increased the amount of epicuticular wax deposited on the leaf surface, wheat (Clarke and Richards, 1988; Araus et al., 1991), carnation (Reed and Tukey, 1982) and barley (Giese, 1975). The stimulatory effect of radiation on epicuticular wax deposition was identified when dark grown barley plants dramatically increased epicuticular wax load (EWL) within the first 24 hours after being exposed to light (Giese, 1975). High temperature, irradiation and water deficit also influence the composition of epicuticular waxes by altering the quantity of hydrocarbons present in structure (Baker, 1974; Giese, 1975; Araus et al., 1991; Kim et al., 2007). Epicuticular waxes can reduce the amount of radiation entering the leaf and thus the plants thermal load through its function, reflecting excess light and by providing a barrier against water loss through epidermal transpiration in hot and dry environments (Reicosky and Hanover, 1978; Clarke and Richards, 1988; Araus et al., 1991; Sánchez et al., 2001; Samdur et al., 2003). Durum wheat genotypes, with the lowest adaptation of flag leaves to sunny-dry conditions, are still more productive because they are able to better maintain their water status than bread wheat (Araus et al., 1998).

The effect of environmental factors on photosynthesis and photosynthetic capacity has been studied using chlorophyll fluorescence (ChFl) as a physiological indicator.

Fluorescence is the re-emittance of light energy, red and far-red light, by chlorophyll. Thus, changes in fluorescence emission are complementary to changes in heat dissipation and a first line of defense to protect photosynthetic function during stress. Photosynthetic metabolism and gas exchange of a leaf is closely related to ChFl, providing a doorway to study the effects of environmental stresses on photosynthesis (Smillie and Nott, 1982; Delieu and Walker, 1983; Ireland et al., 1984). When exposed to environmental stress, a decrease in the ratio, F_v/F_{max}, indicate damage to the photosynthetic apparatus has occurred, possibly from photoinhibition (Keck and Boyer, 1974; Baker et al., 1983; Strand and Öquist, 1985). F_0 is minimum level of fluorescence, F_v is the difference between F_{max} and F_0 and F_{max} is the maximum level of light fluoresced in the induction curve. Epicuticular wax appears to be an adaptation that reduces photoinhibition in plants, offering protection via increased reflectance of high solar irradiation (Barker et al., 1997; Roháček and Barták, 1999; Mohammadian et al., 2007; Yang et al., 2009).

The ability of a plant to limit water loss through the leaf epidermis is essential to survive severe water deficit conditions (Araus et al., 1991). Epicuticular wax has been reported to improve water-use efficiency and reduce transpiration in wheat (Richards et al., 1986; Clarke et al., 1991). Drought stress also reduces transpiration rate while increasing epicuticular wax load in peanuts (Samdur et al., 2003), bread wheat (Clarke and Richards, 1988; Clarke et al., 1991; Elham et al., 2012), durum wheat (Araus et al., 1991), *Eucalyptus regnans* (England and Attiwill, 2011), and sesame (Kim et al., 2007). In a previous study, canopy temperatures were reduced in pea plants with higher

stomatal transpiration during HT stress (Sánchez et al., 2001), while EW and glaucousness combined to produce a greater effect on reducing transpiration than either alone in wheat (Clarke and Richards, 1988) with a high association between increased EW and reduced transpiration occurring in water stressed wheat plants. A thicker EW layer may be advantageous in limiting transpiration by partially plugging stomata (Araus et al., 1991). In young leaves, transpiration rate was low, but gradually increased during leaf expansion and subsequently declined as leaves senesced (England and Attiwill, 2011). Removal of EW from *Jatropha* leaves resulted in a lower transpiration rate compared to leaves with intact epicuticular wax (Figueiredo et al., 2012), possibly due to the loss of transpiration control.

Incident irradiation is either absorbed, reflected or transmitted unabsorbed by leaves (Lichtenthaler et al., 1998). As mentioned, EW reduces incident irradiation, however, during stress changes in the photosynthetic pigments do occur and can be detected through changes that occur in the reflectance signatures of leaves (Lichtenthaler et al., 1998). These changes in optical reflectance can be used to determine spectral reflectance indices (SRI) based on specific wavebands in the visible and near-infrared region (400-1200 nm) (Penuelas et al., 1995a; Araus et al., 2001; Babar et al., 2006a), each of which is diagnostic for unique but overlapping physiological functions. The SRI are divide into three main groups, vegetative indices, water based indices and pigment-based indices (Prasad et al., 2009). As such, SRI are ideal diagnostic tools to examine the role increase EW plays in improving physiological functions linked to a plants' water status and water use efficiency. The most commonly studied vegetative indices, the

normalized difference vegetative index (NDVI) and the simple ratio (SR), measures canopy biomass and canopy photosynthetic active area, respectively (Gitelson et al., 1996; Penuelas and Filella, 1998). The water indices, normalized water index-1, normalized water index-2, normalized water index-3, normalized water index-4, and the water index (WI), are the most common used to estimate canopy water status (Penuelas et al., 1993). The photochemical reflectance index (PRI), the most common pigment based index, measure changes in the xanthophyll cycle associated with changes in photosynthetic radiation use efficiency (Penuelas et al., 1995b). Normalized phaeophytinization index (NPQI), another pigment-based index, measures the degradation of chlorophyll inversely (Penuelas et al., 1995a). Previous studies reported significant associations between SRIs and grain yield in various crops exposed to various environmental stresses (Babar et al., 2006a; Prasad et al., 2007b; Prasad et al., 2009; Gutierrez et al., 2010).

This study was conducted to determine the relation between increased epicuticular wax and leaf temperature depression and stomatal conductance and improved photosynthetic function in terms of chlorophyll fluorescence, NDVI, SR, PRI, NPQI) and water status (WI). Additionally, we wanted to determine if an early deposition of EW prior to the onset of heat stress rather than in response improved tolerance in terms of physiology and yield.

2.2. Materials and methods

2.2.1. Plant material

Twelve wheat cultivars, seven spring and five winter, were grown in a greenhouse under optimal growth conditions. The cultivars and their attributes are listed in Table 1. The winter cultivars were vernalized for 6 weeks at 4 ^{0}C. The seedlings were transplanted at two per pot and replicated 30 times in 11 x 15 cm pots containing Metro Mix 900 potting soil (45-50% composted pine bark, vermiculite, Canadian sphagnum, peat moss, perlite and dolomitic limestone) (Sun Gro Horticulture, Canada LTD). This resulted in three single plant replications for each sampling period for both control (optimal conditions) and high temperature stress treatments. Only one of the two plants in each pot was sampled per pot for each stage. The experiment followed a randomized complete block design with three replicates for each stage of sampling. Initially, plants were grown under optimal conditions in greenhouses under natural sunlight at ~21^{0}C/18^{0}C day/night cycles with a 12 h photoperiod from 7 a.m. to 7 p.m. with supplemental light of 600 μmol m^{-2} s^{-1} PAR. At Feekes 10.5 or full emergence of the primary inflorescence stage half the pots for each cultivar were subjected to high temperature stress conditions under natural sunlight at ~40 ^{0}C/20 ^{0}C day/night cycles with a 12 h photoperiod from 7 a.m. to 7 p.m. with supplemental light of 600 μmol m^{-2} s^{-1} PAR. Measurements were collected from seven growth stages for both the high temperature and control treatments in replicates of three. The growth stages were defined as (1) full emergence of spike, (2) 3 days after full emergence, DAFE, (3) 6 DAFE, (4) 9 DAFE, (5) 10 days after pollination (DAP), (6) 13 DAP and (7) 15 DAP.

Table 1 The name, source and attributes of evaluated wheat cultivars.

Cultivar	Adaptation	Maturity	Source	Attributes
Siete Cerros (7C)	Spring	Medium	Mexico	semi-dwarf, rust resistant, excellent yields
Australith	Winter	Early	Israel	strip rust partially resistant, good yield
Cutter	Winter	Late	United States	high temperature sensitive
Fang 60	Winter	Medium	Thailand	boron tolerant, semi-dwarf
Halberd	Spring	Medium	Australia	heat tolerant, good yield
Kauz	Spring	Medium	Mexico	high yields, disease resistant, high temperature tolerant
Len	Spring	Medium	United States	semi-dwarf, good agronomic traits,
Ogallala	Winter	Medium	United States	semi-dwarf, high yields, leaf rust resistant
Seri 82	Spring	Medium	Mexico	stripe rust resistant, high yields, moderately drought tolerant,
Tam 111	Winter	Medium	United States	high yields, semi-dwarf, drought resistant,
Tam 112	Winter	Medium	United States	high yields, greenbug resistant, leaf rust resistant, drought tolerant,
Tam 401	Winter	Early	United States	good yields, drought and high temperature tolerant,

2.2.2. Leaf and spike temperature

The effect of long term high temperature stress on leaf temperature, spike temperature and epicuticular wax load were assessed on the glumes of the primary inflorescence and flag leaf. Leaf and spike temperature were determined by measuring the temperature of each flag leaf and spike glumes of the primary inflorescences using a portable handheld infrared thermometer (Fluke 566 series, Everett, Washington, USA). The thermometer was held at 45^0 to the flag leaf and spike. Temperatures were collected at the seven growth stages for both control and high temperature stressed plants. The air temperature was recorded using a data logger (EL-USB-2_LCD, Lascar Electronics, PA, USA) hanging just above the canopy. Temperature depression was calculated as the difference in air temperature and flag leaf and spike temperatures. All measurements were collected between 1:00pm to 3:30 pm on the day the sample was collected. The measurements were collected on leaves and spike glumes of three plants per sample stage in both environments.

2.2.3. Leaf gas exchange

Transpiration, stomatal conductance (g_s) of attached flag leaves was measured using a handheld leaf porometer (Model Sc-1, Decagon Services Inc, Pullman, WA). The clip was placed at the center of each flag leaf and the abaxial and adaxial side of the leaf was measured. Measurements were collected on plants at three growth stages, 3 DAFE, 10 DAP and 15 DAP for both control and high temperature stresses plants. The measurements were performed on the flag leaves of the primary inflorescence three

plants per sample stage in both environments. Epicuticular waxes were removed from intact leaves by submersion in chloroform for 10 seconds in a glass vial. The dip in chloroform almost completely removed the EW from the leaf (Eberhart and Russell, 1966; Mayeux and Jordan, 1984) as determined by the colorimetric assay described below (Ebercon et al., 1977). The stomatal conductance (g_s) was measured after a period of 15-20 minutes post EW removal.

2.2.4. Chlorophyll fluorescence

Chlorophyll fluorescence (F_v/F_m) was measured with a handheld fluorometer (Fluoropen FP100, Photo systems Instruments, Czech Republic). Measurements were taken of the adaxial (upper) side of the flag leaf with three readings along the middle section of each leaf. Measurements were collected on plants at three growth stages, 3 DAFE, 10 DAP and 15 DAP for both control and high temperature stress plants. The measurements were performed on fully expanded leaves of three plants per sample stage in both environments.

2.2.5. Spectral reflectance

Spectral reflectance was measured using a UniSpec-SC spectrometer (PPsystems, UN1007, Amesbury, MA USA) by emitting a beam of light through the adaxial leaf surface. An absorbance curve was recorded based on light reflectance for each flag leaf sampled. Measurements were taken at the center of each flag leaf at 3 DAFE, 10 DAP and 15 DAP for both control and high temperature stresses plants. The measurements were performed on the flag leaves of the primary inflorescence of three plants per sample stage in both environments. Epicuticular waxes were removed from intact leaves by submersion in chloroform for 10 seconds in a glass vial as described above. The spectral reflectance was measured after a period of 15-20 minutes post EW removal. The spectral reflectance data was used to determine vegetative, pigment and water indices (Table 2).

Table 2 Description and abbreviations of spectral reflectance indices employed in this study.

Reflectance Index	Abbreviation	Formula	Author
Simple ratio	SR	R_{900} / R_{680}	Gitelson et al., 1996
Normalized difference vegetation index	NDVI	$(R_{900} - R_{680}) / (R_{900} + R_{680})$	Peñuelas et al., 1997b
Photochemical reflectance index	PRI	$(R_{531} - R_{570}) / (R_{531} + R_{570})$	Peñuelas et al., 1995b
Water Index	WI	R_{900} / R_{970}	Peñuelas et al., 1993,1997
Normalized phaeophytinization Index	NPQI	$(R_{415} - R_{453}) / (R_{415} + R_{453})$	Peñuelas et al., 1995c

2.2.6. Wax extraction and quantification

Leaf discs for wax analysis were collected from flag leaves using a disc punch with a 1 cm diameter drum (Rabbit Tool USA, Rock Island IL USA). Three 1cm (diameter) leaf punches was collected from the primary inflorescence leaf per plant and used to determine EW concentrations using the colorimetric method (Ebercon et al., 1977). Leaf EW was extracted by submerging leaf discs in 1 ml HPLC grade chloroform for 30 seconds, the submersion time previously determined to completely remove the epicuticular wax from the leaf (Mayeux and Jordan, 1984). The resulting mixture was transferred to a clean 1.8 ml glass GC vial (VWR Auto sampler Vial, Radnor, PA) and chloroform removed under a stream of nitrogen gas. The resulting extract was oxidized by adding 300µl acidified potassium dichromate and heated for 30 minutes in a water bath at 100 ^{0}C. After boiling, vials were allowed to cool and 700µl of deionized water was added to each vial, allowing color development for a 1 hr. period. A spectrophotometer (PHERAstar plus, BMG LABTECH, Offenburg, Germany) was used to determine the optical density for each sample at 590 nm. Samples were loaded in 96 well polystyrene, untreated, clear flat bottom plates (Greiner Bio-One, Monroe, NC, USA). A standard curve was developed from randomly selected wheat flag leaves. Flag leaves were placed in large glass vials and 20 ml HPLC grade chloroform was added to remove EW. The resulting chloroform-wax solution was proportioned based on the serial dilution technique. The standard curve was used to calculate wax levels based on leaf area.

2.2.7. Data analysis

Data was analyzed using the generalized linear model (PROC GLM) to estimate analysis of variance (ANOVA), with cultivars and environments considered as fixed effects and replication as a random effect using the statistical software SAS (SAS v9.2, SAS institute Inc. Cary, NC, USA). Least square means (LSmeans) and Waller-Duncan test with Bonferroni correction was used to contrast means, at a significance of 5% ($P \leq 0.05$), when differences were detected, Pearson's correlations were used to identify associations between treatment, epicuticular wax, leaf temperature depression, chlorophyll fluorescence and transpiration.

2.3. Results

2.3.1. Epicuticular wax load

Significant differences were observed between cultivars for epicuticular wax (EWL). The interaction between cultivars and environments was highly significant ($P \leq 0.001$) for EW of flag leaves (Table 3). Surprisingly, stage was not significant for EW, but cultivar was significant within individual stages (data not shown). EWL values were between 0.28 to 1.76 mg dm^{-2} in the control environment and 0.52 to 2.57 mg dm^{-2} in HT treatment (Fig. 2). The highest EW was observed at 13DAP in control treated flag leaves, but at 15DAP in HT treatment. Len, a moderately heat susceptible cultivar, produced the highest amount of EWL when combined across reproductive stages in the HT treatment, whereas, SeriM82, a high temperature tolerant cultivar, produced the highest amount in control conditions. There was a substantial increase of 29.35% in

EWL on average across the reproductive stages, in response to the HT stress. However, only two of the twelve cultivars registered a significant increase in EWL in HT stress compared to their counterpart in control conditions (Fig. 2). The cultivars that exhibited a significant increase in EW due to HT were Len and Halberd. Increase in crop age during the reproductive stage, coincided with a steady increase in EWL in the HT stress treatment, but fluctuated in control conditions (Fig. 2A). On average, a 23.78% increase occurred between the initial stage and 3DAFE, 17.5% between 6DAFE and 9DAFE, 14.53% between 10DAP and 13DAP, with an overall increase of 38.21% between 6DAFE and 15DAP. EWL significantly and negatively associated with chlorophyll fluorescence (ChFl) ($P \leq 0.05$, $r=0.23$) and abaxial stomatal conductance (SC_Ab) ($P \leq 0.05$, $r=0.21$), indicating that increased EWL plays a role in reducing the effect of high temperature on the overall plant health, by reducing ChFl and SC_Ab.

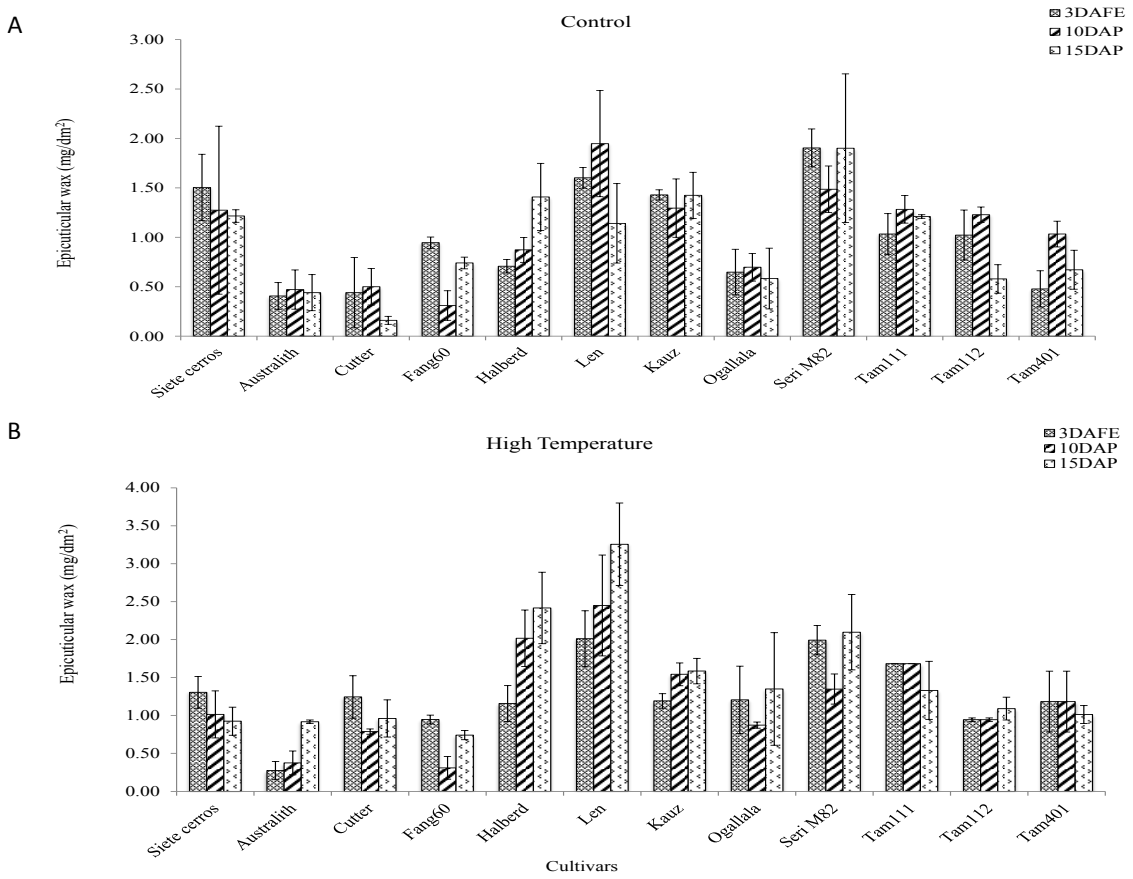

Fig. 2 Change in epicuticular wax load (EWL) of cultivars from tiller emergence to grain filling. (A) epicuticular wax levels of cultivars at reproductive stages at 3 days after full emergence of tiller (3DAFE), 10 days after pollination (10DAP) and 15 days after pollination (15DAP) under control conditions. (B) epicuticular wax levels of cultivars at reproductive stages at 3 days after full emergence of tiller (3DAFE), 10 days after pollination (10DAP) and 15 days after pollination (15DAP) under high temperature conditions (~40^0C/20^0C day/night). Values are the means of three replicates of individual plants for each cultivar at respective reproductive stages. Error bars represent standard error of the means.

Table 3 Mean squares of interactions for the ANOVA F-test for physiological traits and spectral reflectance indices indicating the effects of environment, cultivar and reproductive stages. *, **, *** significant at p≤ 0.05, 0.01 and 0.001 respectively. NS not significant. Cul cultivar, trt treatment, env environment, rep replication, stage reproductive stage.

Interactions		Traits										
Source	DF	LTD	STD	EWL	ChFl	SC_Ad (upper)	SC_Ab (lower)	WI	SR	PRI	NDVI	NPQI
cul	11	6.923***	4.786*	3.748***	0.010***	15980.416*	38135.062***	0.002^{NS}	2.281**	0.002***	0.006^{NS}	0.015***
trt	1	-	-	-	-	1292495.907***	171695.355***	0.060***	8.489**	0.008**	0.052**	0.006^{NS}
rep	2	1.090^{NS}	2.320^{NS}	0.314^{NS}	0.002^{NS}	2405.089^{NS}	6304.678^{NS}	0.001^{NS}	1.311^{NS}	0.000^{1NS}	0.006^{NS}	0.001^{NS}
env	1	17.931**	37.371***	3.458***	0.114***	19005.244^{NS}	13007.850^{NS}	0.003^{NS}	0.793^{NS}	0.014***	0.021*	0.019**
stage	1	15.577**	13.017*	0.143^{NS}	0.060***	2301.002^{NS}	51066.376*	0.000^{NS}	3.222^{NS}	0.000^{NS}	0.024*	0.004^{NS}
env*cul	11	10.983***	15.584***	0.795***	0.011***	16269.192*	6643.635^{NS}	0.002^{NS}	1.931*	0.001^{NS}	0.005^{NS}	0.002^{NS}
stage*cul	11	4.308*	8.159***	0.373^{NS}	0.004^{NS}	20641.163**	14333.961^{NS}	0.005**	1.689^{NS}	0.003***	0.005^{NS}	0.004*
stage*env	11	3.577^{NS}	4.816^{NS}	0.544^{NS}	0.044***	6865.356^{NS}	3797.700^{NS}	0.000^{NS}	0.773^{NS}	0.003^{NS}	0.008^{NS}	0.000^{NS}
trt*cul	1	-	-	-	-	16412.819*	2143.125^{NS}	0.002^{NS}	0.891^{NS}	0.001^{NS}	0.004^{NS}	0.002^{NS}
env*trt	1	-	-	-	-	1300.001^{NS}	11333.014^{NS}	0.009*	0.414^{NS}	0.001^{NS}	0.000^{NS}	0.015**
stage*env*cul	11	4.039^{NS}	6.754**	0.233^{NS}	0.004^{NS}	19622.575**	5748.085^{NS}	0.004*	1.550^{NS}	0.002**	0.004^{NS}	0.002^{NS}
env*trt*cul	11	-	-	-	-	8816.486^{NS}	16563.510^{NS}	0.002^{NS}	0.606^{NS}	0.002*	0.003^{NS}	0.002^{NS}
stage*env*trt*cul	24	-	-	-	-	9694.393^{NS}	16569.072^{NS}	0.002^{NS}	0.475^{NS}	0.002**	0.002^{NS}	0.001^{NS}

Key: Leaf temperature depression (LTD), spike temperature depression (STD), epicuticular wax load (EWL), chlorophyll fluorescence (ChFl), stomatal conductance adaxial (SC_Ad), stomatal conductance abaxial (SC_Ab), normalized phaeophytinization Index (NPQI), normalized difference vegetative index (NDVI), simple ratio (SR), photochemical reflective index (PRI), water index (WI).

2.3.2. Leaf and spike temperature

Leaf temperature depression during reproductive stages differed significantly among cultivars for both the treatments. It was lower in control treatment (0.13 to 4.00 ^{0}C) compared to HT stress (1.92 to 4.9 ^{0}C). The only interactions with significance were environment x cultivar, stage x cultivar and stage x environment x cultivar (Table 3). At anthesis (~3DAFE), Seri M82, a high temperature tolerant cultivar, had the highest LTD or lowest canopy temperatures, whereas, Halberd, also a high temperature tolerant cultivar, had the highest LTD during the early stages of grain filling under HT conditions. The high LTD of Halberd could possibly be a result of the high EWL naturally present on its flag leaves providing a cooler leaf environment (Fig. 3B). The correlation between LTD and EWL was significant and positive ($P \leq 0.01$, $r = 0.27$) under HT conditions (Table 4). Spike temperature depression (STD) also differed significantly among cultivars across environments and was lower in control (0.43 to 3.72 ^{0}C) compared to HT stress (1.63 to 5.7 ^{0}C) environment. Surprisingly, no significant correlation between LTD and ChFl, SC_Ad and SC_Ab was identified in this experiment (Table 4).

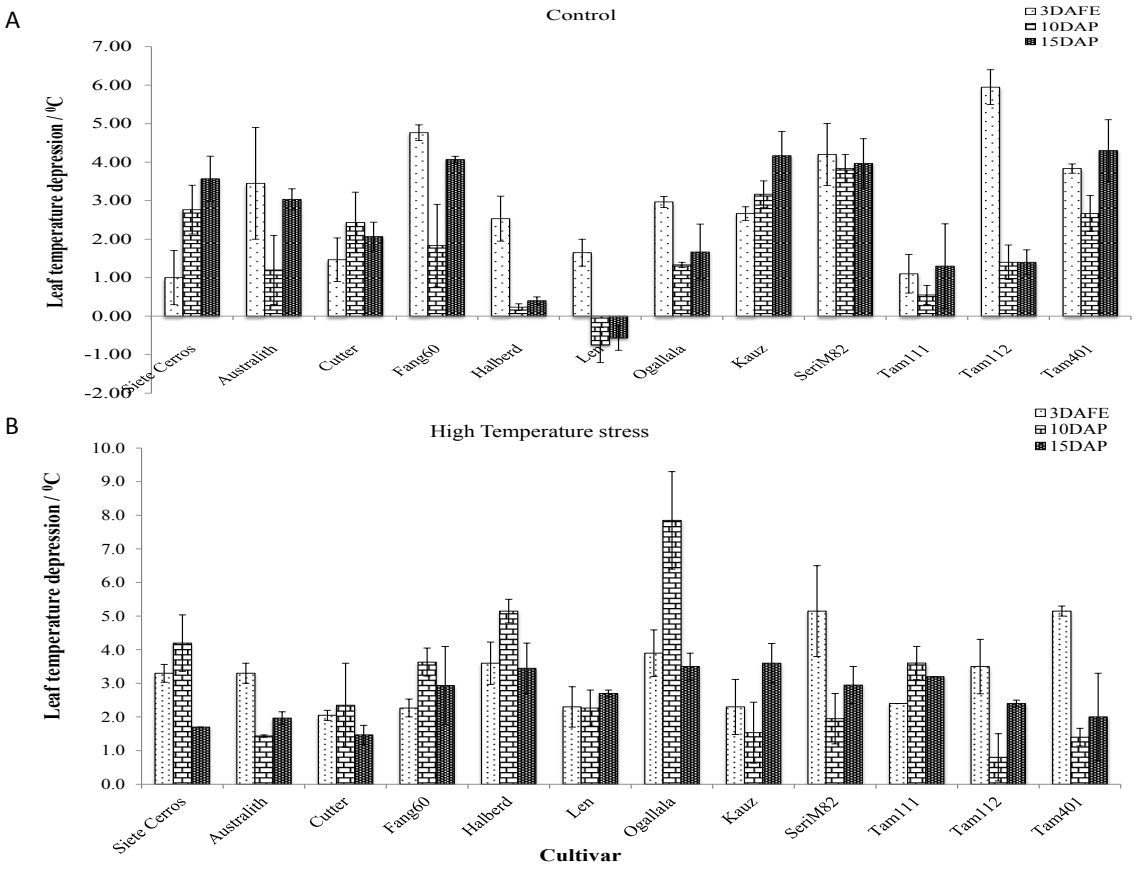

Fig. 3 Effect of epicuticular wax load (EWL) on surface leaf temperature for cultivars. (A) leaf temperature depression of flag leaves at the reproductive stages of 3 days after full emergence of tiller (3DAFE), 10 days after pollination (10DAP) and 15 days after pollination (15DAP) under control conditions. (B) leaf temperature depression of flag leaves at the reproductive stages of 3 days after full emergence of tiller (3DAFE), 10 days after pollination (10DAP) and 15 days after pollination (15DAP) under high temperature conditions (~40^0C/20^0C day/night). Temperature depression was measured as the difference between air temperature and leaf surface temperature. Values are the means of three replicates of individual plants for each reproductive stage. Error bars represent standard error of the means.

Table 4 Pearson's correlation coefficients of epicuticular wax load (EWL), stomatal conductance (SC), chlorophyll fluorescence (ChFl), leaf temperature (LTP), leaf temperature depression (LTD), spike temperature depression (STD), and heat susceptibility index (HSI) for plants under high temperature treatment control conditions (ct), high temperature conditions (ht). *, **, *** significant at p≤ 0.05, 0.01 and 0.001 respectively. NS not significant.

Traits	ChFl	SC_Ad	SC_Ab	EWL	Yield (ct)	Yield (ht)
SC_Ad	0.05^{NS}					
SC_Ab	0.20^{NS}	0.45***				
EWL	-0.23*	-0.05^{NS}	-0.21*			
LTP	-0.24*	-0.26*	-0.35*			
LTD	0.02^{NS}	-0.04^{NS}	0.05^{NS}	0.27**		
STD	-0.11^{NS}	-0.05^{NS}	0.18^{NS}	0.31**		
Yield (ct)	-0.11^{NS}	0.09^{NS}	0.10^{NS}	0.06^{NS}		
Yield (ht)	0.27^{NS}	-0.28^{NS}	0.04^{NS}	0.40*	0.38*	
HSI	-0.24^{NS}	0.36*	0.05^{NS}	-0.33^{NS}	0.15^{NS}	-0.73***

2.3.3. Chlorophyll fluorescence

The effect of HT on photosynthetic activity, measured as ChFl, was significant for cultivars. (Table 3). Cultivars in control treatment (0.70 to 0.74 F_v/F_m) had significantly higher ChFl values than those in HT stress treatment (0.58 to 0.74 F_v/F_m) (Fig. 4B). However, there were significant differences identified among cultivars in HT treatment unlike the control treatment. In the control conditions, ChFl significantly differed at 3DAFE and 10DAP, however, in HT, significant differences were observed at 10DAP and 15DAP among cultivars (Fig. 4). The most prominent differences for ChFl were detected during 10DAP. Evidently, ChFl decreased gradually as leaves experienced

aging through the reproductive stage. ChFl was significant and negatively correlated with EWL, but had no significant association with LTD (Table 4).

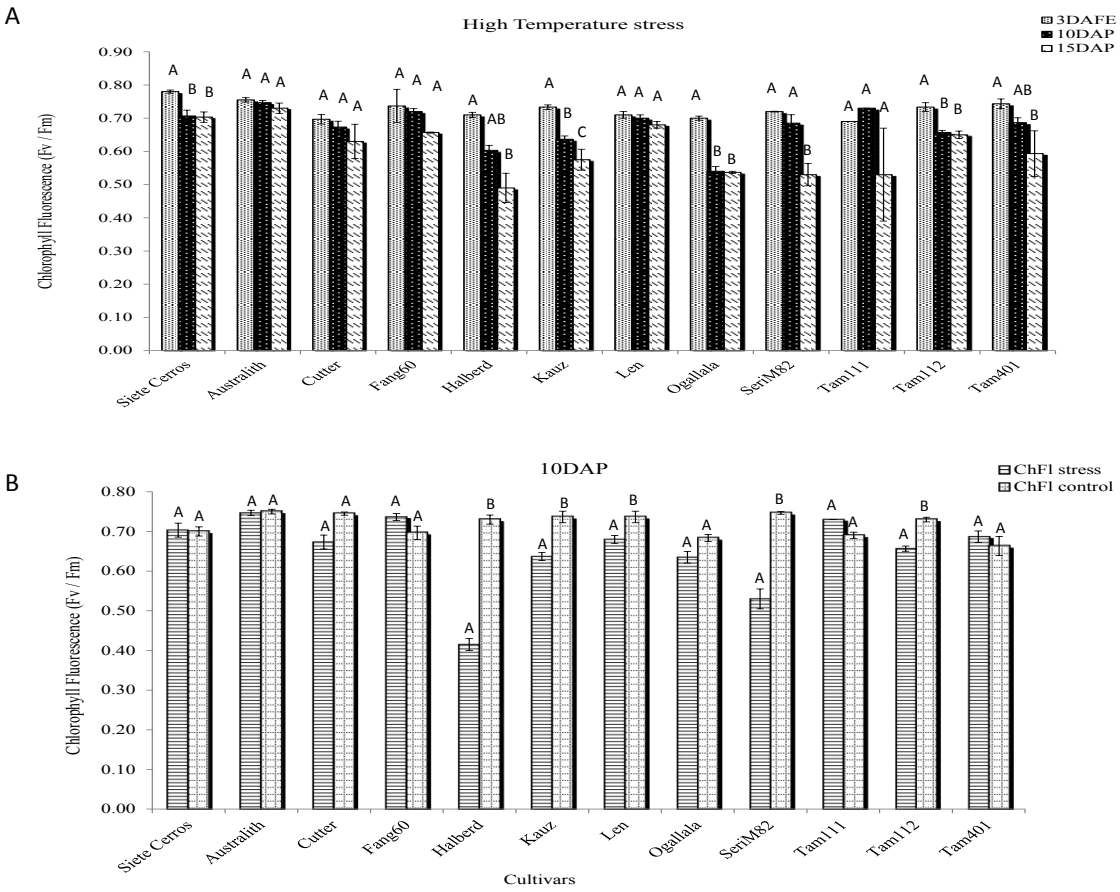

Fig. 4 Chlorophyll fluorescence (ChFl) of cultivars under high temperature and control conditions. (A) ChFl at the three reproductive stages (3DAFE, 10DAP, 15DAP) under high temperature stress conditions. (B) comparison of cultivars at10 days after pollination (10DAP) for high temperature and control conditions. Error bars represent standard error of the means of the each cultivar at reproductive stages and the both conditions. Different letters above bars represent significant differences of the means between treatments by Duncan multiple range comparison test.

2.3.4. Leaf gas exchange

Stomatal conductance (SC), g_s, varied significantly on the abaxial (lower) surface (SC_Ab) of leaves but was consistent on the adaxial (upper) surface (SC_Ad) ($p \leq 0.001$). Significant differences were identified between reproductive stages within environments ($p \leq 0.001$) both for leaves devoid of EW and those with wax (Fig. 5). Environment x treatment interaction was not significant but environment x cultivar, stage x cultivar and environment x stage x cultivar was significant for SC_Ad (Table 3). For SC_Ab, no interaction was significant, however, reproductive stage and cultivar were significant (Table 3). Lower g_s was observed after removal of EW than when a waxy layer was present in control and HT treatments. Abaxial leaf surface, devoid of EW, during 3DAFE, 10DAP, and 15DAP experienced lower g_s under HT stress compared to control (Fig. 2). No significant correlation was observed between the SC_Ad and EWL, however, it was significant and negatively correlated with leaf temperature ($P \leq 0.05$, $r=0.26$) (Table 4). Interestingly, there was no significant association observed between SC_Ad and LTD. The SC_Ab was significant and negatively correlated with EWL ($P \leq 0.05$, $r = 0.21$) and CTP ($P \leq 0.05$, $r=0.35$) (Table 4). There was no significant correlation observed between SC_Ab and LTD. Furthermore SC_Ad and SC_Ab were positively correlated with each other ($P \leq 0.001$, $r=0.45$) (Table 4).

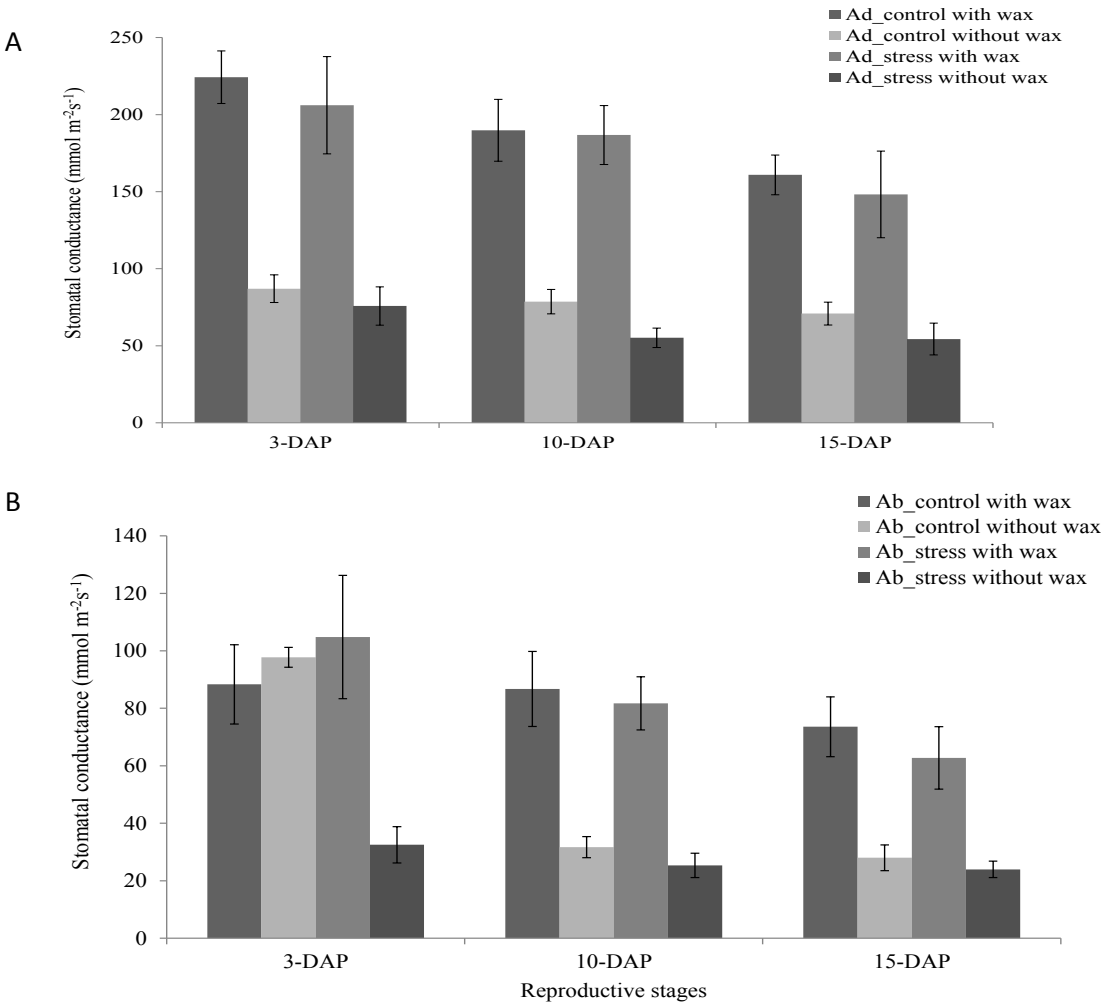

Fig. 5 The effect of epicuticular wax on stomatal conductance (g_s) of wheat cultivars exposed to control and high temperature stress conditions. Plants received water continually on a daily basis until completion of the grain filling stage in both environments. Epicuticular wax was stripped from leaves by dipping leaves directly in chloroform contained in a glass vials for 10 seconds. Each value represents the mean of all cultivars for three replicates at each reproductive stage. Error bars represent standard error of the means. Adaxial surface (Ad), abaxial surface (Ab), leaves stripped of epicuticular wax (without wax), leaves with intact wax (with wax).

2.3.5. Spectral reflectance indices

Significant differences were observed for stage x cultivar, stage x environment x cultivar and stage x environment x cultivar x treatment interactions for the pigment based index, PRI (Table 3). The other pigment index, NPQI, had significant differences for stage x cultivar and environment x treatment interactions (Table 3). Significant differences were observed for stage x cultivar, environment x treatment and stage x environment x cultivar interactions for the water based index, WI (Table 3). Reproductive stages were significant for the vegetative indices in both environments and NPQI was significant for stages only under HT conditions. The other pigment-based index, PRI, was significant for reproductive stages only in the HT environment. The water index was not significant for reproductive stages under either environmental condition (Table 3). Simple ratio and PRI were significant for leaves devoid of wax across stages under HT and control conditions, respectively, but NDVI, NPQI and WI showed no significant differences. There was a general decrease from control to high temperature conditions in NPQI, NDVI and PRI with leaves without wax. SR was observed to increase from control to HT conditions in leaves devoid of wax. For leaves with wax, all SRI values were lower in the HT environment compared to control conditions. The WI was similar in both environments for leaves with and without wax. Under HT conditions significant phenotypic correlations between EWL and the spectral reflectance indices were not observed at 10DAP and 15DAP. However, only one cultivar differed statistically in the WI value within a reproductive stage (Fig. 6A). The WI value for TAM401 was significantly less under HT than control conditions at 10 DAP (Fig.

6A) but was not statistically different at 15 DAP (Fig. 6B). A few significant correlations between EWL and spectral reflectance indices were observed at the 3DAFE stage. The WI, SR and NPQI were negatively associated with EWL. The association of EWL with SRI was consistently positive at 10DAP and 15DAP but negative at 3DAFE. When the association was tested using average of stages and EWL, only NPQI was significant, but no significant associations were observed under control conditions. The SRI were observed to be significantly associated with each other except for NDVI and PRI (Table 5). The water index was negatively associated with NDVI and NPQI, but SR and PRI were negatively associated with each other (Table 5).

Table 5 Pearson's correlation coefficients of epicuticular wax and the individual spectral reflectance indices of plants under high temperature and control conditions. *, **, *** significant at p≤ 0.05, 0.01 and 0.001 respectively. Normalized phaeophytinization Index (NPQI), normalized difference vegetative index (NDVI), simple ratio (SR), photochemical reflective index (PRI), water index (WI), epicuticular wax present on leaf (EW), epicuticular wax stripped from leaf (NW).

Trait	NPQI	NDVI	PRI	SR	WI
NPQI					
NDVI	0.3438***				
PRI	0.4730***	0.0355^{NS}			
SR	0.3506***	0.8869***	-0.1447**		
WI	-0.2435***	0.2939***	-0.2023***	0.3653***	
EW	0.20*	0.10^{NS}	0.08^{NS}	0.11^{NS}	0.04^{NS}

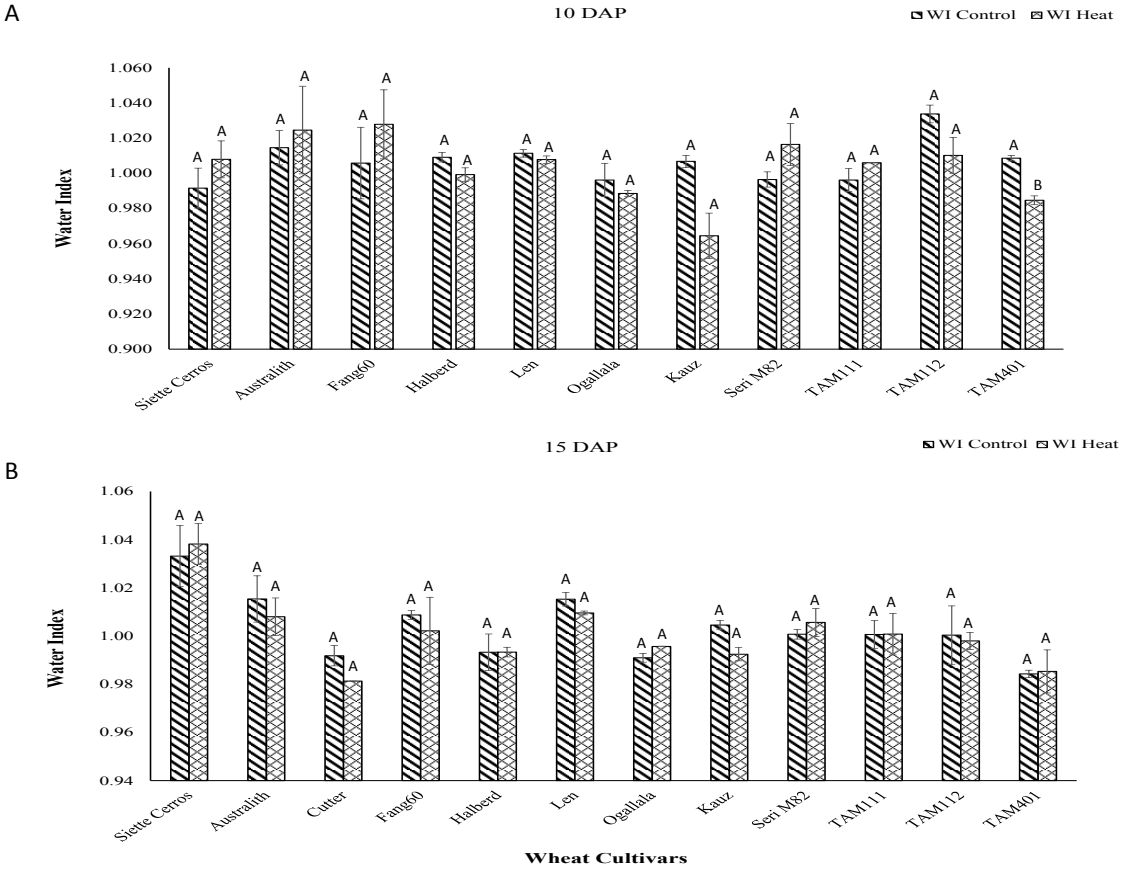

Fig. 6 The effect of epicuticular wax (EWL) on spectral reflectance represented in terms of the water index (WI). (A) water index values of cultivars at 10 days after pollination (10DAP) under control and high temperature conditions. (B) water index values of cultivars at 15 days after pollination (15DAP) under control and high temperature conditions. Values are the means of three replicates measured from individual plants of each cultivar. Error bars represent standard error of the means. Different letters above bars represent significant differences of the means between treatments by Duncan multiple range comparison test.

2.4. Discussion

2.4.1. Epicuticular wax load

Epicuticular wax load is a product not only of genetics but is also influenced by the environment and its concentration and composition varies tremendously within species and across species (Clarke and Richards, 1988; Araus et al., 1991; Sánchez et al., 2001; Oliveira et al., 2003; Samdur et al., 2003; Kim et al., 2007; Elham et al., 2012). Significant differences in EWL have been observed after plants were exposed to water deficit stress (Oliveira et al., 2003; Kim et al., 2007; Elham et al., 2012) and HT stress (Richards et al., 1986; Clarke and Richards, 1988; Araus et al., 1991). In wheat, under water deficit stress, genotypes produce greater EWL than those under well watered conditions, increasing by as much as 20% (Elham et al., 2012). Similarly, tobacco plants exposed to varying water deficit stress produced approximately 2.5 fold more EWL than well-watered plants (Cameron, 2005). A substantial and significant increase of about 20% in EWL was reported in peanuts under water deficit conditions and observed that genotype x environment interaction for EWL differed significantly (Samdur et al., 2003). In the present study, EWL was highly significant for cultivars and for cultivar x genotype interactions (Table 3), similar to findings reported in peanuts (Samdur et al., 2003). The observed response in Len to high temperature was somewhat surprising, continually increasing its EWL as it progressed through reproductive development. This response could be due to its late maturity nature, hence exposing itself to higher temperatures and irradiation compared to other cultivars. It is a reasonable assumption to think that cultivars with higher EWL would have lower transpiration rates, higher light

reflectance and cooler canopies. In the present study, EWL significantly correlated with ChFl, g_s, and LTD (Table 4). Similar studies in pea (Sánchez et al., 2001), wheat (Clarke and Richards, 1988; Araus et al., 1991) and caatinga (Oliveira et al., 2003) observed similar correlations with EWL. The lack of correlation between g_s of leaves with EW and leaves devoid of EW support previous findings that EW plays an important role in regulating g_s (Araus et al., 1991). The adaxial surfaces of wheat flag leaves were visually less glaucous than the abaxial surface and had higher g_s in both environments (Fig. 5). The lack of glaucousness or waxy covering on the adaxial surface is a common occurrence that has been observed on wheat flag leaves (Johnson et al., 1983). EWL seemingly increased as flag leaves aged, especially between the inflorescence emergence and early grain filling. Similarly in peanuts, EWL increased between from an early reproductive stage through maturity with an average increase of 86% (Samdur et al., 2003).

2.4.2. Leaf and spike temperature

High temperature stress has similar effects on epicuticular waxes as water deficit stress. In fact, the effects of these two stress conditions tend to be confounded in naturally hot and dry environments (Lombardini, 2006). High temperature stress during the reproductive stage of wheat can result in significant yield loss (Wardlaw et al., 1989; Zahedi et al., 2003; Hays et al., 2007). In previous water deficit studies, it was reported that significant increases in EWL was observed in stressed plants compared to well irrigated plants of wheat (Clarke and Richards, 1988), sesame (Kim et al., 2007), and

peanut (Samdur et al., 2003). In this study, significant genotypic variation for LTD was observed in both the control and HT environments (Table 3). It gave a strong relationship with EWL in the HT environment but was not significant for control conditions (Table 4). The LTD was higher in the HT environment than in control conditions (Fig. 3). The positive relationship indicates that an increase in EWL may decrease the temperature of leaf microenvironment by efficiently reducing the amount of incident irradiation. The association between LTD and EWL was highest under HT conditions. Previous studies have reported that a high association exist between CTD and grain yield (Reynolds et al., 1998; Gutierrez et al., 2010), but in this study a non-significant positive correlation was identified between LTD and the primary tiller yield. A higher leaf temperature was observed in plants under HT stress conditions with most cultivars having a lower micro-environmental temperature than the air temperature. The arrangement and structure of epicuticular waxes may play a role in the differences observed in cultivars in internal temperature and may also influence light reflection and transpiration (Araus et al., 1991).

2.4.3. Chlorophyll fluorescence

Chlorophyll fluorescence provides a fast nondestructive viable option for estimating damage to photosystem II (PSII) processes cause by environmental stresses (Bolhar-Nordenkampf, 1989; Krause and Weis, 1991; Yang et al., 2009). It was observed to decrease as the age of the leaf increased, declining steadily from 3DAFE to 15DAP under HT conditions (Fig. 4A). Previous studies have also observed a decline in

ChFl in various crops in HT environments with increase in maturity (Araus et al., 1998; Garty et al., 2001; Sayed, 2003). A decline in ChFl could possibly be due to decreased leaf chlorophyll content under HT conditions, causing unfavorable effects on quantum yield, thereby reducing chlorophyll content (Rosyara et al., 2010; Souza et al., 2010). Phenology likely plays a role in the difference observed in ChFl across cultivars under HT conditions, although some variability in genetics can explain these differences (Araus et al., 1998). Halberd and other high temperature stress tolerant cultivars had a much lower F_v/F_m in the HT environment compared to their counterpart in control conditions. This is an expected observation since the effect on quantum yield can only be reduced, as reported by Rosyara *et a*l (2010), where HT stress tolerant genotypes were photosynthetically stable. This could indicate that heat tolerant lines under control conditions have low ChFl due to a higher SR in the photosynthetic wavelengths and thus have a lower need o dissipate excess light via ChFL. In a previous study, a negative correlation between ChFl and g_s was observed in plants grown in semiarid climate (Del Blanco et al., 2000), however, in this study, a poor correlation was observed. Glaucousness or epicuticular wax has been reported to aid plants to cope with abiotic stress, by protecting against photoinhibition and preventing damage to PSII system (Blum, 1988; Clarke and Richards, 1988; Barker et al., 1997; Roháček and Barták, 1999; Kim et al., 2007). Mohammadian et al (2007) reported that epicuticular wax appeared to reduce photoinhibition even though the effects on reflectance could not be measured. Photoinhibition can occur as ChFl is insufficient to reduce excess photosynthetic irradiance and as such, supports our results that higher EW reduces excess

photosynthetic irradiation into the plant, thus resulting in reduced ChFl.

2.4.4. Leaf gas exchange

The results indicate that EWL may play a role in changes that occur in g_s, under HT conditions. Stomatal conductance decreased for both the adaxial and abaxial surfaces as the leaves matured. This agrees with previous studies that have reported a similar decrease in g_s as leaves age (Mohammadian et al., 2007; England and Attiwill, 2011; Figueiredo et al., 2012). Variation in g_s of young leaves could possibly be related to the environment rather than age or a combination of both and increased photosynthesis during leaf expansion may be associated with increased g_s (England and Attiwill, 2011). Stomatal conductance was observed to negatively correlate with EWL suggesting that an increase in EWL may be associated with reduced g_s. Cuticular transpiration was observed to decrease with increased leaf EWL and is a possible consequence of stomatal closure (England and Attiwill, 2011). Decreases in g_s might result from the occlusion of the stomatal opening by EW as leaves mature or result from reduced heat dissipation resulting from EW role in light reflectance and reducing the absorption of excess photosynthetic energy (Turner and Heichel, 1977). Plants devoid of wax had a lower g_s than plants with a waxy layer in control and HT environments. This agrees with similar findings of Figueiredo et al (2012), who reported that well watered plants whose leaves were devoid of wax had a lower g_s than those under water deficit conditions. Suggesting that loss of EW reduces the transpirational water loss, which is compensated for by reduced stomatal conductance. Under Mediterranean conditions, it was observed that

flag leaves with EW and flag leaves rinsed with chloroform did not differ in g_s across genotypes (Araus et al., 1991). In the present study, a higher g_s was observed in leaves devoid of wax under control conditions than in those under high temperature conditions (Fig. 2), which is consistent with Figueiredo et al (2012). The contrasting observations in the two studies may be due to the amount of EW present on leaves of the different species as well as reproductive stage, thus explaining the differences observed (Jordan et al., 1984; Araus et al., 1991). In this study, leaves of control plants had a higher g_s than HT stressed leaves with and without EW (Fig. 5). Increased EWL and reduced stomatal conductance under HT stress conditions, suggest that when stomata close to reduce gas exchange, EW provide an effective barrier to reduce heat dissipation and transpiration. HT tolerant cultivars or species possess thicker EW than those lacking tolerance (Shepherd and Griffiths, 2006). Plants generally cool their canopies by reducing HT stress through transpiration, which occurs by way of stomata when soil water is available (Sánchez et al., 2001). Efficient reproductive development require higher gas exchange, which occur by way of open stomata (Souza et al., 2004), putting plants in a constant battle for survival against severe environments.

2.4.5. Spectral reflectance indices

Plant canopy reflectance and its association with aboveground biomass have been used in numerous crops to predict yield (Reynolds et al., 1994; Bort et al., 2005; Babar et al., 2006a; Prasad et al., 2009). The vegetative indices, SR and NDVI, progressively decreased through reproductive stages (5.573 to 4.819 and 0.693 to 0.633, respectively)

under HT conditions, which suggest a decline in plant health. These two SRI were observed to decreased in durum wheat as plants advanced through the reproductive stage, possibly due to a decline of green leaf area (Bort et al., 2005). Interesting, in the present study, SR and NDVI values increased during early grain filling. Leaves devoid of EW, experienced a similar pattern for the two vegetative indices, even though the values were much lower when compared to leaves with a waxy layer. Lower values were generally observed for leaves stripped of EW than leaves with an intact waxy layer for all SRI except, PRI, which had higher values for leaves devoid of EW. In stressed conditions, PRI is expected to increase compared to non-stressed conditions, indicating a reduction in radiation use efficiency (Penuelas et al., 1995a; Bort et al., 2005; Babar et al., 2006a). In this study, PRI values did decrease as leaves matured, thus confirming similar observations where PRI declined as plants transitioned from anthesis to maturity (Aparicio et al., 2000). Although the values for WI between reproductive stages were not significant, it was highly significant for cultivars at 15DAP under HT conditions. Cultivars possessed lower WI values after HT stress, suggesting that they were able to maintain a good canopy water status (Peñuelas et al., 1997; Babar et al., 2006a; Prasad et al., 2009). NPQI decreased in HT conditions indicating increased chlorophyll damage, remembering that NPQI is inversely proportion to chlorophyll degradation (Penuelas et al., 1995b; Bort et al., 2005). In this experiment, a few SRI correlated with EW 3DAFE (around anthesis). The subsequent decrease of the SRI of leaves devoid of EW in HT conditions, suggest that epicuticular wax plays a role in reducing the amount of irradiation and light that a leaf microenvironment encounters, by efficiently reflecting

incident irradiation.

2.5 Conclusions

Epicuticular wax offers advantages in protecting the plant from both biotic and abiotic stresses. Under HT conditions, EWL can reduce chlorophyll fluorescence by reflecting excess irradiation and also reduce stomatal conductance g_s helping to regulate the rate of transpiration. Leaves stripped of EW lose their protection against high temperature and irradiation causing a decline in stomatal conductance in an effort to limit transpiration. Epicuticular wax tempers the leaf microenvironment to HT, indicated by the positive relationship observed between EWL and LTD. This reduction is presumably due to increase reflectance at the leaf surface. The results indicate that HT tolerant cultivars can effectively use EW levels to adjust stomatal conductance, chlorophyll fluorescence and leaf temperature. Tolerant cultivars such as Halberd and SeriM82 were able to maintain their seed weight in HT stress conditions. Although environment affects EW deposition, stomatal conductance and chlorophyll fluorescence, it is important to remember that they are also affected leaf phenology.

CHAPTER III

DEFINING THE ROLE OF EPICUTICULAR WAX ON YIELD STABILITY USING PARAMETRIC AND NON-PARAMETRIC STABILITY STATISTICS IN A RECOMBINANT INBRED LINE POPULATION OF BREAD WHEAT (*TRITICUM AESTIVUM* L.) ACROSS DIVERSE ENVIRONMENTS

3.1. Introduction

Wheat is one of the most widely adapted cereal crops with a total harvested area of 220 million hectares in 2011 and a total harvest of 704 million tons (FAOSTAT. 2012). The total production in 2013 is expected to be 4.3% higher than in 2012, which would be the second largest production total on record. The expected increase is due to the recovery of the wheat industry that was devastated the past two years by extreme high temperatures and drought (FAOSTAT. 2012). The development of adaptive wheat germplasm has allowed for growth in sub-tropical environments even though it is primarily of temperate origin. It has been reported that moderate stress of 30 ^{0}C results in a 30% reduction in grain weight (Zahedi et al., 2003). Grain yield is a rather complex trait that is highly dependent on the environment and is a product of many intricate physiological processes (Graybosch, 2001; Quarrie et al., 2005; Barnabás et al., 2008; González and Ayerbe, 2009; Bennett et al., 2012a).

In wheat, high temperature result in many physiological and metabolic changes, destroying photosynthetic processes, as well as disruption in hormone homeostasis, causing a decline in its rate at a much earlier stage than other processes (Al-Khatib and

Paulsen, 1999; Barnabás et al., 2008). One of the main physiological changes is modification of membranes resulting in altered membrane fluidity (Barnabás et al., 2008). The effect of drought and high temperature stress on yield has been investigated in a number of agriculturally important crops. In a study of sesame, a 37% loss was reported for seed yield after exposure to drought stress post-flowering (Kim et al., 2007). In wheat, a RIL population of Seri and Babax under drought stress resulted in reduced grain yield of 35% to 82% (Olivares-Villegas et al., 2007). A number of physiological traits have been reported to correlate with yield during high temperature and water, including maturity, height, awns, grain yield and canopy temperature (Trethowan et al., 2002; Quarrie et al., 2005; Olivares-Villegas et al., 2007; Bennett et al., 2012a).

Photosynthesis only requires about half of the light that a plant receives to carry out the necessary reactions required (Shepherd and Griffiths, 2006). Additionally, a leaf will exchange 100% of its water in 1 hour on a hit sunny day. Diverse mechanisms, including leaf epicuticular wax, canopy temperature, stomatal conductance and transpirational cooling may contribute to high temperatures and drought stress tolerance (Araus et al., 1991; Reynolds et al., 1994; Ayeneh et al., 2002). The thickness of the cuticle wax layer alters the amount of light reflected by plants, while also reducing water loss (Shepherd and Griffiths, 2006). The composition of epicuticular wax of common bread wheat at unique developmental stages has been investigated, laying the foundation for the characterization of wheat epicuticular wax composition under certain environmental stresses (Bianchi and Corbellini, 1977; Koch et al., 2005). Recent evidence suggests that drought stress provokes an increase in leaf epicuticular wax

resulting in reduce water loss and maintenance of photosynthetic as well as stomatal conductance rates in tobacco (Cameron, 2005), barley (González and Ayerbe, 2009), sunflower (Kim et al., 2007), and transgenic alfalfa (Jiang et al., 2009).

Wheat breeding programs specifically aim to develop new cultivars that are high yielding, usually selecting the best genotype for a specific environment or that are stable and high yielding across diverse environments. Breeding programs have achieved tremendous gains in grain yield, quality and tolerance to abiotic and biotic stresses over the past two decades, however, yield has fluctuated over the past years due to genotype by environmental interactions (GxE) (Reynolds et al., 1994; Stone and Nicolas, 1994; Kang, 1997; Reynolds et al., 2009; Mohammadi et al., 2012). Selecting for stability across locations, particularly in environments with low precipitation or high temperature, may be a useful method of selecting for stress tolerance (Kang, 1997). This idea of stability led breeding programs to place greater emphasis on phenotypic consistency and yield performance across diverse environments (Lin et al., 1986; Akcura and Kaya, 2008). To determine the genetic efficiency of a genotype, various statistical methods have been developed that effectively partition environmental effects (Akcura and Kaya, 2008; Rafii et al., 2012; Tabrizi, 2012). Increased epicuticular wax may be an ideal adaptive trait that could contribute to increase yield stability in stress environments.

Crop yield stability can be measured by several statistical procedures. These procedures fall into two categories, parametric and non-parametric analyses. The parametric methods relies on a few assumptions, mainly normal distribution of the population and the means and standard deviations, whereas non-parametric methods

have no assumption about the distribution of the population and are not affected by the removal of observations (Lin et al., 1986; Hussein et al., 2000; Asghar et al., 2006; Akcura and Kaya, 2008). Parametric analyses include the environmental variance (S_i^2) and coefficient of variance (CV %) (Francis and Kannenberg, 1978), Wricke's ecovalence (W_i^2) (Wricke, 1962), the regression coefficient (b_i) (Eberhart and Russell, 1966), stability variance (σ^2) (Shukla, 1972), Perkins and Jenks' regression coefficient (β_i) (Perkins and Jinks, 1968), coefficient of determination (R^2) (Pinthus, 1973), and Lin and Binns superiority measure (P_i) (Lin et al., 1986). The most common non-parametric stability statistics include Huhn's $S_i^{(2)}$, $S_i^{(3)}$ and $S_i^{(6)}$ (Nassar and Huehn, 1987; Hühn and Nassar, 1989; Lu, 1995), Kang's rank-sum (RS) (Kang et al., 1991) and Fox's TOP (Fox et al., 1990). Stability analyses have shown that they are successful in separating genotypes that produce high yields across multiple environments in many field-based trials. Stability studies have been done in wheat (Akcura and Kaya, 2008; Mohammadi et al., 2012), barley (Bantayehu, 2009), oil palm (Rafii et al., 2012), sunflower (Tabrizi, 2012), and lentils (Asghar et al., 2006).

The objectives of this study were to identify the association of yield stability with epicuticular leaf wax and relationships among various parametric and non-parametric stability statistics, and greater stability.

3.2. Materials and methods

3.2.1. Plant material

The population was developed from a cross between 'Halberd', a heat tolerant Australian spring wheat cultivar and 'Len', a moderately heat susceptible USA spring wheat cultivar. The 'Halberd' cultivar is more glaucous and has visibly more wax on its flag leaves, stems and spikes than the 'Len' cultivar. The initial cross was developed in the greenhouse in 2003. The population consists of 180 $F_{2:6}$ recombinant inbred lines (RILs) that were derived through single seed decent. The population was derived from a subset of a larger population to be uniform in flowering within 7 days. The F_8 and F_9 families were evaluated in 2011 and 2012, respectively, for drought and heat tolerance under field conditions for leaf epicuticular wax and yield stability.

3.2.2. Field trials

The entire RIL population (180 lines) and the two parent cultivars 'Halberd' and 'Len' were planted in the field in 2011 and 2012 under irrigated and drought stressed conditions. The population was sown as a delayed planting in January in Chillicothe (TX), College Station (TX) Obregon (Mexico) and Uvalde (TX) in 2011 and College Station (TX) and Uvalde (TX) in 2012. The population was planted in an alpha-lattice design with two replications per RIL per treatment. The seeds were normalized to 1800 kernels per plot and planted in 0.5 x 3 meter plots. All plots were uniformly surface irrigated (drip irrigation) to 90% of field capacity (0-0.25m depth) equivalent to 30 mm irrigation or sufficient saturation to promote uniform emergence after planting. Control

and drought stress plots were watered identically until stem elongation (Feekes 5) at which time irrigation was withheld from the drought stressed trial, leaving the population to rely on moisture received from rainfall. The irrigated treatment (control), received water (drip irrigation) until the dough stage of grain development (Feekes 11), continually receiving water every two weeks (to 90% of soil available moisture) or available soil moisture falls below 50% (in the 0-1m profile). The drought environments in Chillicothe, College Station and Uvalde received approximately 175mm of irrigation whereas the high temperature environments received approximately 376mm. Drip irrigation zones consisted of 0.6 meter spaced drip tape (T-Tape, 10 ml, 0.3m emitter spacing, 0.46 GPM/100ft, at 8PSI). At planting, 22.68kg NPK as (13:13:13) fertilizer was applied and 22.68kg/Ha (Urea Ammonium Nitrate-UAN3200) at Feekes 7. To separate confounding effects of pests from high temperature and drought stress, the trials were treated with fungicides and insecticides. The minimum and maximum temperatures for the reproductive months are represented in Table 6.

Table 6 Minimum, maximum and mean temperatures during the reproductive stages months for each location in 2011 and 2012.

Temperature/^{0}C	Chillicothe 2011			College Station 2011			Uvalde 2011				Obregon 2011		
Month	Apr11	May11	June11	Apr11	May11	June11	Apr11	May11	June11	July11	Apr11	May11	June11
Maximum	38.38	42.22	45.56	35.56	37.22	40.00	37.78	40.56	41.67	37.78	35.6	40.0	40.0
Minimum	0.56	2.78	18.89	5.00	8.33	20.56	3.89	7.78	20.00	20.56	10.6	12.8	17.8
Mean	21.11	23.89	32.78	24.44	25.56	30.56	25.56	26.11	29.44	29.44	23.3	25.0	30.0

Temperature/^{0}C	College Station 2012			Uvalde 2012		
Month	Apr11	May11	June11	Apr11	May11	June11
Maximum	32.78	34.44	41.11	37.78	36.67	42.22
Minimum	10.00	16.11	20.00	10.56	13.89	20.00
Mean	22.78	26.11	29.44	23.33	25.00	30.00

3.2.3. Leaf epicuticular waxes

A modified colorimetric method was used to quantify epicuticular wax concentration present on the leaf surface (Ebercon et al., 1977; Koch et al., 2005) and to determine differences between irrigated and non-irrigated treatments. Four flags leaves from spikes that were 10 DAP chosen randomly from the middle of each plot of the RIL population grown under both irrigated and non-irrigated (drought) conditions. Twelve leaf discs, 1 cm in diameter, were punched into glass vials from the collected leaves using a rabbit tool (Rabbit Tool USA, Rock Island IL USA). Leaf discs were collected at ten days after pollination (10DAP) from each plot with at least fifty percent anthesis and placed in glass vials. Leaf discs were dried then stored at -20 ^{o}C until waxes were extracted. Leaf epicuticular wax was extracted from the leaf discs by complete submersion in HPLC grade chloroform for 30 seconds. The resulting mixture was transferred to a clean GC vial and chloroform allowed to evaporate in a fume hood. The resulting extract was oxidized with 300µl acidified potassium dichromate ($K_2Cr_2O_7$) in a water bath at 100 ^{0}C for 30 minutes. 700µl of deionized water was added to each vial after cooling, allowing for color development for a one hour period. A spectrophotometer (PHERAstar plus, BMG LABTECH, Offenburg, Germany) was used to determine the optical density for each sample at 590 nm. Samples were loaded in 96 well polystyrene, untreated, clear flat bottom plates (Greiner Bio-One). A standard curve was developed from randomly selected wheat leaves and used to determine leaf wax concentrations, calculated based on leaf area and expressed in mg/dm^2 units (Ebercon et al., 1977).

3.2.4. Agronomic trait measures and yield components

To estimate yield components, fifty heads (spikes) were randomly collected from within an entire plot. The yield components that were estimated are single kernel weight (skw), kernel number per spike (kns), mean single head weight (mshw), thousand kernel weight (thkw) and spikes per meter squared (spm2). Height was measured from the center of each plot by placing a meter rule at the base of the plants at the soil surface and measuring to the top of the plot excluding awns.

3.2.5. Yield stability estimates

Yield stability estimates were calculated across nine diverse environments. The stability statistics included five parametric and five non-parametric stability statistics. The parametric statistics used were based on environmental variance (S_i^2) (Francis and Kannenberg, 1978), ecovalence, which uses the genotypic by environmental effects for the genotypes across all environments (W_i^2) (Wricke, 1962), stability variance, measures stability as the variance of a genotype across all environments (σ_i^2) (Shukla, 1972), regression coefficient, measures a genotype response to environmental changes (b_i) (Eberhart and Russell, 1966) and superiority measures the difference between the genotype response and the maximum response over environments (P_i) (Lin and Binns, 1988) (Table 7). The non-parametric stability statistics are Nassar and Huhn's $S_i^{(2)}$ which measures between ranks variance across environments, $S_i^{(3)}$ which sums absolute deviations of squares of ranks for each genotype, and $S_i^{(6)}$ that uses sums of squares of ranks for each genotype relative to mean ranks (Nassar and Huehn, 1987; Hühn and

Nassar, 1989). The other two non parametric statistics are rank-sum (RS) that considers both yield and Shukla's stability variance (Kang et al., 1991) and TOP which ranks genotypes in a stratified manner based on ranking of each genotype in each environment (Fox et al., 1990) (Table 7). Having a large population of 180 individuals presented a few obstacles in identifying stable lines based on each stability measure. To deal with such large number a subset of the population, the top 15% of each stability statistic was selected as stable. The bottom 15% of each stability statistic was selected as the least stable. In the top 15%, lines present in five or more of the stability statistics were deemed as stable across multiple environments.

3.2.6. Statistical analysis

The locations were treated as separate environments for the duration of the field trials. The generalized linear model was used to estimate analysis of variance (ANOVA), with genotypes and environments considered as fixed effects and replication as a random effect. The REML model was used to estimate the least square means (LSMEANS) for each trait in the statistical software SAS (SAS v9.2, SAS institute Inc. Cary, NC, USA). Spearman correlation was used to estimate the associations for all traits using JMP (JMP v10.0, SAS institute Inc. Cary, NC, USA). Stability analysis measures were calculated using SAS (SAS v9.2, SAS institute Inc. Cary, NC, USA) and AGROBASE Generation II (Agronomix software Inc. Winnipeg Manitoba Canada).

Table 7 A summary of authors and equations of the parametric and non-parametric stability statistics used in this study.

Stability Equation	Symbol	Name	Author
$\sum_{j=1}^{q} (X_{ij} - \bar{X}_{i.})^2 / (q-1)$	S_i^2	Environmental Variance	Francis and Kannenberg (1978)
$[p / (p-1)(q-1)]\ W_i - SSGE / (p-1)(q-2)(q-1)$	σ_i^2	Interaction Variance	Shukla (1972)
$\Sigma_j(yij - \bar{y}_{i.} - \bar{y}_{.j} + \bar{Y})$	W_i^2	Ecovalence	Wricke (1962)
$1+[\ \Sigma(y_{ji} - \bar{y}_i - \bar{y}_{.j} + \bar{y}_{..})(\bar{y}_{.j} + \bar{y}_{..}) / \Sigma(\bar{y}_{.j} - \bar{y}_{..})^2]$	β_i	Regression Mean Square	Eberhart and Russell (1966)
$\sum_{j=1}^{n} (x_{ij} - M_j)^2 / 2E$	P_i	Superiority Index	Lin and Binns (1988)
$\sum_{j=1}^{N} (r_{ij} - \bar{r}_{i.})^2 / (N-1)$	$Si^{(2)}$	Between rank variance	Huhn (1979)
$\sum_{j=1}^{N} (r_{ij} - \bar{r}_{i.})^2 / \bar{r}i$	$Si^{(3)}$	Sum of absolute Deviation	Nassar and Huehn (1987)
$\sum_{j=1}^{N} \lvert r_{ij} - \bar{r}_{i.} \rvert / \bar{r}_i$	$Si^{(6)}$	Sum of Squares of Ranks	Nassar and Huehn (1987)

3.3. Results

3.3.1. Agronomic data

Chillicothe had a temperate environment with cold winters and hot summers. College Station and Uvalde display subtropical environments with mild winters and hot summers. Obregon has a hot desert like environment annually. During the reproductive stages (flowering to grain filling) average temperatures (early May to mid June) were 28^0C in Chillicothe, 28^0C in College Station 2011 and 2012, 28^0C in Uvalde 2011 and 27^0C in Uvalde 2012. Maximum temperatures during this time reaching as high as 46^0C in Chillicothe 2011, 40^0C in College Station 2011, 36^0C in College Station 2012, 42^0C in Uvalde 2011 and 2012 (weatherunderground.com). Table 6 show the maximum, minimum and mean temperatures during the flowering and grain filling months for each location.

The combined analysis of variance was performed to determine the effects of the RIL population (G), environment (E), and genotype by environment interactions (GxE) on grain yield. The analysis detected highly significant differences for the RIL population, environment, and (GxE) for grain yield as well as the yield components, kernel number per spike, mean single head weight and thousand kernel weight (Table 8). The significant difference of the GxE interactions for both yield and yield components indicates an adaptive response of the RILs to changes in environmental conditions. Grain yield varied between 1.51 tons ha^{-1} and 3.27 tons ha^{-1} for RIL HL167 and HL29, respectively when combined across locations. Kernel number per spike ranged between 14.51 (HL34) to 26.40 (HL31), mean single head weight ranged between 0.49g (HL102)

to 0.79g (HL58), and thousand kernel weight varied between 25.33g (HL63) to 37.72g (HL173) for the RIL population when combined across locations. Spm2 varied between 152 (HL155) to 398 (HL112) when combined across locations. The Halberd parent produced higher grain yield, KNS, MSHW and THKW compared to the Len parent (Table 9).

3.3.2. Leaf epicuticular wax

The environment with the highest temperatures had the greatest effect on epicuticular wax load (EWL) in the parental cultivars. The parents in three of the eight environments had significantly different amounts of EWL, Uvalde 2011 under water deficit conditions, in College Station 2012 and Obregon 2011 under irrigated (high temperature) conditions. The Obregon environment resulted in the highest amount of EWL for Halberd and Len producing 10.89 mg dm^{-2} and 6.90 mg dm^{-2}, respectively.

Table 8 Combined analysis of variance (ANOVA) of 180 RILs and parental wheat genotypes for grain yield, single kernel weight, kernel number, mean single head weight, thousand kernel weight and spike per meter squared across multiple environments.

		Kernel no. spike^{-1}	Mean single head weight	Thousand kernel weight	Yield	Spike m^{-2}
Source	DF	Mean Squares	Mean Squares	Mean Squares	Mean Squares	Mean Squares
Env (E)	8	12320.87***	12.90***	49046.15***	124.76***	2568607.23***
Rep	1	47.37^{NS}	0.02^{NS}	1.13^{NS}	0.01^{NS}	2949.36^{NS}
Var (G)	181	71.25***	0.07***	66.39***	0.58***	3515.05^{NS}
Rep (Env)	8	243.50***	0.25***	55.44***	8.84***	15426.02*
Var*Env (GxE)	1424	19.29***	0.02***	13.49***	0.31**	4040.54^{NS}
Error	1533	12.38^{NS}	0.01^{NS}	9.70^{NS}	0.25^{NS}	4375.58^{NS}

***, **, significant at probability level $p \geq 0.001$ and 0.01 respectively, and ^{NS} not significant.
Key: Environment (Env), replication (Rep), recombinant inbred line (Var).

Table 9 Combined physiological and phenological trait means for the parents Halberd and Len and range for the RIL population grown under water deficit and high temperature environments across locations.

Trait	Abbr.	Units	Drought				Heat			
			Avg	Range	Halberd	Len	Avg	Range	Halberd	Len
Canopy Temperature	CTP	^{0}C	34.58	24.65 - 37.88	32.92	31.99	31.49	25.03 - 33.37	32.68	31.17
Epicuticular wax	EWL	mg dm^{-2}	4.25	2.238 - 6.487	6.18	4.28	3.61	2.417 - 5.529	3.33	3.27
Kernel per spike	KNS	Number	18.23	11.60 - 27.19	20.68	17.02	20.14	11.95 - 32.05	21.46	20.64
Mean single head weight	MSHW	g	0.587	0.403 - 0.806	0.69	0.58	0.666	0.407 - 0.870	0.72	0.69
1,000 kernel weight	THKW	g	26.65	37.72 - 25.33	29.00	28.13	28.03	18.00 - 30.88	28.80	28.98
Spike per m^2	Spm2	g m^{-2}	211†	95 - 510†	148†	227†	255	149 - 499	267	277
Grain yield	Yield	tons ha^{-1}	2.29	1.18 - 3.32	2.71	2.10	2.13	0.93 - 3.80	2.83	1.90

† - data of one location from one year.

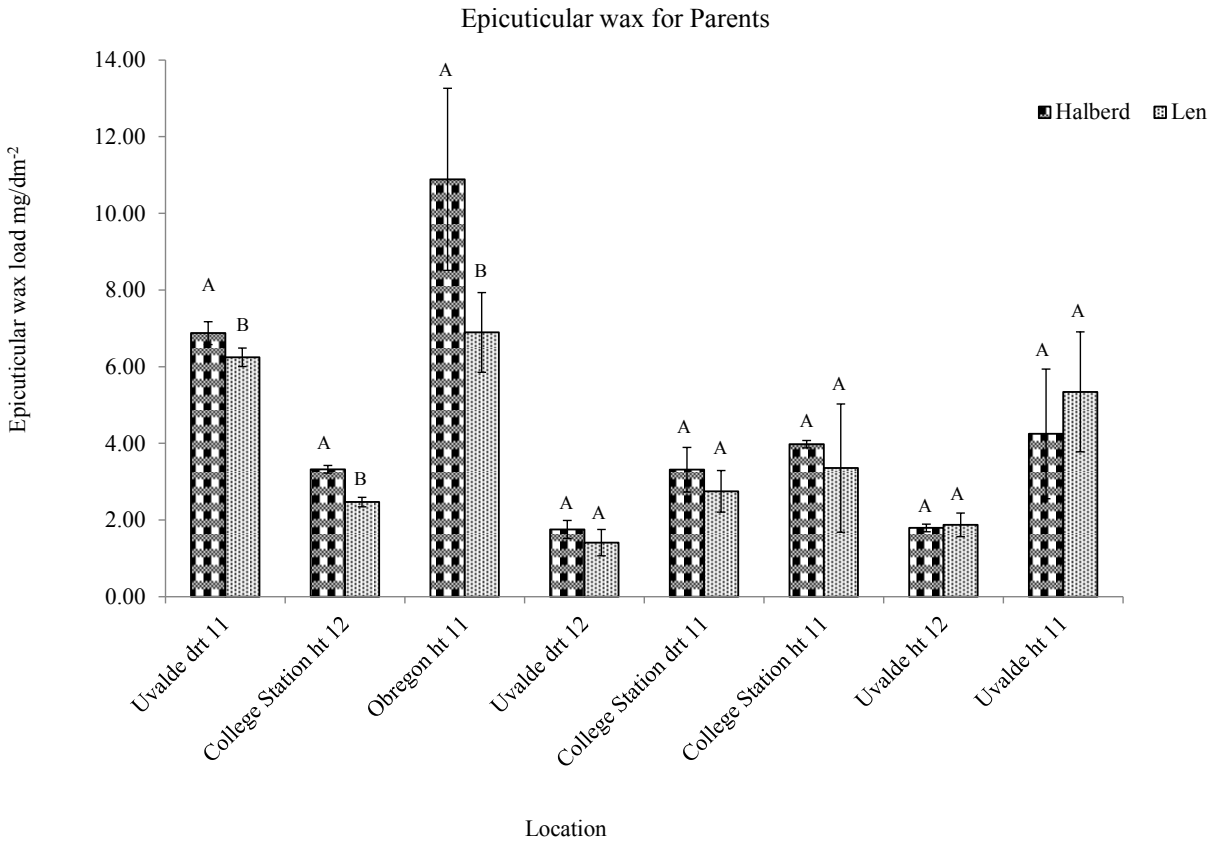

Fig. 7 Epicuticular wax load for the parents Halberd and Len for eight environments across two years of field trials. The epicuticular wax values are the mean of two replications in each environment for each parent. The bar represent the standard error of the mean (±) and the letters represent significant differences between parents based on the Waller-Duncan and Ryan-Einot-Gabriel-Welsch Q (REGWQ) multiple comparison tests.

The lowest amount of EWL was produced in Uvalde 2012 under irrigated (high temperature) conditions (1.79 mg dm^{-2}) for Halberd, whereas Len produced the lowest EWL in Uvalde 2012 under water deficit conditions (1.41 mg dm^{-2}) (Fig. 7). Epicuticular wax load in the RIL population varied from 2.64 mg dm^{-2} (HL136) to 5.12 mg dm^{-2} (HL170) across environments (Table 9). The parental cultivars, Halberd and Len, on average produced 4.16 mg dm^{-2} and 3.71 mg dm^{-2} of EWL, respectively.

3.3.3. Correlation between EWL, physiological and yield traits

Grain yield under high temperature and water deficit treatments were not correlated with each other. Grain yield under high temperature treatments was positive and significantly correlated with the yield components THKW, KNS and MSHW ($r = 0.29$, $r = 0.73$ and $r = 0.35$, respectively, $P \leq 0.001$). Grain yield under water deficit treatments was positive and significantly correlated with the yield components THKW and MSHW ($r = 0.17$ and $r = 0.19$, respectively, $P \leq 0.001$) (Table 10). In water deficit treatments, grain yield, THKW, KNS and MSHW negatively correlated with CTP ($r = -0.63$, $r = -0.56$, $r = -0.48$ and $r = -0.59$, respectively, $P \leq 0.001$) (Table 10). However, under high temperature treatments, KNS, THKW, MSHW, and Spm2 negatively associated with CTP and were significant. MSHW significantly and negatively associated with Spm2, whereas grain yield and KNS were significant and positively associated with Spm2 under high temperature conditions (Table 10). In both the high temperature and water deficit treatments, CTP positively and significantly correlated with EWL ($r = 0.26$ and $r = 0.29$, respectively, $P \leq 0.001$).

Table 10 Pearson's correlation of physiological traits measured for the Halberd x Len RIL population grown in water deficit and high temperature environments in multiple years.

	Kernel no. per spike Ht	Mean single Weight Ht	1000 kernel weight Ht	EWL Ht	Canopy temperature Ht	Yield Ht
Mean single Weight Ht	0.65***					
1000 kernel weight Ht	0.18***	0.58***				
EWL Ht	-0.38***	-0.36***	-0.25***			
Canopy temperature Ht	-0.15***	-0.34***	-0.55***	0.26***		
Yield Ht	0.73***	0.35***	0.29***	-0.30***	0.06^{NS}	
Spike m^{-2}	0.13**	-0.36***	0.02^{NS}	-0.17***	-0.12*	0.29***

	Kernel no. per spike Drt	Mean single Weight Drt	1000 kernel weight Drt	EWL Drt	Canopy temperature Drt	Yield Drt
Mean single Weight Drt	0.75***					
1000 kernel weight Drt	0.45***	0.59***				
EWL Drt	-0.50***	-0.77***	-0.56***			
Canopy temperature Drt	-0.48***	-0.59***	-0.56***	0.29***		
Yield Drt	0.05^{NS}	0.19***	0.17***	0.52***	-0.63***	

***, **, *, significant at $p \geq 0.001$, 0.01 and 0.05 respectively.
Key: Canopy temperature (CTP), epicuticular wax load (EWL), plot yield (yield).
Letters after traits represent environments, Ht (high temperature) and Drt (water deficit).

Grain yield under high temperature conditions negatively correlated with EWL (r = -0.30, P≤0.001), but positively correlated with EWL under water deficit treatments (r = 0.52, P≤0.001) (Table 10). When combined, EWL had no significant association with yield and yield components, as well as with canopy temperature.

3.3.4. Correlation between EWL, yield traits and stability indices

The associations of epicuticular wax, grain yield, yield components and the parametric and non-parametric stability statistics were evaluated using the Spearman's rank correlation test. Epicuticular wax had no significant association with any of the stability indices and the associations were very low (Table 11). Mean grain yield was highly significant and positively correlated with b_i, S_i^2, $S_i^{(3)}$, and $S_i^{(6)}$ and TOP statistics. Furthermore, mean grain yield was also highly significantly with P_i and RS but the relationship was negative (Table 11). There was no significant relationship observed between mean grain yield and W_i^2, σ_i^2, and $S_i^{(2)}$ (Table 11). Kernel number spike^{-1} had significant negative associations with W_i^2, σ_i^2, P_i, $S_i^{(2)}$, $S_i^{(3)}$,and $S_i^{(6)}$, and Kang's rank-sum (RS) and positively associated with (b_i) and TOP. Although significantly and positively associated with S_i^2, the association was quite low. Thousand kernel weight and MSHW had significant and negative associations with W_i^2, σ_i^2, P_i, $S_i^{(2)}$, $S_i^{(3)}$,and $S_i^{(6)}$, and Kang's rank-sum (RS) but was positively associated with (b_i) and TOP. However, there was no significant association observed with the stability indices S_i^2 for both yield components (Table 11). Spike per m^{-2} had significant associations only with b_i, and the relationship was positive (Table 11).

Table 11 Spearman's correlation of epicuticular wax, canopy temperature, mean grain yield, thousand kernel weight, mean single head weight, kernel number per spike, and parametric and non-parametric stability statistics for the Halberd x Len RIL population across multiple environments.

	Yield	Thkw	Mshw	Kns	Spm2
b_i	0.54***	0.82***	0.94***	0.92***	0.45***
W_i^2	0.04^{NS}	-0.80***	-0.94***	-0.92***	0.09^{NS}
S_i^2	0.45***	-0.11^{NS}	0.11^{NS}	0.15*	0.10^{NS}
σ_i^2	0.04^{NS}	-0.80***	-0.91***	-0.95***	0.08^{NS}
P_i	-0.95***	-0.82***	-0.95***	-0.93***	-0.13^{NS}
$S_i^{(2)}$	-0.13^{NS}	-0.81***	-0.93***	-0.91***	0.08^{NS}
$S_i^{(3)}$	0.33***	-0.82***	-0.93***	-0.91***	0.05^{NS}
$S_i^{(6)}$	0.62***	-0.83***	-0.93***	-0.91***	0.05^{NS}
TOP	0.75***	0.83***	0.94***	0.92***	0.13^{NS}
RS	-0.70***	-0.79***	-0.95***	-0.92***	0.01^{NS}
EWL	-0.07^{NS}	0.00^{NS}	-0.04^{NS}	0.07^{NS}	0.08^{NS}
CT	0.11^{NS}	0.07^{NS}	0.14^{NS}	0.11^{NS}	-0.03^{NS}

***, **, *, significant at $p \geq 0.001$, 0.01 and 0.05 respectively. Regression coefficient of Eberhart and Russell (b_i), environmental variance (S_i^2), ecovalence (W_i^2), stability variance (σ_i^2), superiority measure (P_i), Nassar and Huhn's $S_i^{(2)}$, $S_i^{(3)}$,and $S_i^{(6)}$, Kang's rank-sum (RS) and Fox's TOP, thousand kernel weight (Thkw), mean single head weight (Mshw), kernel number per spike (Kns), spike per meter squared (Spm2), plot yield (Yield).

3.3.5. Rank of Halberd x Len lines

Stability analysis is usually performed on a set of 50 individuals or less but with such a large set of 180 individuals it becomes difficult to present findings from the analysis. To combat this, an ideal representation of the data was devised allowing for an overall analysis opposed to a singular view. The top stable 15% of each individual stability statistics was selected for yield and each yield component separately. After selection, only the RILs (≤10) that were present in five or more of the stability statistics were considered and are represented in Table 12. The most stable RILs for grain yield were determined to be HL81, HL65, HL85, HL39 and HL86, as they were present as the most of the stability statistics (Table 12). In fact, HL39 was present in the most stable RILs for both mean single head weight and thousand kernel weight and was the only RIL present in the most stable for grain yield and the yield components. HL152 and HL31 were the only RILs present all three yield component measures, kernel number per spike, mean single head weight and thousand kernel weight (Table 12). HL47, HL71, and HL126 were present in kernel number per spike and mean single head weight, whereas, HL87 was present in mean single head weight and thousand kernel weight components (Table 12). The least stable RILs for grain yield were identified as HL9, HL35, HL67, HL83 and HL105 appearing in most of the stability measures (Table 12). The RIL HL67 was identified as a least stable line in both grain yield and mean single head weight, whereas, HL105 and HL150 were identified in grain yield and thousand kernel weight component (Table 12). Although, HL151 was identified as stable RIL for grain yield, the three yield components identified it as a least stable individual.

Table 12 Recombinant inbred lines of the Halberd x Len cross that were identified as stable by 5 parametric and 5 non-parametric stability statistics for mean yields, kernel number, mean single head weight and thousand kernel weight. The RILs represented in the table are those that were present in 5 or more of the stability statistics from a subset of the lines that were considered as stable (top 15%). Epicuticular wax load, grain yield and yield components values are the means of individual RILs across all locations.

	Line	KNS	EWL	Line	MSHW (g)	EWL	Line	THKW (g)	EWL	Line	Yield (tons hec^{-1})	EWL
1	HL47	17.49	4.47	HL166	0.67	4.03	HL17	27.22	3.59	HL81	2.20	4.90
2	HL71	19.42	4.17	HL31	0.75	3.12	HL96	25.28	3.64	HL65	2.45	4.79
3	HL98	18.14	3.62	HL39	0.65	3.60	HL152	29.40	3.16	HL85	1.83	4.72
4	HL167	19.11	3.64	HL47	0.56	4.47	HL4	29.39	4.36	HL39	2.56	3.60
5	HL22	16.81	3.67	HL71	0.61	4.17	HL16	28.33	4.94	HL86	2.21	4.02
6	HL24	22.31	4.21	HL84	0.51	4.18	HL31	28.30	3.12	HL129	2.31	4.13
7	HL126	17.55	4.53	HL87	0.70	3.56	HL39	26.94	3.60	HL142	2.05	2.90
8	HL31	26.40	3.12	HL95	0.54	4.76	HL50	27.44	4.16	HL158	1.76	3.64
9	HL152	24.66	3.16	HL126	0.58	4.53	HL79	26.28	3.63	HL162	2.42	3.03
10				HL152	0.73	3.16	HL87	27.39	3.56	HL170	2.23	5.12
	HL181	21.12	4.16	HL181	0.71	4.16	HL181	28.89	4.16	HL181	2.35	4.16
	HL182	17.76	3.71	HL182	0.60	3.71	HL182	27.00	3.71	HL182	1.97	3.71
	RILs	14.51 - 26.40	2.64 - 5.12	RILs	0.49 - 0.79	2.64 - 5.12	RILs	25.33 – 37.72	2.64 - 5.12	RILs	1.51 - 3.27	2.64 - 5.12
1	HL48	18.15	2.96	HL48	0.62	2.96	HL105	29.22	3.63	HL9	2.85	3.60
2	HL58	25.99	4.35	HL155	0.77	4.11	HL7	28.39	4.57	HL35	2.68	3.97
3	HL151	16.17	4.18	HL168	0.62	4.04	HL72	25.78	3.85	HL67	2.88	3.55
4	HL155	19.00	4.11	HL29	0.72	3.56	HL80	28.94	3.72	HL83	1.82	4.07
5	HL168	18.23	4.04	HL82	0.62	3.67	HL150	24.78	4.16	HL105	1.88	3.63
6	HL7	22.70	4.57	HL107	0.54	4.25	HL174	30.61	3.75	HL174	2.63	3.75
7	HL29	22.96	3.56	HL111	0.62	2.98	HL34	29.89	4.34	HL11	2.46	3.68
8	HL41	17.72	3.64	HL151	0.59	4.18	HL151	29.39	4.18	HL60	2.47	4.58
9	HL127	17.58	3.84	HL67	0.71	3.55	HL73	29.00	3.27	HL116	2.35	4.27
10	HL100	18.27	3.94	HL157	0.64	3.60	HL163	29.61	3.74	HL150	1.86	4.16

Key: grain yield (yield), kernel number spike^{-1} (KNS), mean single head weight (MSHW) and thousand kernel weight (THKW), Epicuticular wax load (EWL) recombinant inbred line (Line).

3.4. Discussion

3.4.1. Agronomic and physiological traits

Breeders currently have a slew of methods available to them to assess the performance of cultivars in specific environments but find it challenging to select the ideal method depending on the situation (Eskridge, 1990). Selecting the best model depends on numerous factors not limited to the size of the data set, available environments, concept of stability and adaptability and most important environmental variation (Farshadfar et al., 2012). The implementation of stability statistics in breeding programs may help to identify cultivars with positive responses to improved environmental conditions and predictable performance. The measure of performance using stability statistics is a suitable method for developing high temperature and water deficit tolerant cultivars that not just fit into a specific niche but multi-environmental. It is imperative to incorporate methods that combine both stability and yield performance since the most stable may not by the highest yielding genotype (Kang, 1993). Stability, though defined in numerous ways, can be characterized as a parallel response in yield to environmental variance, hence, there is a small contribution to GxE resulting in constant yield (Lin et al., 1986; Farshadfar et al., 2012; Mohammadi et al., 2012). This allow cultivars to be evaluated in numerous countries, such as Africa and East Asia, in various locations, allowing for identified cultivars to consistently produce high grain yield. The RIL population had differential response to the various environments indicated by the highly significant G x E interaction for grain yield and the yield components (Table 8).

Combined analysis of variance for the nine environments revealed that the RIL

population responded differentially to the diverse environments indicated by the highly significant difference for grain yield, kernel number, mean single head weight and thousand kernel weight among lines and environments (Table 8). The impact of the environment on grain yield for this wheat population can be inferred by the highly significant G x E interaction. This effect was also observed for the yield components kernel number, mean single head weight and thousand kernel weight (Table 8). Previous studies have cataloged the effect of environment, especially, water deficit and high temperature conditions, on grain yield in wheat (Reynolds et al., 1994; Del Blanco et al., 2000; Olivares-Villegas et al., 2007), barley (González and Ayerbe, 2009), and sesame (Kim et al., 2007). Within the population some lines indicated that they were widely stable and adapted across diverse environments.

Epicuticular wax is a complex trait that is controlled by numerous biosynthetic pathways and in highly environmentally influenced (Jenks et al., 1992; Sánchez et al., 2001; Kunst and Samuels, 2003; Broun et al., 2004). In the Halberd x Len population epicuticular wax load varied within the population and across environments. The RILs that were considered to be stable by the stability statistics possessed intermediate or high EWL amounts (Table 12). Epicuticular wax did not have the overall expected effect of increasing yield stability instead it varied throughout the population and the environments. The RIL HL170, having the greatest EWL, was considered to be stable for grain yield across environments but HL142 was also considered to be stable even though its' EWL was quite low (Table 12). The parents showed significant differences in their EWL only in three environments of which two were considered as high temperature

stressed (Fig. 7). Two of the environments were in 2011, which experienced a shortage of rainfall creating a hot dry climate in the growing locations. The highest EWL produced by the parents was at the Ciudad be Obregon location in Mexico which is consistently dry and hot year round, receiving less than 15 mm annually. The parents produced higher EWL on average in locations of the dryer year, 2011, than the same location in the much wetter year, 2012 (Fig. 7)

3.4.2. Correlations of EWL, physiological and yield traits

Water deficit and high temperature cause changes in the composition of epicuticular wax that persist after stress playing a role in acclimation to present and ensuing encounters (Kim et al., 2007). In this study EWL and yield in water deficit conditions were positively and significantly associated (Table 10). The positive relationship suggests that as EWL increases yield also increases. Previous studies have reported similar observations with EW and total grain yield (Sánchez et al., 2001; Samdur et al., 2003). Under water deficit conditions, yield and CT associated negatively and significantly, indicating the adverse effect that increasing temperatures have on grain yield (Reynolds et al., 1994; Olivares-Villegas et al., 2007). Previous studies involving water deficit and high temperature conditions reported increased EWL, with an increase as much as 20% under water deficit conditions (Sánchez et al., 2001; Samdur et al., 2003). Interestingly, yield and EW under high temperature conditions was negative and significantly associated, suggesting that increased EW decreases yield. Increased EW has been shown to decrease canopy temperatures in water deficit and high

temperature environments in various crop plants, including pea (Sánchez et al., 2001), sesame (Kim et al., 2007) and peanut (Samdur et al., 2003). However, in this study under high temperature conditions, a significant positive association was observed between EW and CT, indicating increased EW may increase CT. This is similar to a previous study that reported increase EW keep plants at higher temperatures than plants without EW (Mohammadian et al., 2007). The difference in response between the two treatments, could be due to the shorter period of available moisture for the population under water deficit conditions compared to the high temperature treatment and can also be a consequence of the confounding effects of high temperature with water deficit when they occur together (Ayeneh et al., 2002; Lombardini, 2006; Kirigwi et al., 2007; Mason et al., 2011; Bennett et al., 2012a).

The associations between several agronomic and physiological traits were examined using Pearson's correlation. The positive association between grain yield and KNS MSHW, THKW and Spm2 suggests that overall yield is affected by these attributes. Previous studies have also reported similar observations in wheat exposed to varying environmental conditions (Del Blanco et al., 2000; Cuthbert et al., 2008; McIntyre et al., 2010). Single kernel weight was observed to be negatively associated with spm2 under high temperature, which suggests that skw is dependent on the amount of tillers a cultivar produces. The higher the number of tillers a plant produces the lower the weight of kernels since the source-sink ratio will be greatly affected. A previous study have also reported similar observations under irrigated and rain-fed conditions (del Moral et al., 2003; McIntyre et al., 2010). In general, there were significant associations

between agronomic traits and physiological under both environmental conditions.

3.4.3. Yield stability statistics

Selecting favorable genotypes is not as simple as identifying those that are considered to stable by stability statistics but combine both performance and high mean yield. Stability can be defined by three different concepts, type I, type II, and type III. Type I statistics include environmental variance (S_i^2) and coefficient of variance (CV) (Francis and Kannenberg, 1978). Wricke's ecovalence (W^2) and Shukla's stability variance (σ_i^2) are of type II and Eberhart and Russell's regression coefficient (b_i) is apart of type III. The type I concept defines stability as a genotype having small among environment variance. Type II concept defines a genotype as stable if the mean and environmental responses are parallel. A small residual mean square in a regression model defines the type III concept (Lin et al., 1986; Hussein et al., 2000; Tabrizi, 2012). Type I is considered to be a response of the plants biological system and is seldom used by breeders (Becker, 1981). Type II stability is defines as an agronomic response to environment and conclusions are usually restricted to the set of genotypes being tested (Lin et al., 1986). The type III stability concept sub divide the response to environment into predictable and non predictable components (Lin et al., 1986). Breeders interested in multi-environment stability, type I statistics should be deployed and for those interested in among comparisons, type III are best. Stability statistics each measure a different dynamic of stability and to satisfactorily measure and select genotypes on performance, a combination of measures are required across multiple environments (Flores et al.,

1998).

The stability statistics differently classify a genotype as stable according to the values calculated. A genotype is considered stable if it has low contributions to GxE interactions or has high yields with low (W_i^2), (σ_i^2), (P_i), (S_i^2), $S_i^{(2)}$, $S_i^{(3)}$, $S_i^{(6)}$, and RS stability values but genotypes with the high TOP values are considered stable (Asghar et al., 2006; Akcura and Kaya, 2008; Mohammadi et al., 2012; Tabrizi, 2012). Eberhart and Russell's regression coefficient (b_i) considers a genotype as stable if its value is 1 or close to 1 and not stable when its value approaches zero. Nassar and Huhn's $S_i^{(2)}$ considers zero variance to indicate maximum stability, $S_i^{(3)}$ and $S_i^{(6)}$ determine stability as genotype mean rank by combining stability and yield rank in each environment (Hühn, 1979; Nassar and Huehn, 1987), Kang's rank sum combines stability variance and yield to determine a genotype as well adapted (Kang, 1988) and Fox's TOP considers a genotype to be well adapted if it appears in the top third across environments (Fox et al., 1990).

Recombinant inbred line HL29 and HL50 were the top yielding but HL29 appeared in the top 5 most stable lines in only one of the stability statistics, (P_i), whereas, HL50 appeared in (P_i) and TOP statistics if considered on an individual basis. HL30 and HL50 were the most adaptable and stable RILs according to the TOP statistic that incorporates a stratified ranking of performance in each environment. RILs considered as stable HL170 in Eberhart and Russell's (b_i), HL102 in Shukla's stability variance (σ_i^2) and Wricke's ecovalence (W_i^2), HL135 in environmental variance (S_i^2), HL81 and HL85 in Nassar and Huhn's $S_i^{(2)}$, and $S_i^{(3)}$, HL158 and HL85 in $S_i^{(6)}$.

According to mean yield performance only HL29 and HL50 were present in the top twenty highest yielding RILs. To better assess the stability of lines in such a large population the top 15% of each stability statistic that was considered stable was selected and from this set only those present in five or more of the statistics were deemed stable across environments. The purpose of such an approach was two-fold, it allowed for inferences to be made about a smaller set of individuals and it helped to combine the individual concepts of stability. The RILs identified as stable by these methods were usually in the upper third of mean grain yield with a few falling in the lower third. Yield is a complex trait that is controlled by many genes and to get an overall view a combination of many factors associated with yield should be considered. Yield components provide another view when assessing yield, especially since one of these factors, kernel number, is set by the end of growth stage 2 (GS2) of wheat allowing only for decreases thereafter (Shpiler and Blum, 1986; Acevedo, 1991). The use of yield and yield component stability may present an intriguing method to identify stable cultivars. In this study, HL39 was identified as stable in yield, mean single head weight and thousand kernel weight, having high MSHW and relatively good grain yield (Table 12). Two RILs, HL31 and HL152 were identified in the three yield components stability statistics but were not present in grain yield. These two RILs both had high KNS and MSHW, with HL31 having the highest KNS value of the population but a low THKW (Table 12). HL31 yield total across environments was intermediate but HL152 had a low mean yield adding another wrinkle in the ongoing process of identifying stable genotypes in diverse environments.

The significant positive association of mean grain yield with (b_i), (S_i^2), $S_i^{(3)}$, and $S_i^{(6)}$ and TOP statistics suggests that increasing these stability statistics could change the selection process for increased grain yield (Table 11). Similar association of grain yield with these stability statistics were reported in wheat (Akcura and Kaya, 2008) and chickpea (Farshadfar et al., 2012) genotypes. In fact, the relationship between grain yield and the non parametric statistics of $S_i^{(3)}$, and $S_i^{(6)}$ and TOP support previous observations of Becker and Leon (1988). The significant but negative associations of grain yield with (P_i) and RS suggests that lower values of these statistics may aid in selecting for higher grain yields. They incorporate the variance response of a genotype compared to the overall response (Kang, 1988; Lin and Binns, 1988). Akcura et al (2008) also reported a similar association of grain yield with Kang's rank sum. Shukla's stability variance and Wricke's ecovalence, although positively associated with grain yield, had low probability values and was not significant (Table 11). This association fails to support a similar study in chickpea that found a significant and positive correlation with high probability values (Farshadfar et al., 2012). The stability variance and ecovalence had an association value of one (r = 1.00), indicating that there is no difference in their ability to determine stability. On closer examination, Shukla's stability variance is a derivative of Wricke's ecovalence, presenting the environmental variance or deviation mean square linearly, explaining why RILs in both methods were in the same ranking.

The non parametric statistics $S_i^{(3)}$, and $S_i^{(6)}$ measures stability by ranking genotype yield environmental by merging stability and yield, whereas, $S_i^{(2)}$, is a function of stability only (Huehn, 1990b). In this experiment, these statistics were positive and

highly associated with each which is expected since they are related to the static concept of stability, with similar associations previously reported (Flores et al., 1998; Akcura and Kaya, 2008; Farshadfar et al., 2012). Although $S_i^{(2)}$ conveys the static concept of stability it fails to account for environmental changes unlike it counterparts $S_i^{(3)}$, and $S_i^{(6)}$. Therefore, all three can assess stability but only the $S_i^{(3)}$, and $S_i^{(6)}$ are ideal for selecting genotypes that respond to varying environmental conditions. The positive associations among the non-parametric statistics indicate that they can similarly classify genotypic stability in various environmental conditions. Although non-parametric statistics provide little information about adaptability unlike parametric statistics, are not biased by outliers and data need not be normally distributed making them suitable alternatives.

3.5. Conclusion

Breeders seek to identify genotypes with potential to produce maximum yield in diverse environments. In a large and varied population such as a recombinant inbred population, identifying one genotype, as the most stable become a complex task. Stability is characterized as yield performance as well as adaptability to varying environments, therefore both mean yield and stability must be considered when determining a genotype as stable. The parametric and non-parametric stability measures implemented in this study present viable methods for analyzing stability. The positive and highly significant associations among the non-parametric statistics suggest that one or two can be used to determine stability. Considering the overall analysis of the

population for yield and yield components it can be concluded that HL32, HL39 and HL152 are the most stable recombinant inbred lines and will perform relatively well in diverse environments. High EWL may promote stable yields but its sensitivity to environmental conditions makes it challenging to definitively point to it as a source of improved stability. Further study is required to gain more insight to the phenomena that is stability and epicuticular wax.

CHAPTER IV

QTL MAPPING OF LEAF EPICUTICULAR WAX LOAD, AND ITS INFLUENCE ON CANOPY TEMPERATURE AND YIELD STABILITY IN A RECOMBINANT INBRED LINE POPULATION OF HALBERD/LEN IN *TRITICUM AESTIVUM* L. UNDER WATER DEFICIT AND HIGH TEMPERATURE CONDITIONS DURING REPRODUCTIVE STAGES

4.1. Introduction

Wheat breeding programs specifically aim to develop new cultivars that are high yielding, usually selecting the best genotype for a specific environment. Breeding programs have achieved tremendous gains in grain yield, quality and tolerance to abiotic and biotic stresses over the past two decades (Reynolds et al., 1994; Stone and Nicolas, 1994; Reynolds et al., 2009). However, yield has fluctuated over the past decade due to genotype by environmental interactions (GxE) (Kang, 1997; Mohammadi et al., 2012). This fluctuation is due in part to the complexity, that yield is controlled by numerous quantitative trait loci (QTL) with both major and minor effects (Cuthbert et al., 2008). Yield components, spikes per meter squared, thousand kernel weight, kernel number per spike and single head weight, can also be sensitive to environment and are controlled by numerous genes (Reynolds et al., 1994; Cuthbert et al., 2008). When evaluating stability of yield across diverse environments it is imperative to evaluate yield components as well, since they contribute to overall yield. To detect QTL for specific traits of interest, mapping populations are usually created from a bi-parental cross, since the generated

population will segregate for the traits of interest (Marza et al., 2006; Bennett et al., 2012a). A number of physiological traits have been reported to correlate with high temperature and water deficit, including maturity, height, awns, grain yield and canopy temperature (Trethowan et al., 2002; Quarrie et al., 2005; Olivares-Villegas et al., 2007; Bennett et al., 2012a).

High temperatures and water deficit stress are two factors that limit food production worldwide (Barnabás et al., 2008). In plants, response to high temperature and water deficit stress takes place at the physiological, molecular and cellular levels and has been associated with effectiveness in suppressing reactive oxygen species (Sairam and Saxena, 2000; Barnabás et al., 2008). High temperature and water deficit stresses during reproductive stages of wheat result in many physiological and metabolic changes, especially the disruption in hormone homeostasis (Barnabás et al., 2008). Grain filling rate increases due to high temperatures, which adversely reduces the grain filling period (Yin et al., 2009). These environmental conditions cause a reduction in yield by affecting yield components, especially thousand kernel weight and kernels per spike (Hossain et al., 2012). Canopy temperature (CTP) and canopy temperature depression (CTD) are highly associated with grain yield, indicating that high temperatures play a vital role in overall yield (Reynolds et al., 1994; Olivares-Villegas et al., 2007; Mason et al., 2011). Plants that are better able keep their leaf microenvironment temperature low in respect to the air temperature, maintaining a high CTD had higher grain yields (Ayeneh et al., 2002). Epicuticular wax (EW), although a product of genetics, like grain yield, is influenced by environmental interactions (Clarke and Richards, 1988; Araus et

al., 1991; Sánchez et al., 2001; Samdur et al., 2003; Kim et al., 2007; Elham et al., 2012). High temperature and water deficit stress significantly affect epicuticular wax load (EWL), resulting in increased deposition on leaf surfaces (Richards et al., 1986; Araus et al., 1991; Oliveira et al., 2003; Kim et al., 2007). The reaction to these environmental stresses played a key role in reducing CTP contributing to the maintenance of high yields (Clarke and Richards, 1988; Samdur et al., 2003; Kim et al., 2007; Elham et al., 2012). As stated, reduced CTP resulted in increased grain yield, raising the question, if epicuticular wax and canopy temperature interact with each and if so, how?

Selecting for stability across locations, particularly in environments with low precipitation or high temperature, may be a useful method of selecting for stress tolerance (Kang, 1997). This idea of stability led breeding programs to place greater emphasis on phenotypic stability and yield performance across diverse environments (Lin et al., 1986; Akcura and Kaya, 2008). To determine the efficiency of a genotype, statistical methods have been developed that are able to effectively partition environmental effects (Akcura and Kaya, 2008; Rafii et al., 2012; Tabrizi, 2012). These statistical analyses consider a genotype as stable if it has low contributions to GxE interactions (Asghar et al., 2006; Akcura and Kaya, 2008; Mohammadi et al., 2012; Tabrizi, 2012). The methods used to determine stability are based on parametric and non-parametric analyses. Parametric methods are the most commonly used analyses and are based on genotypic distribution assumptions (normality) and the non-parametric analyses have few assumptions (Lin et al., 1986; Hussein et al., 2000; Asghar et al.,

2006; Akcura and Kaya, 2008). Stability analyses have demonstrated that they are successful in separating genotypes based on high yields across multiple environments in numerous field trials. This includes studies in wheat (Akcura and Kaya, 2008; Mohammadi et al., 2012), barley (Bantayehu, 2009), oil palm (Rafii et al., 2012), sunflower (Tabrizi, 2012), and lentils (Asghar et al., 2006).

Approximately 2 to 10% of phenotypic variations in crops are accounted for by QTL associated with yield and yield related traits (Quarrie et al., 2005). One of the most critical factors when breeding for wide adaptation and yield stability is the stable expression of a QTL in numerous environments (Quarrie et al., 2005). Epistatic interactions between numerous QTL, influence of one loci over another, frequently complicate grain yield and its corresponding agronomic traits (Kusterer et al., 2007; Li et al., 2007). Traits such as early flowering, grains per spike, harvest index and grain number are known to associate with higher yields (McIntyre et al., 2010). The recent studies aimed at identify these complex interactions have resulted in the detection of major and minor QTL that closely associate with grain yield and yield components. Five major QTL associated with grain yield were identified in a wheat RIL population, located on chromosomes 2A, 2D, 3B and 6A (Li et al., 2007). In this same study, twenty-seven QTL associated with yield components were detected, 9 for thousand kernel weight (THKW), 7 for kernel number per spike (KNS) and 11 for spike number (Li et al., 2007). Another study involving a Chinese wheat RIL population, identified 99 putative QTL associated with grain yield and yield components, with pleiotropic effects of yield detected across 13 loci located on chromosomes 1A, 1B, 1D, 2A, 2B, 2D, 3A,

3B, 4B, 4D, 5B, 6D and 7D (Wang et al., 2008). Drought treatment of durum wheat revealed 32 distinct major QTL, 16 QTL associated with grain yield, 15 for heading date and 11 associated with plant height (Maccaferri et al., 2008). In yet another study, seventeen yield component QTL were mapped to chromosomes 2D, 3A, 4A, 5A, and 6B from an identified 56 QTL with environmental association (Börner et al., 2002).

Canopy temperature QTL have been detected across 11 loci mapped to chromosomes 1B, 2B, 3A, 3B, 4A, 5A, 5B, and 7A (Pinto et al., 2010; Bennett et al., 2012a). CTD QTL have been identified across 9 loci located on chromosomes 2D, 3B, 5A, 5D, and 7A in a RIL population of wheat (Mason et al., 2011; Mason et al., 2013). Some of these QTL overlapped with QTL also regulating yield. To date, few QTL regulating epicuticular wax QTL have been identified in major grain crops, with 2 in rice (*Oryza sativa* L) on chromosome 3 and 8 under water deficit conditions (Srinivasan et al., 2008). The QTL on chromosome 3 explained 22.9 % of the phenotypic variation and the one on chromosome 8 explained 9.6% of the phenotypic variation. In wheat, major QTL for flag leaf glaucousness were mapped to chromosome 1D, 2B, 2D, 3A, 4D, 5A, 5B, and 6A (Mason et al., 2010; Bennett et al., 2012b). Minor glaucous QTL were also identified on chromosomes 2B, 3B, 3D, and 7D.

The objectives of this study was to construct a genetic linkage map from a cross between a heat tolerant line (Halberd) and a heat susceptible line (Len) with SNP molecular markers and identify QTL for increased epicuticular wax and its increase while decreasing canopy temperature under water deficit and high temperature conditions.

4.2. Materials and methods

4.2.1. Plant material

The population was developed from a cross between 'Halberd' a heat tolerant Australian spring wheat cultivar and 'Len' a heat susceptible USA spring wheat cultivar. The Halberd parent is more glaucous, has visibly more wax on its flag leaves, stems and spikes than the Len parent. The initial cross was developed in the greenhouse in 2003, producing a population consisting of 180 $F_{6:8}$ recombinant inbred lines (RILs) derived through single seed decent. The population was derived from a subset of a larger population to be uniform in flowering time within 7 days. The F8 and F9 families were evaluated in 2011 and 2012, respectively, for water deficit and high temperature tolerance under field conditions for leaf epicuticular wax and yield stability.

4.2.2. Field trials

The entire RIL population (180 lines) and the two parents, Halberd and Len, were planted in the field in 2011 and 2012 under irrigated and non-irrigated conditions. The population was sown as a delayed planting in January in Chillicothe (TX), College Station (TX) Obregon (Mexico) and Uvalde (TX) in 2011 and College Station (TX) and Uvalde (TX) in 2012 to impose high temperature stress. The lines were planted in an alpha-lattice design with two replications per RIL per treatment. The seeds were normalized to 1800 kernels per plot and planted in 0.5 x 3 meters plots with 0.3 m spacing between plots. All plots were uniformly surface irrigated (drip irrigation) to 90% of field capacity (0-0.25m depth) equivalent to 30 mm irrigation or sufficient saturation

to promote uniform emergence after planting. Water deficit conditions were applied at stem elongation, Feekes 5, by withholding irrigation (drip irrigation), leaving the population to rely only on moisture received from rainfall. The irrigated treatment, received irrigation (drip irrigation) until Feekes 11, the dough stage of grain development, continually receiving water every two weeks (to 90% of soil available moisture) or available soil moisture falls below 50% (in the top 0-1m soil profile). Drip irrigation zones consisted of 0.6 meter spaced drip tape (T-Tape, 10 mil, 0.3m emitter spacing, 0.46 GPM/100ft, at 8PSI). At planting, 22.68kg NPK as (13:13:13) fertilizer was applied and 22.68kg/Ha (Urea Ammonium Nitrate-UAN3200) at Feekes 7. To separate confounding effects of pests from high temperature and drought stress, the trials were treated with fungicides and insecticides. Due to the occurrence of bird damage in Uvalde for year 2011, plots were not harvested for either treatment. Chillicothe yield plots were not harvested in 2011 due to uneven plot stands.

4.2.3. Canopy temperature

Canopy temperatures were taken at approximately ten days after pollination (10DAP) from each plot with at least fifty percent anthesis for both irrigated and non-irrigated treatments. Temperatures were recorded using a portable handheld infrared thermometer (Fluke 566 series, Everett, Washington, USA) between 1100 and 1500 hours. The temperature for the canopy was recorded for each plot by holding the thermometer at a 45% angle to the horizontal and vertical distance from the plot canopy. Average plot temperature was recorded from one end of the plot to the other along its'

length. The thermometer recorded all temperature readings of individual leaves and then calculated the average for the plot.

4.2.4. Leaf epicuticular waxes

A modified colorimetric method was used to quantify epicuticular wax concentrations present on the leaf surface (Ebercon et al., 1977; Koch et al., 2005) and to determine differences between irrigated and non-irrigated treatments. Four flags leaves at approximately 10DAP, were randomly chosen from the middle of each plot of the RIL population for both treatments. Eight leaf discs were punched from the four leaves with a rabbit tool (Rabbit Tool USA, Rock Island IL USA) into a glass vial attached o the tool. Leaf discs measured 1 cm in diameter. Leaf epicuticular wax was extracted from the leaf discs by complete submersion in HPLC grade chloroform for 30 seconds. The resulting mixture was transferred to a clean GC vial and chloroform allowed to evaporate in a fume hood. The resulting extract was oxidized with 300µl acidified potassium dichromate ($K_2Cr_2O_7$) in a water bath at 100 ^{0}C for 30 minutes. To this solution 700µl of deionized water was added to each vial after cooling, allowing for color development for 1 hour. A spectrophotometer (PHERAstar plus, BMG LABTECH, Offenburg, Germany) was used to determine the optical density for each sample at 590 nm. Samples were loaded in 96 well polystyrene, untreated, clear flat bottom plates (Greiner Bio-One). A standard curve was developed from randomly selected wheat leaves and used to determine leaf wax concentrations, calculated based on leaf area and subsequently converted to mass expressed in mg/dm^2 units (Ebercon et al., 1977).

4.2.5. Agronomic trait measures and yield components

To estimate yield components, fifty heads (spikes) were randomly collected from the center of an entire plot. The yield components kernel number per spike (KNS), mean single head weight (MSHW), spikes per meter squared (Spm2) and thousand kernel weight (THKW) were determined. Height was measured from the center of each plot by placing a meter rule at the base of the plants at the soil surface and measuring to the top of the plot. The phenological and physiological traits and environments for the field study are summarized in Table 13 with accompanying abbreviations, as well as the method or technique used to measure the various response variables. The fifty heads and height were collected at the end of maturity, when 90 % of the plot had senesced. Each plot was harvested using a combine harvester to measure total plot grain yield when plots were completely senesced.

Table 13 Summary of agronomic and physiological traits measured in the Halberd/Len RIL mapping population grown in water deficit and high temperature environments.

Trait	Abbreviation	Environment Evaluated[b]	Method of Measurement
Grain Yield	Yield	CS11, OBR11, UVL12, UVLD12	Weight of grain harvested per unit area (tons ha^{-1})
Spike m^{-2}	Spm2	CS11, UVL12, UVLD12	Plot yield (g m^{-2}) divided by single head weight
Thousand kernel weight	THKW	CH11, CS11, CS12, OBR11, UVL11, UVL12	Weight of 100 grain sample x 10 (g)
Mean single head weight	MSHW	CH11, CS11, CS12, OBR11, UVL11, UVL12	Weight of 50 random heads from each plot divided by 50 (g)
Kernel number per spike	KNS	CH11, CS11, CS12, OBR11, UVL11, UVL12	mean single head weight divided by single kernel wieght
Epicuticular wax	EWL/wax	CS11, CS12, OBR11, UVL11, UVL12	Assessed using four 1cm leaf disk from two different flag leaves in each plot (mg dm^{-2})
Canopy Temperature	CTP	CS11, CS12, OBR11, UVL11, UVL12	Average temperature of plot (^{0} C)

[b] Environment codes: CS-College Station, CH-Chillicothe, OBR-Cuidad Obregon, UVL-Uvalde, 11-2011, 12-2012.

4.2.6. Yield stability estimates

Yield stability estimates were calculated across nine diverse environments. The stability statistics included five parametric and five non-parametric stability statistics. The parametric statistics used were based on environmental variance (S_i^2) (Francis and Kannenberg, 1978), ecovalence (W^2), which use the genotypic by environmental effects for the genotypes across all environments (Wricke, 1962), stability variance (σ_i^2), which measures stability as the variance of a genotype across all environments (Shukla, 1972), regression coefficient (b_i), which measures a genotype response to environmental changes (Eberhart and Russell, 1966), and superiority measure (P_i), which measures the difference between the genotype response and the maximum response over environments (Lin and Binns, 1988). The non parametric stability statistics are Nassar and Huhn's $S_i^{(2)}$ which measures between ranks variance across environments, $S_i^{(3)}$ which sums absolute deviations of squares of ranks for each genotype, and $S_i^{(6)}$ that use sums of squares of ranks for each genotype relative to mean ranks (Nassar and Huehn, 1987). The other two non-parametric statistics are rank-sum (RS) that considers both yield and Shukla's stability variance (Kang et al., 1991) and TOP which ranks genotypes in a stratified manner based on ranking of each genotype in each environment (Fox et al., 1990).

4.2.7. QTL analysis

Genomic DNA was extracted from leaves collected from plants grown from F_8 generation seeds. The leaves were about 2-3 weeks in age when collected and were

subjected to a modified CTAB DNA extraction protocol (Doyle, 1987; Cullings, 1992; Stein et al., 2001). Molecular marker analysis was performed at the USDA-ARS Biosciences research laboratory located in Fargo, North Dakota by Dr. Shiaoman Chao. High throughput genotyping was performed using a 90,000 single nucleotide polymorphism chip (Golden Gate) that uses the Infinium Assay II from Illumina. The genotyping performed was based on diversity arrays technology (DArT) that is able to detect changes in single base pairs (Jaccoud et al., 2001). Clustering and genotype calling was performed using Illumina's Genome Studio genotyping module. Polymorphic and co-dominant markers identified were assigned map positions in linkage groups using Haldane mapping function in the computational software JoinMap 4 (Van Ooijen, 2006). A total of 2,700 SNP polymorphic markers were identified but 2,565 were used to construct a linkage genetic map. These markers were used to define 22 linkage groups except for 2D, 3D, and 4D. Previously identified SSR markers, five to ten, with known chromosomal locations were anchored to the data set and used as an initial framework to create the linkage map. Multiple QTL Mapping (MQM) analysis was conducted in individual environments and across environments to detect main effect QTLs using MapQTL 6 (Van Ooijen, 2011). This method uses markers as cofactors, thus, reducing residual variance to determine QTL positions. The procedure was performed using the program default values, with a significance level of 0.05 and 10,000 permutations to determine the maximum likelihood of odds (LOD) score threshold. A QTL was determined to be present if the LOD score was greater than 3.0. Graphical presentation of linkage maps and QTLs were performed using MapChart 2.2 software

(Voorrips, 2002). To obtain a 95% coverage, the LOD drop-off method was used to calculate a support interval of 2-LOD (Lander and Botstein, 1989).

4.2.8. Statistical analysis

The locations were treated as separate environments for the duration of the field trials. The generalized linear model was used to estimate analysis of variance (ANOVA), with genotypes and environments considered as fixed effects and replication as a random effect. The REML model was used to estimate the least square means (LSMEANS) for each trait in the statistical software SAS (SAS v9.2, SAS institute Inc. Cary, NC, USA). Stability statistics were estimated using SAS (SAS v9.2, SAS institute Inc. Cary, NC, USA) and AGROBASE Generation II (Agronomix software Inc. Winnipeg Manitoba Canada). To analyze parametric and non-parametric stability statistics a comprehensive SAS code was used for yield and yield components (Hussein et al., 2000).

4.3. Results

4.3.1. Agronomic data

The water deficit and high temperature experiments were performed side by side in both 2011 and 2012. To impose high temperature stress, seeds were late sown allowing for later anthesis and grain filling periods. Water deficit was imposed at stem elongation by withholding irrigation from the experiment. The maximum temperature during anthesis and grain filling was seen in Chillicothe in 2011 and Uvalde during the 2012 season (Table 14). Uvalde 2011 and Ciudad de Obregon 2011 had the highest maximum

temperatures during the anthesis and grain filling periods, while College station 2011 and 2012 had the highest minimum temperatures during the same period (Table 14). College Station 2011 and 2012 had the highest number of days with average daily temperatures > 30 ^{0}C (Table 14). The temperatures recorded were sufficiently high to consider the experiment as temperature stressed. The drought environments in Chillicothe, College Station and Uvalde received approximately 175mm of irrigation whereas the high temperature environments received approximately 376mm. This amount of water was sufficient to impose water deficit conditions, moderate drought stress (Bennett et al., 2012a). The water deficit environments achieved similar average yields to the high temperature environments across the RILs (2.29 and 2.13 tons ha^{-1}, respectively) (Table 9). Halberd (2.71 ha^{-1}) yielded higher than Len (2.10 ha^{-1}) in the water deficit experiment and the high temperature experiment (2.83 and 1.90 ha^{-1} respectively) but the difference was not statistically significant (Table 9).

Table 14 Minimum, maximum and mean temperatures during anthesis and grain filling periods and the number of days with average daily temperature above 30 ^{0}C for each location in 2011 and 2012.

Experiment	April			May			Number of days with maximum		
	Min	Max	Avg	Min	Max	Avg	>30 ^{0}C	>35 ^{0}C	Avg. >30 ^{0}C
Chillicothe 2011	12.0	29.5	21.1	15.6	31.7	23.4	20	14	8
College Station 2011	17.2	31.1	24.4	18.9	31.7	25.6	24	10	19
Obregon 2011	16.7	30.0	23.3	16.7	33.9	25.0	28	15	0
Uvalde 2011	17.8	33.3	25.6	18.9	33.3	26.1	27	15	11
College Station 2012	17.2	28.3	22.8	20.6	31.1	26.1	24	0	21
Uvalde 2012	15.6	30.0	23.3	18.9	31.1	25.0	24	10	1

4.3.2. QTL mapping analysis

The Halberd x Len RIL population originally consisted of 180 individuals but was reduced to 176 individuals since DNA from four lines was not extracted. The genetic linkage map was constructed with 2,565 single nucleotide polymorphic markers spanning approximately 3, 778 cM with average spacing of 1.4 cM across 22 linkage groups. A total of 44 QTL were detected across the nine environments for EWL, canopy temperature and yield and yield component stability statistics. QTL were detected on chromosomes 1A, 1B, 2A, 2B, 3A, 3B, 4B, 6B, and 7A for EWL, canopy temperature and yield and yield component stability statistics but no significant QTL was detected on any group D chromosomes. In total, 9 loci across 5 chromosomes associated with leaf EWL, with each QTL explaining between 7.7 and 12.1 % of the phenotypic variation. LOD scores for the observed EWL QTL ranged from 2.90 to 4.90 (Table 15). Nine loci across 5 chromosomes were associated with CTP, each QTL explaining between 7.3 and 16.1% of the phenotypic variation. Observed CTP QTL had LOD scores between 2.90 to 6.90 (Table 15). Yield stability statistic QTL were identified for 10 loci across 6 chromosomes, each QTL explaining between 7.3 and 9.9% variation with LOD scores between 3.08 and 3.97 (Table 15). Three loci across 3 chromosomes, 4B, 6B and 7A, associated with the yield component KNS stability statistic with LOD scores between 2.80 to 5.24 and accounted for 7.3 to 12.8% of the variation observed (Table 15). Three loci on chromosome 7A associated with the yield component MSHW stability statistic. The QTL LOD scores were between 3.40 and 4.64 and accounted for 8.5 to 11.4% of the variation observed (Table 15). THKW stability statistic loci were identified on 3

chromosomes (1A, 3A, and 4B) and were associated with 3 loci having LOD scores between 3.43 and 4.17, accounting for 7.4 to 10.3% of the variation observed (Table 15).

Halberd alleles were associated with increased yield stability at 5 loci located on chromosomes 1B, 2A, 2B, and 4B, while Len had favorable alleles on 2B (Table 15). Halberd alleles also associated with increased KNS stability at 2 loci located on chromosomes 6B and 7A, while Len had favorable alleles on chromosome 4B (Table 15). For THKW stability, Halberd alleles were associated with 2 loci identified on chromosomes 1A, and 4B, while Len had the favorable alleles on chromosome 3B (Table 15). The yield component MSHW had increased stability at 3 loci identified on chromosome 7A, with Len contributing all the favorable alleles (Table 15). QTL for CTP during grain filling was detected in both high temperature and water deficit treatments in the same region on chromosome 2B. The CTP QTL that accounted for the largest percentage of genetic variation was identified on chromosome 2B (*Qtp.tam-2B.2*, R^2 = 16.1%) with Halberd being the contributor of the favorable alleles and was detected under water deficit conditions (Table 15).

Table 15 Summary of QTL detected in the Halberd x Len RIL population for canopy temperature and epicuticular wax co-localizing with yield and yield components stability statistics across environments.

QTL	Co-localization	Marker	LOD	R^2	Additive[b]	Favorable Allele
Epicuticular wax load						
QWax.tam-2B.1	*QYieldss.tam-2B.1*	BS00094373_51	3.97	9.9	2.215	Halberd
QWax.tam-2B.2	*Qtp.tam-2B.1*	Excalibur_rep_c108225_302	2.94	7.4	-0.008	Len
QWax.tam-2B.3	*QYieldss.tam-2B.3*	BS00004120	3.09	7.8	-0.193	Len
QWax.tam-6B	*Qknsss.tam-6B*	wsnp_Ex_c8011_13584847	3.24	8.1	45.786	Halberd
QWax.tam-7A.1	*Qmshwss.tam-7A.1*	wsnp_Ku_c19943_29512612	3.40	8.5	-0.066	Len
Canopy temperature						
Qtp.tam-1B	*QYieldss.tam-1B*	Tdurum_contig50988_500	3.28	8.2	0.050	Halberd
Qtp.tam-2B.2	*QYieldss.tam-2B.2*	wsnp_Ex_rep_c68386_67199155	3.08	7.7	0.189	Halberd
Qtp.tam-2B.4	*QYieldss.tam-2B.3*	BS00004120	3.09	7.8	-0.193	Len
Qtp.tam-3B	*Qthkwss.tam-3B*	wsnp_BE498786B_Ta_2_1	4.17	10.3	-17.606	Len
Qtp.tam-7A.1	*Qmskwss.tam-7A.2*	Jagger_c319_99	4.64	11.4	-0.075	Len
Qtp.tam-7A.2	*Qmskwss.tam-7A.3*	wsnp_Ra_c31751_40835513	3.12	7.8	0.498	Halberd
Yield Stability						
QYieldss.tam-4B	*Qknsss.tam-4B*	RAC875_rep_c105718_430	5.10	12.5	26.507	Halberd
QYieldss.tam-4B	*Qthkwss.tam-4B*	RAC875_rep_c105718_430	3.43	8.6	25.863	Halberd
Significant and unco-localized QTL						
Qthwss.tam-1A		Tdurum_contig13937_2280	4.06	10.1	27.720	Halberd
QWax.tam-1A.1		Excalibur_rep_c107144_405	3.17	8.3	-0.150	Len
QWax.tam-1A.2		wsnp_Ex_rep_c103028_88077819	3.14	8.0	0.014	Halberd
Qtp.tam-4B		Excalibur_c52517_464	2.91	7.3	-0.030	Len
Qknsss.tam-7A		RAC875_c100339_541	5.24	12.8	-26.607	Len
QWax.tam-7A.2		BS00034689_51	4.94	12.1	-0.078	Len

Key: *Wax* epicuticular wax load, *tp* canopy temperature, *kns* kernel number per spike, *mshw* mean single head weight, *thkw* thousand kernel weight, *Yield* plot yield, *ss* stability statistic. [b] additive effect of allele substitution

Similarly, EWL QTL was detected in both treatments in the same region on chromosome 2A. The EWL QTL that accounted for the largest percentage of genetic variation was identified on chromosome 7A (*QWax.tam-7A.2*, R^2 = 12.1 %) with Len contributing the favorable alleles and was detected in the high temperature treatments (Table 15). The Len alleles were mostly associated with increased stability for the yield components. The QTL with the largest percentage of genetic variation for KNS stability was located on chromosome 7A (*Qknsss.tam-7A,* R^2 = 12.8 %). The QTL with the largest percentage of genetic variation for MSHW stability was located on chromosome 7A (*Qmshwss.tam-7A.2*, R^2 = 11.4 %) and that for THKW, *Qthkwss.tam-3B,* (R^2 = 10.3 %), was identified on chromosome 3B .The QTL *QYieldss.tam-2B.1*, (R^2 = 9.9 %), was associated with the largest percentage of genetic variation for yield stability and was contributed by Halberd (Table 15).

The yield stability QTL located on 1B (*QYieldss*.tam-1B) co-located with the QTL for CTP (Table 15; Fig. 8). In fact, five different yield stability statistics identified the QTL in this region and associated with the same molecular marker (BS00094373_51). Another yield stability QTL identified on chromosome 2B co-located with QTL for CTP, also co-localized with an EWL QTL (Table 15; Fig. 8). On chromosome 3B, another yield stability QTL co-localized with a EWL QTL (Table 15; Fig. 8).

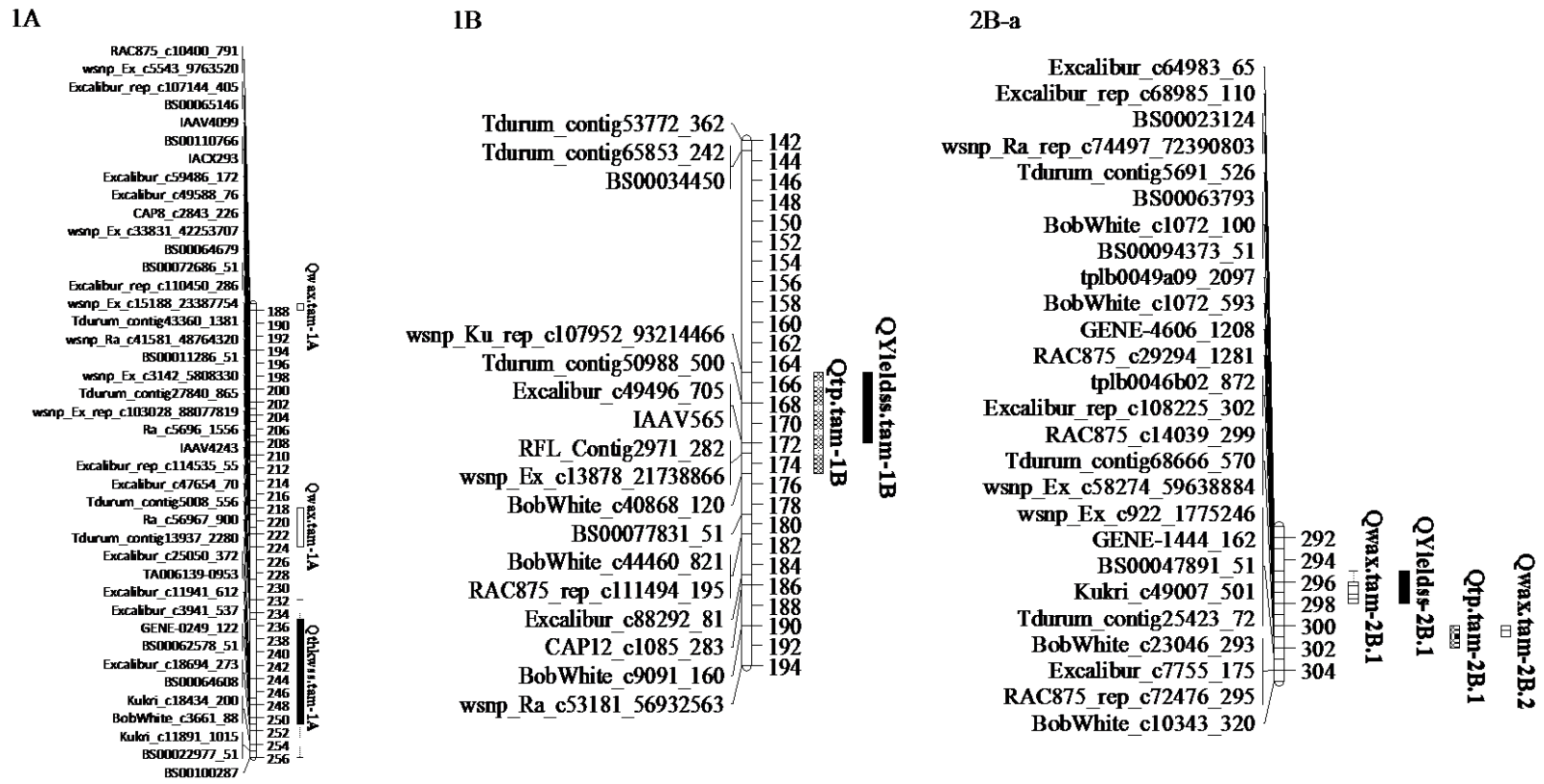

Fig. 8 Linkage map and quantitative trait loci for canopy temperature, epicuticular wax, yield stability and yield component stability in the Halberd/Len RIL population across water deficit and high temperature experimental environments. Kosambi mapping function was used to determine marker positions that are presented in cM. QTL for canopy temperature (dotted bars), epicuticular wax (white bars) and yield and yield components stability (black bars) are determined at a threshold of 0.05 level of significance and presented as 2 LOD intervals.

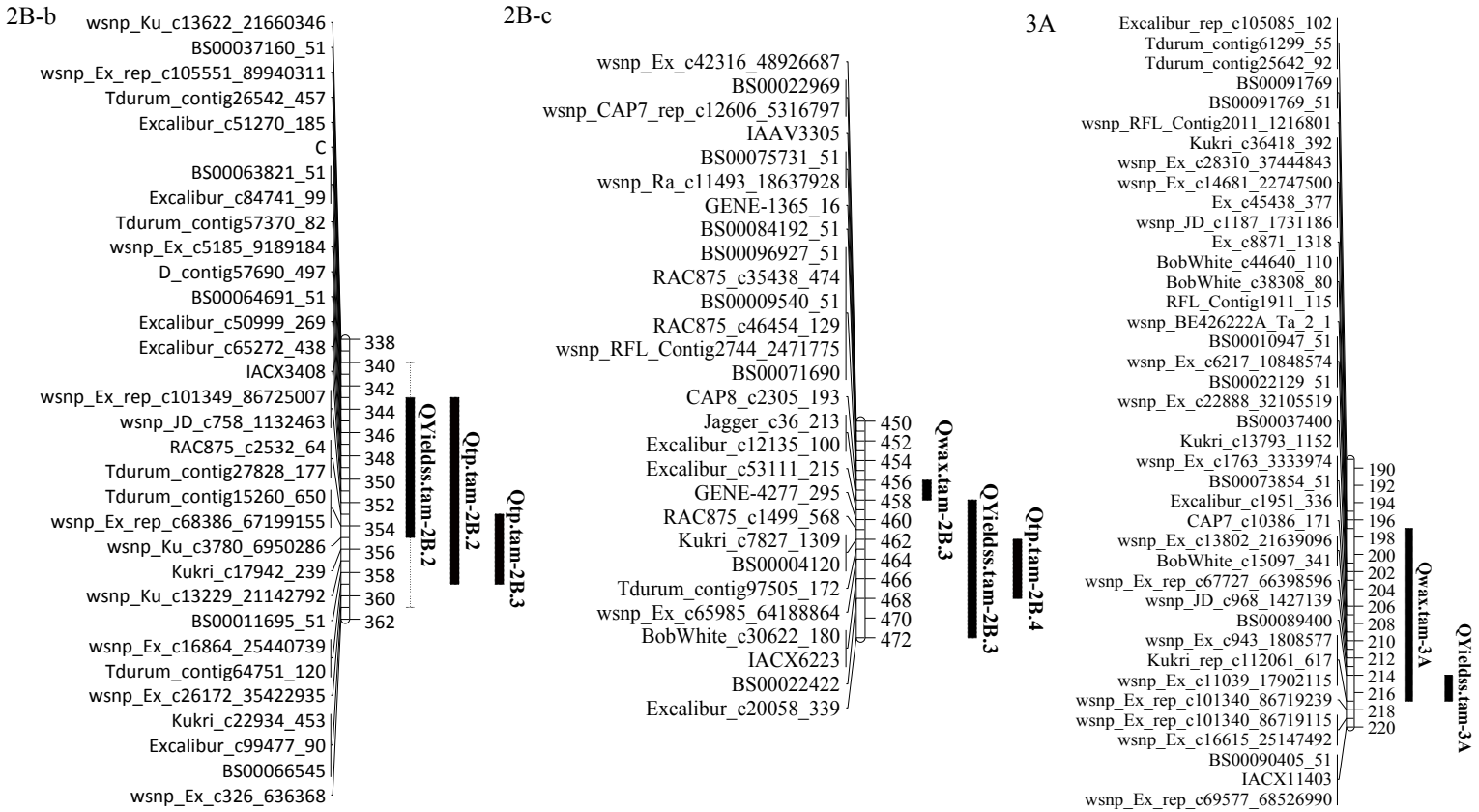

Fig.8 Continued

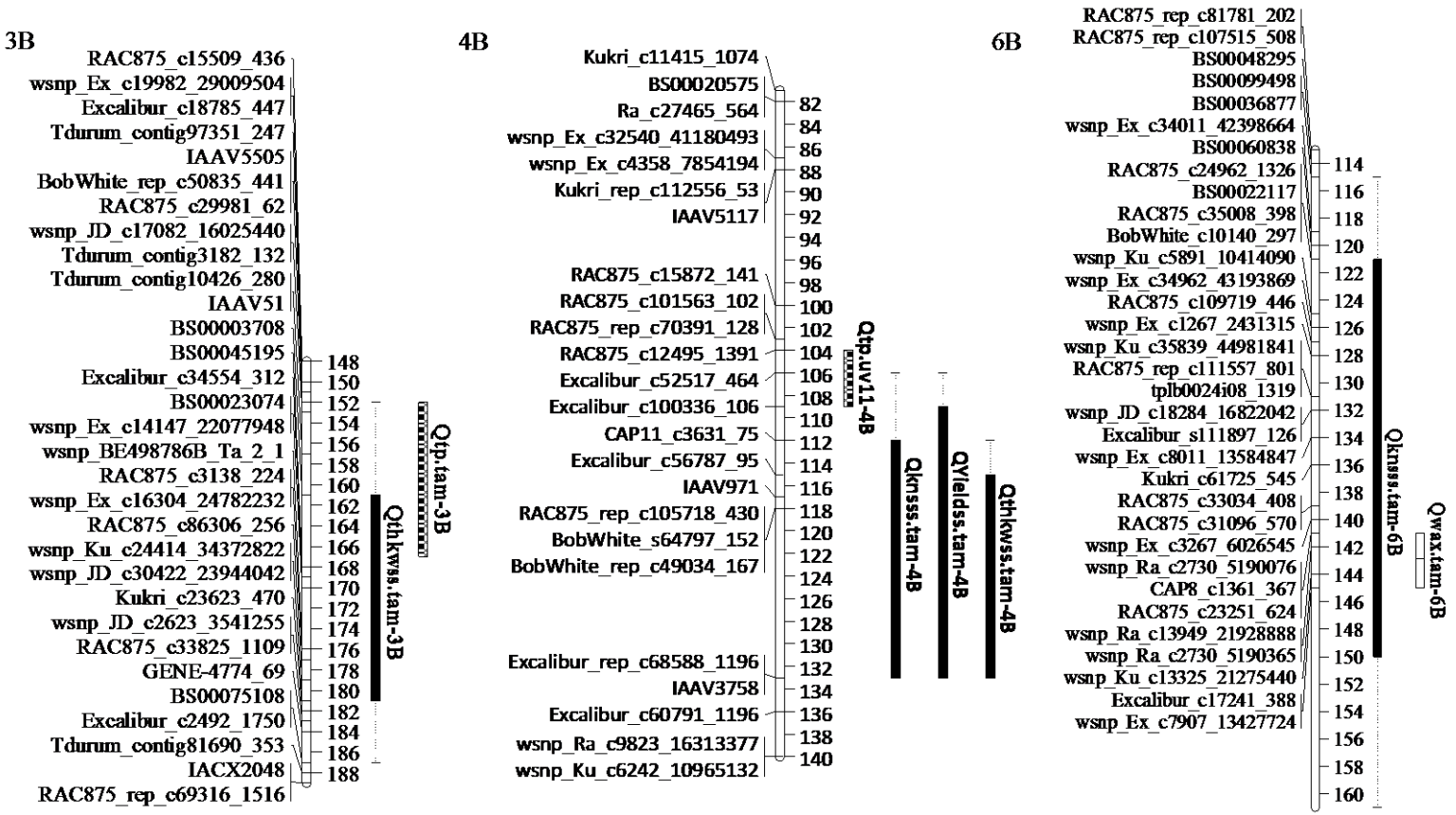

Fig.8 Continued

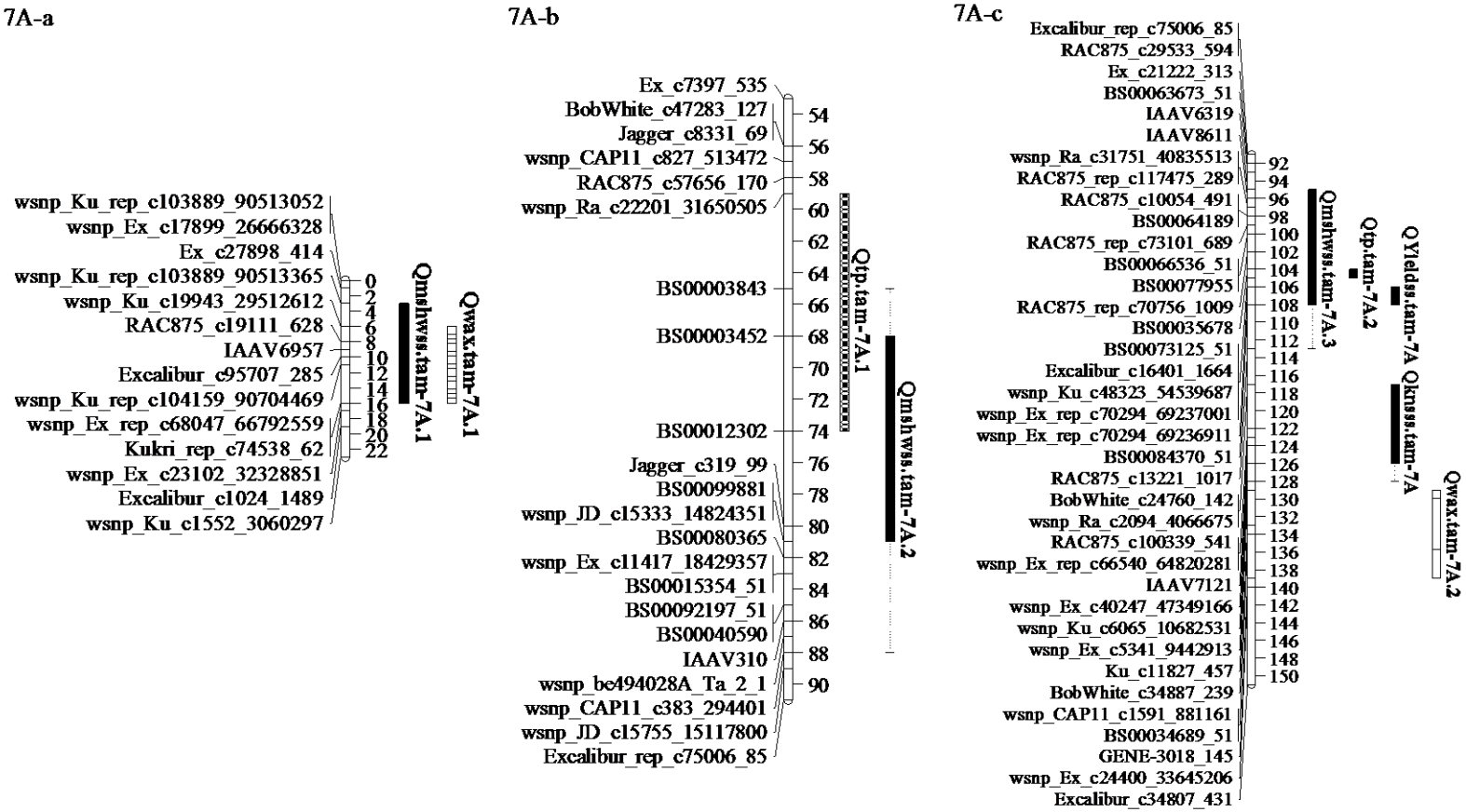

Fig.8 Continued

Furthermore, QTL for yield stability were co-located with QTL for CTP in conjunction with SKW, KNS and THKW stability QTL on chromosome 4B, and MSHW stability QTL on chromosome 7A (Table 15; Fig. 8). The KNS stability QTL located on chromosome 6B was co-located with a QTL for EWL, while a MSHW stability QTL located on chromosome 7A co-located with EWL, CTP and yield stability QTL (Table 15; Fig. 8). Moreover, a THKW stability QTL located on chromosome 3B was co-located with a CTP QTL (Table 15; Fig. 8). A number of the yield stability statistics QTL co-located with other yield component statistics QTL on chromosomes 1B, 4B, 6B, and 7A (Fig. 8). Furthermore, other non co-localized QTL for EWL, CTP, yield stability and yield component stability were identified on chromosomes 1A, 1B, 2B, 3A, 3B, 4B, 6B and 7A. The yield and yield components stability QTL with high LOD scores were identified on a few chromosomes compared to CTP and EWL QTL that were identified on all chromosomes of the A and B genome (Fig. 9).

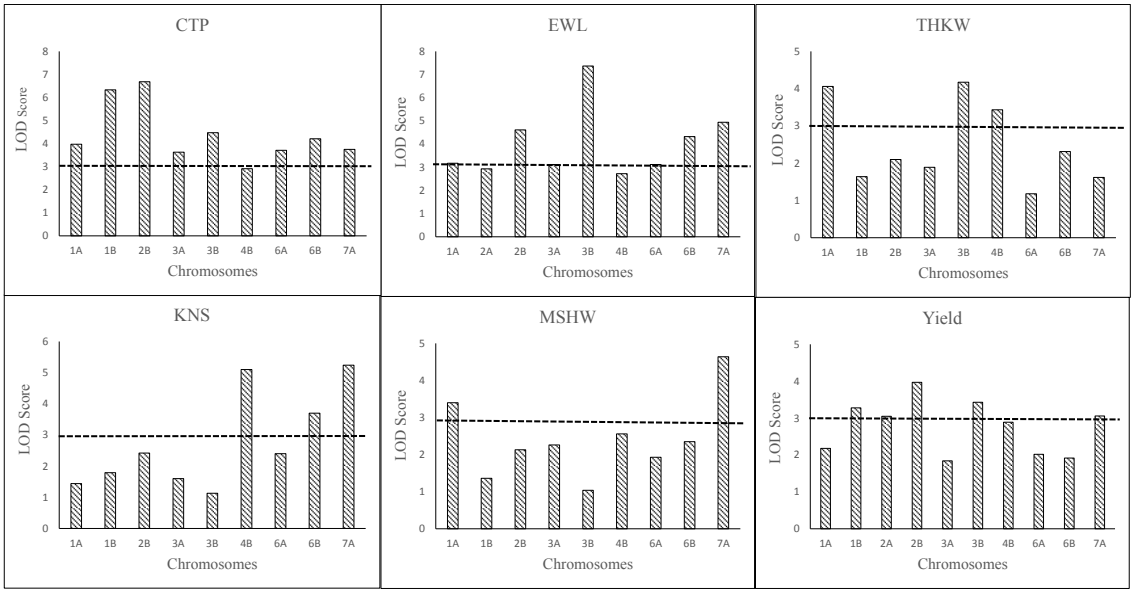

Fig. 9 Maximum LOD scores for canopy temperature, epicuticular wax yield stability and yield component stability statistics per chromosome measured on the Halberd/Len RIL population across five locations. The LOD threshold, 3.0, is represented by the horizontal dashed line to indicate a QTL.

4.4. Discussion

4.4.1. Agronomic and physiological traits

Developing new water deficit and high temperature tolerant cultivars requires a better understanding of the genetic and physiological variance present within the wheat genome. Evaluating beneficial traits to these abiotic stresses require good water management practices and the ability to expose plants to high temperature under field conditions. A late sowing time present an ideal solution for allowing plants to experience high temperatures during anthesis and grain filling, while drip irrigation provides a viable option for monitoring water levels. Employing these two strategies created two distinct environments for the Halberd x Len recombinant inbred population in multiple

locations and years (Table 13). The present study sought to identify QTL associated with increase leaf epicuticular wax and their overlap with decreased CTP and improved yield and yield components stability. Although the yield for the two treatments were similar, the high temperature environment had the lowest average yield for the RIL population of the two treatments, despite receiving twice as much water as the water deficit experiment (Table 9). Although not significant, Halberd produced greater yields under both conditions than the Len parent. In a doubled-haploid population, the lowest yields for the population was recorded under water deficit conditions, unlike the current study, but yield under water deficit and high temperature was similar and not significant, similar to the current study (Bennett et al., 2012a). The RIL population had more epicuticular wax load on average under water deficit than high temperature conditions (Table 9). The parent Halberd had higher EWL under both water deficit and high temperature conditions, producing almost 2 mg dm^{-2} more than Len under water deficit, but similar amounts under high temperature conditions (Table 9). These observations suggest that water deficit and high temperatures combined to have a more profound effect on leaf EWL than high temperature environments. Canopy temperature for both parents in water deficit and high temperature conditions were similar, but the RIL population had higher CTP on average, about 3 ^{0}C, under water deficit than high temperature conditions (Table 9). EWL was also higher under water deficit than high temperature conditions for the RIL population. The yield components, KNS and MSHW, were higher in the Halberd parent than the Len parent under both treatments, with Halberd having approximately 3 kernels per spike more than Len under water deficit

conditions (Table 9). These observations are consistent with the characteristics of the Halberd parent, a high temperature tolerant cultivar, with an ability to produce high yields.

4.4.2. QTL mapping results for agronomic traits

In the present study, QTL associated with yield stability, yield components stability, canopy temperature and epicuticular wax were identified (Table 15). The environments where the traits were evaluated were mostly free of disease limiting any effects on yield or the possibility of identifying disease QTL. In wheat, previous studies have identified genetic variation associated with a number of physiological traits under water deficit and high temperature environments (Olivares-Villegas et al., 2007; Cuthbert et al., 2008; Pinto et al., 2010; Bennett et al., 2012a), but few studies have investigated QTL associated with yield stability or yield component statistics, along with their overlap with QTL regulating increase EW. In barley (*Hordeum vulgare*), stability statistics QTL for yield and heading-date have been identified in a recombinant chromosome substitution lines population and spring population (Kraakman et al., 2004; Emebiri and Moody, 2006; Inostroza et al., 2008). These studies demonstrated the possibility and usefulness of stability statistics in selection and breeding for complex traits such as yield.

The discovery of QTL associated with yield stability and yield components stability had minor and major effects, but only major QTL will be discussed. Eight CT QTL mapped to five chromosomes, 1B, 2B, 3B, 4B and 7A (Table 15; Fig. 8). Previous

studies have identified CT QTL on chromosomes 1B, 2B, 3B, 4A, 5A, and 7A with major effects for water deficit and high temperature conditions (Pinto et al., 2010; Bennett et al., 2012a). QTL regulating CT associated with yield stability or yield stability components across all chromosomes with yield stability the most associated trait. The yield stability QTL identified on chromosome 1B (*QYieldss.tam-1B*) was consistent across environments and co-localized with a CT QTL under water deficit conditions in the same region and identified by the same molecular marker (Table 15; Fig. 8; Fig. 10d).

In a wheat study, a grain yield QTL was identified on chromosome 1B and was associated with a grain per meter squared QTL (Pinto et al., 2010). However, the yield stability QTL was identified in a higher region just above the position of the grain yield QTL. In this study yield stability QTL mapped to various loci throughout the length of chromosome 2B, with a total of four significant QTL identified. Three of the four yield stability QTL co-localized with other QTL for EWL and CTP at two and three regions, respectively (Table 15; Fig. 8).

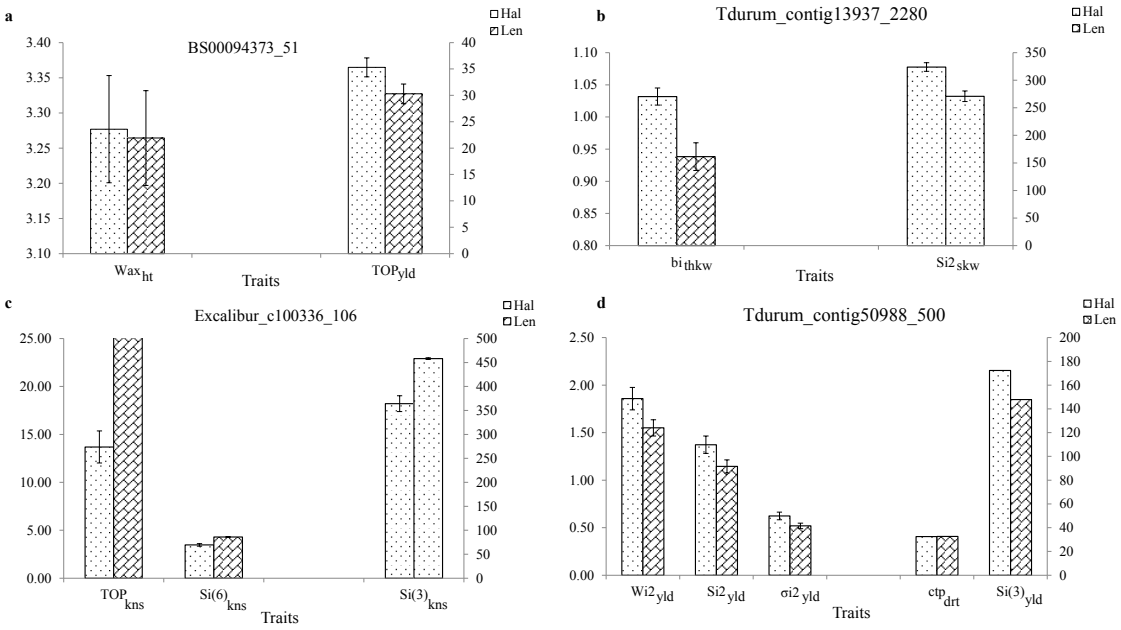

Fig. 10 Contrast analysis for allelic variants showing the pleiotropic effects between canopy temperature, epicuticular wax and yield traits across environments. a) traits on chromosome 2B, b)traits on chromosome 1A, c) traits on chromosome 4B, d) traits on chromosome 1B. Key: grain yield (yld), kernel number spike^{-1} (kns), mean single head weight (mshw) and thousand kernel weight (thkw), Epicuticular wax load (wax), canopy temperature (ctp). Regression coefficient of Eberhart and Russell b_i (B), environmental variance S_i^2 (BCV), ecovalence W_i^2 (W), stability variance σ_i^2 (Shuk), Nassar and Huhn's $S_i^{(3)}$ (Si3) and $S_i^{(6)}$ (Si6) and Fox's TOP.

In fact, the QTL *QYieldss.tam-2B.1* co-localized with two EWL QTL (Fig. 8), one related to water deficit treatment and the other to high temperature environment, and also sat adjacent to a CTP QTL. The EWL loci in this region have the potential to be used in developing better water deficit and high temperature tolerant cultivars with improved grain yields since they exhibit some association. Another yield stability QTL (*QYieldss.tam-2B.2*) at a lower region co-located with CTP for both water deficit and high temperature treatments. The other yield stability QTL (*QYieldss.tam-2B.3*) co-

located with the two traits EWL and CTP (Table 15; Fig. 8). The 2B chromosome seems to play an important role in maintaining grain yield and improved production under water deficit and high temperature environments. Grain yield QTL have been previously mapped to this chromosome, as well as other physiological traits including CTP (Marza et al., 2006; Bennett et al., 2012a). These multiple regions may be pleiotropic given the numerous associations identified between the yield stability statistics and physiological traits. The stability statistics for yield at these regions, where the Len allele contributed to increased stability, identified with the lower values for the stability statistics (Fig. 10d). Yield stability QTL located on chromosome 3A and 7A co-localized with EWL and the yield stability component, MSHW, respectively. The favorable allele for the two QTL located on chromosome 3A, were contributed by the Halberd parent, confirming its water deficit and high temperature stress nature (Table 15). Another yield stability QTL located on chromosome 7A was co-localized both with CTP and MSHW stability with the favorable alleles contributed by the Len parent (Table 15; Fig. 8). Interestingly, a QTL for the senescence related trait (stay-green), time to maximum rate of senescence, mapped to the region similar to the CTP QTL in this study (Vijayalakshmi et al., 2010). The CTP QTL identified on chromosome 7A (7A-c) is located in the same region as the Sr22 gene for stem rust resistance (Kerber and Dyck, 1973; The, 1973; Periyannan et al., 2011). Grain yield QTL have been identified in a similar region to the yield stability QTL on chromosome 3A, however, the QTL on 7A, although not in the same region, did associate with the yield component KNS (Bennett et al., 2012a). A recent study mapped a QTL for heat susceptibility index of single kernel weight and another for heat

susceptibility index in the region similar to the yield stability and MSHW stability QTL (Mason et al., 2010; Mason et al., 2013). Furthermore, the KNS stability QTL identified on chromosome 7A overlapped with QTL for grain filling duration, heading date, heat susceptibility index and canopy temperature depression under high temperature conditions (Mason et al., 2013). Two yield components, KNS and THKW stability, had QTL that was co-located with a yield stability QTL on chromosome 4B. Although not located in the same regions, the yield stability QTL that were identified on chromosome 1B, 2A, 2B, 3A, 4B, and 7A in this study, were similar to the yield QTL identified on the same chromosomes in previous studies (Börner et al., 2002; Marza et al., 2006; Cuthbert et al., 2008; Pinto et al., 2010; Bennett et al., 2012a). The identification of the stability QTL for grain yield in similar regions of chromosomes suggest that these QTL loci work in conjunction to maintain yield and are possibly inherited together.

A number of QTL were identified for yield stability components, KNS, MSHW, and THKW on various chromosomes. Three QTL were identified for each of the yield components on different chromosomes except for MSHW, which had all three loci located on one chromosome (Fig. 8). The THKW stability QTL identified on chromosomes 3B and 4B co-located with a CTP QTL as well as a yield stability QTL, respectively (Fig. 8). The parent Halberd contributed the favorable alleles for the QTL on 1A and 4B, whereas, the Len parent contributed the favorable allele on 3B. In several studies, THKW QTL were mapped to these chromosomes but outside the regions identified in this study. The co-localization of CTP and yield QTL was previously reported on chromosome 4B, furthermore, the THKW QTL in a previous study was

located in a similar region of the THKW stability QTL identified in this study (Börner et al., 2002; Cuthbert et al., 2008; Pinto et al., 2010). The co-localization of THKW stability QTL and a CTP QTL on chromosome 3B, was mapped to a similar region where THKW QTL and CTP QTL also co-located (Cuthbert et al., 2008; Pinto et al., 2010). Numerous QTL for KNS have previously been mapped to regions of chromosome 1A, 1D, 2A, 2B, 2D, 3A, 4A, 4B, 4D, 5A, 6D, 7A and 7D with major effects across multiple locations and environments (Börner et al., 2002; Kumar et al., 2007; Cuthbert et al., 2008; Wang et al., 2008). Only three KNS stability QTL were identified and mapped to chromosomes 4B, 6B, and 7A with favorable alleles contributed by the Halberd parent (Table 15). The QTL located on chromosome 4B co-located with CTP, whereas the QTL on 6B and 7A co-located with EWL (Fig. 8). The region on 7A where the KNS stability QTL was identified is similar to the region where KNS, MSHW, THKW and grain yield QTL mapped in previous studies (Kumar et al., 2007; Cuthbert et al., 2008; Bennett et al., 2012a). Although not abundant in this study, MSHW QTL were identified on chromosomes 1A, 2A, 2D, 3B, 4A, 4B, 4D, 5A, 5B, 5D, 6A, 6B, 7A, and 7B in previous water deficit and high temperature stress studies (Börner et al., 2002; Cuthbert et al., 2008; Wang et al., 2008). Moreover, the MSHW stability QTL identified in this study mapped to only one chromosome, 7A, at three different loci and one of these region (~80 cM) coincided with a MSHW and KNS QTL previously identified in a similar region (Börner et al., 2002; Cuthbert et al., 2008). Interestingly, two of the MSHW stability QTL co-located with two different CTP QTL in high temperature stress conditions (Fig. 8). However, the other MSHW stability QTL

identified co-located with an EWL QTL. Interestingly, a recent study of wheat under high temperature conditions mapped a flag leaf width QTL in this region (Mason et al., 2010). The yield component stability QTL identified in this study present a fascinating view into the complexity that is yield and how it is affected by differing environments.

A number of QTL for Spm2 have been identified in previous studies on chromosomes 1A, 1B, 1D, 2A, 2D, 3B, 4B, 5A, 5B and 7D (Campbell et al., 2003; Marza et al., 2006; Li et al., 2007; Cuthbert et al., 2008). However, in this study when stability measures were applied to Spm2, no significant QTL were identified. In a wheat study, grain yield and Spm2 QTL co-located with each other on chromosomes 2D and 3B, and agreed with the simple correlation that was identified between the two traits (Li et al., 2007). Spikes per meter squared is sensitive to high temperature and water deficit stress between stem elongation and anthesis (del Moral et al., 2003). It is affected by nitrogen availability and spacing (Simane et al., 1993). One possibly explanation for the lack of Spm2 stability QTL is that it is more influenced by the environment than genetics and it could also be due to the lack of sufficient environments to detect enough variation.

Although several studies in wheat have mapped glaucous (waxy) QTL, relatively few have mapped QTL for epicuticular wax load (Börner et al., 2002; Mason et al., 2010; Mason et al., 2011; Bennett et al., 2012a; Bennett et al., 2012b). Glaucous QTL were identified on chromosomes, 1D, 2A, 2B, 2D, 3A, 4A, 4D, 5A, 5B, 6A, and 7A under water deficit and high temperature conditions in multiple environments. In this study, EWL QTL mapped to chromosomes 2A, 2B, 3A, 6B and 7A in multiple locations under water deficit and high temperature conditions (Table 15). Interestingly, the EWL

QTL identified on chromosome 7A was located in the region similar to a glaucous QTL mapped on this chromosome, suggesting that EWL may contribute to the glaucousness effect (Bennett et al., 2012a). This EWL QTL was also identified in the same region as the Wx-A1 gene on chromosome 7A (Chao et al., 1989; Nakamura et al., 1993; Yamamori et al., 1994). It is important to note that EWL QTL associated with CTP QTL only on chromosomes 2B, but at two different regions, also associating with yield stability (Fig. 8). No other glaucous QTL was identified in similar regions as EWL QTL on any of the chromosomes. The observations in this study and previous studies provides evidence that glaucousness and epicuticular wax, though related or similar, may be controlled by different sets of genes or may be a result of cascading gene effect.

4.5. Conclusion

Through QTL analysis and physiological traits, yield stability and yield components stability has been genetically dissected for a 180 RIL population under water deficit and high temperature conditions across multiple years and locations. Although there were mixed relationships with yield performance and environments, the stability statistics QTL provide strong evidence that genetic variation may be heritable and could have implications for breeding programs targeting a set of environments rather than a single environment. The CTP QTL located on chromosomes 1B and 2B had the largest genetic variation with the Halberd parent contributing the favorable alleles and co-located with yield stability and EWL QTL. The region on chromosome 2B should be further dissected to identify candidate genes, arming breeders with another tool in the

continuous battle to improve grain yield under water deficit and high temperature environments. The EWL QTL on chromosome 7A had the largest genetic variation, with the Len parent contributing the favorable alleles, co-locating with yield stability and MSHW stability QTL. The CTP QTL identified on chromosome 7A (7A-c) is located in the same region as the Sr22 gene for stem rust resistance, thus presenting the possibility that this loci may play a role in high temperature tolerance. Further efforts should be conducted to identify potential genes underlying this locus potentially adding more ammunition to the fight for yield improvement. Subsequent marker development of identified genes will provide breeders with another tool to improve grain yield in high temperature prone areas through marker-assisted breeding.

CHAPTER V

SUMMARY

Breeders seek to identify genotypes with potential to produce maximum yield in diverse environments. In a large and varied population such as a recombinant inbred population, identifying one genotype, as the most stable become a complex task. Stability is characterized as yield performance as well as adaptability to varying environments, therefore both mean yield and stability must be considered when determining a genotype as stable. Through QTL analysis and physiological traits, yield stability and yield components stability has been genetically dissected for a 180 RIL population under water deficit and high temperature conditions across multiple years and locations. Although there were mixed relationships with yield performance and environments, the stability statistics QTL provide strong evidence that genetic variation may be heritable and could have implications for breeding programs targeting a set of environments rather than a single environment.

Epicuticular wax or glaucousness offers advantages in protecting the plant from both biotic and abiotic stresses. It help plants to cope with abiotic stress, by protecting against photoinhibition and preventing damage to PSII system (Blum, 1988; Clarke and Richards, 1988; Barker et al., 1997; Roháček and Barták, 1999; Kim et al., 2007). Under HT conditions, EWL can reduce stomatal conductance g_s helping to regulate the rate of transpiration. Decreases in g_s might result from the occlusion of the stomatal opening by EW as leaves mature or result from reduced heat dissipation resulting from EW role in

light reflectance and reducing the absorption of excess photosynthetic energy (Turner and Heichel, 1977). EWL reduced chlorophyll fluorescence by reflecting excess irradiation, which is indicated by the strong and significant relationship between them in the HT environment. Chlorophyll fluorescence was observed to decrease as the age of the leaf increased, declining steadily from 3DAFE to 15DAP under HT conditions, as was he case in previous studies of various crops (Araus et al., 1998; Garty et al., 2001; Sayed, 2003).

HT tolerant cultivars or species possess thicker EW than those lacking tolerance providing an effective barrier to reduce heat dissipation and transpiration (Shepherd and Griffiths, 2006). Epicuticular wax accomplishes this by tempering the leaf microenvironment to HT, indicated by the positive and significant relationship observed between EWL and LTD. The positive relationship indicates that an increase in EWL may decrease the temperature of the leaf microenvironment by efficiently reducing the amount of incident irradiation. This reduction in incident irradiation is presumably due to increase reflectance at the leaf surface and thus, indicate that HT tolerant cultivars can effectively use EW levels to adjust stomatal conductance, chlorophyll fluorescence and leaf temperature. The vegetative indices, SR and NDVI, progressively decreased through reproductive stages under HT conditions, suggesting a decline in plant health. The decline in plant health is possibly due to a decrease in green leaf area (Bort et al., 2005). The subsequent decrease of the SRI of leaves devoid of EW in HT conditions, suggest that epicuticular wax efficiently reflect incident irradiation from the microenvironment of a leaf. Although environment affects EW deposition, stomatal

conductance and chlorophyll fluorescence, it is important to remember that they are also affected leaf phenology.

The CTP QTL located on chromosomes 1B and 2B had the largest genetic variation with the Halberd parent contributing the favorable alleles and co-located with yield stability and EWL QTL. Canopy temperature QTL also associated with other yield and yield component stability QTL on chromosomes 3B, 4B and 7A. The EWL QTL on chromosome 7A had the largest genetic variation, with the Len parent contributing the favorable alleles, co-locating with yield stability and MSHW stability QTL. Epicuticular wax load QTL also associated with other yield and yield component stability QTL on chromosomes 3A and 6B. Furthermore, the CTP QTL identified on chromosome 7A (7A-c) is located in the same region as the Sr22 gene for stem rust resistance, thus presenting the possibility that this loci may play a role in high temperature tolerance. The 2B and 7A chromosomes possess regions where physiological and agronomic traits interact providing a gateway to better understand the influence of environmental on yield and yield-associated traits. Thus these regions on chromosome 2B and 7A should be further dissected to identify candidate genes, arming breeders with another tool in the continuous battle to improve grain yield under water deficit and high temperature environments.

The goal of this research was to investigate the nature of QTLs affecting agronomic performance in a RIL population and their interactions with the environment allowing for a connection between phenotypic variation and DNA sequence. High EWL may promote stable yields but its sensitivity to environmental conditions makes it

challenging to definitively point to it as a source of improved stability. Further efforts need to be conducted to identify potential genes underlying this locus potentially adding more ammunition to the fight for yield improvement. Subsequent marker development of identified genes will provide breeders with another tool to improve grain yield in high temperature prone areas through marker-assisted breeding.

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