

**THE ROLE OF LEAF EPICUTICULAR WAX AN IMPROVED ADAPTATION
TO MOISTURE DEFICIT ENVIRONMENTS IN WHEAT**

A Dissertation

by

SUHEB MOHAMMED

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

Chair of Committee,	Dirk B. Hays
Co-Chair of Committee,	Amir Ibrahim
Committee Members,	William L. Rooney
	Russel W. Jessup
Head of Department,	David D. Baltensperger

May 2014

Major Subject: Plant Breeding

Copyright 2014 Suheb Mohammed

ABSTRACT

Water deficiency is the primary reason for decreasing wheat (*Triticum aestivum*) yields globally, causing a nearly 50-90% yield reduction on at least 60 Mha of land in developing countries. Previous studies have identified associations in genomic regions for cooler canopies, heat susceptible index, and grain yield components in winter wheat. This project aims to define the role that leaf epicuticular wax (EW) plays as a drought adaptive trait in terms of yield stability. A spring wheat Len/Halberd recombinant inbred line population was used to test this question. The RIL population exhibits significant segregation for leaf EW, canopy temperature (CT), awns, and drought susceptible index (DSI) yet has been selected. An alpha lattice design with 180 recombinants and 2 replications was used with two distinct treatments (water deficit and control conditions) at each of 5 environments. The inheritance of leaf EW was low (15%) due to a high environmental influence. The RILs grown under water deficit produced significantly higher EW content (19 to 30%) when compared to control. The leaf EW load significantly correlated with plot yield ($r=32\%$), DSI ($r=-40\%$), and leaf CT ($r=-32\%$) under water deficit conditions. In addition, EW and CT correlated with higher yield stability using DSI and across environments using Eberhart stability under water deficit. Novel and robust co-localized QTLs for the leaf EW, cooler canopies, DSI, and grain attributes were detected on 2B, 3B, 5A, 5B, 6B, 7A, and 7B. High LOD scores and co-localization of CT and DSI along with independent EW loci explaining 35%, 41%, and 31% phenotypic variation respectively were detected on chromosome 4A. Chromosome

3B was investigated with closed association of leaf EW and canopy temperature all across the chromosomal length. Chromosome 6B had significant SNPs associated with cooler canopies in Halberd (2.4°C) compared to Len. The Halberd parent played a role in donating major alleles for moisture stress tolerance whereas, Len donates major yield allelic variants. Many novel and robust QTLs were identified to dissect the crop performance under moisture stress conditions. These identified genetic loci conducive potential tools in strategic breeding approaches.

This dissertation is dedicated to my beloved parents, Mohammed Hussain & Rabiya Begum, and also to my loving and supportive wife, Nida Khan. The sacrifices that you made have provided the opportunity to achieve this honor. To all those who have supported and contributed to this achievement, I dedicate this work to you

ACKNOWLEDGEMENTS

I would like to extend a heartfelt thank you to Drs. Dirk B Hays and Amir Ibrahim for not only serving as my graduate advisors, but also as teachers. I also extend a warm appreciation to Drs. Bill L Rooney and Russell W Jessup for serving as members of my committee. I am grateful to Drs. Hays and Ibrahim for treating me as colleague and not just as a student. Drs. Hays, Amir Ibrahim, and Bill Rooney are excellent teachers and are responsible for imparting the knowledge to be a fortified plant breeder.

NOMENCLATURE

DSI	– Drought susceptible index
HSI	– Heat susceptible index
EW	– Epicuticular wax
CT	– Canopy temperature
MSHW	– Mean single head weight
KNS	– Kernel number per spike
TKW	– Thousand kernel weight
QTL	– Quantitative trait loci
RIL	– Recombinant inbred lines

TABLE OF CONTENTS

	Page
ABSTRACT	ii
DEDICATION	iv
ACKNOWLEDGEMENTS	v
NOMENCLATURE	vi
TABLE OF CONTENTS	vii
LIST OF FIGURES	ix
LIST OF TABLES	x
CHAPTER I INTRODUCTION AND LITERATURE REVIEW	1
1.1. Wax biosynthesis	3
1.2. Wax as a light reflective agent	4
1.3. Role of leaf epicuticular wax as drought tolerance trait	5
1.4. Leaf epicuticular wax vs canopy temperature	6
1.5. Wax as an environmental adaptation	8
1.6. Wax quantitative trait loci under moisture and heat stress	10
1.7. Rationale and objectives of the project	12
CHAPTER II THE ROLE OF LEAF EPICUTICULAR WAX IN IMPROVED ADAPTATION TO DROUGHT STRESS	13
2.1. Introduction	13
2.2. Materials and methods	16
2.2.1. Parents pedigree	16
2.2.2. Population development and experimental design	17
2.2.3. Agronomic and physiological measurements	18
2.2.4. Leaf EW quantification	18
2.2.5. Yield quantification	19
2.2.6. Statistical analysis	21
2.2.7. Stability analysis	22
2.3. Results	23
2.3.1. Precipitation	23

	Page
2.3.2. Wax inheritance.....	24
2.3.3. Leaf EW and CT vs yield potential.....	25
2.3.4. Leaf EW and CT vs yield stability.....	26
2.4. Discussion.....	30
2.4.1. Variation among RILs and heritability of leaf EW.....	30
2.4.2. Leaf EW and CT to improve potential yield.....	34
2.4.3. Leaf EW and CT to improve yield stability.....	36
2.5. Conclusions.....	37
CHAPTER III MAPPING THE GENETIC LOCI REGULATING DROUGHT ADAPTIVE TRAITS; LEAF EPICUTICULAR WAX, CANOPY TEMPERATURE, AND DROUGHT SUSCEPTIBLE INDEX IN <i>TRITICUM AESTIVUM</i> L.....	39
3.1. Introduction.....	39
3.2. Materials and methods.....	42
3.2.1. Population development.....	42
3.2.2. Phenotype measurements.....	43
3.2.3. Canopy temperatures.....	44
3.2.4. Yield measurements.....	44
3.2.5. Statistical analysis.....	45
3.2.6. Molecular analysis.....	46
3.2.7. Genetic linkage and mapping.....	47
3.3. Results.....	48
3.3.1. Genetic mapping.....	49
3.4. Discussion.....	60
3.4.1. Genetic linkage for agronomic and physiological traits.....	60
3.4.2. Genetic dissection of EW and CT.....	63
3.4.3. Potential SNPs for marker assistance selection.....	68
3.5. Conclusions.....	70
CHAPTER IV CONCLUSIONS.....	71
REFERENCES.....	73

LIST OF FIGURES

	Page
Fig. 1 Growing season precipitation data during 2011 and 2012 across three locations for water deficit and irrigation treatments for 180 Len X Halberd spring wheat RILs.....	24
Fig. 2 Epicuticular wax content and leaf canopy temperature were plotted against plot yield and mean single head weight with Regression analysis (R^2) and Pearson's correlations (r) for 180 RILs of Len X Halberd population during 2011 and 2012. MSHW – Mean single head weight, KNS-Kernel number per spike, TKW- Thousand kernel weight, HAL- Halberd, UV - Uvalde, grain yield and canopy temperature was taken from the Uvalde 2012 environment.	29
Fig. 3 Mean grain yield, leaf CT (Uvalde), and leaf epicuticular wax contents plotted against yield stability index with regression analysis and Pearson's correlations for 180 RILs of Len X Halberd population during 2011 and 2012. MSHW – Mean single head weight, KNS- Kernel number per spike, DSI – Drought susceptible index, Leaf CT-Leaf Canopy temperature, UVL - Uvalde. Grain yield and canopy temperature was taken from Uvalde 2012 environment.	32
Fig. 4 Genetic linkage groups constructed in the 180 RIL population derived from Len and Halberd cultivars for F8 & F9 generations, during 2011 & 2012 growing seasons. Identified co-localized QTLs were traced across different linkage groups of wheat genome with > 3 LOD scores and 10,000 permutations.	53
Fig. 5 The estimated positions of SNP markers on linkage group 4A associated with canopy temperature (CT), drought susceptible index - mean single head weight (DSIM), and colorimetric wax in 180 RILs derived from Len X Halberd cross. The high LOD score QTLs were detected in different locations (College station-CS and Uvalde-UVD) years (2011 and 2012).....	61
Fig. 6 QTLs contrast analysis on different chromosomes for wax content, canopy temperature (CT), drought susceptible index (DSI) for mean single head weight and thousand kernel weight (TKW) in a recombinant inbred line (RIL) population developed from a cross between drought tolerant line Halberd and drought susceptible line Len. Note that allelic variation at QTL identified for wax (Qwax), cooler canopies, DSI, and yield components (MSHW & TKW) were donated by Halberd.	62

LIST OF TABLES

	Page
Table 1 Combined mean square variance of entries and entry X environment interactions over five environments and broad sense heritability of agronomical and physiological traits for 180 Len X Halberd under water deficit during 2011 and 2012.....	27
Table 2 Pearson’s correlation coefficient between agronomic and physiological traits for individual and combined locations and years (2011 & 2012) for Len X Halberd RIL population.....	28
Table 3 Mean values and Eberhart’s stability describes five top and bottom Len X Halberd moisture stress treated RILs across 5 environments during 2011 and 2012.....	31
Table 4 Means of parents and range of RIL (Len X Halberd) across five environments for agronomic and physiological traits under moisture deficit conditions during 2011 and 2012.	50
Table 5 QTLs co-localized for yield components (MSHW-mean single head weight, TKS-thousand kernel weight, KNS-kernel number per spike, plot yield) and physiological traits (leaf colorimetric wax content, leaf canopy temperature (CT), drought susceptible index-Mean single head weight –DSI-MSHW) in the 180 Len X Halberd RILs mapping population across 5 environments during 2011 and 2012.	52
Table 6 QTL identified in more than one environments for agronomic and physiological traits in 180 Len X Halberd population during 2011 and 2012.	58
Table 7 Summary of significant QTLs detected for agronomic and physiological traits, linkage groups, positions and phenotypic variations across 5 environments during 2011 and 2012.	67
Table 8 Contrast analysis for QTLs with significant difference between moisture stress and control treatments across different environments and chromosomal linkage groups for different traits in 180 Len X Halberd RILs population during 2011 and 2012.	69

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Wheat (*Triticum aestivum*) is the staple food in many regions of the world. It is grown in a wide range of climates that includes tropical, subtropical, and temperate regions. The average temperature during reproductive and grain filling stages in the US great plains can reach 28 to 30⁰C (Assad and Paulsen 2002). Wheat is comprised of 10 to 20% proteins, 80% carbohydrates, providing 40 – 60% calories in the daily diet of developed nations. Water stress which occurs during grain filling is less harmful to yield reduction compared to water stress that occurs pre-anthesis (Musick and Dusek 1980). Heat stress (Hays et al. 2007) and moisture stress (Saeedipour and Moradi 2011) limit export and transport of sucrose and invertase leading to zygote abortion, reduction in kernel weight, kernel number, and subsequent yield loss. Wheat utilizes about 0.64 to 0.76 cm of moisture during the grain filling stage (Herbek and Lee 2009). The ideal situation to increase yield potential and stability under drought conditions would be to increase drought tolerance during the reproductive and grain filling period. Drought stress has wide impact on yield by reducing each of the following: the number of days to heading, grain filling period, number of days to maturity, plant height, number of heads m⁻², head length, number of grains per head, 1000 grain weight, protein content, and SDS sedimentation (Kilic and Yagbasanlar 2010). Water deficiency is the primary constraint in decreasing wheat yield potential globally. It is responsible for a 50-90% yield reduction on at least a 60 million ha area worldwide (Reynolds et al. 2000). By

2020, global wheat demand will rise by 40% while wheat production resources will decline (Pingali and Rajaram 1999; Rosegrant et al. 2001). Regionally, water deficit during 2011 resulted in the loss of 240 M bushels of winter wheat in the Southern Great Plains (Rudd 2011). Year to year yield fluctuations, due to water deficiency and high temperature stress, is very common in drought prone environments. In addition, scarce and erratic precipitation, decreasing water tables, increasing pest and disease infestation, weeds, and poor soils are major constraints to global wheat yields. Water deficit stress accompanied with heat stress leads to confounding effects followed by increased yield losses (Shah and Paulsen 2003).

Under water-deficit conditions, the survival of crops depend on the capacity of the aerial regions to resist dehydration. Various adaptations such as an elongated root system, reduced leaf area (with reduced turgor pressure), reduced stomatal conductance, reduced transpiration, reduced leaf abscission, and many other physiological traits in wheat lines have been selected to improve periodic water-deficit tolerance. Higher and more stable wheat yields have been reported to be associated with cooler canopies under high temperature stress (Rashid et al. 1999). Leaf epicuticular wax (EW) is one of the physiological drought adaptive mechanisms to improve moisture stress tolerance partially through reducing canopy temperatures during reproductive stages (Mondal 2013), pea (*Pisum sativum*) (Sánchez et al. 2001), and peanut (*Arachis hypogea*) (Samdur et al. 2003).

1.1. Wax biosynthesis

Wax is a complex mixture of very long-chain fatty acids, alkanes, aldehydes, primary and secondary alcohols, ketones, esters, triterpenes, sterols, and flavonoids. The biosynthesis of the leaf EW is a complicated and dynamically regulated process (Jenks et al. 2002). The initial process in wax biosynthesis begins with the elongation of the C₁₆ to C₁₈ fatty acid precursors with 2 carbons donated by malonyl-CoA to form long chain fatty acids. Elongase is the activities of an acyl chain undergoing the sequential reactions of condensation, reduction, dehydration, and a second reduction for each two carbon elongation (Post-Beittenmiller 1996). Very long chain fatty acids are hydrolyzed to free fatty acids by CUT1 enzymes (cuticular wax) which are further derivitized to alkanes, secondary alcohols, and ketones through decarbonylase enzymatic pathway or condensing primary alcohols with free fatty acids to form esters, used to generate the leaf EW in *Arabidopsis* (*Arabidopsis thaliana*) (Millar et al. 1999). The leaf EW in wheat is compose of hydrocarbons alkanes (9%), esters (9%), alcohols (17%), acids (3%), β-diketones (36%), hydroxyβ-diketones, and unidentified materials (17%) (Tulloch and Hoffman 1971). In one wheat study, a homologous series of wax components were analyzed and n-hentriacotane was found to be the largest component (>75%) followed by alcohols and fatty acids (<10%) (Cameron et al. 2006). Another study involving wheat wax by gas chromatography and mass spectrometry identified octacosan-1-ol as the most abundant organic chemical (Koch et al. 2006).

1.2. Wax as a light reflective agent

High irradiation differences in bean (*Phaseolus vulgaris* cv. Labels) resulted in large differences in total dry weight, specific leaf weight, and pigment concentrations (Deckmyn et al. 1994). The UV and longer wavelengths can either be reflected, absorbed, or transmitted and unabsorbed through the leaf surface (Woolley 1971). The absorption of visible wavelength (300 - 700 nm) depends on the amount of leaf chlorophyll, carotenoid, and anthocyanin pigments, but most of the high energy wavelength radiations (700 - 1300 nm) are not absorbed and much of it is reflected by leaf and soil surfaces (Knipling 1970). The leaf EW is a primary component that reflects and dissipates heat energy on leaf surfaces. The genetic variation of the leaf EW with different genes has been reported to have a significant impact on light reflectance and transmittance in sorghum (Grant et al. 1995). In addition, differences in reflectance and transmittance were found to be related to differences in canopy temperature and radiation (Grant 1987; Grant et al. 1995). In sorghum bloom and bloomless type, genetic lines had significant differences between transpiration and photosynthesis rate, with a 10 to 32% decrease in transpiration rate in bloom type compared to bloomless type (Chatterton et al. 1975). In wheat grass (*Thinopyrum intermedium*), hybrids contrasting glaucousness had a significant impact on leaf water potential and leaf reflectance of high radiation and canopy temperature within a crop micro environment (Jefferson 1994). Screening for drought tolerant lines is a difficult process. In addition, it is expensive to quantify the agronomic and physiological traits related to moisture and heat stress tolerance. Spectral reflectance indices and canopy temperature are considered to be a

potential new tool for the early generation selection of heat and moisture deficit tolerant lines (Babar et al. 2006; Olivares-Villegas et al. 2007). The leaf canopy temperature shown to be associated with increase water indices (WI) (Peñuelas et al. 1997) and grain yield (Babar et al. 2006) across many different genetic backgrounds. However; the role of leaf EW plays in cooling canopies and increasing water indices has yet to be studied.

1.3. Role of leaf epicuticular wax as drought tolerance trait

Final grain yield may be affected by various factors, including agronomic and phenological traits (González et al. 2007), physiological factors (González et al. 2008), planting geometry (Mohammed et al. 2012), among other factors. Some of the physiological factors, such as stomatal closure, leaf senescence, stay green, epicuticle thickness, photosynthetic rate, osmotic adjustments, reduced transpiration, and others can improve water stress and heat tolerance. The physiological trait with a bluish-green blanket of wax on the leaf epicuticle improves drought and heat tolerance in various crops (Baenziger et al. 1983; Sangam et al. 1998). EW plays a vital role in improving other physiological mechanisms such as increased water use efficiency, reduced transpiration, and also partly through increased light reflectance can increase overall yield under moisture stress conditions (Johnson et al. 1983). The leaf EW was shown to influence transpiration rate, canopy temperature, and harvest index during abiotic stress in the peas (Sánchez et al. 2001). Studies also show that plants adapted to hot climatic conditions possess a thick cuticle with reduced transpiration rates (Ristic and Jenks

2002). Epicuticular wax load was found to be higher in non-irrigated conditions compared to irrigated conditions in numerous crops. Total wax amount and its chemical components, specifically alcohols and aldehydes increased significantly under moisture stress conditions in sesame (*Sesamum indicum* L.) (Kim et al. 2007a) and soybean (Kim et al. 2007b). Also, the periodic drying of tobacco (*Nicotiana glauca* L. Graham) leaves resulted in an increase of total wax load by 1.5 – 2.5 fold and an increase of 6-fold for lipid transfer protein gene transcripts in the extracellular matrix (Cameron et al. 2006). Further drought studies on barley (*Hordeum vulgare* ssp. *spontaneum*) showed a 9% increase in mean leaf EW deposition in water stressed lines compared to non-water stressed lines while the mean residual transpiration rate increased by 20% in irrigated lines compared to non-irrigated lines (González and Ayerbe 2010).

1.4. Leaf epicuticular wax vs canopy temperature

Canopy temperature (CT) is a physiologically integrated trait associated with drought and heat tolerance in wheat (Mason et al. 2011), rice (*Oryza sativa*) (Srinivasan et al. 2008), and sorghum (Arnold et al. 1988). It is highly influenced by cloudy and windy weather. It is a very sensitive, simple, rapid, highly heritable, and practical tool that breeders can use to screen early generations and advance lines (Araus et al. 2002; Mason et al. 2011; Rees et al. 1993; Richards 2000). It is significantly and consistently correlated with grain yield across environments (Olivares-Villegas et al. 2007). CT is genetically and phenotypically highly correlated with stomatal conductance across

environments (Rebetzke et al. 2012), and residual transpiration rate in pea (Sánchez et al. 2001), and therefore can serve as a breeding tool for indirect selection of leaf porosity, stomatal conductance and grain yield. The CT relationships with the colorimetric leaf EW content has not been well documented in wheat or other crops.

The leaf glaucousness, a visible expression of EW composition, as a physiological adaptation to water deficiency has been reported in several plant species. Leaf EW preserves water loss by reflecting and reducing solar radiation on the leaf surface of sorghum (*Sorghum bicolor*) (Blum 1975). A thick EW layer and glaucousness in pea cultivars has been shown to reflect UV radiation (400-700 nm wave lengths) from the leaf surface and therefore, reduce the leaf and transpiration rate under water deficit conditions (Sánchez et al. 2001). Wax coated leaves have a significant effect on decreasing CT in pinus seedlings (*Pinus sp*) (Thames 1961). A significant association exists between leaf glaucousness, reduced leaf CT, and grain yield has been reported in wheat (Clarke et al. 1994; Johnson et al. 1983).

Leaf EW and reduced CT may improve the potential grain yield and yield stability, that is otherwise highly unstable under water stress conditions (Smith 1982; Sojka et al. 1981). CT with significant trait segregation has a high inheritance and potential marker-assisted selection tool, and has been shown to be linked to increase yield or high yield stability across stress environments (Olivares-Villegas et al. 2007; Rashid et al. 1999). Canopy temperature depression was found to be robustly associated with grain yield and co-localized with yield QTLs (Mason et al. 2013). Other physiological traits, including osmotic adjustments have a dual role of improving

potential yield and yield stability across environments (Eberhart stability) (Fischer et al. 2005). Leaf EW may be a component of CT that has synergistic effects on improving potential yield and maintaining stability under moisture deficit condition.

Variation in yield between stress and non-stress moisture environments have been used to calculate a drought susceptibility index (DSI). DSI when used functions as a yield stability indicator within an environment (Fischer and Maurer 1978). This yield stability indicator, obtained due to difference between environments, can also be estimated using a joint linear regression method (Eberhart and Russell 1966; Finlay and Wilkinson 1963). Eberhart's stability is determined based on a regression slope between higher and lower values across environments. Then an environmental stability index value can be calculated for each individual genotype. DSI has been shown to be negatively correlated with grain yield under stress environments and only slightly positively correlated under non-stress environments in barley (Ceccarelli 1987). The smaller the DSI values the better the yield stability and drought tolerance. The relationships of the DSI, CT, and leaf EW have not been reported in wheat, however, grain yield has been shown to be directly correlated with wax content under water stress conditions in barley (González and Ayerbe 2010).

1.5. Wax as an environmental adaptation

The leaf EW load is highly influenced by environment, such as in barley where a genotype's EW load varies across years and locations (González and Ayerbe 2010).

Little is known about the inheritance of leaf EW load. Some studies indicate that non-glaucousness based scoring is controlled by a single dominant gene but glaucousness is controlled by the same genes or tightly linked genes (pleiotropy or linkage). The inheritance of wax load across generations was found to be poor and not well understood in durum wheat (*Triticum turgidum* L., *vardurum*) (Clarke et al. 1994). The inheritance of glaucousness in spikes and foliar tissue is separate and was influenced by different environmental factors (Johnson et al. 1983). The genetic variance for sorghum leaf EW was stable and the narrow sense heritability was 0.36, but the EW was highly adapted to environmental conditions (Jordan et al. 1983). In other studies in rice (*Oryza sativa*) (Haque et al. 1992), leaf EW trait has been shown to be polygenic, controlled by many genes that give an additive effect .

A study of physiological mechanisms relating leaf EW as a drought adaptive trait with increased yield stability under moisture deficit conditions is important. Genes segregating for colorimetric leaf EW concentration in various individuals with common genetic backgrounds may decrease the genetic complexity and may improve the determination of genetic variance and wax inheritance. The relationship between colorimetric leaf EW, CT, DSI, and yield stability (Eberhart's stability) across multiple environments has yet to be elucidated. Such knowledge would lead to a better understanding of one key physiological drought adaptive mechanism.

1.6. Wax quantitative trait loci under moisture and heat stress

Numerous water deficit and heat stress studies have been done to identify the genes (QTL) related to various physiological mechanisms, such as leaf glaucousness (Bennett et al. 2012b), cooler canopies (Olivares-Villegas et al. 2007; Pinto et al. 2010), and high energy wavelength reflectance and transmittance (Babar et al. 2006). Studying QTLs related to moisture deficit environment has been challenging and has produced confounding results due to irregular conditions. In addition, plant phenology (maturity, tillers), water use efficiency (Blum 2005), plant geometry (Mohammed et al. 2012), completion of life cycle before onset of moisture deficit (Chaves et al. 2003), remobilization of photosynthetic assimilates (Turner 1979), floral infertility (Passioura 2007), and seed abortion (Hays et al. 2007) may all deflect genotypic and phenotypic trait associations. In spite of all these challenges moisture deficit associated yield QTL have been identified, but few genes have been cloned or deployed to date. Dissecting yield under moisture deficit through varied physiological and agronomic traits under accurate moisture deficit conditions would be a conducive strategy to screen for potential markers (Fleury et al. 2010).

Many attempts to study the leaf EW genetic variability and stability under different genetic backgrounds have been conducted in wheat (Mondal 2013), sorghum, and rice (Srinivasan et al. 2008). The inheritance of leaf glaucousness in wheat (*WI*), is dominant over non-waxy genes (*Iw1*) with an epistatic effect, and were located on the short arm of the chromosome 2D (Driscoll 1966; Tsunewaki and Ebana 1999; Watanabe et al. 2005). A solitary novel QTL for flag leaf glaucousness with positive allelic effect

from the heat tolerant Halberd was identified on chromosome 5A (Mason et al. 2010). Another novel and robust waxy QTL was localized on the 3A chromosome of a RIL population explaining 52% of phenotypic variations (Bennett et al. 2012b). EW trait was assumed to be correlated with CT and NDVI, significant co-localized loci for these traits were detected on 3B, 4A, and 1B genetic linkage groups (Pinto et al. 2010). A recent study in our program has identified two additive effects of leaf EW loci on 5A and 1B that co-localized with leaf temperature depression and heat susceptibility index (HSI) for kernel weight and main single spike weight (Mondal 2013).

The phenotypic relationships and genetic overlap among EW, CT, DSI, and grain yield is not well documented and sparsely studied. Mapping QTL in bi-parental populations is one method to dissect the relationship between these complex traits (Marza et al. 2006). Earlier studies using bi-parental populations have identified QTL related to HSI on chromosomes 1A, 2A, 2B, and 3B that co-localized with visual wax glaucousness (Mason et al. 2010). In another wheat study, 7 distinct loci were identified to co-localize for yield and temperature depression (Mason et al. 2011). Interestingly, recent studies reported the cloned wheat Lr34 and Yr36, two adult plant resistance genes for pathogenically diverse leaf, stripe, and stem rust may be associated with wax genes (protein related lipid transfer domains) (Fu et al. 2009; Krattinger et al. 2009) though the connection has not been proven. The pleiotropic effects of leaf EW with biotic and abiotic traits could be a potentially useful tool to aid in screening for drought adaptive traits with high yielding lines.

1.7. Rationale and objectives of the project

Breeding wheat for water stress tolerance by integrating complex traits such as leaf EW and stable grain yields, has proven difficult as such polygenic traits are challenging to phenotype and are influenced by environment (Smith et al. 1990). Studies to elucidate the behavior of leaf EW layer and how its complex structure reduces the effect of water and heat stress on wheat physiology have shed little light on the relationship. *We hypothesize that increased leaf epicuticular wax results in cooler canopies and is critically adaptive trait that can be used to improve wheat moisture stress tolerance. We further hypothesize that leaf wax and cooler canopies can improve yield potential and stability under moisture deficit conditions across environments.*

The objectives of this study are:

- 1. Determine the relationship between increased leaf epicuticular wax with cooler canopies and increased potential-stable yields.*
- 2. Identify QTL associated with increased leaf epicuticular wax and its overlap with potential-stable yield during heat and water deficit stress.*

CHAPTER II
THE ROLE OF LEAF EPICUTICULAR WAX IN IMPROVED ADAPTATION
TO DROUGHT STRESS

2.1. Introduction

Wheat (*Triticum aestivum* L.) is globally grown over large areas that cover the tropical and temperate regions of the world with an approximate production of 695 MT in 2013. The world wheat production in 2013/14 is projected to be 701 MT, or 7% more than the current production (FAO 2013). World water demand has tripled over the past 50 years, while water tables are depleting at a faster rate in, heavily irrigated regions of the US Southern Great Plains. Aquifer depletion is faster at 31 to 76 m depths, as a 4 m depletion leads to a 9% reduction in aquifer storage (USGCRP 2009). Depletion of the water table and inadequate precipitation have had the greatest impact on the potential production of wheat. Pre-anthesis, increased maturation, inhibition of starch movement to seed synthesis, seed abortion, and poor seed settings have been identified in local elite wheat cultivars susceptible to heat and water-deficit conditions (Bhullar and Jenner 1985; Hays et al. 2007; Weldearegay et al. 2012). The demand for wheat production has been increasing yearly, but few genetic gains in heat- and water-stressed tolerance have been achieved. An improvement in drought tolerance would help to maintain socioeconomic stability across the globe.

Leaf epicuticular wax (EW) is a bluish-green covering on the adaxial and abaxial leaf surfaces that appears during the early reproductive stage and is associated

with increased drought tolerance in rice (*Oryza sativa*) (Haque et al. 1992), maize (*Zea mays*) (Meeks et al. 2012), barley (*Hordeum vulgare*) (Febrero et al. 1998), wheat (Bennett et al. 2012a), sorghum (*Sorghum bicolor*) (Jordan et al. 1983), and many other crops (Baenziger et al. 1983). In addition to the leaf surface, it is present on the peduncle, leaf sheath, stem sheath, and other parts of the plant. It acts as a hydrophobic barrier between the leaf epicuticle and the surrounding environment (Bird et al. 2007). It may resist the movement of moisture flow, and impede leaf-feeding insects, and pathogenic fungi (Eigenbrode and Espelie 1995). Leaf EW and its varied composition have significant impacts on stomatal or epidermal conductance in wheat (Araus et al. 1991) and relative water content or decreased transpiration in *Jatropha mallissima* (Figueiredo et al. 2012) and improved water-use efficiency in peanut (*Arachis hypogea*) (Samdur et al. 2003) and wheat (Johnson et al. 1983). It also influences canopy light reflectance of high-energy wavelength; studies in barley conclude that photosynthetically active regions (PARs) have a high percentage of reflectance difference at 560-nm wavelength between glaucous and non-glaucous lines (Febrero et al. 1998).

The conjecture exists that because EW is interrelated with the different physiological traits, it may also decrease leaf canopy temperature (CT) and drought susceptible indices (DSI) while increasing yield stability. Leaf CT is an integrated trait that is being used as an early generation selection tool (Olivares-Villegas et al. 2007; Pinto et al. 2010), and that is also significantly correlated to DSI (Blum et al. 1989; Rashid et al. 1999) and leaf EW (Mondal 2013). In pea cultivars, EW influences grain yield indirectly

by improving harvest index, and decreasing residual transpiration rates, and leaf CTs under water-deficit conditions (Sánchez et al. 2001). Lower CTs also strongly associated with increased grain yields, serving as a high throughput phenotyping tool for mapping populations under moisture stress conditions (Olivares-Villegas et al. 2007). Increased leaf EW, may compensate for increased stomatal conductance, to increase leaf temperature depression and yield stability under heat stress conditions (Mondal and Hays 2007). Yield stability can be determined within an environment (DSI and HSI) (Blum et al. 1989; Fischer and Maurer 1978) and between different environments (Eberhart and Russell 1966). The possible phenotypic correlations among EW, CTP, DSI, and stable yields may be expressed in the co-localized QTLs. These pleiotropic loci may provide marker assisted selection tools and help in the rapid advancement of water-deficit and heat-tolerant wheat cultivars. The inheritance of glaucous and non-glaucous which is an expression of EW composition is influenced by a single co-dominant allele in durum wheat (Clarke et al. 1994). In another study, non-glaucousness was shown to be controlled by a single dominant allele (Liu et al. 2007). The additive gene action of glaucousness was also identified in durum wheat (Clarke et al. 1994) and bread wheat (Stuckey 1972). In rice, EW was determined to be inherited as a polygenic trait (Haque et al. 1992). In maize, the heritability of leaf EW based on leaf area was estimated to be 0.41 in (inbred) and 0.59 (hybrid lines) (Meeks et al. 2012).

EW content was shown to increase under water-deficit conditions when compared to controlled conditions in some crops such as peanut (Samdur et al. 2003), oat (*Avena sativa*) (Bengtson et al. 1978), wheat (Johnson et al. 1983), rice (Haque et al.

1992), and sorghum (Blum et al. 1989). The abaxial leaf surface has a higher wax content and lower stomatal conductance when compared to the adaxial leaf surface and it is also highly influenced by environmental factors (Araus et al. 1991).

The present study aims (i) to evaluate the genetic variability and inheritance of leaf EW and (ii) to determine the drought-adaptive role of increased leaf EW under different water-deficit conditions and its correlation with lower leaf CT, DSI, and increased yield stability in a recombinant inbred lines (RILs) population.

2.2. Materials and methods

2.2.1. Parents pedigree

RILs of 180 individuals were derived from two parents, Halberd and Len. Halberd is an Australian spring wheat (*Triticum aestivum* L) donor cultivar developed at Roseworthy Agricultural College in 1969 with the pedigree Scimitar/Kenya/C6042/Bobin/2/Insignia49 (Paull et al. 1998). Halberd was one of the dominant Australian cultivars during twentieth-century wheat production with boron tolerance (Paull et al. 1992), durable rust resistance alleles (Bariana et al. 2007), drought tolerant, and ability to maintain carbohydrate accumulation during moisture stress (Ji et al. 2010). Len is a hard red spring wheat cultivar developed in North Dakota in 1979 with the pedigree ND499/3/Justin/RL4205/W1261 that was originally called ND543 (Grain Genes database). Len was evaluated as moderate leaf and stem rust resistant and developed

from the sister line Thatcher with adult plant resistance enhanced by the *Lr34* gene (Kolmer et al. 2011). Len is semi-dwarf that is drought and heat susceptible (Hossain et al. 2012), with low glossy leaves and good agronomic traits. The two parents were chosen due to similarities in flowering period and maturity.

2.2.2. Population development and experimental design

The US elite line Len as a female was crossed with abiotic stress-tolerant cultivar Halberd as the donor male parent. The RIL were advanced via single seed descent in a greenhouse environment to the F₅ generation. Seeds from the F₅ generation were bulked to develop 180 F_{5:6} RILs. The F₆ lines were advanced in the field for yield trials and were evaluated during 2010 as an F_{5:7} generation. During 2011 and 2012, generations F₈ and F₉ were used, respectively, to conduct experiments. Yield trials were conducted at Uvalde, College Station, Chillicothe (USDA TX-ARS research stations), and International Maize and Wheat Improvement Center (CIMMYT northwestern Mexico) Ciudad de Obregon during 2011. During 2012, two trials were conducted at the Uvalde and College Station Agrilife research stations with two treatment, control and water-deficit conditions, with each treatment replicated twice. Halberd, Len, and the RIL were randomized within each replication and treatment. Irrigation was similar for both control and water-deficit treatments until the initiation of stem elongation, at which point irrigation was stopped for the water-deficit treatment (Fig. 1). The water that was supplied for the control treatment was ≈ 366 mm, and the water-deficit treatment was \approx

170 mm at all environments. Irrigation was supplied with a drip irrigation system (I – tape) with a 2.54-cm-diameter and with 0.15-m emitter spacing. Fertilizer was supplied as weeds, diseases, pests, and birds damaged were controlled. Plots were 1.5 × 3 m in dimension with 6 rows, and spaced at 10 cm. Each plot planting was standardized to 1800 kernel number. The RILs were randomized uniformly with an alpha lattice design (13 × 24) in each replication within control and water-deficit treatments independently. Mean average precipitation and total amount of water applied at each of the locations were recorded during 2011 and 2012 (Fig. 1).

2.2.3. Agronomic and physiological measurements

Physiological traits measured at 10 DAP (days after pollination) are leaf canopy temperature (CT) and leaf epicuticular wax (EW). The leaf CT was measured using a portable infrared thermometer (Fluke 561 IR). The thermometer gun was focused at the lateral sides of the plot canopy at a 45° angle horizontally for 30 s. Measurements were always taken at a specific time between 1 PM and 3 PM. The CT measurements were recorded during hot, sunny, non-cloudy, and low windy days.

2.2.4. Leaf EW quantification

Flag leaf discs of 1-cm diameter were punched at approximately 10 DAP. The leaf disc punches were collected into vials without disturbing adaxial and abaxial leaf

EW content, and the sample vials were air dried to avoid pathogen infections and stored at -20°C before wax extraction.

The colorimetric method is based on exchange of wax color produced based on a reaction with an acidified $K_2Cr_2O_7$ reagent (Ebercon et al. 1977). To extract wax, the samples were immersed within 1 ml chloroform for 30 sec and transferred into a separate 2-ml vial. The chloroform was air dried in a hood. Then, 300 μ l of the acidic potassium dichromate volume was added to each vial, and the vial was heated at 100°C in a water bath for 30 min. Next, 700 μ l of deionized water was added to each of the vials, and the color was allowed to develop for 1hr. The optical density of the sample was then measured at 590 nm using a plate reader (BMG-Labtech PHERAstar^{plus}). Each sample of 100- μ l volume was replicated 3 times and then loaded and recorded in a 96-well U-shaped ELISA microplate (Greiner bio-one GmbH). A standard curve was prepared using a serial dilution technique from 20 randomly selected Halberd flag leaves. The resulting linear standard curve equation was used to determine the wax concentration of samples.

2.2.5. Yield quantification

Plant height was measured in cm by placing a ruler in the center of each plot from the ground to the top of main spike head. Measurements were taken after complete plant maturity, when leaves were completely dry and grain ready to harvest. Awn data

was taken followed by plant stand (1-10) base scale and bird damage (%) (co-variate data).

Kernel plot yield (gm^{-2}), thousand kernel weight (g) (TKW), and kernel number per spike (KNS) were estimated by harvesting 50 heads at each plot. Mean single head weight (MSHW) was the average weight of seed from 50 heads harvested from each plot. Main heads were harvested from the central region of the plot area uniformly for all RILs, excluding secondary tiller heads. Grain weight for 100 kernels was measured using a seed-counting machine (SeedBuro TM 801 Count-a-Pak) and weighed to calculate the TKWs (g).

DSI was calculated based on the individual and mean grain yield RILs under control and water-deficit conditions (Fischer and Maurer 1978) for MSHW and TKW. The individual RILs with a $\text{DSI} < 1$ are considered drought resistant (good yield stability), and the RILs with $\text{DSI} > 1$ are considered as drought susceptible (poor yield stability). The DSI was calculated using the following formula:

Equation 1 $\text{DSI} = [1 - (Y_{s1}/Y_{p1}) / 1 - (Y_{s2}/Y_{p2})]$

Where Y_{s1} = grain yield under water-deficit treatment (stressed environments)

Y_{p1} = grain yield under control treatment (non-stressed environments)

$1 - (Y_{s2}/Y_{p2})$ = Stress intensity calculated based on the mean of grain yield under stressed (Y_{s2}) and non-stressed (Y_{p2}) environments.

2.2.6. Statistical analysis

PROC CORR (Pearson's correlation method) (SAS Institute Inc., Cary, NC, USA) was used to analyze the agronomic and physiological trait correlations across different environments, as Pearson's correlation is preferred for parametric data (Isobe et al. 1986). The trait uniform frequency distributions across population were analyzed using Proc Univariate SAS codes. A test for normality was done for each of the traits across individual locations and years, variance across environment were homogeneous, combined analysis was performed together across all environments. PROC GLM and PROC MIXED models were used to perform the analysis of variance test. Variance components—mean square of genotype (σ_g^2), mean square of genotype \times environment (σ_{gxe}^2) interactions, model error/experimental mean square error (σ_{error}^2)—were used to calculate the broad sense heritability (2008 SAS version 9.1). Entry and environment were considered as random model. The broad sense heritability was calculated based on an entry mean basis method using the following formula:

Equation 2
$$H^2 = \frac{\sigma_g^2}{\sigma_{error}^2 + \sigma_{gxe}^2 + \sigma_g^2}$$

2.2.7. Stability analysis

To analyze the water-deficit tolerance and the stability of different genotypes across environments (locations and years), the (Eberhart and Russell 1966) equation was used to calculate beta regression slope (β) values for each individual RIL for different yield components and leaf EW. AGROBASE system software and SAS 9.3 version (SAS 2011) were used to analyze the different stability indices for each trait across environments. The stability of each genotype is judged by the least variation in yield loss across the environments. It is determined as the regression slope of each individual entry by the mean yield of all entries recorded at various moisture levels of all different locations. Individuals with a stability index value ($\beta = 1$) are more stable than are individuals with $\beta >/< 1$ (Eberhart and Russell 1966) (Table 3). Individuals with a value of $\beta = 1$ and $sd = 0$ (Lin et al. 1986) will be rated as 10 (high stability) and individuals with β and sd values deviating from 1 and 0 respectively, are considered to be less stable. The Eberhart index calculates RIL stability between environments, and the DSI calculates stability within an environment for different moisture regimens. The following equation was used to calculate Eberhart stability;

Equation 3 Deviation from regression mean square (Lin et al. 1986)

$$\delta_i^2 = \left[\frac{1}{(q-2)} \right] [(Y_{ji} - \bar{Y}_i)^2 - b_i^2 \sum (\bar{Y}_{.j} - \bar{Y})^2]$$

Equation 4 Regression coefficient (Ali et al. 2012; Finlay and Wilkinson 1963)

$$\beta = \frac{1 + [\sum(y_{ji} - \bar{y}_i - \bar{y}_j + \bar{y}_{..})(\bar{y}_j + \bar{y}_{..}) / \sum(\bar{y}_j - \bar{y}_{..})^2]}{2}$$

Y_{ij} = Response variable for individual i in j th environment, b = Regression slope,
 q = number of environments, $\bar{y}_{.i}$ = the mean of i th family, $\bar{y}_{.j}$ = the mean of the j th
environment, \bar{Y} = predicted variable, $\bar{y}_{..}$ = overall mean

2.3. Results

2.3.1. Precipitation

The year of 2011 was the best water-deficit experimental year; ≤ 5 cm precipitation at Uvalde and Chillicothe, ≤ 10 cm at College Station, and 0 cm precipitation at Obregon were recorded during the crop-growing season (Fig. 1). Maximum air temperature during the reproductive stage ranges between 34°C and 43°C at Obregon, 35°C and 40°C at Uvalde, 28°C and 39°C at Chillicothe, and 34°C and 37°C at College Station. In addition to drought, bird damage impacted plot yield in Uvalde during 2011. Substantial significant differences were observed between the water-deficit (170 mm) and the control irrigation (366 mm) treatments for physiological and agronomic variables (Fig. 1) (Jefferey 1995).

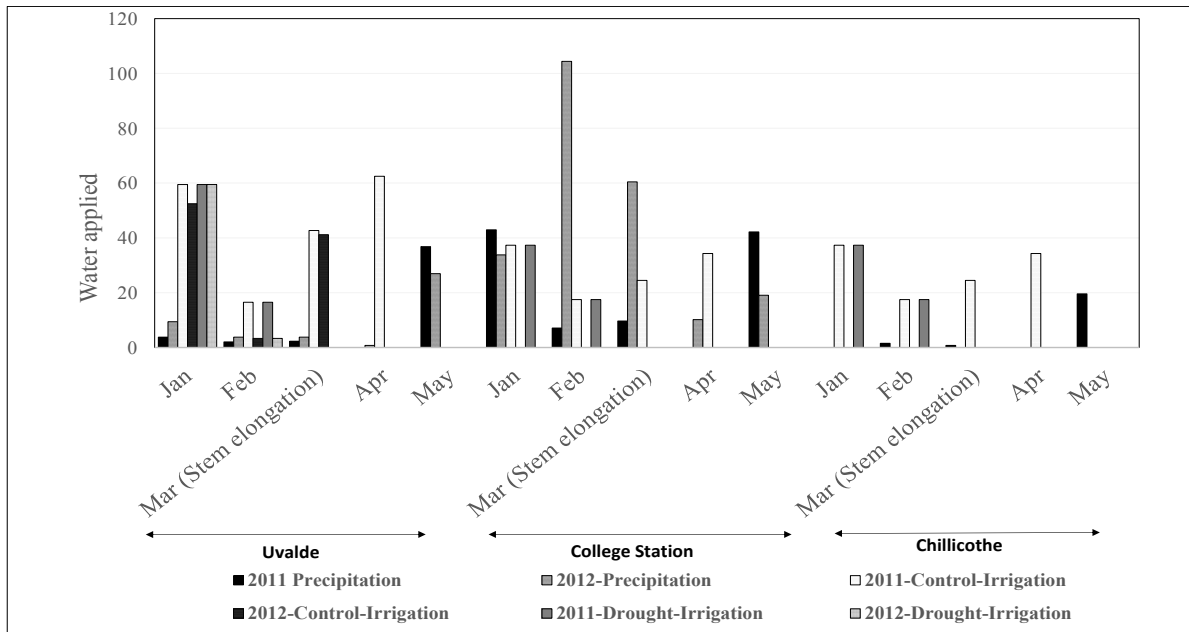


Fig. 1 Growing season precipitation data during 2011 and 2012 across three locations for water deficit and irrigation treatments for 180 Len X Halberd spring wheat RILs.

2.3.2. Wax inheritance

The RILs across years and locations varied significantly for all traits measured. Under water deficit, plot yield and EW showed transgressive segregation for over an approximate 15 RILs and lower DSI was recorded in 27 and more individuals compared to the mean of the better parent (Halberd) (Table 1). The DSI of MSHW (6.09 to -8.3) and plot yield (1.8 to -0.5) had higher range values at Uvalde in 2011 (Table 1). Broad sense heritability (H^2) was calculated based on entry mean basis for phenotypic traits under water stress and irrigated treatments by considering (replications, environments, entry \times environments) as random and entries as random (Table 1). The trait heritabilities under the control treatment were higher when compared to the water stress treatment

(data not mentioned), which may be due to the significant entry \times environment interaction. The leaf EW deposition did not show significant entry \times year interactions, although 2011 was drier and produced more wax than 2012 (Fig. 2I). The EW has an additive effect with poor broad sense heritability (0.15), since the mean squares entry \times environment was higher (Table 1). Thus, major entry \times environment interactions would mask the effect of genetic variance, and the correlations between genotype and phenotype would be reduced (Romagosa and Fox 1993). Even the EW Eberhart stability index value increases with the increase of EW content on leaf surface and explains the unstable nature of the wax across environments (Fig. 2F), thus elucidating the environmental adaptive nature of the leaf EW content. All other traits (MSHW – 0.39, KNS – 0.39, TKW – 0.39, height – 0.80, and CT – 0.40) displayed low to high inheritance across environments with significant entry \times environment interactions (Table 1).

2.3.3. Leaf EW and CT vs yield potential

The leaf EW and cooler canopies correlate negatively with each other ($r=-0.32$, $P\leq 0.001$) and even associate and influence the potential yield significantly under moisture stress conditions (Table 2). The RILs with an intermediate wax amount in the center ranges (3.5 to 5 mg dm⁻²) resulted in an increased total yield and yield components with a consistent positive response (Table 2 and Fig. 2A, B, E, F). These intermediate wax amounts have a significant impact on yield and are even more stable

across different environments. The RILs with low and high amounts of EW are unstable across environments and had a lower influence on the yield components (Figs. 2 and 3).

In addition to EW, cooler canopies which is partly regulated by EW also acts as a drought-adaptive trait increasing different yield components under moisture stress (Table 2 and Fig. 2C, D, H). Thus, leaf EW and CT both have a synergistic impact on yield increment under moisture-deficit conditions. The mean of EW for the top 6 RILs increased the MSHW with an improvement of yield stability. However the top 6 RILs for wax are less stable than bottom 6 RILs for wax Eberhart stability (Table 3).

2.3.4. Leaf EW and CT vs yield stability

Yield stability index values within a location (DSI) and across locations (Eberhart's) for most of the entries lay between 0 and 1 (Fig. 3). The RILs with intermediate wax load may not have a greater influence or adaptability to environment, as they are more stable and have values closer to 1 and even result in cooler canopies and higher mean yields (Fig. 2 and 3). The genetic stability of the leaf EW varied significantly and highly adapted to environmental conditions in sorghum (Jordan et al. 1983)

Table 1 Combined mean square variance of entries and entry X environment interactions over five environments and broad sense heritability of agronomical and physiological traits for 180 Len X Halberd under water deficit during 2011 and 2012.

	df	Mean square			Broad Sense Heritability (H ²)
		Entry	Entry X Environments	Error	
MSHW	181	0.039***	0.024**	0.015**	0.39
TKW	181	31.94***	24.69**	11.01**	0.23
Height	181	304.56***	50.45**	43.00**	0.80
KNS	181	38.91***	25.81**	18.04	0.39
Head Number	181	8901ns	9263ns	10060ns	-
CT	181	5.10***	3.07**	2.22**	0.40
EW	181	3.03**	2.66*	2.28**	0.15

*** Significance at < 0.001, ** significance at < 0.01, * significance at < 0.05, MSHW – Mean Single Head Weight, TKW – Thousand kernel weight, KNS- Kernel number per spike, CT – Canopy temperature, EW – Leaf epicuticular wax.

Table 2 Pearson's correlation coefficient between agronomic and physiological traits for individual and combined locations and years (2011 & 2012) for Len X Halberd RIL population.

Location	Year	EW- MSHW	EW- TKW	EW- KNS	EW-DSI MSHW	EW -CT	CT- MSHW	CT- KNS	MSHW-DSI MSHW	Yield – Head No.	SKW- Head No.
UV	2011	ns	-----	-----	ns	-0.32**	ns	-----	-----	0.38**	-0.10ns
	2012	0.19**	0.25**	0.33**	-0.30**	-0.28**	-0.26**	-0.10ns	-0.48**	0.89***	0.07ns
CS	2011	ns	ns	0.27**	-0.17*	-----	-----	-----	-0.53**	0.36**	-0.02ns
	2012	0.15*	0.18*	0.20*	-----	-0.19*	-0.40**	-0.38**	-----	-----	-----
Combined	2011-2012	0.26**	0.26**	0.32**	-0.40**	-0.28**	-0.36**	-0.20**	-0.35**	0.58***	-0.45***
UV-Yield	2012	0.27**	-----	-----	-----	-0.32**	-0.42**	-----	-0.74**	-----	-----
OB-Yield	2011	0.30**	-----	-----	-----	-----	-0.23**	-----	-----	-----	-----

** Significance at < 0.0001, * significance at < 0.001, ns – non-significant, ___ no data available. (MSHW – Mean single head weight, CT – Leaf canopy temperature, DSI – Drought susceptible index, EW – Leaf epicuticular wax, DSIMSHW – Drought susceptible index for mean single head weight, TKW – Thousand kernel weight, DSI-TKW – Drought susceptible index thousand kernel weight, KNS – Kernel number per spike. SKW – Single kernel weight, Head No. – Number of heads per m²).

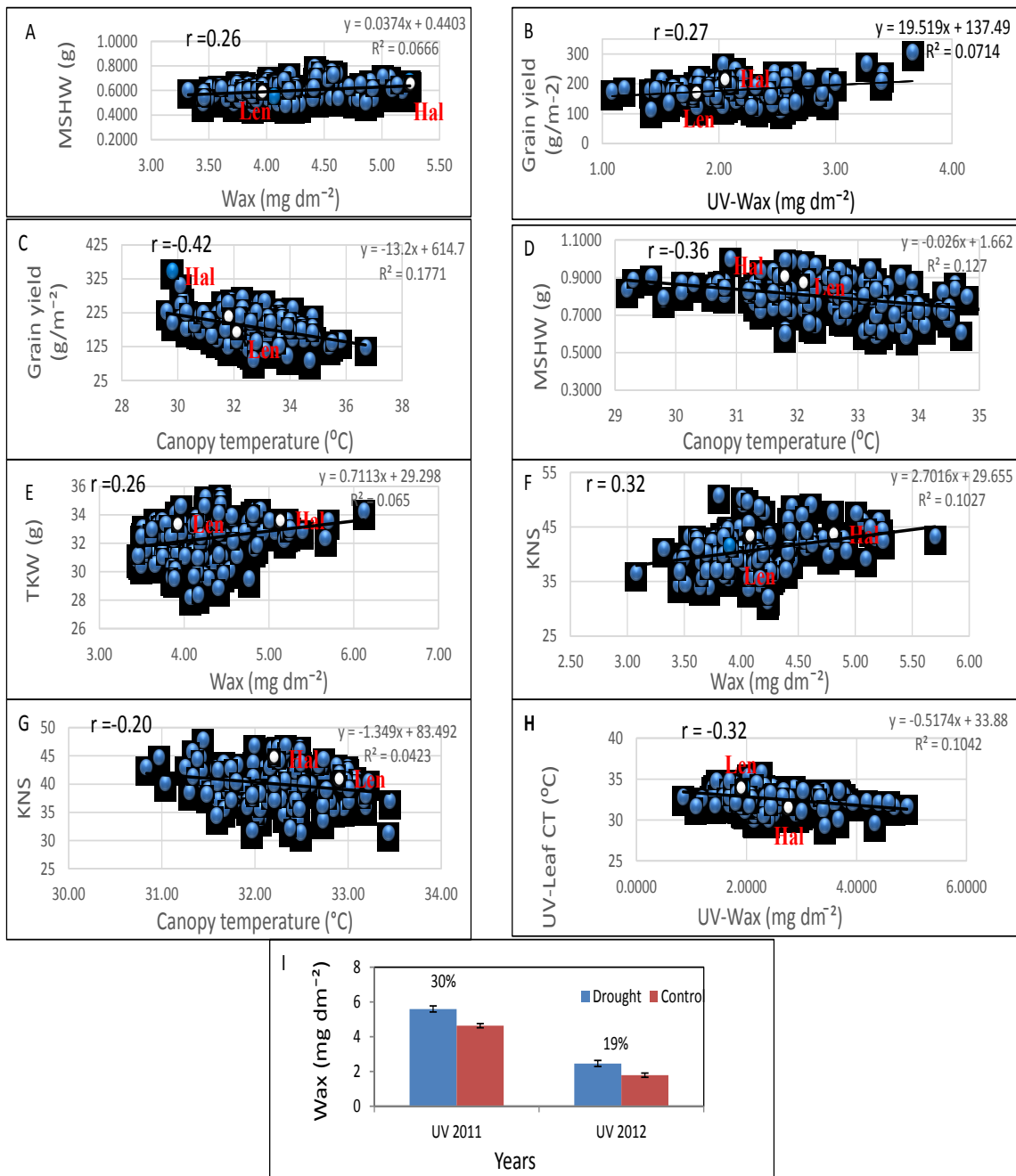


Fig. 2 Epicuticular wax content and leaf canopy temperature were plotted against plot yield and mean single head weight with Regression analysis (R^2) and Pearson's correlations (r) for 180 RILs of Len X Halberd population during 2011 and 2012. MSHW – Mean single head weight, KNS-Kernel number per spike, TKW- Thousand kernel weight, HAL- Halberd, UV - Uvalde, grain yield and canopy temperature was taken from the Uvalde 2012 environment.

Halberd illustrated higher yield stability index when compared to Len within and across environmental conditions and under water deficit and control treatments (Figs. 2 and 3) (Hays et al. 2007; Mason et al. 2011; Mason et al. 2010; Mondal and Hays 2007). The RILs with higher yield potential displayed higher yield stability index within (DSI) and across environments (Eberhart's) (Fig. 3A, B). The leaf EW correlated negatively with the DSI of MSHW ($r=-0.40$, $P\leq 0.001$) and with the Eberhart stability index of MSHW ($r=-0.26$, $P\leq 0.001$) and KNS ($r=-0.24$, $P\leq 0.001$) (Fig. 3C, D, E), combining better moisture conservation with improved grain setting and grain filling formation during reproductive stages. In addition to the leaf EW content, leaf CT improved yield stability and correlated positively with yield according to Eberhart's stability index ($r=0.24$, $P\leq 0.001$) and DSI ($r=0.37$, $P\leq 0.001$) (Fig. 3H). The RILs with the intermediate leaf EW content are considered to be more stable for different yield components such as MSHW, KNS, and TKW (Fig. 3C, D, E).

2.4. Discussion

2.4.1. Variation among RILs and heritability of leaf EW

A RIL population from a cross between spring wheat lines Halberd and Len has been developed to investigate phenotypic and genotypic correlations between physiological and agronomic traits under water-deficit conditions in Southern Texas and Northern Mexico. Halberd exhibits significantly ($P\leq 0.01$) higher EW

Table 3 Mean values and Eberhart's stability describes five top and bottom Len X Halberd moisture stress treated RILs across 5 environments during 2011 and 2012.

Rank	Entry	CT (°C)	MSHW- β	MSHW (g)	EW- β	EW (mg dm ⁻²)
Top						
1	LXH_6	33.17	4	0.79	6.5	4.42
2	LXH_118	32.89	5	0.71	4	5.10
3	LXH_115	31.60	7	0.76	2.5	4.56
4	LXH_5	31.44	7	0.68	6	4.44
5	LXH_1	32.78	7	0.73	8.5	4.20
	Mean	32.23		0.74		4.53
	SE	0.32		0.02		0.12
Bottom						
1	LXH_17	33.00	7	0.57	9	3.64
2	LXH_18	31.45	8	0.65	8	3.82
3	LXH_144	32.75	6	0.53	8	3.49
4	LXH_127	31.40	8	0.59	8	3.45
5	LXH_91	32.00	8	0.73	8	3.44
6	LXH_93	32.30	7	0.50	10	3.32
	Mean	32.28		0.57		3.48
	SE	0.39		0.02		0.05

LXH- Len X Halberd, CT- Canopy temperature, MSHW- Mean single head weight, EW- Leaf epicuticular wax, SE – standard error
 β – Eberhart's stability index consider a line with values of $\beta = 1$ and deviation means squares $sd = 0$ as stable and scored as 10.

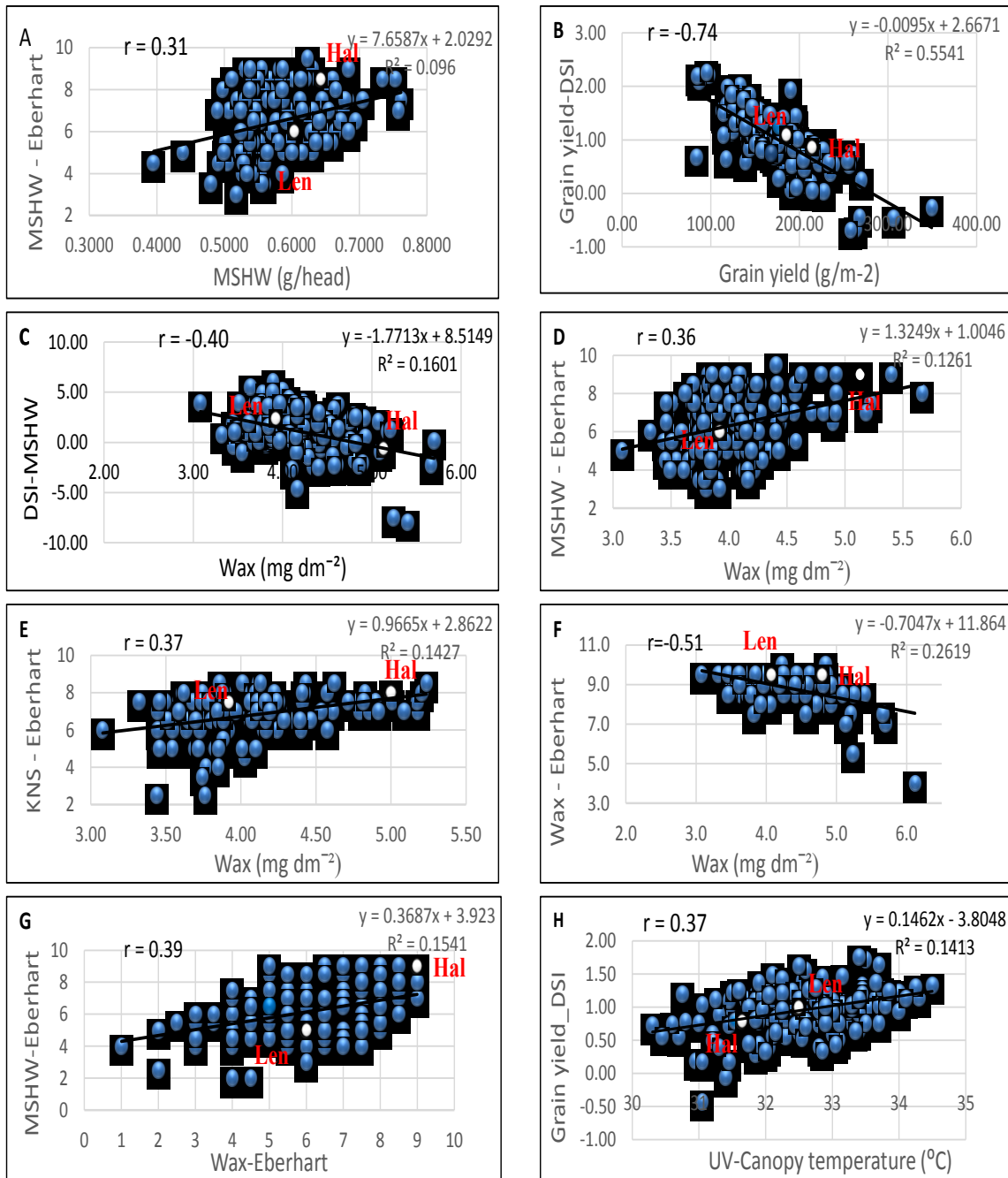


Fig. 3 Mean grain yield, leaf CT (Uvalde), and leaf epicuticular wax contents plotted against yield stability index with regression analysis and Pearson's correlations for 180 RILs of Len X Halberd population during 2011 and 2012. MSHW – Mean single head weight, KNS- Kernel number per spike, DSI – Drought susceptible index, Leaf CT-Leaf Canopy temperature, UVL - Uvalde. Grain yield and canopy temperature was taken from Uvalde 2012 environment.

load and lower CT with relatively higher yield stability compared to Len (Fig. 2 & 3). Although the data has not taken, Halberd parent and the RIL entries with medium to higher EW depositions are little stressed, prevent leaf rolling, and maintain high leaf rigidity when compared to Len and the RILs with lower leaf EW deposition.

The present investigation revealed significant genetic variability and trait segregation for the leaf EW (Table 1) (Araus et al. 1991; Uddin and Marshall 1988). The leaf EW did correlate significantly with yield and yield stability, but it was highly influenced by the environment (Fig. 3F and Table 1). The high environmental interaction and low genetic stability decreased the EW inheritance ($H^2 = 0.15$) across locations and years (Table 1). The low inheritance of leaf EW was also noted in different crops such as maize (0.17) (Meeks et al. 2012), wild rye grasses (*Elymus triticoides*) (Jefferson 1994), alfalfa (*Medicago sativa*) (0.35) (Jefferson et al. 1989), sorghum (0.36) (Jordan et al. 1983), etc. The leaf EW content on the leaf surface under water stress treated RILs was statistically (0.19 to 0.30 mg dm⁻²) greater than that in control treatment (Fig. 2I). Similarly, a previous study revealed a significant increase of 6-fold lipid transfer protein (LTP) gene transcripts and 1.5- to 2-fold increase in wax accumulation in tobacco (*Nicotiana glauca* L. *Graham*) leaves exposed to increased periodic drying (Cameron et al. 2006). The LTP's in wheat seeds were identified to be associated with a wheat gene (TdPR61) (Kovalchuk et al. 2012). The greater the water stress, the higher the EW produced. Obregon was the hottest location with 0 mm precipitation during crop growing season, resulted in the highest leaf EW load range (2.99 to 12.81 mg dm⁻²), and followed by Chillicothe and Uvalde. Additionally, EW was reported to produce different

amounts within plant growth stages in peanut at 45, 75, and 95 days after sowing (DAS), with 95 DAS (2.0 mg dm^{-2}) producing maximum wax amount (Samdur et al. 2003). EW production between plants growth stage is highly variant and distinct during reproductive stages; thus, wax should be collected at a specific growth stage.

2.4.2. Leaf EW and CT to improve potential yield

The association between the leaf EW and the grain yield depends on the intensity of water deficit and the interaction of environment with EW in the field. The locations Uvalde and Obregon, had better correlations between plot yield and EW (Fig. 2B and Table 2). Glaucous lines reflect high energy radiation, maintain water use efficiency, and improved mean yields in dryland relative to irrigated conditions (Febrero et al. 1998). Although glaucousness maintains mean yield through wax load, not much is related to EW quantity and its effect on yield (Blum et al. 1989; Johnson et al. 1983). The cooler canopy is the result of increased EW load under water-deficit; cooler canopies confirm the association ($r=-0.26$, $P\leq 0.001$) between EW load and leaf CT across environments or within environments (Table 2). The negative correlations between the EW and CT ranges from -0.19 to -0.32. With an increase of wax load from 1 mg dm^{-2} to 4.5 mg dm^{-2} , there is a decrease of 1.8°C in temperature (Fig. 2C). The greater the water stress, the stronger the correlation observed; Uvalde was the driest test location in Texas and it had stronger correlation ($r=-0.32$, $P\leq 0.001$) between cooler canopies and EW, followed by College Station (Table 2). Similar negative correlations were identified under heat

treatment in winter wheat (Mason et al. 2013). In addition to plot yield, EW correlated positively and significantly with different yield components, such as MSHW, KNS, and TKW, across all environments (Fig. 2A, E, F and Table 2). Similar trait correlations and genetic overlap prevails between staying green, leaf EW, and canopy temperature in sorghum (Awika 2013; Ehleringer 1980; Mkhabela 2012), and wheat (Araus et al. 1991; Fischer and Wood 1979; Mondal and Hays 2007). Genotypes with greater wax load restrict residual water transpiration in pea (Sanchez et al., 2001) and reflect higher light energy (Vanderbilt et al. 1991). In another study, different wax-rich sorghum genotypes reflected high energy radiation and reduced transpiration rate (Premachandra et al. 1994). Reduced leaf CT effectively increased final grain yield, as a 1°C decrease in canopy temperatures may increase the grain yield components by 4 mg in grain weight (Ishag et al. 1998; Olivares-Villegas et al. 2007). Wheat drought-susceptible genotypes under water and heat stress were identified with poor maintenance of photosynthesis, chlorophyll content, and biomass content, with an early transition to the dry seed stage and poor seed set (Yang et al. 2002). In pea plants, leaf EW positively correlated with harvest index and negatively correlated with CT, where increased wax load on leaf epicuticle reflects high energy radiation, prevents photo-oxidative damages, and reflects photosynthetically active radiation (Sánchez et al. 2001). These waxy cuticular layers may affect transpirational cooling needs and stomatal conductance acting as a reflective surface to high temperatures, high energy radiations, and water-deficit conditions, reducing unnecessary water loss yet cooling canopies during reproductive stages. Thus, it might be inferred that leaf EW and cooler canopies during the reproductive stage act as

important integrated components of drought-adaptive traits. Leaf EW conditions cooler canopies and can be effectively incorporated into a high yielding genetic background.

2.4.3. Leaf EW and CT to improve yield stability

The role of leaf EW in maintaining stable yields across different water-deficit environments has been poorly documented. This study reveals a consistent negative correlation of leaf EW content with Eberhart stability index of different yield components such as MSHW ($r=-26\%$, $P\leq 0.01$), KNS ($r=-0.24$, $P\leq 0.01$), TKW ($r=-0.25$, $P\leq 0.01$), and DSI ($r=-0.40$, $P\leq 0.001$) clearly indicating the importance of leaf EW in maintaining yield stability across different water-deficit conditions (Fig. 3). Another physiological trait, leaf CT, also correlates positively with yield DSI ($r=0.40$, $P\leq 0.001$) (Ishag et al. 1998) and Eberhart yield stability index ($r=0.24$, $P\leq 0.001$), concentrating most of the RIL stability index values close to 1 (Fig. 3). In another study of wheat genotypes, the contribution of cooler canopies to keep DSI close to 1 under water-deficit environments has been observed (Rashid et al. 1999). Increased leaf EW and its influence on cooling canopies acts as an important target to improve yield and yield stability under water-deficit (Fig. 2 & 3 and Table 2). Almost 75% of the RIL population showed Eberhart stability index ≤ 1 across environments and 61% of individual RILs showed ≤ 1 for DSI within an environment (Fig. 2C, D). The most stable individual RILs, had an intermediate EW content and had enhanced yield under water-deficit treatments (Fig. 3A, B). A positive and significant correlation exists between leaf EW Eberhart stability

and MSHW Eberhart stability index, with most RILs concentrating toward the center ($r=0.33$, $P\leq 0.001$) (Fig. 3G). The top five and the bottom five RILs for EW has significant difference for MSHW, EW, and EW Eberhart stability index (Table 3). This elucidates that the RILs with higher wax stability have higher yield stability. To conclude, leaf EW is integrated with cooler canopies and a lower DSI and Eberhart stability index as such is an important trait in imparting water deficit tolerance. Similar to EW other physiological trait, osmotic adjustment improves yield stability within the environment (DSI) (Khanna-Chopra 1999) and in between different environments (Eberhart stability) (Moinuddin et al. 2005).

2.5. Conclusions

The goal of this study was to investigate drought-adaptive traits with significant genetic variation. The significant phenotypic correlations of agronomic and physiological traits provide a clue about the existence of genetic linkage for drought-adaptive and potential yield attributes across different environments. The RILs with intermediate leaf EW content have a significant impact on yield increment and maintaining potential yield under moisture-deficit conditions. The leaf EW has a significant association with cooler canopies, thus reflecting high energy wavelengths and dissipating excess heat energy on the leaf surface. Integrating genetic loci that regulate high levels of leaf EW and cooler canopies in the genetic background of drought- and heat-susceptible elite lines can be feasible. These multiple genes pyramiding on a high-

yielding genetic background wheat cultivar would be instrumental to improve yield under moisture stress conditions.

CHAPTER III

MAPPING THE GENETIC LOCI REGULATING DROUGHT ADAPTIVE TRAITS; LEAF EPICUTICULAR WAX, CANOPY TEMPERATURE, AND DROUGHT SUSCEPTIBLE INDEX IN *TRITICUM AESTIVUM* L

3.1. Introduction

Water stress during wheat (*Triticum aestivum*) reproductive stages is a primary constraint that limits grain yields on at least 40 and 25 Mha in developed and developing countries, respectively (Byerlee and Moya 1993). Additionally, significant yield and revenue losses, affects 50% and 70% of wheat growing areas in the developing and developed countries respectively (Trethowan and Pfeiffer 2000). Soil water deficit conditions combined with high temperatures (dry air and soil) during vegetative and reproductive stages also increase seed abortion (Hays et al. 2007) and limit overall grain yield in wheat and oats (*Hordeum vulgare*) (Hossain et al. 2012). In the Southern Great Plains, the Ogallala aquifer has declined 266 million acre-foot from 1940 to 2012, with an average water loss of 10 million acre-foot per year between 2000 and 2007 (Stanton et al. 2011). Without adequate irrigation, dryland wheat in the U.S. High Plains is severely limited due to poor soil moisture and irregular and inadequate precipitation. As such, improvements in drought adaptive (water deficit tolerance) traits such as transpiration efficiency, water use efficiency, and cooler canopies in new cultivars adapted to this region is needed.

Molecular plant breeding could be effective tool to select, fix, and improve physiological traits adapted to water deficit and high temperature conditions. Enhanced exploitation of the wheat genome and interdisciplinary activities, may offer the potential to dissect different factors limiting grain yield under water deficit conditions (Tuberosa and Salvi 2006). An integrated trait, leaf canopy temperature depression has wide genetic variation, is easily selected and has moderate to high heritability and robust association with grain yield components, is one physiological heat and drought adaptive trait (Reynolds et al. 2007; Richards 2000). Leaf epicuticular wax (EW) has been shown to be one of the contributing factors conferring cooler leaf canopy temperature (CT). In pea (*Arachis hypogea*) increased wax resulted in decreased canopy temperatures (Awika 2013) and an increased harvest index (Sánchez et al. 2001). Epicuticular wax is expressed as whitish blanket like structure, present on the surface of leaves. It acts as a light reflective agent for certain wave lengths and prevents leaf burning from high energy wavelength, such as in *Leucadendron lanigerum* (Proteacea) (Mohammadian et al. 2007) and provides protection against photo-inhibition such as in *Cotyledon orbiculata* (Barták et al. 2004). Leaf EW has been found to be associated with many physiological traits, such as increased residual water content, reduced transpiration in pea (Sánchez et al. 2001), increased water use efficiency (Samdur et al. 2003), increased light reflectance and reduced heat susceptibility index (HSI) (Mondal 2013).

Genomic regions associated with grain yield parameters, such as drought susceptible index (DSI) and heat susceptibility index (HSI), were previously identified in various crops including wheat (Mondal 2013) and soybean (Du et al. 2009). The

association of two or more phenotypic traits with a common genomic region (QTL) is known as pleiotropy or linkage effects (Huang et al. 2004). Recent studies have found that heat and drought stress adaptive QTL have associated with different grain yield components such as grain number (chromosomes 3B, 4A, 6B), average test weight (chromosomes 4A) and kernel weight per main spike (chromosome 3B) (Mason et al. 2011; Pinto et al. 2010).

Breeding for complex traits requires consideration of various factors such as trait segregation, genetic variance, interaction of traits, physiological and molecular basis, and trait interaction with the environment. Leaf EW in sorghum (*Sorghum bicolor*) has stable genetic variance, yet is highly influenced by environment making it challenging to breed and study (Jordan et al. 1983). Genetic variance and inheritance of glaucous genes have been reported in wheat with dominant and epistatic effects on the short arm of chromosome 2B (Tsunewaki and Ebana 1999). Mason et al. (2010) reported QTL for flag leaf glaucousness or visual wax content on chromosome 5A from the heat tolerant Australian spring cultivar Halberd with a positive additive allelic effect. In another heat study, stable QTLs on wheat chromosomes 5A and 1B regulating EW content co-localized with leaf temperature depression and a lower HSI for kernel weight and single kernel weight, were reported (Mondal 2013). Also a new flag leaf glaucousness QTL was identified on chromosome 3A that explained 52% of the genetic variation in warm temperatures (Bennett et al. 2012a). Sorghum lines with stay green traits were found to have significantly cooler canopies compared to non-stay green lines. In addition, stay green QTL were reported to co-localize with QTL increased leaf EW on sorghum

chromosome 1 (Awika 2013). Recently, in a study of heat and water stress treatments, stable QTL explained 14% and 28% of phenotypic variations were identified on chromosome 3B. Quantitative trait loci regulating cooler canopies were found to co-localize with yield potential (Pinto et al. 2010).

The wheat cultivar Halberd has shown to be a source of heat tolerance and has donated numerous QTL for better HSI and higher canopy temperature depression when crossed to local elite wheat cultivars (Mason et al. 2011; Mason et al. 2010; Mondal 2013). The objective of the present study was to identify the QTL regulating increase epicuticular wax content and determine their pleotropic overlap with cooler canopies, drought tolerance, and grain yield components. This study also sought to identify robust QTL associated moisture stress conditions. The specific objectives of this study were: (1) to identify the effect of moisture stress on wax production and investigate the genetic loci that strongly regulate the levels of leaf EW content; and (2) to determine the genetic associations between higher leaf wax deposition, lower canopy temperature, stable and potential yield under moisture deficit environments.

3.2. Materials and methods

3.2.1. Population development

A recombinant inbred population derived from an initial cross between an Australian spring wheat cultivar, ‘Halberd’ (Scimitar/Kenya/C6042 /Bobin/2/Insignia49)

and a North Dakota elite hard red spring wheat cultivar, 'Len' (ND499/3/Justin/ RL4205 //W1261) was used in this study. The parent lines were randomly selected based on their differential responses to control and drought stress treatments and their similarity in maturity. The F₆ derived recombinant inbred line (RILs) population consisted of 180 individuals. Which varied significantly in leaf epicuticular wax (EW) content, leaf canopy temperature (CT), DSI, and grain yield components (mean single head weight, thousand kernel weight, and kernel number per spike). The F₆ derived F₈ and F₉ family RILs were planted as two identical trails with two replications each in 2011 and 2012 at three Texas A&M Agrilife research stations (College station, Chillicothe, Uvalde-TX) and CIMMYT in Ciudad de Obregon, Mexico. In both 2011 and 2012 growing seasons, phenotypic trait such as leaf EW content, CT, and yield components were collected under two differing moisture regimes. In Texas both trails received identical water treatment until Feekes 6. At Feekes 6 water was withheld from the drought stress trial while irrigation continued at regular intervals on the control trial.

3.2.2. Phenotype measurements

Flag leaf discs 1 cm diameter were punched using a custom leaf punching tool (Rabbit Toole USA www.rabbittool.com). Four leaf discs were punched from randomly selected flag leaves in the center of each plot and collected in glass vials. One ml chloroform was add to the leaf discs for 30 s. The resulting chloroform solution containing dissolved waxes was transferred into a clean GC 2 ml vials. The chloroform

was dried under N₂ gas. The wax samples were then analyzed using the colorimetric method (Ebercon et al. 1977). Concentrated potassium dichromate solution was used to dissolve and quantify the wax based on reaction between wax and the dichromate solution. The leaf EW samples extracted were analyzed through optical density at 590 nm wavelength using a plate reader BMG-Labtech PHERAstarplus. Each sample with 100µl volume and 3 replications was loaded into a 96-well capacity U-shape Elisa microplate (Greiner bio-one GmbH). Halberd flag leaves from the field were used to develop a standard curve equation through serial dilution techniques to quantify extracted colorimetric wax.

3.2.3. Canopy temperatures

Leaf canopy temperatures (CT) were recorded at 10 DAP. Measurements were taken using a portable infrared thermometer (Fluke 561 IR). The infrared thermometer was focused at a 45° angle to the lateral canopy of each plot. Data was collected on a cloudless day with low wind between 1 PM to 3:30 PM.

3.2.4. Yield measurements

At maturity 50 spikes were harvested randomly from the center of each plot to determine mean single head weight (MSHW). Before harvesting for plot yield, awn data and plant height (cm) were recorded. Stand count and bird damage was also recorded to

standardize plots across replications. Thousand kernel weight (TKW) was estimated using a seed counter (SeedBuro TM 801 count-a-pak), and kernel number per spike (KNS) was also calculated using MSHW and TKW data. Drought susceptibility index (DSI) was calculated using the individual and mean grain yield values under moisture stress and irrigated treatments (Fischer and Maurer 1978).

The following formula was used to calculate the DSI for each individual RIL:

Equation 5 $DSI = [1 - (Y_{S1}/Y_{P1}) / 1 - (Y_{S2}/Y_{P2})]$

Y_{S1} = Grain yield under the water deficit treatment (stressed environments)

Y_{P1} = Grain yield under the irrigated treatment (non-stressed environments)

$1 - (Y_{S2}/Y_{P2})$ = Stress intensity calculated based on the mean yields of stressed (Y_{S2}) and non-stressed (Y_{P2}) environments. The DSI was calculated for MSHW, plot yield, and TKW across all five environments.

3.2.5. Statistical analysis

Statistical analysis was carried out using the PROC MIXED model procedure (SAS v9.3) (SAS 2011). The genetic variance of the yield components and physiological traits were calculated by considering the irrigation treatments as fixed and genotypes, years, and replications as random effects. Simple contrast analysis was performed on QTL associated with parental alleles to determine phenotypic means of different traits.

3.2.6. *Molecular analysis*

DNA extraction was performed on the 180 RIL population of F₈ generation including the parents using the DArT method (Doyle 1990; Jaccoud et al. 2001). Extraction buffer stock (0.35M sorbitol, 0.1 M Tris HCl, 5mM EDTA), lysis buffer stock (0.2M Tris HCl, 0.05 M EDTA, 2 M NaCl, and 2% CTAB) and sarcosyl stock 5% (w/v) solutions were prepared accordingly. In addition, a fresh solution of 0.5% w/v sodium disulfite, 2% w/v PVP-40 (Polyvinylpyrrolidone) (sigma chemicals) was added to the extraction, lysis, and sarcosyl buffers. Fresh leaf tissue of 2 week old RIL seedlings were harvested and placed in 2 ml eppendorf tubes. Then 1ml of the freshly prepared extraction buffer solution at 65°C was added and the tissue was disrupted using a Fastprep -24 homogenizer at 4.0 Movement/s for a 2 min period. The resulting mixtures were incubated in a water bath at 65°C for 1 hr. After cooling, 1 ml of chloroform:isoamyl alcohol (24:1) mixture was added to the samples and then centrifuged at 10,000 rpm for 20 min. The supernatant of each tube was transferred into new 2 ml eppendorf tubes, and then an equal volume of ice cold isopropanol was added. Tubes were then centrifuged at 10000 rpm for 30 min to precipitate the DNA. The supernatant was discarded, and the precipitate pellet was washed with 1.5 ml 70% ethyl alcohol. The resulted nucleic acid pellet was air dried and then dissolved in 200 µl of 1 X TE (10mM TrisHCl pH 8.0, 1 mM EDTA pH 8.0).

3.2.7. Genetic linkage and mapping

The DNA of the RIL population was genotyped using silica bead chips containing 90K SNPs (Single nucleotide polymorphism) array through Illumina Infinium Golden Gate assay using next generation sequencing (Akhunov et al. 2009; Cavanagh et al. 2013) at the USDA-ARS, Fargo, ND. The SNPs clustering and annotations were analyzed using GenomeStudio v2011.1 software. Each SNP was annotated based on the clustering of individual alleles across the population. After scoring and annotating of 90K SNPs, SNPs that showed monomorphic clustering, SNPs showing more than 20% missing points, SNPs with vague calling, and SNPs that had a minor allele frequency $< 10\%$ were discarded. The resultant data set of 2,700 polymorphic SNPs was exported from GenomeStudio. The software JoinMap version 4.0 (Van Ooijen 2006) was used to create a linkage map using recombinant events and the different reference population maps, such as a map from 9K SNPs (Gregersen et al. 2005), Avalon X Cadenza (Nelson et al. 1995), Savannah X Rialto (Snape et al. 2007), and Synthetic X Opeta (Allen et al. 2011). Finally, 22 linkage groups were identified at a significance level of 0.05 and 10,000 permutations across the wheat genome. These linkage groups were mapped with phenotypic data across five environments to identify possible QTL using MapQTL v6 (Van Ooijen 2004). The traits (agronomic and physiological) with significant segregation/genetic variations or low genetics by environment interactions or normally distributed populations were utilized for QTL mapping. The Kosambi function was used to calculate the recombinant event distances with a critical LOD score value of 3.0. The mapping method MQM (multiple QTL mapping) was used, where markers of non-

linkage groups were used as cofactors and reduce noise on the genetic background (Jansen and Stam 1994). Co-localized QTL with major effects identified across the wheat genome for yield, moisture stress and control treatment traits were represented graphically using the software map chart (Voorrips 2002).

3.3. Results

The year 2011 was considered dry compared to 2012 in the Uvalde and College Texas A&M Research Stations. Minimum and maximum air temperature and total precipitation during crop growing season were recorded. At Ciudad de Obregon, Mexico, 0 mm precipitation and air temperature ranging between 34°C to 43°C during crop growing season were recorded. The Ciudad de Obregon environment had the highest temperatures and thus produced the maximum amount of leaf EW content compared to other environments (Table 4). As mentioned irrigation under moisture deficit treatment was ceased at Feekes 6 or during the initiation of stem elongation in an effort to impose moisture stress during flowering at the Texas research stations. Substantial and significant differences for moisture regimes were observed between control (366 mm) and moisture stress (170 mm) treatments. The moisture stress differences between the irrigation treatments were intensified resulting in significantly different amounts of leaf EW productions, with moisture deficit regions producing higher amounts compared to the control treated population.

'Halberd' and 'Len' were not significantly different under field moisture deficit conditions for agronomic (MSHW, TKW, KNS) and physiological traits (CT, leaf EW). However, Halberd did produce higher mean yield and EW content across the five moisture deficit environments (Table 4). There was also a significant difference ($P \leq 0.001$) between parents for DSI for MSHW and TKW. 'Halberd' was significantly more tolerant than 'Len' under moisture stress conditions. In Ciudad de Obregon and Uvalde Halberd had significantly higher leaf EW compared to the Len (Table 4). Transgressive segregation was also observed in the RIL population for various traits, such as leaf EW, CT, yield components, and DSI (Table 4). Significant genetic variance was identified for EW ($P \leq 0.05$), CT ($P \leq 0.001$), DSI ($P \leq 0.001$), and different yield components ($P \leq 0.001$) across 5 environments (Table 4). Broad sense heritability was calculated for leaf EW (0.12), CT (0.40), MSHW (0.39), TKW (0.23), and KNS (0.39) using the variance components and entry mean basis method (Table 1).

3.3.1. Genetic mapping

A total of 2565 SNPs that were co-dominant and polymorphic between parents were assembled to identify genetic linkages based on reference chromosomal information and recombinant events. The constructed genetic map covers a 3778 cM genetic distance with each marker spaced at an average distance of 1.47 cM. The B genome had the largest number (1502) markers followed by the A genome (963) while the D genome had only 100 markers.

Table 4 Means of parents and range of RIL (Len X Halberd) across five environments for agronomic and physiological traits under moisture deficit conditions during 2011 and 2012.

Combined	Halberd		Len		RILs range	Means	Probability Trait Segregation
	Moisture deficit	Control	Moisture deficit	Control			
MSHW (g)	0.729a	0.790a	0.664a	0.590a	0.534– 0.867	0.612	***
TKW(g)	33.57a	31.00a	32.57a	28a	24.22– 40.62	32.32	***
KNS	22.4a	19.0a	22.4a	18.0a	16 – 26	20.00	***
DSI (MSHW)	-0.219b	-	2.105a	-	-15.53 – 12.3	0.855	***
DSI (TKW)	-0.217b	-	4.401a	-	-22.32 – 12.44	0.982	**
Height (cm)	66a	78a	67.2a	76a	50 – 76.87	64.97	***
CT (° C)	34.04a	30a	34.03a	30a	28.5 – 35.45	30.32	***
Wax (mg dm ⁻²)	3.69a	2.7b	3.17a	2.3b	2.57 – 6.25	3.66	**
Obregon wax (mg dm ⁻²)	-	10.89a	-	6.89b	2.99 – 12.81	6.07	***
Uvalde wax (mg dm ⁻²)	6.07a	4.37a	5.52b	4.11a	2.63 – 5.91	3.99	**

*** Significance at < 0.001, ** significance at < 0.01. Numbers followed by same letters are insignificant, MSHW- Mean single head weight, TKW – Thousand kernel weight, KNS – Kernel number per spike, DSI – Drought susceptible index, CT – Canopy temperature.

In particular, the D genome chromosomes 1D, 5D, 7D, and 6D covered approximately 1.6%, 0.7%, 0.4%, 0.2% of the total A and B genome, respectively. The genetic linkages for the chromosomes 2D, 3D, and 4D were not identified. The chromosome 2B was the largest linkage group with the most number of markers (17.6%) at a 558 cM distance and a (1.22cM), average marker intervals followed by 5B (376.4 cM) and 1A (279.28 cM). The genetic groups on chromosomes 2A, 3B, 7D, and 6D had poor coverage possessing 0.9%, 0.6%, 0.4%, and 0.5% of the total SNPs, respectively.

The MQM mapping method detected 53 significant QTL associated with leaf EW, of which, 31 QTL explained 7 to 31% of phenotypic variation in irrigated or the

control treatment. Twenty-two QTL explaining 8 to 16% phenotypic variation were detected under moisture stress conditions only. Thirty nine QTL for leaf CT were detected with 25 QTL explaining 7.5-25% variation under control conditions, whereas, 14 QTL explained 7.6-35% variation under moisture stress conditions. Drought tolerance traits, DSI-MSHW and DSI-TKW were associated with 30 and 20 QTL, explaining maximum phenotypic variations of 41% and 17%, respectively. Interestingly, a larger percentage of yield QTL were detected under moisture deficit compared to control treatment. The aforementioned QTL were located on chromosomes 1A, 1D, 2B, 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, 7A, and 7B (Table 5). On seven of these chromosomes, co-localization of leaf EW, CT, DSI, and yield components occurred at peak LOD score of 3 (Table 5; Fig. 4). At most of the loci, leaf EW associated with cooler canopies, moisture stress tolerance, and yield components (Fig.1). At most of the genetic loci, the Halberd parent contributed favorable QTL for leaf EW content, DSI, and cooler canopy, thus, contributing to moisture stress tolerance and heat tolerance (control) in the RIL population (Table 6). Previous studies have shown that Halberd donates significant alleles related to heat tolerance in other biparental mapping populations (Mason et al. 2011; Mason et al. 2010). Similar studies for drought tolerance involving 'RAC875', reported that it donated more alleles related to wax glaucousness and moisture-heat stress tolerance to biparental mapping population (Bennett et al. 2012a). More QTL related to yield components (TKW, KNS, plot yield, and MSHW)

Table 5 QTLs co-localized for yield components (MSHW-mean single head weight, TKS-thousand kernel weight, KNS-kernel number per spike, plot yield) and physiological traits (leaf colorimetric wax content, leaf canopy temperature (CT), drought susceptible index-Mean single head weight –DSI-MSHW) in the 180 Len X Halberd RILs mapping population across 5 environments during 2011 and 2012.

Traits co-localized	Wax QTL	Marker	LOD	R ²	σ_a^2	Allele
DSIS,TKW,CT,KNS,MSHW	Qwax.tam-2B	wsnp_Ra_rep_c74497_72390803	4.61	0.11	0.274	Halberd
		wsnp_Ex_c42316_48926687	4.62	0.11	0.275	Halberd
MSHW,TKW,KNS,DSIM,DSI CT,DSIS,MSHW,KNS	Qwax.tam-7A Qwax.tam-3B	BS00034689_51	4.94	0.12	-0.078	Len
		BobWhite_c33344_143	4.64	0.11	-0.015	Len
		CAP12_c2400_438	3.53	0.09	-0.047	Len
		wsnp_Ex_c3096_5709369	7.37	0.17	-0.029	Len
DSIM,MSHW,KNS,TKW DSIM,TKW,MSHW,KNS,CT TKW,KNS,DSIM,MSHW MSHW,KNS,TKW,Yield	Qwax.tam-5A Qwax.tam-5A Qwax.tam-5B QWax.tam-7Ba	Ku_c23179_1250	4.74	0.11	0.220	Halberd
		wsnp_Ex_c19647_28632894	4.89	0.12	0.058	Halberd
		wsnp_Ex_rep_c68269_67060931	3.53	0.09	-0.016	Len
		BS00108020	4.08	0.10	0.073	Halberd
		wsnp_Ex_c15972_24385702	3.10	0.08	0.002	Halberd
Traits co-localized with CT	CT QTL					
Wax, MSHW, DSIM, TKW	QCT.tam-2B.1	wsnp_Ex_rep_c67411_65994109	15.94	0.343	0.051	Halberd
Wax, MSHW, DSIM, TKW	QCT.tam -2B.2	wsnp_Ex_rep_c67411_65994109	3.38	0.085	-0.022	Len
TKW, MSHW, DSIM	QCT.tam -2B.3	wsnp_Ku_c3780_6950286	3.2	0.080	0.009	Halberd
Wax, DSIM, TKW, MSHW, KNS	QCT.tam -6B.1	wsnp_Ra_c2730_5190076	3.16	0.079	-1.169	Len
Wax, DSIM, TKW, MSHW, KNS	QCT.tam -6B.2	wsnp_Ex_c25505_34771897	3.14	0.079	-0.996	Len
Wax, DSIM, TKW, MSHW, KNS	QCT.tam -6B.3		3.22	0.081	-1.182	Len
MSHW,TKW,KNS,DSIS	QCT.tam-4B	Excalibur_c52517_464	2.94	0.073	-0.029	Len
DSIS, MSHW	QCT.tam-3B	Excalibur_c2492_1750	9.26	0.215	0.061	Halberd
Wax, MSHW, DSIS	QCT.tam-7Bb	wsnp_Ku_c10355_17149304	6.94	0.167	0.079	Halberd
Traits co-localized with DSIM	DSIM QTL					
MSHW, DSIS, Wax, CT	QDSIM.tam-2B.1	Excalibur_c48957_395	3.04	0.077	-0.589	Halberd
TKW, DSIS	QDSIM.tam -2B.2	Excalibur_c21117_99	5.66	0.138	1.073	Len
Wax, CT	QDSIM.tam -5B.1	BobWhite_c41725_430	6.79	0.164	0.374	Len
DSIS, TKW, Wax, MSHW, KNS	QDSIM.tam-5B	BS00010213	19.76	0.411	-0.008	Halberd
		BS00023161	16.08	0.350	-0.025	Halberd
Wax	QDSIM.tam -5A	Excalibur_rep_c69282_651	3.22	0.081	0.265	Len
TKW, MSHW, DSIM	QDSIM.tam -2Be	RAC875_c12803_916	5.39	0.132	0.052	Halberd

MSHW-Mean Single head weight, DSI- Drought susceptible index, TKW- Thousand kernel weight, KNS- Kernel number per spike, CT- Leaf canopy temperature, QTL- Quantitative trait loci.

^aLOD thresholds were estimated in MapQTL 6 using 10,000 permutations

^bR² Percent of phenotypic variation explained

^c Additive effect of allele substitution

UV-Uvalde, CS-College Station, CH-Chillicothe, MSHW- Mean single head weight, KNS- Kernel number per spike, TKW- Thousand kernel weight, DSIM- Drought susceptible index Mean single head weight, DSIS- Drought susceptible index-Thousand kernel weight. R² - Percent of phenotype variance explained by the QTL, C - Control, D - Moisture deficit.

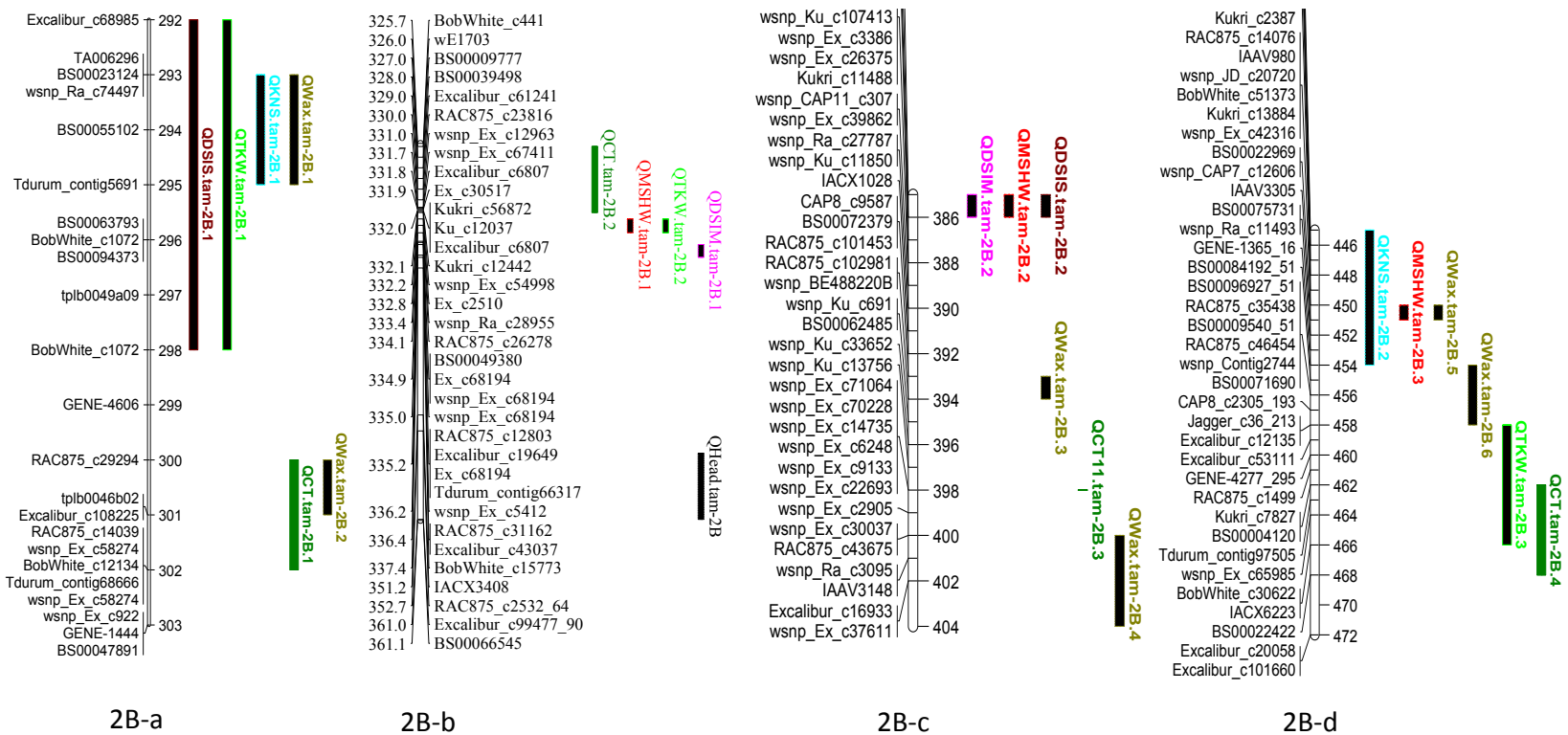


Fig. 4 Genetic linkage groups constructed in the 180 RIL population derived from Len and Halberd cultivars for F8 & F9 generations, during 2011 & 2012 growing seasons. Identified co-localized QTLs were traced across different linkage groups of wheat genome with > 3 LOD scores and 10,000 permutations.

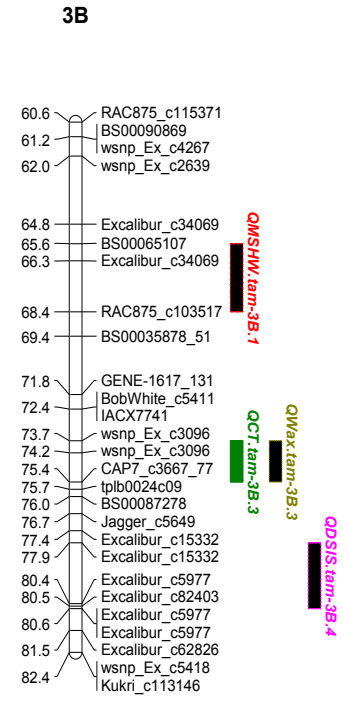
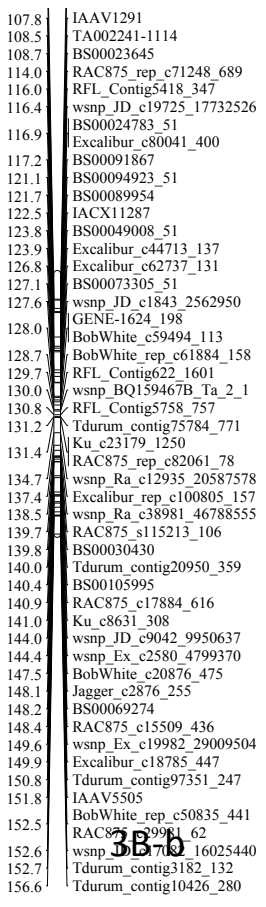
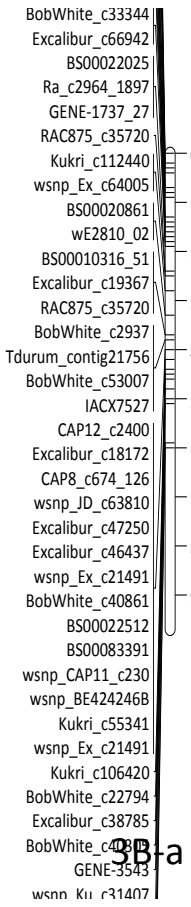


Fig. 4. Continued

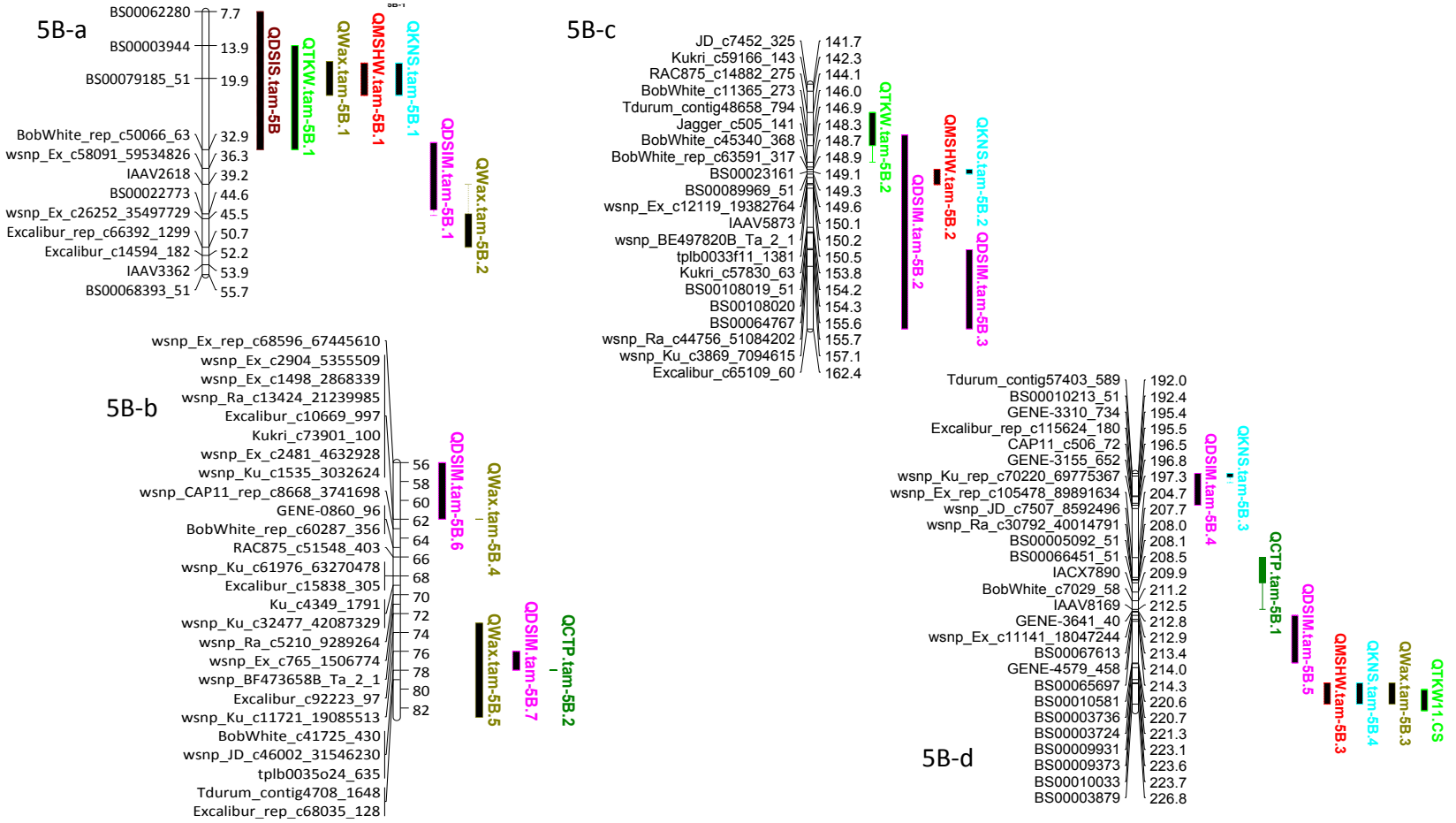


Fig. 4 Continued

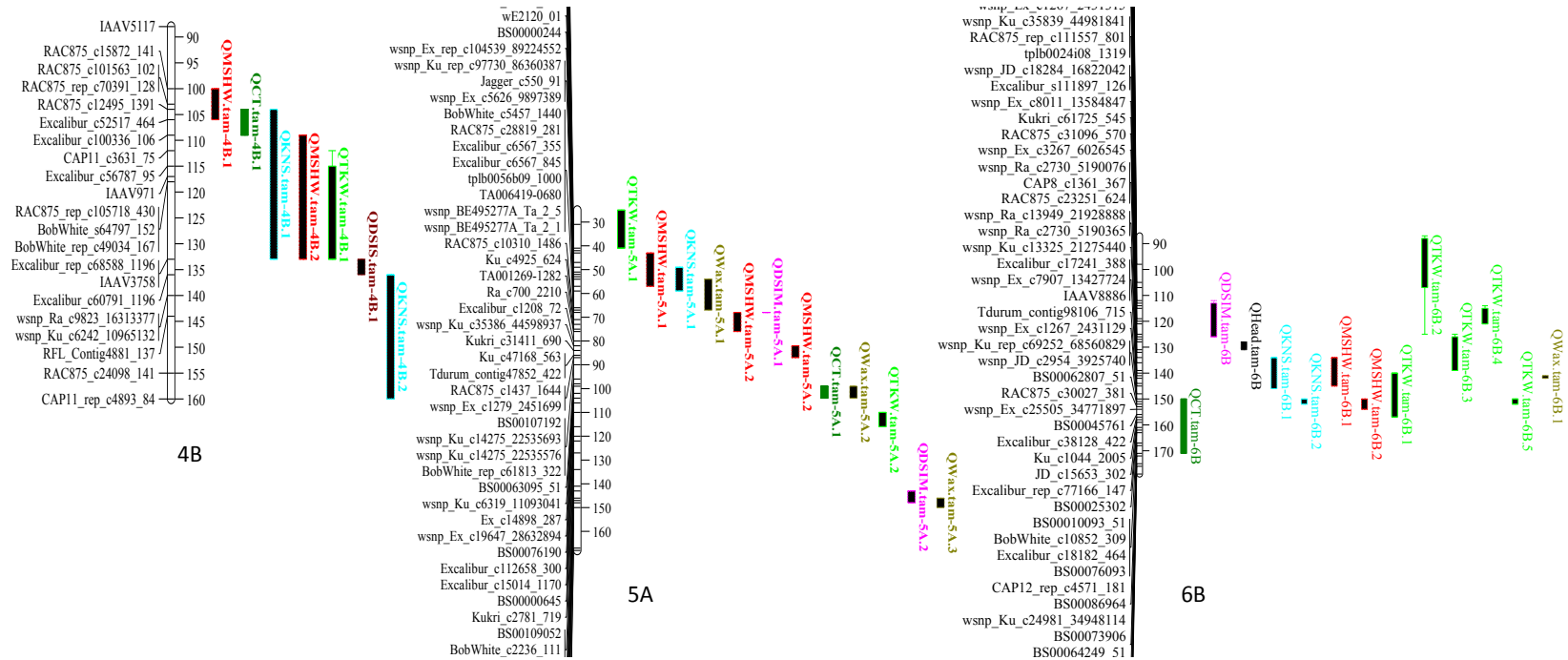


Fig. 4 Continued

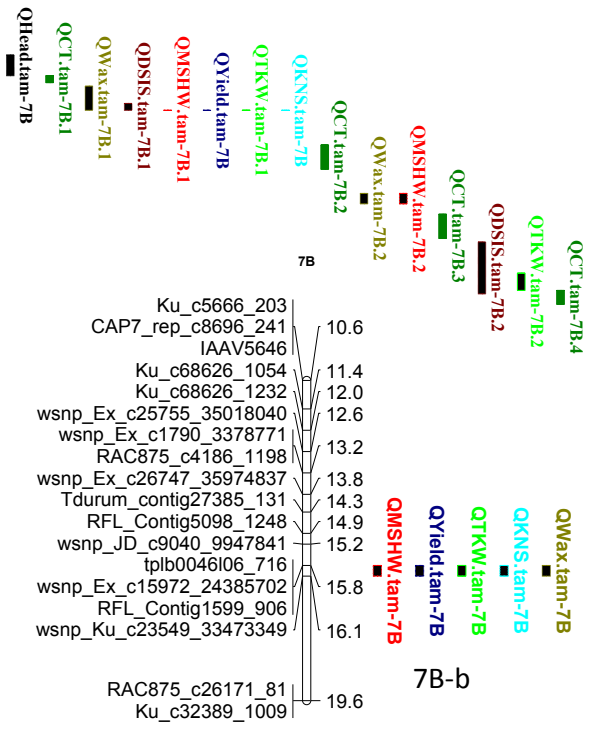
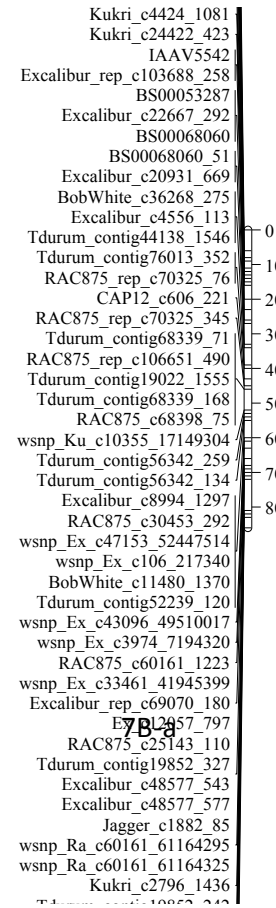
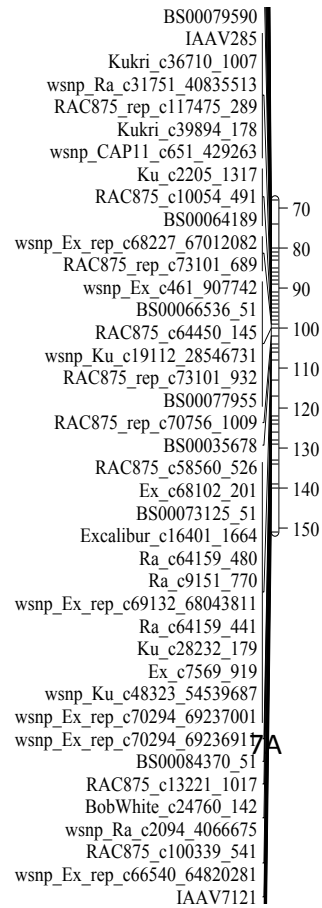


Fig. 4 Continued

Table 6 QTL identified in more than one environments for agronomic and physiological traits in 180 Len X Halberd population during 2011 and 2012.

Traits	Chromosomes	Stable markers	Environment	LOD	R ²	Additive variance
DSIS	7A	RAC875_c22792_672	UV11_D	3.04	0.08	0.061
			CH11_D	3.54	0.09	0.012
MSHW	7A	wsnp_be494028A_Ta_2_1	UV11_D	10.51	0.08	0.063
			CH11_D	3.43	0.10	0.023
			CH11_C	5.15	0.15	0.027
TKW	7A	BS00034689_51	UV12_C	8.47	0.14	0.194
			CS11_D	6.1	0.08	0.185
			CS11_C	8.47	0.08	0.194
KNS	7A	Tdurum_contig49723_593	UV11_D	5.42	0.21	1.675
			CH11_D	3.01	0.13	0.753
			CH12_C	7.06	0.20	1.222
DSIM	1B	Tdurum_contig15593_407	CS11	19.76	0.41	-0.004
			CH11	3.04	0.12	-0.023
			UV11	4.55	0.08	-0.131
CT	2B	wsnp_Ex_rep_c67411_65994109	UV11_C	15.94	0.34	0.051
			UV11_D	3.07	0.08	0.269
Wax	5B	CAP7_c11288_109	UV12_D	3.01	0.07	-0.026
			CS11_D	4.34	0.10	0.045
			UV12_C	3.31	0.08	-0.199
MSHW	7B	wsnp_Ku_c19112_28546731	CS11_D	8.76	0.09	-0.010
			CS11_C	5.12	0.09	-0.014
KNS	7B	wsnp_Ku_c19112_28546731	CS11_D	8.32	0.08	-0.119
			CS11_C	3.5	0.11	-0.048
DSIM	6A	BS00011962	CS11_C	3.45	0.10	0.073
			CH11_C	3.85	0.08	0.145
MSHW	6A	BS00003818	CH11_D	2.99	0.08	-0.021
			UV11_C	3.34	0.09	-0.015
TKW	6B	RAC875_c23251_624	UV12_C	3.34	0.08	-1.068
			CS11_C	3.34	0.10	-1.068
			CS11_D	9.07	0.15	-0.665
KNS	6B	RAC875_c23251_624	CS11_D	3.2	0.20	0.098
			CS11_C	3.47	0.12	0.161
			UV11_D	3.18	0.14	-0.032
MSHW	4B	RAC875_c12495_1391	UV11_C	4.13	0.08	-0.036
			UV12_D	3.56	0.08	-1.094
KNS	4B	RAC875_c12495_1391	UV_D	3.14	0.21	-0.964
			UV11_C	3.82	0.13	-1.327
			UV12_D	3.7	0.20	-1.190
KNS	4B	Excalibur_c100336_106	UV11_C	4.99	0.09	-1.691
			UV12_C	3.36	0.09	-1.038
			CS11	7.33	0.18	-0.088
DSIS	4A	tplb0033c09_1345	CH11	2.98	0.08	-0.013
			CS11	7.28	0.17	-0.064
			Kukri_c29142_473	CS11	7.28	0.17
Head No.	1B	GENE-0142_178	CS11_C	8.21	0.20	3.447
			wsnp_Ex_rep_c69986_68942834	UV12_D	3.01	0.08

across the RIL population were donated by Len (82) parent compared to Halberd (48). The QTL detected under control treatment were also detected under moisture stress treatment on chromosomes 7A, 7B, 6A, 6B-2, 5B, and 4B (Table 6 & 7).

A major and novel leaf CT QTL (*QCT.tam-4A*) detected on chromosome 4A co-localized with DSIM QTL (*QDSIM.tam-4A*), with the favorable alleles contributed by Halberd (Fig. 5). The CT and DSI-MSHW QTL were detected by the SNP *Excalibur_C7034_234* and *RAC875_c35453_201* with LOD scores of 16.4 and 19.9, respectively. Another major QTL for increased wax (*QWax.tam-4A*), with a LOD score 14.3, was detected by SNP *wsnp_Ex_c15801_24178779*, at the distal end of chromosome 4A with favorable alleles donated by Len (Fig. 5). Most of the novel and stable QTL were identified from chromosomes 7A, 4A, 3B, and 4B under different environments (Table 6; Fig. 5). The novel and robust QTL for leaf EW were detected on 2B, 3A, 3B, 7B, and 5B (Table 5). The genetic linkage groups at 1B and 5B consistently mapped novel QTL for DSI-MSHW in more than 2 environments with in a 1cM genetic interval (Table 6). Although, the leaf EW was unstable across environments, a major leaf EW QTL co-localized with the traits DSI-TKW, TKW, CT, KNS, and MSHW, designated as *Qwax.tam-2B*. It was associated with the SNP marker *wsnp_Ex_c42316_48926687*, and found to be consistent across 3 environments (Table 5 & 6). Numerous robust and repeatable QTLs with significant LOD scores were detected on 2B, 3B, 5B, 7B, and 7A linkage groups (Table 6 & 8).

3.4. Discussion

3.4.1. Genetic linkage for agronomic and physiological traits

The mean values for MSHW, TKW, and KNS for all RILs were significantly higher when they had the Halberd versus the Len allele. However, Len contributed more yield QTLs than Halberd. Len exhibited a positive mean DSI for all yield components, except in a few environments, and has relatively high DSI values compared to Halberd, indicating that Halberd possess more genes for moisture stress tolerance (Fig. 6). Halberd has previously been shown to be a heat tolerant cultivar with low HSI (Hays et al. 2007; Ji et al. 2010; Mason et al. 2011; Mason et al. 2010; Mondal 2013; Mondal and Hays 2007). Therefore, study helps to confirm that Halberd, in addition to being heat tolerant, is also moisture stress tolerant and may possess improved water holding capacity by reflecting high energy wavelengths and attenuating excess light and heat radiation from leaf surfaces. Strong phenotypic correlations exist among the unique traits EW, CT, HSI, harvest index, and yield components under both control and moisture deficit conditions (Mondal 2013; Sánchez et al. 2001). Studies of waxy blue spruce lines reported high levels of wax reflectance compared to non-waxy lines (Grant et al. 1995; Jenks et al. 1992; Reicosky and Hanover 1978). Thus, the presence of wax on the leaf epicuticle may act as a barrier to excess heat and moisture stress. Although, Len showed good phenological resistance to moisture stress, although data was not taken, more shriveled seeds were evident in comparison to Halberd. The QTLs identified under moisture stress conditions were similar in number to those found in irrigated conditions.

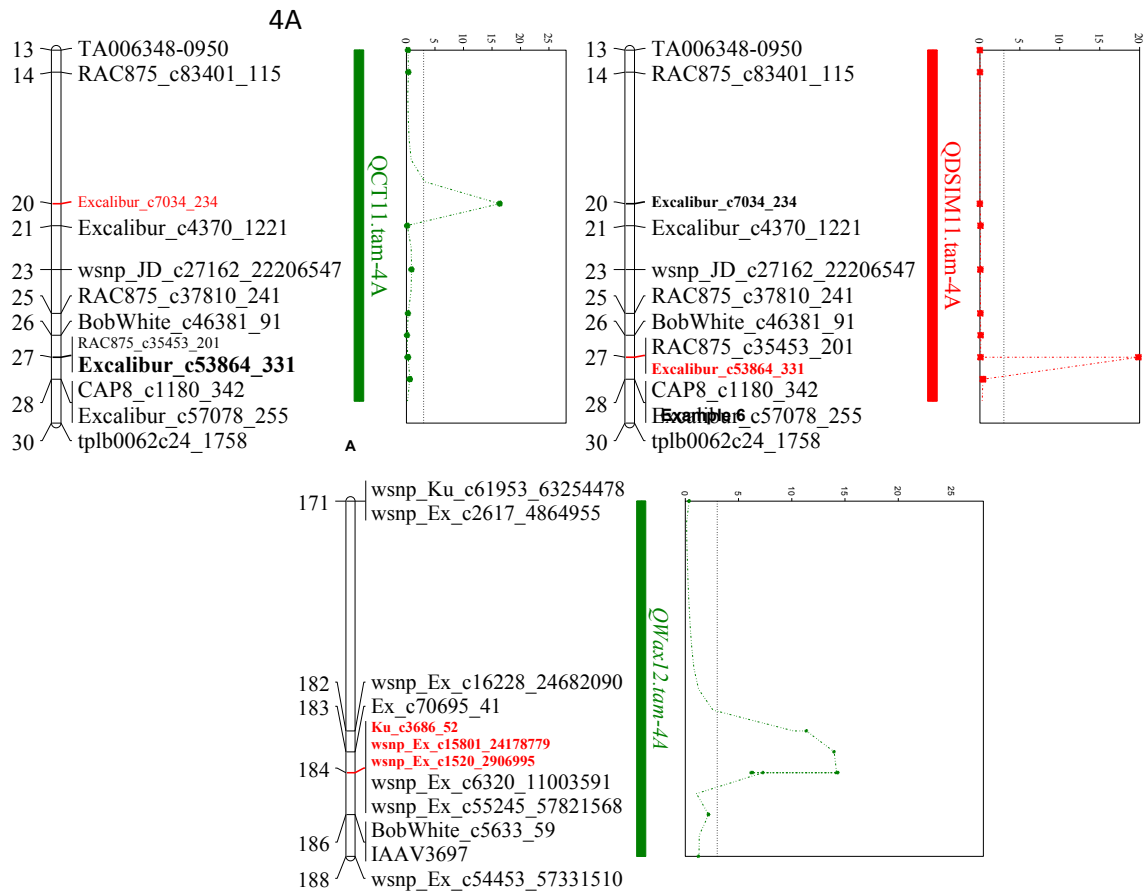


Fig. 5 The estimated positions of SNP markers on linkage group 4A associated with canopy temperature (CT), drought susceptible index - mean single head weight (DSIM), and colorimetric wax in 180 RILs derived from Len X Halberd cross. The high LOD score QTLs were detected in different locations (College station-CS and Uvalde-UVD) years (2011 and 2012).

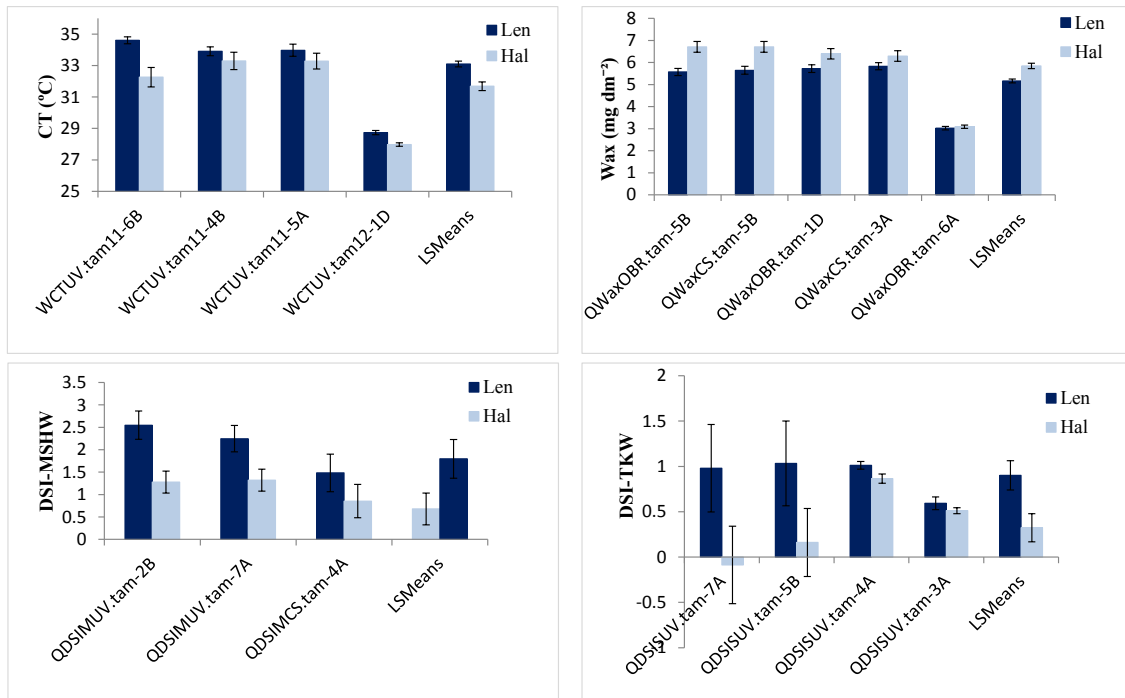


Fig. 6 QTLs contrast analysis on different chromosomes for wax content, canopy temperature (CT), drought susceptible index (DSI) for mean single head weight and thousand kernel weight (TKW) in a recombinant inbred line (RIL) population developed from a cross between drought tolerant line Halberd and drought susceptible line Len. Note that allelic variation at QTL identified for wax (Qwax), cooler canopies, DSI, and yield components (MSHW & TKW) were donated by Halberd.

These observations of low LOD scores and phenotypic variations could be a result of high genotype by environment interactions, suggesting that traits for environmental adaptation or minimum effect QTLs, will be difficult to select for (Romagosa and Fox 1993).

3.4.2. Genetic dissection of EW and CT

Most physiological traits confer significant genetic variation (water soluble carbohydrates, carbon isotopes discrimination, osmotic potential, etc.) yet few genes (QTL) that benefit production have been detected under moisture stress conditions to date (Rebetzke et al. 2008). And yet all loci identified suitable for breeding have been cloned and few have been deployed using marker assisted selection (Collins et al. 2008). Stable QTL co-localizing for various traits and identified across multiple environments would be of high value. This study identified novel regions on the A and B genomes of wheat similar to those reported in previous studies (Bennett et al. 2012a; Pinto et al. 2010). The lack of significant QTLs identified on the D genome is likely due to insufficient marker coverage. Pleiotropic QTL were identified for different traits such as leaf EW, CT, DSI, and yield components on 3B chromosome (Fig. 4), and similar to the study of Serim82 and Babax RIL population in which CT and yield components were phenotypically correlated and genetically co-localized on chromosome 3B (Pinto et al. 2010). The chromosomes 3B, 1A, and 7D were identified with stay green QTLs donated by Cheriya 3 explaining a phenotypic variation of 38.7% (Kumar et al. 2010). The co-localization of leaf EW and CT were not identified, although cooler leaf canopies have a unique and direct impact on yield increase across different environments (Olivares-Villegas et al. 2007; Pinto et al. 2010).

High temperature stress has detrimental effects on photosynthesis (Mathur et al. 2011). A thick waxy layer may reflect and dissipate excess heat and light from the leaf surface, preventing detrimental effects during reproductive stages (Grant 1987). Large

genotypic variations in leaf EW has been reported while EW in wheat and sorghum have also been shown to be responsive to the environment (Jordan et al. 1983). The RILs with Intermediate leaf EW content have been found to be highly stable across various environments. Wheat cultivars with higher wax content improved surface reflectance (Johnson et al. 1983) increased water use efficiency, improved flag leaf green area, and reduced CT (Richards et al. 1986). In this study, a novel and robust QTL (*QWax.tam-7A*) for leaf EW detected on the 7A chromosome co-localized with DSI and yield components (Fig. 4). Previous studies also identified pleiotropic QTLs for spectral reflectance indices normalized difference vegetative index (NDVI), co-localized and co-localizing for canopy temperature on chromosome 7A (Pinto et al. 2010). In another moisture stress study of wheat tetraploids QTLs regulating osmotic potential and chlorophyll content were detected on chromosome 5A, 5B, 6A and 6B (Peleg et al. 2009). We identified similar chromosomal loci for leaf EW and DSI, indicating the existence of moisture stress adaptive QTLs. Another closely linked genetic loci regulating canopy temperature and DSI-MSHW (14.34) was identified on chromosome 4A with LOD score of (19.87) explaining 35% and 41% of phenotypic variance, respectively. A similar position also associated with yield and CT showed a strong negative allelic correlation (Pinto et al. 2010). Also was reported a significant and strong negative correlation existing between the leaf EW and canopy temperature among pea cultivars, phenotype across different environments (Sánchez et al. 2001). The phenotypic correlations between leaf EW and CT were validated by co-localized loci on 2B, 3B, 5A, 6B, and 7B. Of these, four co-localized loci were detected throughout chromosome 3B

with phenotypic variation explaining between 9 to 16% (Fig. 4). Similar results were reported on chromosome 3B, for loci reducing leaf CT that co-localized with other physiological traits such as NDVI, water soluble carbohydrates (WSC), carbon isotope discrimination, and yield components (Bennett et al. 2012b; Pinto et al. 2010). In addition, leaf temperature depression QTL was identified on chromosome 3B that co-localized with HSI for mean single kernel weight where the major alleles were also donated by Halberd (Mason et al. 2013; Mason et al. 2011). These genetic loci on chromosome 3B could be given priority for fine mapping and candidate gene deployment or used for marker-assisted selection (MAS) to screen early generations for abiotic stress tolerance.

DSI for MSHW and TKW traits segregated uniformly across the population and were detected on various chromosomes 2B, 3B, 4A, 5A, 5B, 7A, and 7B (Table 4 & 5; Fig. 4). These chromosomal positions were previously reported for co-localization of HSI for mean single kernel weight and other yield components in another RIL populations with the same heat tolerant parent Halberd donating major alleles (Mason et al. 2011; Mason et al. 2010), thus, validating Halberd as drought tolerant and also for donating more alleles for moisture and heat stress tolerance. Both parents donated equal alleles for stable and co-localized yield QTLs across five environments (Table 7). Surprisingly, Halberd performed better than Len for yield components under moisture stress conditions though not significantly different (Table 4).

Information on leaf EW QTLs is lacking to date, with only a couple of genetic loci detected at 2B and 2D (King and von Wettstein-Knowles 2000). In some other

studies, 6 major and robust QTLs were detected across 3 environments for wax glaucousness with additive effects ranging from 0.5 to 1.1 (Bennett et al. 2012a). In this study, 12 robust and stable QTLs for leaf EW were found on 2B, 1A, 3A, 3B, 5A, 5B, 4A, and 7B with phenotypic variation ranging between 7.8 to 25% with 8 having an additive effect (0.01 to 0.55) from Halberd and 4 additive effect (-0.01 to -0.23) QTLs coming from Len. Apart from moisture or heat stress tolerance in Halberd, previous QTL studies revealed the existence of late maturity alpha amylase loci at 7B and 3B (Mrva and Mares 2001), yellow leaf spot disease resistance traced at 5B (Cheong et al. 2004), adult plant resistance and leaf rust severity at 3D and 7B (Bariana et al. 2007), flour quality traits at 7A and 3B (Mares and Campbell 2001), longer seedling and greater seedling vigor at 6A (Spielmeyer et al. 2007), and boron tolerance at 7B (Jefferies et al. 2000). Halberd has been associated with various positive QTLs at 5B, 7A, 7B, and 3B for biotic and abiotic stress tolerance. The novel SNPs marker such as *w SNP_Ra_c2730_5190076*, *Excalibur_c17241_388*, and *w SNP_Ex_c3267_6026545* have showed a significant difference for alleles between Halberd and Len for CT. Alleles from Halberd produced a cooling effect of 2.4°C on leaf canopy temperature during reproductive stages compared to Len under moisture stress conditions (Table 5 & 6). These loci were first traced close to each other at the distal region of chromosome 6B and were also identified in the mapping population KleinProteo x KleinChaja developed by Jorge Dubcovsky (Cavanagh et al. 2013). Halberd also showed QTL with high levels of leaf EW content on 6B compared to the susceptible cultivars Len (Table 6), Karl92 and Cutter (Mondal et al., 2013).

Table 7 Summary of significant QTLs detected for agronomic and physiological traits, linkage groups, positions and phenotypic variations across 5 environments during 2011 and 2012.

Linkage groups	DSI-MSHW	DSI-TKW	EW		CT		MSHW		TKW		KNS	
			C	D	C	D	C	D	C	D	C	D
2B	4	-	3	4	4	2	-	2	1	4	-	4
1A	3	2	1	1	2	1	3	-	-	2	-	-
3A	-	1	3	1	1	-	-	1	-	2	-	2
1B	5	-	2	-	-	1	2	1	-	1	-	-
7A	2	1	2	1	1	-	6	7	2	1	4	6
3B	-	2	3	3	2	1	1	2	-	2	1	3
6B-1	-	-	-	-	1	1	-	-	-	-	-	-
5B-1	4	4	2	4	1	-	-	1	-	4	-	3
5B-2	3	-	4	1	-	-	-	-	-	-	-	2
4A	1	3	1	2	2	2	2	1	-	-	1	1
7D	1	-	-	-	-	-	-	1	-	-	-	2
7B	-	4	2	1	2	3	2	4	2	4	1	1
6A	2	-	4	-	1	1	1	1	-	1	1	1
6B-2	2	1	1	1	2	-	1	2	4	2	1	1
5A	2	-	2	3	2	-	-	2	-	2	-	2
4B	1	-	-	-	1	-	2	2	2	-	1	1
1D	-	1	1	-	2	2	-	-	-	-	-	-
2A	-	1	-	-	-	-	-	-	-	-	-	-
6D	-	-	-	-	-	-	-	-	1	-	-	-
Total QTLs	30	20	31	22	25	14	20	27	12	25	10	29
Max %R ²	41	17	31	16	25	35	21	27	20	34	22	21

Bold and italics : QTLs with main effects and repeating in more than one environment, classified as stable QTLs traced in particular chromosomes with more than an year or location

Shaded regions: Chromosomes with shade across moisture stress (D) and Control (C) environment are co-localized for particular SNPs

Unshaded and not bold: These regions did not showed any consistency for more than an environment

Except CT (3 environments) all other traits were measured across 5 environments during 2011 and 2012.

Stable QTLs across environments and treatments (Hot and moisture deficit) in each linkage groups are not far apart, less than 10 cM.

3.4.3. Potential SNPs for marker assistance selection

The QTL for moisture stress tolerance consistently identified in different environments with high LOD scores were *QWax.tam-5B*, *QDSI-MSHW.tam-1B*, and *QCT.tam-2B* (Table 7). Interestingly; high mean leaf EW, low leaf CT and DSI trait combinations were also detected in the susceptible parent Len, for a few loci such as *Qwax.tam-4A*, *QCT.tam-2B*, and *QDSIM.tam-1B* (Fig. 4). Numerous stable and consistent QTLs regulating high levels of leaf EW were identified under both moisture stress and irrigation conditions. Yield components and DSI have been genetically dissected under moisture stress and irrigated treatments through QTL mapping. The moisture stress treatment increased leaf EW content. Robust QTLs detected across multiple environments could have a significant impact as MAS tools for developing moisture stress tolerant wheat lines. The EW QTL designated as *Qwax.tam-2B* (*BS00071690*) with a LOD score of 11.2 contributed by Halberd explained 25% of phenotypic variation and also co-localized with other traits such as MSHW, KNS, TKW, DSI, and CT at multiple trail locations (Fig. 4). Fine mapping loci could provide breeders with a selection tool to improve moisture stress tolerance in wheat for multiple stress environments. In addition, to improving physiological marker tools the QTL on 7A and 7B (*wsnp_Ex_rep_c68227_67012082* at 7A and *wsnp_Ex_c15972_24385702* at 7B) were significant loci for different yield components under both moisture stress and irrigated conditions (Table 8). Common QTLs were identified for drought and control treatment across different chromosomal regions (Table 8).

Table 8 Contrast analysis for QTLs with significant difference between moisture stress and control treatments across different environments and chromosomal linkage groups for different traits in 180 Len X Halberd RILs population during 2011 and 2012.

Trait	SNP	Chromosome	Environment	A	B	Significance level
DSIS	RAC875_c29533_594	7A	UV11	1.28	2.56	*
	RAC875_c22792_672	7A	CH11	1.33	2.54	*
MSHW	BS00040992	7A	CH11_D	0.391	0.279	*
	BS00040992		CH11_H	0.798	0.763	*
TKW	BS00034689_51	7A	CS11_D	48.99	49.21	*
	BS00034689_51		CS11_H	57.30	56.63	*
KNS	BS00034689_51	7A	CS11_D	13.08	12.71	*
	BS00034689_51		CS11_H	13.35	13.06	*
Wax	CAP7_c11288_109	5B	UV12_D	2.39	2.49	*
	CAP7_c11288_109		UV12_H	1.58	1.99	*
DSIS	wsnp_Ra_c26091_35652620		UV11	1.32	2.36	*
DSIM	Ex_c41873_341		UV12	0.73	1.26	*
MSHW	wsnp_Ku_c19112_28546731	7B	CS11_D	0.607	0.589	*
			CS11_H	0.860	0.862	*
KNS	wsnp_Ku_c19112_28546731		CS11_D	13.08	12.66	*
			CS11_H	13.66	12.85	*
DSIM	BS00011962	6A	CS11	0.64	0.49	*
			CH11	1.10	0.81	*
TKW	RAC875_c23251_624	6B	CS11_D	48.39	49.71	*
			CS11_H	55.47	57.60	*
KNS	RAC875_c23251_624		CS11_D	13.08	12.66	ns
			CS11_H	13.66	12.85	ns
MSHW	RAC875_c12495_1391	4B	UV11_D	0.801	0.843	*
			UV11_H	0.881	0.883	*
KNS	BobWhite_rep_c49034_167		UV12_D	25.00	27.27	*
			UV12_H	26.39	28.29	*
CT	Excalibur_c84741_99	2B	UV11_D	34.5	34.4	ns
	wsnp_Ku_c3780_6950286		UV11_H	33.7	33.7	ns

* - Significant, ns - Non-significant, DSIS- Drought susceptible index-Thousand kernel weight, MSHW- Mean single head weight, TKW- Thousand kernel weight, KNS- Kernel number per spike, DSIM- Drought susceptible index for MSHW, CT- Canopy temperature. A- Halberd allele, B- Len allele.

The genes with good marker and trait associations will be further dissected by using heterogenous inbred families (HIFs).

3.5. Conclusions

The significant genetic variance in Len/Halberd population across five environments identified novel and stable genetic loci associated with yield components, yield stability, DSI, canopy temperature, and EW. Loci 2B, 5B, 3B, 4A, 6B, and 7A which showed significant co-localization of cooler canopies, increased EW, and indicate potential genetic regions for fine mapping and marker assisted selection. Our study results also conclude that leaf EW is controlled by many genes, may have additive effects unlike wax glaucousness with 1 or 2 genes (Tsunewaki 1966; Tsunewaki and Ebana 1999). Many robust SNPs detected across environments will be ideal MAS tools to improve leaf EW and may also result in cooler canopies and higher yields.

CHAPTER IV

CONCLUSIONS

The research work described in this dissertation has first focused on quantifying and describing the variation in leaf epicuticular wax production and canopy temperature in response to moisture stress in wheat under field conditions. Moreover it helps to understand the role of EW to decrease canopy temperature and its impact on yield potential and stability across moisture stress conditions. This study also explains the role of leaf EW as drought adaptive trait in improving the potential and stability of grain yield components. The leaf EW load significantly correlated with plot yield ($r=32\%$), DSI ($r=-40\%$), and leaf CT ($r=-32\%$) under water-deficit conditions. In addition, EW and CT correlated with higher yield stability using DSI and across environments using Eberhart stability during water deficit. This study explains the interrelationship between leaf EW and cooler canopies in improving yield potential and stability under water-deficit conditions in wheat. The co-localized loci identified serve as potential target regions to screen for water-deficit tolerant lines in wheat germplasm and for marker-assisted selection.

Analysis of Len X Halberd population under field conditions help to identify novel and stable QTLs identified in more than one environment for MSHW, TKW, KNS, head number m^{-2} , DSI, leaf EW, and canopy temperature. Novel and robust co-localized QTLs for the leaf EW, cooler canopies, DSI, and grain attributes were detected on 2B, 3B, 5A, 5B, 6B, 7A, and 7B chromosome loci. 4A was detected with high LOD

co-localization of CT and DSI along with independent EW loci explaining 35%, 41%, and 31% phenotypic variation respectively. 3B was shown to have a close association between leaf EW and canopy temperature all across the chromosomal length. 6B was identified with significant SNPs (*wsnp_Ra_c2730_5190076*, *Excalibur_c17241_388*, and *wsnp_Ex_c3267_6026545*) associated with 2.4°C cooler canopy temperatures in Halberd compared to Len. Halberd parent played role in donating alleles for moisture stress tolerance and Len donates yield allelic variants. The loci identified in this study would be a good source for marker assisted selection of leaf EW and other drought tolerant traits.

REFERENCES

- Akhunov E, Nicolet C, Dvorak J (2009) Single nucleotide polymorphism genotyping in polyploid wheat with the Illumina golden gate assay. *Theor Appl Genet* 119:507-517
- Ali A, Masood MA, Zahid MA (2012) Identifying the most promising genotypes in lentil for cultivation in a wide range of environments of Pakistan using various yield stability measures. *Pakistan Journal of Botany* 44:1919-1922
- Allen AM, Barker GL, Berry ST, Coghill JA, Gwilliam R, Kirby S, Robinson P, Brenchley RC, D'Amore R, McKenzie N (2011) Transcript-specific, single-nucleotide polymorphism discovery and linkage analysis in hexaploid bread wheat (*Triticum aestivum* L.). *Plant Biotechnology Journal* 9:1086-1099
- Araus J, Slafer G, Reynolds M, Royo C (2002) Plant breeding and drought in C3 cereals: What should we breed for? *Annals of Botany* 89:925-940
- Araus JL, Febrero A, Vendrell P (1991) Epidermal conductance in different parts of durum wheat grown under Mediterranean conditions: The role of epicuticular waxes and stomata. *Plant, Cell & Environment* 14:545-558
- Arnold RB, Ghera C, Sanchez R, Fernandez AG (1988) The role of fluctuating temperatures in the germination and establishment of *Sorghum halepense* (L.) Pers. Regulation of germination under leaf canopies. *Functional Ecology* 2:311-318
- Assad M, Paulsen G (2002) Genetic changes in resistance to environmental stresses by US Great Plains wheat cultivars. *Euphytica* 128:85-96
- Awika H (2013) Determining genetic overlap between staygreen, leaf wax and canopy temperature depression in sorghum RILs. *Soil and Crop Sciences*. Texas A&M University, College Station, TX, p 120
- Babar M, Reynolds M, Van Ginkel M, Klatt A, Raun W, Stone M (2006) Spectral reflectance to estimate genetic variation for in-season biomass, leaf chlorophyll, and canopy temperature in wheat. *Crop Science* 46:1046-1057
- Baenziger P, Wesenberg D, Sicher R (1983) The effects of genes controlling barley leaf and sheath waxes on agronomic performance in irrigated and dryland environments. *Crop Science* 23:116-120
- Bariana H, Miah H, Brown G, Willey N, Lehmensiek A (2007) Molecular mapping of durable rust resistance in wheat and its implication in breeding. *Wheat Production in Stressed Environments* 12:723-728

Barták M, Hájek J, Vráblíková H, Dubová J (2004) High-light stress and photoprotection in *Umbilicaria antarctica* monitored by chlorophyll fluorescence imaging and changes in zeaxanthin and glutathione. *Plant Biology* 6:333-341

Bengtson C, Larsson S, Liljenberg C (1978) Effects of water stress on cuticular transpiration rate and amount and composition of epicuticular wax in seedlings of six oat varieties. *Physiologia Plantarum* 44:319-324

Bennett D, Izanloo A, Edwards J, Kuchel H, Chalmers K, Tester M, Reynolds M, Schnurbusch T, Langridge P (2012a) Identification of novel quantitative trait loci for days to ear emergence and flag leaf glaucousness in a bread wheat (*Triticum aestivum* L.) population adapted to southern Australian conditions. *Theor Appl Genet* 124:697-711

Bennett D, Reynolds M, Mullan D, Izanloo A, Kuchel H, Langridge P, Schnurbusch T (2012b) Detection of two major grain yield QTL in bread wheat (*Triticum aestivum* L.) under heat, drought and high yield potential environments. *Theor Appl Genet* 125:1473-1485

Bhullar S, Jenner C (1985) Differential responses to high temperatures of starch and nitrogen accumulation in the grain of four cultivars of wheat. *Functional Plant Biology* 12:363-375

Bird D, Beisson F, Brigham A, Shin J, Greer S, Jetter R, Kunst L, Wu X, Yephremov A, Samuels L (2007) Characterization of Arabidopsis ABCG11/WBC11, an ATP binding cassette (ABC) transporter that is required for cuticular lipid secretion. *The Plant Journal* 52:485-498

Blum A (1975) Effect of the Bm gene on epicuticular wax and the water relations of *Sorghum bicolor*. *Israel Journal Botany* 24:1-24

Blum A (2005) Drought resistance, water-use efficiency, and yield potential are they compatible, dissonant, or mutually exclusive? *Crop and Pasture Science* 56:1159-1168

Blum A, Shpiler L, Golan G, Mayer J (1989) Yield stability and canopy temperature of wheat genotypes under drought-stress. *Field Crops Research* 22:289-296

Byerlee D, Moya P (1993) Impacts of international wheat breeding research in the developing world, 1966-1990. CIMMYT, Elbatan, Mexico

Cameron KD, Teece MA, Smart LB (2006) Increased accumulation of cuticular wax and expression of lipid transfer protein in response to periodic drying events in leaves of tree tobacco. *Plant Physiology* 140:176-183

Cavanagh CR, Chao S, Wang S, Huang BE, Stephen S, Kiani S, Forrest K, Saintenac C, Brown-Guedira GL, Akhunova A (2013) Genome-wide comparative diversity uncovers multiple targets of selection for improvement in hexaploid wheat landraces and cultivars. *Proceedings of the National Academy of Sciences* 110:8057-8062

Ceccarelli S (1987) Yield potential and drought tolerance of segregating populations of barley in contrasting environments. *Euphytica* 36:265-273

Chatterton N, Lee D, Powell J, Hanna W (1975) Photosynthesis and transpiration of bloom and bloomless sorghum. *Canadian Journal of Plant Science* 55:641-643

Chaves MM, Maroco JP, Pereira JS (2003) Understanding plant responses to drought from genes to the whole plant. *Functional Plant Biology* 30:239-264

Cheong J, Wallwork H, Williams K (2004) Identification of a major QTL for yellow leaf spot resistance in the wheat varieties Brookton and Cranbrook. *Crop and Pasture Science* 55:315-319

Clarke JM, McCaig TN, Depauw RM (1994) Inheritance of glaucousness and epicuticular wax in durum wheat. *Crop Science* 34:327-330

Collins NC, Tardieu F, Tuberosa R (2008) Quantitative trait loci and crop performance under abiotic stress: where do we stand? *Plant Physiology* 147:469-486

Deckmyn G, Martens C, Impens I (1994) The importance of the ratio UV-B/photosynthetic active radiation (PAR) during leaf development as determining factor of plant sensitivity to increased UV-B irradiance: effects on growth, gas exchange and pigmentation of bean plants (*Phaseolus vulgaris* cv. Label). *Plant, Cell & Environment* 17:295-301

Doyle JJ (1990) Isolation of plant DNA from fresh tissue. *Focus* 12:13-15

Driscoll C (1966) Gene-centromere distances in wheat by aneuploid F2 observations. *Genetics* 54:131-135

Ebercon A, Blum A, Jordan W (1977) A rapid colorimetric method for epicuticular wax content of sorghum leaves. *Crop Science* 17:179-180

Eberhart St, Russell W (1966) Stability parameters for comparing varieties. *Crop Science* 6:36-40

Ehleringer J (1980) Leaf morphology and reflectance in relation to water and temperature stress. In: Turner N, Kramer P (eds) *Adaptation of plants to water and high temperature stress* Wiley-Interscience, New York, pp 123-128

- Eigenbrode SD, Espelie KE (1995) Effects of plant epicuticular lipids on insect herbivores. *Annual Review of Entomology* 40:171-194
- FAO (2013) FAO cereal supply and demand brief.
<http://www.fao.org/giews/english/shortnews/cpp09052013pdf>
- Febrero A, Fernández S, Molina-Cano JL, Araus JL (1998) Yield, carbon isotope discrimination, canopy reflectance and cuticular conductance of barley isolines of differing glaucousness. *Journal of Experimental Botany* 49:1575-1581
- Figueiredo KV, Oliveira MT, Oliveira AFM, Silva GC, Santos MG (2012) Epicuticular-wax removal influences gas exchange and water relations in the leaves of an exotic and native species from a Brazilian semiarid region under induced drought stress. *Australian Journal of Botany* 60:685-692
- Finlay K, Wilkinson G (1963) The analysis of adaptation in a plant-breeding programme. *Crop and Pasture Science* 14:742-754
- Fischer R, Maurer R (1978) Drought resistance in spring wheat cultivars. I. Grain yield responses. *Crop and Pasture Science* 29:897-912
- Fischer R, Sayre K, Reynolds M (2005) Osmotic adjustment in wheat in relation to grain yield under water deficit environments. *Agronomy Journal* 97:1062-1071
- Fischer R, Wood J (1979) Drought resistance in spring wheat cultivars. III. Yield associations with morpho-physiological traits. *Crop and Pasture Science* 30:1001-1020
- Fleury D, Jefferies S, Kuchel H, Langridge P (2010) Genetic and genomic tools to improve drought tolerance in wheat. *Journal of Experimental Botany* 61:3211-3222
- Fu D, Uauy C, Distelfeld A, Blechl A, Epstein L, Chen X, Sela H, Fahima T, Dubcovsky J (2009) A kinase-Start gene confers temperature-dependent resistance to wheat stripe rust. *Science* 323:1357-1360
- González A, Ayerbe L (2010) Effect of terminal water stress on leaf epicuticular wax load, residual transpiration and grain yield in barley. *Euphytica* 172:341-349
- González A, Martín I, Ayerbe L (2007) Response of barley genotypes to terminal soil moisture stress: phenology, growth, and yield. *Crop and Pasture Science* 58:29-37
- González A, Martín I, Ayerbe L (2008) Yield and osmotic adjustment capacity of barley under terminal water-stress conditions. *Journal of Agronomy and Crop Science* 194:81-91

Grant L (1987) Diffuse and specular characteristics of leaf reflectance. *Remote Sensing of Environment* 22:309-322

Grant R, Jenks M, Rich P, Peters P, Ashworth E (1995) Scattering of ultraviolet and photosynthetically active radiation by *Sorghum bicolor*: influence of epicuticular wax. *Agricultural and Forest Meteorology* 75:263-281

Gregersen PL, Brinch-Pedersen H, Holm PB (2005) A microarray-based comparative analysis of gene expression profiles during grain development in transgenic and wild type wheat. *Transgenic Research* 14:887-905

Haque MM, Mackill DJ, Ingram KT (1992) Inheritance of leaf epicuticular wax content in rice. *Crop Science* 32:865-868

Hays DB, Do JH, Mason RE, Morgan G, Finlayson SA (2007) Heat stress induced ethylene production in developing wheat grains induces kernel abortion and increased maturation in a susceptible cultivar. *Plant Science* 172:1113-1123

Herbek J, Lee C (2009) A comprehensive guide to wheat management in kentucky. In: Herbek J, Lee C, William B, Green J, John G, James R, Lloyd M, Greg S, David V (eds) US Department of Agriculture, Cooperative Extension Service, University of Kentucky College of Agriculture, Lexington, and Kentucky State University, Frankfort
<http://www.uky.edu/Ag/Grain-Crops/ID125Section2.htm>

Hossain A, Teixeira da Silva J, Lozovskaya M, Zvolinsky V, Mukhortov V (2012) High temperature combined with drought affect rainfed spring wheat and barley in south-eastern Russia: yield, relative performance and heat susceptibility index. *Journal of Plant Breeding and Crop Science* 4:184-196

Huang X, Kempf H, Ganai M, Röder M (2004) Advanced backcross QTL analysis in progenies derived from a cross between a German elite winter wheat variety and a synthetic wheat (*Triticum aestivum* L.). *Theor Appl Genet* 109:933-943

Ishag M, A Mohamed B, HM Ishag K (1998) Leaf development of spring wheat cultivars in an irrigated heat-stressed environment. *Field Crops Research* 58:167-175

Isobe T, Feigelson E, Nelson P (1986) Statistical methods for astronomical data with upper limits. II-Correlation and regression. *The Astrophysical Journal* 306:490-507

Jaccoud D, Peng K, Feinstein D, Kilian A (2001) Diversity arrays: a solid state technology for sequence information independent genotyping. *Nucleic Acids Research* 29:e25-e25

Jansen RC, Stam P (1994) High resolution of quantitative traits into multiple loci via interval mapping. *Genetics* 136:1447-1455

Jefferey M (1995) The weather underground. National weather station, The Weather Channel Interactive Atlanta, Georgia 30339, www.wunderground.com

Jefferies S, Pallotta MA, Paull JG, Karakousis A, Kretschmer JM, Manning S, Islam A, Langridge P, Chalmers K (2000) Mapping and validation of chromosome regions conferring boron toxicity tolerance in wheat (*Triticum aestivum*). *Theor Appl Genet* 101:767-777

Jefferson P, Johnson D, Rumbaugh M, Asay K (1989) Water stress and genotypic effects on epicuticular wax production of alfalfa and crested wheatgrass in relation to yield and excised leaf water loss rate. *Canadian Journal of Plant Science* 69:481-490

Jefferson PG (1994) Genetic variation for epicuticular wax production in Altai wildrye populations that differ in glaucousness. *Crop Science* 34:367-371

Jenks MA, Eigenbrode SD, Lemieux B (2002) Cuticular waxes of Arabidopsis. *The Arabidopsis Book/American Society of Plant Biologists* 1:1-22

Jenks MA, Rich PJ, Peters PJ, Axtell JD, Ashworth EN (1992) Epicuticular wax morphology of bloomless (*bm*) mutants in *Sorghum bicolor*. *International Journal of Plant Sciences* 153:311-319

Ji X, Shiran B, Wan J, Lewis DC, Jenkins CL, Condon AG, Richards RA, Dolferus R (2010) Importance of pre-anthesis anther sink strength for maintenance of grain number during reproductive stage water stress in wheat. *Plant, Cell & Environment* 33:926-942

Johnson DA, Richards RA, Turner NC (1983) Yield, water relations, gas exchange, and surface reflectances of near-isogenic wheat lines differing in glaucousness. *Crop Science* 23:318-325

Jordan W, Monk R, Miller F, Rosenow D, Clark L, Shouse P (1983) Environmental physiology of sorghum. I. Environmental and genetic control of epicuticular wax load. *Crop Science* 23:552-558

Khanna-Chopra R (1999) Osmotic adjustment and yield stability in wheat genotypes and species grown in water limited environments. *Journal of Plant Biology* 26:173-178

Kilic H, Yagbasanlar T (2010) The effect of drought stress on grain yield, yield components and some quality traits of durum wheat (*Triticum turgidum* ssp. durum) cultivars. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* 38:164-170

Kim KS, Park SH, Jenks MA (2007a) Changes in leaf cuticular waxes of sesame (*Sesamum indicum* L.) plants exposed to water deficit. *Journal of Plant Physiology* 164:1134-1143

- Kim KS, Park SH, Kim DK, Jenks MA (2007b) Influence of water deficit on leaf cuticular waxes of soybean (*Glycine max* [L.] Merr.). *International Journal of Plant Sciences* 168:307-316
- King R, von Wettstein-Knowles P (2000) Epicuticular waxes and regulation of ear wetting and pre-harvest sprouting in barley and wheat. *Euphytica* 112:157-166
- Knipling EB (1970) Physical and physiological basis for the reflectance of visible and near-infrared radiation from vegetation. *Remote Sensing of Environment* 1:155-159
- Koch K, Barthlott W, Koch S, Hommes A, Wandelt K, Mamdouh W, De-Feyter S, Broekmann P (2006) Structural analysis of wheat wax (*Triticum aestivum*, cv 'Naturastar'L.): from the molecular level to three dimensional crystals. *Planta* 223:258-270
- Kolmer J, Garvin D, Jin Y (2011) Expression of a thatcher wheat adult plant stem rust resistance qtl on chromosome arm 2BL is enhanced by *Lr34*. *Crop Science* 51:526-533
- Kovalchuk N, Smith J, Bazanova N, Pyvovarenko T, Singh R, Shirley N, Ismagul A, Johnson A, Milligan AS, Hrmova M (2012) Characterization of the wheat gene encoding a grain-specific lipid transfer protein TdPR61, and promoter activity in wheat, barley and rice. *Journal of Experimental Botany* 63:2025-2040
- Krattinger SG, Lagudah ES, Spielmeier W, Singh RP, Huerta-Espino J, McFadden H, Bossolini E, Selter LL, Keller B (2009) A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. *Science* 323:1360-1363
- Kumar U, Joshi AK, Kumari M, Paliwal R, Kumar S, Röder MS (2010) Identification of QTLs for stay green trait in wheat (*Triticum aestivum* L.) in the 'Chirya 3' × 'Sonalika' population. *Euphytica* 174:437-445
- Lin C-S, Binns MR, Lefkovich LP (1986) Stability analysis: where do we stand? *Crop Science* 26:894-900
- Liu Q, Ni Z, Peng H, Song W, Liu Z, Sun Q (2007) Molecular mapping of a dominant non-glaucousness gene from synthetic hexaploid wheat (*Triticum aestivum* L.). *Euphytica* 155:71-78
- Mares DJ, Campbell A (2001) Mapping components of flour and noodle colour in Australian wheat. *Crop and Pasture Science* 52:1297-1309
- Marza F, Bai G-H, Carver B, Zhou W-C (2006) Quantitative trait loci for yield and related traits in the wheat population Ning7840 × Clark. *Theor Appl Genet* 112:688-698

Mason RE, Hays DB, Mondal S, Ibrahim AM, Basnet BR (2013) QTL for yield, yield components and canopy temperature depression in wheat under late sown field conditions. *Euphytica* 194:243-259

Mason RE, Mondal S, Beecher FW, Hays DB (2011) Genetic loci linking improved heat tolerance in wheat (*Triticum aestivum* L.) to lower leaf and spike temperatures under controlled conditions. *Euphytica* 180:181-194

Mason RE, Mondal S, Beecher FW, Pacheco A, Jampala B, Ibrahim AM, Hays DB (2010) QTL associated with heat susceptibility index in wheat (*Triticum aestivum* L.) under short-term reproductive stage heat stress. *Euphytica* 174:423-436

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

Meeks M, Murray SC, Hague S, Hays D, Ibrahim AMH (2012) Genetic variation for maize epicuticular wax response to drought stress at flowering. *Journal of Agronomy and Crop Science* 198:161-172

Millar AA, Clemens S, Zachgo S, Giblin EM, Taylor DC, Kunst L (1999) CUT1, an *Arabidopsis* gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. *The Plant Cell Online* 11:825-838

Mkhabela SM (2012) Genetic variation, heritability estimates, and yield relationship of pre-flowering and post-flowering drought resistant traits in grain sorghum. *Agronomy*. Texas Tech University, Lubbock, TX, p 142

Mohammadian MA, Watling JR, Hill RS (2007) The impact of epicuticular wax on gas-exchange and photoinhibition in *Leucadendron lanigerum* (Proteaceae). *Acta Oecologica* 31:93-101

Mohammed S, Blaser B, Stewart B (2012) Planting geometry and plant population affect dryland maize grain yield and harvest index. *Journal of Crop Improvement* 26:130-139

Moinuddin, Fischer RA, Sayre KD, Reynolds MP (2005) Osmotic adjustment in wheat in relation to grain yield under water deficit environments. *Agronomy Journal* 97:1062-1071

Mondal S (2013) Defining the molecular and physiological role of leaf cuticular waxes in reproductive stage heat tolerance in wheat. *Soil and Crop Sciences*. Texas A&M University, College Station, TX, p 126

Mondal S, Hays D (2007) Leaf epicuticular wax improves heat tolerance in wheat. ASA-CSSA-SSSA 2007 International Annual Meetings. 4–8 November 2007. New Orleans, LA

Mrva K, Mares DJ (2001) Quantitative trait locus analysis of late maturity α -amylase in wheat using the doubled haploid population Cranbrook Halberd. *Crop and Pasture Science* 52:1267-1273

Musick J, Dusek D (1980) Planting date and water deficit effects on development and yield of irrigated winter wheat. *Agronomy Journal* 72:45-52

Nelson JC, Deynze AEV, Sorrells ME, Autrique E, Lu YH, Merlino M, Atkinson M, Leroy P (1995) Molecular mapping of wheat. Homoeologous group 2. *Genome* 38:516-524

Olivares-Villegas JJ, Reynolds MP, McDonald GK (2007) Drought-adaptive attributes in the Seri/Babax hexaploid wheat population. *Functional Plant Biology* 34:189-203

Passioura J (2007) The drought environment: physical, biological and agricultural perspectives. *Journal of Experimental Botany* 58:113-117

Paull J, Chalmers K, Karakousis A, Kretschmer JM, Manning S, Langridge P (1998) Genetic diversity in Australian wheat varieties and breeding material based on RFLP data. *Theor Appl Genet* 96:435-446

Paull J, Nable R, Rathjen A (1992) Physiological and genetic control of the tolerance of wheat to high concentrations of boron and implications for plant breeding. *Plant and Soil* 146:251-260

Peleg Z, Fahima T, Krugman T, Abbo S, Yakir D, Korol AB, Saranga Y (2009) Genomic dissection of drought resistance in durum wheat \times wild emmer wheat recombinant inbred line population. *Plant, Cell & Environment* 32:758-779

Peñuelas J, Isla R, Filella I, Araus JL (1997) Visible and near-infrared reflectance assessment of salinity effects on barley. *Crop Science* 37:198-202

Pingali PL, Rajaram S (eds) (1999) *Global wheat research in a changing world: Options for sustaining growth in wheat productivity*. CIMMYT, Elbatan, Mexico

Pinto RS, Reynolds MP, Mathews KL, McIntyre CL, Olivares-Villegas J, Chapman SC (2010) Heat and drought adaptive QTL in a wheat population designed to minimize confounding agronomic effects. *Theor Appl Genet* 121:1001-1021

Post-Beittenmiller D (1996) Biochemistry and molecular biology of wax production in plants. *Annual Review of Plant Biology* 47:405-430

Premachandra G, Hahn D, Axtell J, Joly R (1994) Epicuticular wax load and water-use efficiency in bloomless and sparse-bloom mutants of *Sorghum bicolor* L. *Environmental and Experimental Botany* 34:293-301

Rashid A, Stark J, Tanveer A, Mustafa T (1999) Use of canopy temperature measurements as a screening tool for drought tolerance in spring wheat. *Journal of Agronomy and Crop Science* 182:231-238

Rebetzke G, Condon A, Farquhar G, Appels R, Richards R (2008) Quantitative trait loci for carbon isotope discrimination are repeatable across environments and wheat mapping populations. *Theor Appl Genet* 118:123-137

Rebetzke GJ, Rattey AR, Farquhar GD, Richards RA, Condon ATG (2012) Genomic regions for canopy temperature and their genetic association with stomatal conductance and grain yield in wheat. *Functional Plant Biology* 40:14-33

Rees D, Sayre K, Acevedo E, Nava Sanchez T, Lu Z, Zeiger E, Limon A (1993) Canopy temperatures of wheat: Relationship with yield and potential as a technique for early generation selection. *Wheat special report no. 10, CIMMYT, Elbatan, Mexico*

Reicosky DA, Hanover JW (1978) Physiological effects of surface waxes I. Light reflectance for glaucous and nonglaucous *Picea pungens*. *Plant Physiology* 62:101-104

Reynolds M, Skovmand B, Trethowan R, Pfeiffer W (2000) Evaluating a conceptual model for drought tolerance. In: Ribaut J, Poland D (eds) *Molecular approaches for the genetic improvement of cereals for stable production in Water-limited environments*, CIMMYT, Elbatan, Mexico, pp 49-53

Reynolds MP, Pierre CS, Saad AS, Vargas M, Condon AG (2007) Evaluating potential genetic gains in wheat associated with stress-adaptive trait expression in elite genetic resources under drought and heat stress. *Crop Science* 47:172-189

Richards R (2000) Selectable traits to increase crop photosynthesis and yield of grain crops. *Journal of Experimental Botany* 51:447-458

Richards R, Rawson H, Johnson D (1986) Glaucousness in wheat: Its development and effect on water-use efficiency, gas exchange and photosynthetic tissue temperatures. *Functional Plant Biology* 13:465-473

Ristic Z, Jenks MA (2002) Leaf cuticle and water loss in maize lines differing in dehydration avoidance. *Journal of Plant Physiology* 159:645-651

Romagosa I, Fox P (1993) Genotype \times environment interaction and adaptation. In: Haywards M, Bosemark N, Romagosa I, Cerezo M (eds) *Plant Breeding*. Springer Netherlands, International Food Policy Research Institute, Washington, DC, pp 373-390

- Rosegrant M, Paisner M, Maeijer S, Witcover J (2001) Global food projections to 2020: Emerging trends and alternative futures. IFPRI, International Washington DC
- Rudd J (2011) AgriLife Research study aimed at reducing drought-stress losses to wheat. Agrilife Today, Amarillo, TX <https://today.agrilife.org/2011/12/14/agrilife-research-study-aimed-at-reducing-drought-stress-losses-to-wheat/>
- Saeedipour S, Moradi F (2011) Comparison of the drought stress responses of tolerant and sensitive wheat cultivars during grain filling: Impact of invertase activity on carbon metabolism during kernel development. *Journal of Agricultural Science* 3:32-44
- Samdur M, Manivel P, Jain V, Chikani B, Gor H, Desai S, Misra J (2003) Genotypic differences and water-deficit induced enhancement in epicuticular wax load in peanut. *Crop Science* 43:1294-1299
- Sánchez FJ, Manzanares Ma, de Andrés EF, Tenorio JL, Ayerbe L (2001) Residual transpiration rate, epicuticular wax load and leaf colour of pea plants in drought conditions. Influence on harvest index and canopy temperature. *European Journal of Agronomy* 15:57-70
- Sangam V, Hanchinal R, Chetti M, Rajgopal B, Prashanthi S (1998) Phenotypic stability of wheat genotypes for epicuticular wax load under heat stress environments. *Crop Research-Hisar* 16:224-228
- SAS (2011) SAS/STAT 9.3 Output delivery system, user's guide. SAS Institute, Cary, North Carolina
- Shah N, Paulsen G (2003) Interaction of drought and high temperature on photosynthesis and grain-filling of wheat. *Plant and Soil* 257:219-226
- Smith E (1982) Heat and drought tolerant wheats of the future. Proc of the National Wheat Res Conf USDA-ARS, Beltsville, Maryland, pp 141-147
- Smith M, Coffman W, Barker T (1990) Environmental effects on selection under high and low input conditions, Department of Agronomy, Louisiana Agriculture Experiment Station, Baton Rouge, LA
- Snape J, Foulkes M, Simmonds J, Leverington M, Fish L, Wang Y, Ciavarrella M (2007) Dissecting gene \times environmental effects on wheat yields via QTL and physiological analysis. *Euphytica* 154:401-408
- Sojka R, Stolzy L, Fischer R (1981) Seasonal drought response of selected wheat cultivars. *Agronomy Journal* 73:838-845

Spielmeyer W, Hyles J, Joaquim P, Azanza F, Bonnett D, Ellis M, Moore C, Richards R (2007) A QTL on chromosome 6A in bread wheat (*Triticum aestivum*) is associated with longer coleoptiles, greater seedling vigour and final plant height. *Theor Appl Genet* 115:59-66

Srinivasan S, Gomez S, Kumar S, Ganesh S, Biji K, Senthil A, Babu R (2008) QTLs linked to leaf epicuticular wax, physio-morphological and plant production traits under drought stress in rice (*Oryza sativa* L.). *Plant Growth Regulation* 56:245-256

Stanton JS, Qi SL, Ryter DW, Falk SE, Houston NA, Peterson SM, Westenbroek SM, Christenson SC (2011) Selected approaches to estimate water-budget components of the High Plains, 1940 through 1949 and 2000 through 2009. US Geological Survey, Scientific Investigations Report, USGS Nebraska Water Science Center, Lincoln, NE, p 79

Stuckey JR (1972) Inheritance of glaucousness in wheat. University of New South Wales, Sydney, Australia

Thames JL (1961) Effects of wax coatings on leaf temperatures & field survival of *Pinus taeda* seedlings. *Plant Physiology* 36:180-182

Trethowan R, Pfeiffer W (2000) Challenges and future strategies in breeding wheat for adaptation to drought stressed environments: A CIMMYT wheat program perspective. In: Ribaut J, Poland D (eds) *Molecular approaches for the genetic improvement of cereals for stable production in water-limited environments*, CIMMYT, Elbatan, Mexico, pp 21-25

Tsunewaki K (1966) Comparative gene analysis of common wheat and its ancestral species. III. glume hairiness. *Genetics* 53:303-311

Tsunewaki K, Ebana K (1999) Production of near-isogenic lines of common wheat for glaucousness and genetic basis of this trait clarified by their use. *Genes & Genetic Systems* 74:33-41

Tuberosa R, Salvi S (2006) Genomics-based approaches to improve drought tolerance of crops. *Trends in Plant Science* 11:405-412

Tulloch A, Hoffman L (1971) Leaf wax of durum wheat. *Phytochemistry* 10:871-876

Turner NC (1979) Drought resistance and adaptation to water deficits in crop plants. In: Mussel H, Staples R (eds) *Stress physiology in crop plants* Wiley, New York, pp 344-372

Uddin MN, Marshall D (1988) Variation in epicuticular wax content in wheat. *Euphytica* 38:3-9

- USGCRP (2009) Global climate change impacts in the United Nations.
http://www.ucsusa.org/assets/documents/global_warming/us-global-climate-change-report-greatplainspdf
- Van Ooijen J (2004) MapQTL® 5. Software for the mapping of quantitative trait loci in experimental populations Kyazma BV, Wageningen, Netherlands
- Van Ooijen J (2006) JoinMap 4. Software for the calculation of genetic linkage maps in experimental populations Kyazma BV, Wageningen, Netherlands
- Vanderbilt VC, Grant L, Ustin SL (1991) Polarization of Light by Vegetation. In: Myneni R, Ross J (eds) Photon-Vegetation interactions. Springer Berlin Heidelberg, pp 191-228
- Voorrips R (2002) MapChart: software for the graphical presentation of linkage maps and QTLs. *Journal of Heredity* 93:77-78
- Watanabe N, Takesada N, Shibata Y, Ban T (2005) Genetic mapping of the genes for glaucous leaf and tough rachis in *Aegilops tauschii*, the D-genome progenitor of wheat. *Euphytica* 144:119-123
- Weldearegay DF, Yan F, Jiang D, Liu F (2012) Independent and combined effects of soil warming and drought stress during anthesis on seed set and grain yield in two spring wheat varieties. *Journal of Agronomy and Crop Science* 198:245-253
- Woolley JT (1971) Reflectance and transmittance of light by leaves. *Plant Physiology* 47:656-662
- Yang J, Zhang J, Liu L, Wang Z, Zhu Q (2002) Carbon remobilization and grain filling in Japonica/Indica hybrid rice subjected to postanthesis water deficits. *Agronomy Journal* 94:102-109