Genome Wide Association Studies for Wheat Kernel Hardness and Related Traits in Response to Heat and Combined Heat-Drought Stresses

（高温および高温・乾燥複合ストレスに対するコムギ種子硬度および関連形質のゲノムワイド関連解析）

Gamila Mohamed Idris Elhadi

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Gamila Mohamed Idris Elhadi

A thesis

submitted to the United Graduate School of Agricultural Sciences, Tottori University, in the partial fulfillment of the requirements for the degree of Doctor of Philosophy in Global Arid Land Science

The United Graduate School of Agricultural Sciences

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2021

Dedication

I dedicate this work for my father how always pray for me, my beloved husband for his endless support and sacrifices, my sweet son Ahmed and sweet daughter Dan, for my sister Tayseer, and for the soul of my mother and my sister.

Gamila Mohamed Idris Elhadi

2021

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*Although, I dedicate this work for my **husband Mohamed Yousif Abubakr** and my **father Mohamed Idris Elhadi**, again I would like to express my gratitude for them; my father keeping pray for me, and my husband for his endless support, encouragement and understanding. He sacrificed with his job, and devoted his time in order to support me, without this support I couldn't reach to this success.*

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LIST OF ABBREVIATIONS

MSD	Multiple synthetic derivatives
OP	Optimum
H	Heat
HD	heat–drought
ANOVA	Analysis of variance
SKCS	Single kernel characterization system
HSI	Heat susceptibility index
HDSI	Heat–drought susceptibility index
BLUP	Best linear unbiased prediction
MTA	Marker trait association
BLUP	Best linear unbiased prediction
GWAS	Genome wide association study
HDI	Combined heat–drought index
HI	Heat index
NAM	No-apical-meristem gene
SEM	Scanning electron microscope

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Wheat importance

Wheat has played an essential role in human civilization and improving food security at global and regional levels. The flour of bread wheat is used to make French bread, Arabic bread, chapatti, biscuits, pastry products and the production of commercial starch and gluten (Tadesse et al. 2019). According to Braun et al. (2010), wheat provides about 19% of the calories and 21% of protein needs of daily human requirements. It is a staple food for 40% of the world's population mainly in Europe, North America, and the western and north parts of Asia. Many of the developing countries that depend on wheat as a staple crop are not self-sufficient in wheat production, and therefore, wheat is their single most important imported commodity. Wheat also accounts for the largest share of emergency food aid (Dixon et al. 2009).

Wheat grain has three major components; those are starch, protein and lipid. Interactions between these three components determine the quality composition of the wheat grain and its suitability.

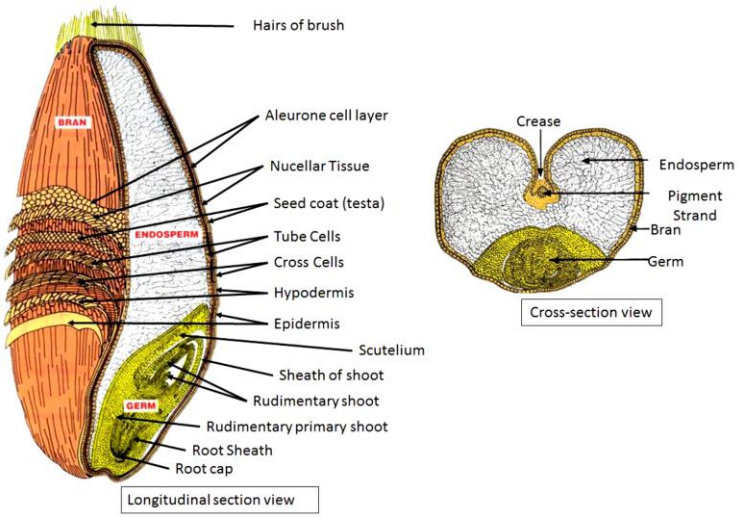


Figure 1.1 Wheat Grain Structure.
Adapted from Corke (2015)

1.2 Hardness as important end-use quality trait

1.2.1 Hardness definition

The word "hardness" is normally defined as the resistance to deformation or fracture properties, also is defined as the level of starch damage after grinding or milling (Anjum and Walker 1991). Hardness is one of the primary commercial classifications in world trade and can be considered the single most important determinant of overall end-use quality of wheat. They contain puroindolines which are unique tryptophan-rich proteins. Puroindolines also was named friabilin to highlight the fact that soft wheat are more friable than hard wheat (Greenwell and Schofield 1989). Whereas, the name "puroindoline" is from puros in Greek (puro for wheat and indoline for the indole ring of tryptophan (Blochet et al. 1993).

At least two proteins, puroindoline-a and puroindoline-b, which are 60% identical, are members of this family. Puroindolines are related to the family basic cystine-rich proteins, which are soluble in the non-ionic detergent Triton X-114 (Blochet et al. 1993). The primary structure of the major component is tryptophan-rich. Puroindoline has a molecular mass of 13 kDa and contains 10 cysteine residues. Puroindolines are lipid-binding proteins that could contribute to the formation and stability of dough foams (Dubreil et al. 1997).

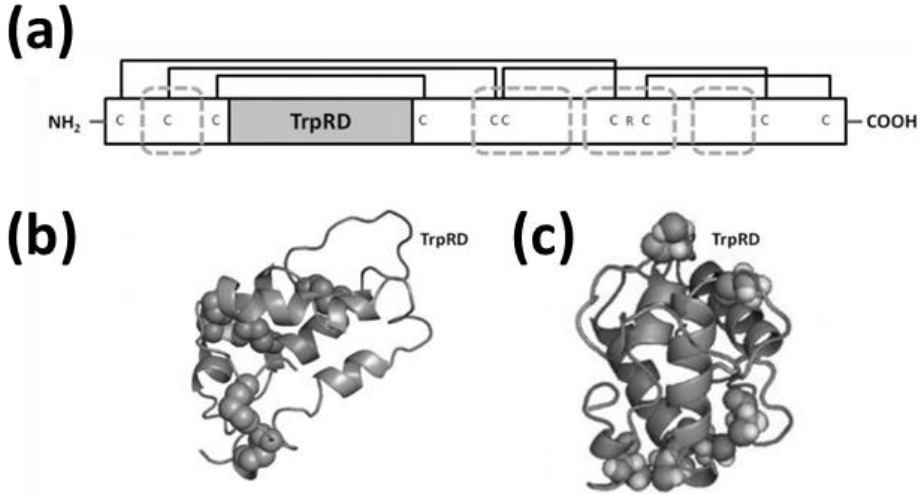


Figure 1.2 Puroindoline structure **(a)** Puroindoline chemical structure **(b)** Puroindoline a 3D structure **(c)** Puroindoline b 3D structure.
Adapted from Pauly et al. (2013)

In mature wheat seeds, puroindolines are located in the starchy endosperm and the aleurone cells (Dubreil et al. 1998). The protein complex friabilin regulates adhesion degree of starch granules to the protein matrix. Also, they are strongly influence the processing quality of flour (Preston 1998, Beecher et al. 2002). Friabilin was suggested to act as a ‘‘non-stick’’ substance that reduced the adhesion of starch granules and protein matrix, therefore, allowing their easier separation.

1.2.2 Hardness importance

Hardness is a key determinant for classification of wheat and end product quality (Campbell et al. 1999). Grain hardness is important for the flour industry because it has significant impacts on milling, baking and qualities of wheat (Bettge and Morris 2000). Hard wheat is suitable for making breads leavened by yeast, as the high levels of damaged starch granules in these flours absorb more water, while soft wheat flour is used for cookies, cakes, pastries and confections (Morris and Rose 1996). Soft kernel wheat has less damaged starch. These kernels easily break down yielding fine powder-like flour having less damaged starch. Hard kernels are difficult to crush and grind, and produce coarser-textured flour with higher levels of starch damage (Ikeda et al. 2005).

Puroindoline proteins possessed antifungal activity as described by Dubreil et al. (1997). Highly purified PINA and PINB reduced growth of several fungi, including *Alternaria brassicola*, *Ascochyta pisi*, *Fusarium culmortun* and *Verticillium dahliae*. Fungal growth was monitored using spectrophotometry. PINB was more active than PINA against sensitive fungi. It was reported that the antifungal activity, when both PINA and PINB were added, has synergistic effect against microbes (Pasha et al. 2010).

1.2.3 Genetic bases for hardness

Hardness is genetically controlled by the Hardness (Ha) locus on the short arm of chromosome 5D. This locus contains the genes Puroindoline a and Puroindoline b (*Pina-D1* and *Pinb-D1*) that encode the main components of friabilin, PINA and PINB. Both Pin genes have been deleted from chromosomes 5A and 5B during the evolution of durum wheat (the contributor of A and B genomes of common wheat).

PIN proteins exhibit a tryptophan-rich domain, which consists of five tryptophan residues in PINA and three residues in PINB (Gautier et al. 1994). The deletion of the *Pina* and *Pinb* genes resulted in the loss of the softness-conferring PIN proteins in durum wheat. As a result, durum has very hard kernel texture. The coding regions of the *Pina-D1* and *Pinb-D1* genes of common wheat are 447 bp long and intronless (Gautier et al. 1994).

Soft texture in common wheat appear through the contribution of *Pina-D1* and *Pinb-D1* genes (Morris 2002). Various mutations in *Pina-D1* and *Pinb-D1* genes have been associated with hard texture in common wheat (Morris and Bhave 2008). In situations where both puroindoline genes are absent. This will lead to 'double-null' mutation of both *Pina-D1* and *Pinb-D1* genes, hence, the kernels are becomes harder similar to those encountered with durum wheat.

The first hardness-associated mutation in *Pina-D1* to be reported was a null mutation, with complete lack of PINA protein. This mutation results in hard kernel, and designated as (*Pina-D1b*) (Morris 2002). Chen et al. (2006), described an SNP that resulted in a premature stop codon replacing Trp43 (*Pina-D1n*). Also, he reported an SNP-based amino acid substitution in PINA (Pro35Ser, *Pina-D1m*) (Morris and Bhave 2008). Chang et al. (2006), also reported a dinucleotide inversion (CA altered to AC) in *Pina* at nucleotide 417–418 (*Pina-D1q*).

For puroindoline b, the most frequent Pin gene mutation among all domesticated wheat cultivars is *Pinb-D1b*. This mutation characterized by guanine to adenine substitution at position 223. This resulting in glycine to serine substitution in PINB at position 46 and changes in the tryptophan-rich domain (Giroux and Morris 1997). The second most frequent alleles found in common wheat cultivars are *Pinb-D1c* and *Pinb-D1d* (Morris and Bhave 2008). *Pinb-D1c* is characterized by thymine to cytosine substitution at position 266, leading to leucine to proline change at position 60 (Lillemo and Morris 2000). In *Pinb-D1d*, thymine to adenine substitution at position 217 causes tryptophan to arginine change at position 44 and leads to changes in the TRD (Lillemo and Morris 2000). An example of the change in reading frame is the allele *Pinb-D1g*, where a single nucleotide change at position 255 (cytosine to adenine substitution) causes a cysteine to stop codon change at position 56 (Morris et al. 2001).

Beside puroindolines that affected hardness, there is other QTLs that affects hardness. Previous studies have shown that there are many other quantitative trait loci (QTL) contributing

to hardness on almost all 21 wheat chromosomes, especially on chromosomes 1B, 2A, 4B, 5A, 5B, 5D and 7D (Bordes et al. 2011; Wang et al. 2012; Li et al. 2016; Chen et al. 2019).

1.2.4 Hardness measurement

Historically, the first measuring of wheat grain texture was developed around 1908 (Roberts, 1910), and it determined the force required to crush the individual kernel's strength. There are some other methods include the distribution of granule by the process of sieving and grinding (Williams et al. 1998). One of the wide approaches for texture measurement was particle size index (PSI), that rely on differences in granularity. This method, quantifies granularity by sifting the ground or milled material and expressing the proportion of material that passes through a sieve of defined hole (Worzella and Cutler 1939). Therefore, a higher number indicates softer texture (due to the lower particle size distribution of soft wheat meals). Another method, near infrared reflectance analysis (NIR). It provides an indirect assessment of particle size through the optical reflectance of ground flour samples (Martin et al. 2001).

One of the important and widely accepted as a standard method to measure grain hardness is Single Kernel Characterization System (SKCS). It provides more precise measurement of hardness, and considered as the best discriminating measure of genetically different wheat based on hardness (Morris et al. 1999). SKCS measures the force required to crush individual grains of a sample between two surfaces, taking into account the weight, diameter and moisture of the grain. The SKCS is well developed system for evaluating the individual wheat kernels quality in terms of a hardness index (Osborne et al. 1997; 2000 Sissons et al. 2000). Figure 1.3 shows SKCS machine and the operating mechanisms.

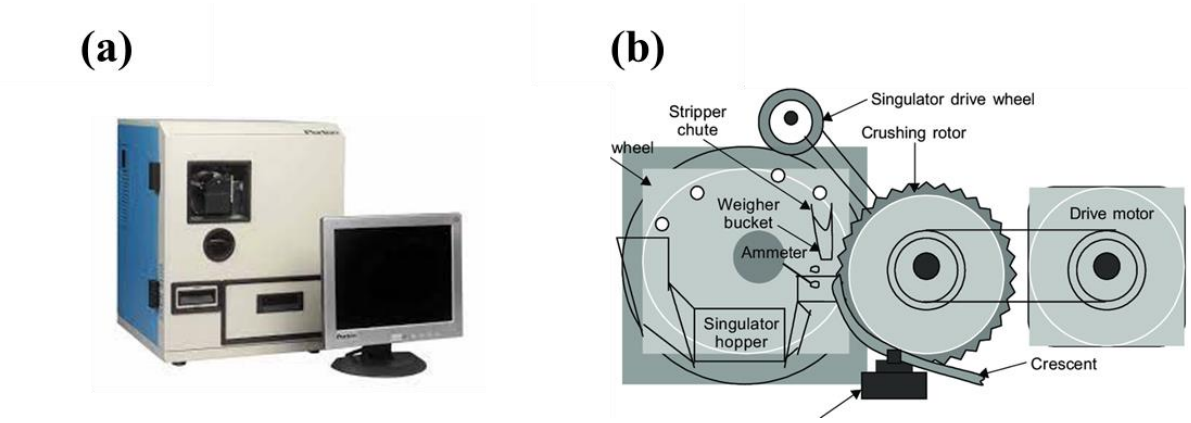


Figure 1.3 Single Kernel Characterization System (SKCS 4100) **(a)**, SKCS operating mechanisms **(b)**.
Adapted from Muhamad et al. (2006)

1.2.5 Relationship between hardness and other quality traits

Grain hardness is influenced by various factors that affecting hardness negatively or positively. One of the important factors, is protein content. Wheat having high protein content is tends to be hard, have strong gluten and thus produce good quality bread. Wheat of low protein content tends to be soft but produce better quality cookies. The higher protein content and density are exhibited only by vitreous kernels than that of those kernels which are starchy or mealy (Tipples et al. 1994).

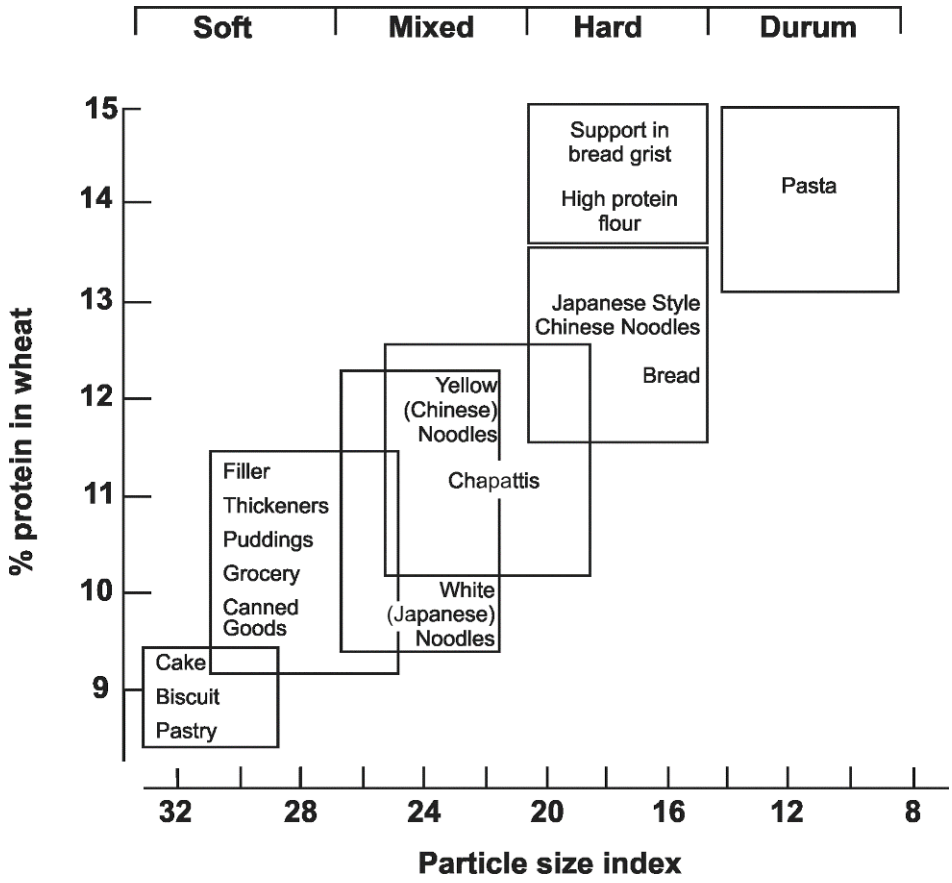


Figure 1.4 Uses of wheat, based on grain hardness and protein content. Adapted from Wrigley (2009).

Another factor that affects hardness is kernel size, which found to have negative correlation, in which soft kernel tend to have bigger kernel size (Hrušková and Švec 2009; Salmanowicz et al. 2012; Szabó et al. 2016). Similarly, significant negative correlation with kernel diameter, kernel length, and kernel width (Sun et al. 2018).

Moreover, seed and flour color are important factors influence hardness. The most common parameters for evaluation of color are L* (lightness), a* (redness) and b* (yellowness), based on the colorimeter. Theoretically, a pure white flour has zero values for a* and b*, and one hundred for L* (Zhang et al. 2009). When grain is normal and light indicates that the grain is solid, thin-crusted and soft, and that it is normal and dark color indicates that the grain belongs to the hard

wheat variety (Kutlu 2018). Whereas, Chen et al. (2019), reported that hardness is positively correlated with b*, and negatively correlated with a*.

Another trait that influences hardness is ash content or in other word mineral content. Kernel hardness in general has positive correlation with ash content (Zhang et al. 2005; Hrušková and Švec 2009).

1.3 Kernel weight as important yield component trait

Improvement of the kernel weight is considered to be an important approach for further improving yield potential. In the past four decades, improvement of grain yield has comes from increased grains per m^2 or larger grain size and weight, due to the utilization of reduced hight (*Rht*) genes in wheat breeding (Calderini and Reynolds 2000). In addition, milling yield could be increased by optimizing kernel weight and size (Gegas et al. 2010). Kernel weight is the most heritable trait among yield components (Su et al. 2016), with heritability reaching as high as 0.87 (Wiersma et al. 2001).

1.4 Kernel weight are highly associated with shape traits

Kernel shape traits are important component of basic plant research, since seed formation and development is a fundamental aspect of plant reproduction. Kernel weight is closely associated with kernel size traits, such as kernel length, kernel width, and kernel diameter (Dholakia et al. 2003). Kernel size traits usually contribute to yield by affecting the kernel weight and can also be associated with milling and processing (Osborne and Anderssen 2003). Therefore, improving kernel weight and size is a prime breeding target for wheat yield potential and end use quality. Grain size and shape are inherited in a stable manner and show higher heritability than overall yield (Kuchel et al. 2007).

1.4.1 Measurement of shape traits

An accurate characterization of grain size and shape traits remains a big challenge due to laborious, time consuming techniques and complex nature of wheat grain shape. Recent advances in the photometric techniques provide more concise, potentially cheaper phenotypic information and can better transfer the function of complex traits into individual genetic components.

Digital image analysis is proving to be a useful tool and can capture the three dimensional shapes of grains using different image orientations (Williams et al. 2013). There is increasing use of image analysis in plant science and agriculture, especially in the field of phenomics. Developments in the availability of image analysis for plant measurement applications have made low cost alternatives.

One of these methods, ImageJ is general purpose image analysis software that is freely available and has been used to analyze seed shape and size parameters in a range of plant species including wheat, rice and Arabidopsis (Li et al. 2009).

Another method, GrainScan, a low cost, high throughput method of robust image capture and analysis for measurement of cereal grain size and color. GrainScan utilizes reflected light to accurately capture color information described in a device independent color space (CIELAB), allowing comparison of color data between scanning devices (Whan et al. 2014).

SmartGrain is another image analysis system that is free to use, and is also based on images captured by consumer level flatbed scanners to extract seed characteristics. SmartGrain builds ellipses on identified grains to establish seed area, perimeter, width and length. SmartGrain automatically recognizes all seeds within a digital image, detects outlines, and then calculates length, width, seed area size, perimeter length, and circularity. Thus, SmartGrain can be used to measure the seeds of various plant species (Tanabata et al. 2012).

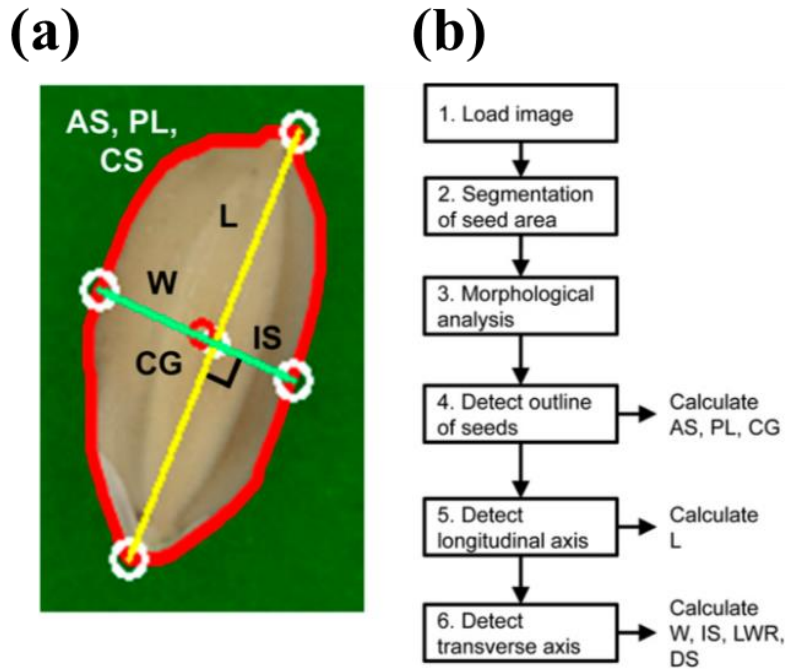


Figure 1.5 SmartGrain software **(a)** Software image **(b)** Steps for image analysis and measurement of seed shape. Adapted from (Tanabata et al. 2012)

1.5 Environment impact on hardness and related traits

Globally, abiotic stresses drastically affect wheat yields and quality raising concerns regarding future food security. Substantial reductions in yield were documented in various important food crops worldwide (Lobell and Field 2007). An annual global yield reduction of 19 million tons in wheat, costing $2.6 billion, due to climate change (Lobell and Field 2007). In the face of climate change, it is important to develop high yielding wheat genotypes combined with high quality to satisfy all stakeholders of the wheat value chain (Fleitas et al. 2020).

Heat and drought stress can affect wheat growth at any developmental stage; however, at the reproductive stage they have a greater impact on grain yields and grain quality (Kumar et al. 2017). Bergkamp et al. (2018) demonstrated that post anthesis heat stress (35°C) shortened the grain filling duration and limited the allocation of resources to grains, leading to lower productivity by 6–51% in wheat plants grown in controlled environments and by 2–27% in plants grown under field conditions. Reproductive processes and grain filling are more sensitive to both these stresses, and have optimum and ceiling temperatures that are relatively lower than those for vegetative

growth and development phases (Prasad and Djanaguiraman 2014). Additionally, Awasthi et al. (2014) reported that drought and heat stress during seed filling causes early senescence and reduces seed filling duration.

Heat and drought stress are identified as a major constraint to wheat quality worldwide. The IPCC (2014) reported that decline in food productivity and quality, primarily due to extreme temperatures and water deficit conditions, poses a serious threat to agriculture (IPCC, 2014). Several studies demonstrated that high temperature along with drought stress, accelerates grain filling, resulting in compresses key events during wheat grain development, like storage protein and starch synthesis in endosperm (Ashraf 2014), which all together affect hardness. Additionally, heat and drought stress affect negatively the yield by reducing grain size and number. While the presence of these stresses during any growth stage can affect final yield, the grain filling stage is crucial for determining average grain weight, size composition and the final quantitative and qualitative yield (Prasad et al. 2017).

1.6 Wheat low diversity

It is pointed out that wheat genetic diversity is narrowed down in extensive breeding programs and that finding new QTLs is indispensable for further wheat improvement (Tilman et al. 2011). Accordingly, in order to improve wheat varieties for yield, quality, and tolerance to abiotic stresses, one of the important factors to achieve this goal is to explore novel genetic resources to discover genes that affect grain yield (Reynolds et al. 2012). Wheat wild relatives *Aegilops tauschii*, D-genome donor of wheat and their derived synthetic hexaploid, are a storehouse for unexploited genetic diversity. There are many reports of *Aegilops* species as well as synthetic hexaploid that have been utilized for improvement of agronomic traits and tolerance against abiotic stresses (Zhang et al. 2015; Rasheed et al. 2914). Wheat multiple synthetic derivatives (MSD), materials of the current study which developed by crossing and backcrossing 43 synthetic wheat lines with the common wheat cultivar ‘Norin 61’ (Gorafi et al. 2018). This material, which harboring genomic fragments from *Aegilops tauschii* can be source for genetic variation, and a pool for diverse valuable genes which in turn can contribute for wheat germplasm enhancement.

1.7 Genome wide association and its importance

Marker assisted selection (MAS) has been widely applied in plant breeding in order to enhance crop yield and tolerance to abiotic stress. Recent advances in genome-wide association study (GWAS) that provides high resolution for the detection of genomic region (Sukumaran and Yu 2014) offers an ultimate MAS tool to accelerate plant breeding and crop improvement.

GWAS is an approach that can be used for identification and high resolution mapping of useful genetic variability from germplasm sets that have resulted from many rounds of historical recombination (Yu and Buckler 2006). Moreover, it facilitates understanding of the genetic bases and dissection of complex genes controlling economic traits such as stress tolerance.

1.8 Research objectives

According to the previously mentioned literature review, kernel hardness is the most important trait that determined end-use quality of wheat. In addition, several traits that affect hardness, among them kernel weight and shape related traits which considered as easy and precise approach to improve yield potential.

Stress conditions such as heat and combined heat-drought are considered the most important factors that affect hardness and shape related traits. Since those stress has not been well addressed for hardness and related traits. Therefore, the current study was conducted to investigate the effect of heat and combined heat-drought stresses upon kernel hardness and shape related traits.

The specific objectives for this study are;

1. To identify the phenotypic and genotypic variation of kernel hardness, kernel weight and shape-related traits among MSD lines under optimum, heat and combined heat-drought conditions.

2. To investigate the association between hardness stabilization and stress tolerance.

CHAPTER TWO

Exploitation of Tolerance of Wheat Kernel Weight and Shape-Related Traits from *Aegilops tauschii* Under Heat and Combined Heat-Drought Stresses

2.1. Introduction

Abiotic stresses such as heat and drought affect yield negatively by reducing grain size and number. While the stresses in any growth stage affect the final yield, those at the grain filling stage are the most crucial (Weldearegay et al. 2012; Prasad et al. 2017). Heat and drought stress in all developmental stages influence important morphological traits (Rad et al. 2020; Gupta et al. 2020). To adapt to these harsh environments, great efforts have been made to produce genetically tolerant plants and to understand the mechanisms behind the stress tolerance (Szliszka et al. 2009; Yousefi et al. 2020);. To identify genomic regions responsible for grain yield under stress conditions, analysis of the yield components is crucial (Schmidt et al. 2020). Kernel weight is considered the most important and heritable trait among these components (Su et al. 2016). This trait is closely associated with kernel shape-related traits, such as kernel length, kernel width and kernel diameter (Dholakia et al. 2003). The improvement of kernel weight and shape-related traits under stressed conditions is a promising approach to increasing wheat production (Würschum et al. 2018). In addition, screening for shape-related traits using image analysis can provide an easy and accurate means to assess yield components.

Wheat genetic diversity has been narrowed down in extensive breeding programs and thus, finding new genetic diversity in wild species is indispensable for improvement (Reynolds et al. 2012; Tilman et al. 2011). Many studies have used *Aegilops* species as genetic resources and reported their tolerance to abiotic stresses (Zhang et al. 2015; Kishii et al. 2019). Among the related wild species, Aegilops tauschii is the most promising species because it has a D genome common with that of bread wheat and because no special cytological technique is needed to induce homologous recombination (Ogbonnaya et al. 2013). In this study, we employed wheat multiple synthetic derivative (MSD) lines, which contain the genetic diversity of a large accession of a wild species, *Aegilops tauschii*, and are suitable materials for genetic analysis (Gorafi et al. 2018).

Great progress has been made in identifying major QTLs for kernel size and shape (Rasheed et al. 2014; Chen et al. 2016; Daba et al. 2018; Su et al. 2018; Desiderio et al. 2019; and Chen et al. 2020), and several candidate genes were identified in wheat. For instance, cytokinin oxidase (encoded by *TaCKX*) reversibly inactivates cytokinin and increases kernel weight (Chang et al. 2015). Cell wall invertase (encoded by *TaGW1*) regulates kernel size by sink tissue development (Song et al. 2012), and RING-type *E3 ubiquitin*–protein ligase (encoded by *TaGW2*) increases kernel weight and size (Bednarek et al. 2012).

These studies describe kernel development under normal conditions, but extensive studies under stress conditions have not been conducted. To breed wheat genotypes that maintain grain yield even under stress conditions, knowledge of genotypic and environment interaction is necessary. Therefore, this study aimed at identifying the phenotypic and genotypic variation of kernel weight and shape-related traits among MSD lines under optimum (OP), heat (H) and combined heat–drought (HD) conditions, and to reveal the genetic mechanism of the productivity under stress, from the view point of kernel traits. Our results revealed a great diversity among the MSD lines in kernel weight and other kernel shape-related traits under all conditions. We identified promising markers and alleles that will contribute to our understanding of productivity under stressed condition and could be used in wheat breeding after validation.

2.2 Materials and Methods

2.2.1. Plant materials

This study used 400 BC_1F_7 multiple synthetic derivative lines developed by crossing and backcrossing the bread wheat (*T. aestivum*) cultivar 'Norin 61' and 43 synthetic wheat lines (Gorafi et al. 2018). The synthetic wheat lines were developed by crossing 43 different *Ae. tauschii* accessions with the durum wheat (*Triticum turgidum* ssp. durum) cultivar Langdon. We evaluated 400 lines under optimum conditions in Japan and 140 selected lines under stress conditions in Sudan. These 140 lines do not require vernalization treatment and are adapted to Sudanese conditions.

2.2.2 Field experiments

Optimum conditions in Japan: the 400 MSD lines were grown in a field of the Arid Land Research Center (35° 32′ N, 134° 13′ E, 11 m asl), Tottori, Japan, in two seasons: 2015‐/16 and 2018‐/19. The soil was sandy (95% sand, 1.3% silt, 3.7% clay) (Fujiyama and Nagai 1989). Before sowing, three commercial fertilizer mixtures, Kumiai Fukugo PKN 366 (MC Ferti-com Co., Ltd . Tokyo. Japan; 60kg), Hitachi Fukugo 1 (Hitachi-kakou Co. Hitachi, Ibaraki prefecture, Japan, Ltd.; 40kg) and granular carbonated magnesium lime (Shimizu Kogyo Co., Ltd. Tokyo, Japan; 100kg) were spread onto the soil. During the tillering stage, Kodo Kasei 444 (Mitsubishi Syoji Agri-service Co., Ltd Osaka, Japan; 50,000 kg ha−1) was spread. The experiment was arranged in an augmented randomized complete block design with eight blocks. We used four replicated checks with 'Norin 61' (the MSD parent), Imam and Tagana (Sudanese heat-tolerant cultivars) and Safedak Ishkashim (a Tajikistan landrace) in each block. Each line was grown in a row of five plants with 0.2 m between plants. The seeds were sown in late October and plants were harvested in mid-June. The average temperature was 11.9 °C in the 2015–/16 and 11.5 °C in the 2018–/19 seasons. The minimum/maximum temperatures were −3.8/26.2 °C in 2015–/16 and 1.8/25.3 °C in 2018–/19; the average temperatures during maturity (May–June) were 20.1 °C and 19.7 °C, respectively.

Heat (H) and heat‐drought (HD) conditions: the lines were grown at the Gezira Re-search Farm (GRF), Agricultural Research Corporation (ARC), Wad Medani, Sudan (14° 24′ N, 29° 33′ E, 407 m asl), in the 2017‐/18 season from November to March. We selected GRF because it is recognized as the global center for heat-tolerance research (Tadesse et al. 2019; Iizumi et al. 2021). The ARC manages it in collaboration with CIMMYT, ICARDA and Tottori University, Japan (SATREPS Project). This farm is within a clay plain and the soil is heavy clay Vertisol (pH 8.5). Before sowing, P was applied at 18.8 kg ha^{-1}. Seeds were treated with Gaucho insecticide (imidacloprid, 35% WP, Bayer Crop Science) at 1 g kg^{-1} seeds and sown at 120 kg ha^{-1} manually in the fourth week of November in an alpha lattice design, with two replications. Plot size was four 1.0-m rows with 0.2 m between rows. In H plots, plants were irrigated every 10–12 days, as recommended by the ARC. In HD plots, water supply was withheld when 50% of the lines reached anthesis. Data loggers (Em50, Decagon Devices, Pullman, WA, USA) connected to sensors (Terso21, Decagon Devices, Pullman, WA, USA) were used to measure soil water potential at 20

cm depth and the plants were re-watered when the potential reached −900 kPa to avoid permanent wilting stress. Nitrogen was applied twice as urea at the three leaf stage (second irrigation) and at the tillering stage (fourth irrigation) at 86 kg ha^{-1}.

2.2.3. Measurement of kernel weight and shape-related traits

From each MSD line in each plot, and each replication in all environments, 100 grains were used for kernel trait measurements. Kernel weight and kernel diameter were measured using a single-kernel characterization system (SKCS 4100, Perten Instruments) at the National Agriculture Research Center for Western Region, Fukuyama, Hiroshima, Japan. Kernel shape parameters (area size, perimeter length, length, width, and circularity) were analyzed in SmartGrain software v. 1.2 Tanabata et al. (2012) with up to 100 intact seeds. Circularity was calculated by an equation, 4π (area size)/ (perimeter length) ^{2}.

2.2.4 Statistical analysis of kernel weight and shape-related traits

Analysis of variance (ANOVA) for an augmented randomized complete block design was performed using Plant Breeding Tools software (PBTools, v.1.4, International Rice Research Institute, Laguna, Philippines http://bbi.irri.org/products). GenStat 18 (VSN International, Rothamsted Research, Harpenden, and Hertfordshire, UK) was used to carry out the ANOVA for alpha lattice design experiments (H and HD conditions). Broad-sense heritability (H^2) was calculated using PBTools. Pearson's correlation coefficients were performed using R software with a custom script in the ggcorplot package (R core team 2021), available at http://www.sthda.com/english/wiki/ggcorrplot visualization of a correlation matrix using ggplot2. Heat susceptibility indices under H (HSI) and HD (HDSI) conditions were calculated for kernel weight, kernel size and kernel diameter as:

$$\text{HSI (or HDSI)} = (1 - Yh/Y)/ (1 - Xh/X)$$

Where Yh is the phenotypic mean of each genotype under H or HD conditions; Y is the phenotypic mean of each genotype under OP conditions; Xh is the mean of all lines under H or HD conditions; and X is the mean of all lines under OP conditions.

2.2.5 Genome-wide association study and candidate gene identification

The MSD lines and 'Norin 61' were genotyped using the DArT-seq platform (Diversity Arrays Technology, Bruce, Australia https://www.diversityarrays.com) (Gorafi et al. 2018). GWAS for kernel weight and shape-related traits was performed with 14,355 DArT-seq markers in TASSEL5 v. 20,151,113 software (Bradbury et al. 2007). We used a mixed linear model (MLM) with PCA and a kinship matrix to account for population structure and cryptic relation-ships. Manhattan plots were generated using −log10 (P). The adjusted $P < 3 \times 10{-}3$ was used as a threshold to determine significant association. To identify the candidate genes, significant markers sequences were used for the search in Unité de Recherche Génomique Info, Versailles, France (URGI: https://urgi.versailles.inra.fr/), with the blast option used for comparisons with the International Wheat Genome Sequencing Consortium, Castanet Tolosan Cedent, France (WGSC) RefSeq V.1 chromosomes. We searched for the candidate genes 0.5 Mb upstream and down-stream of the positions of the significant markers.

2.3 Results

2.3.1. Phenotypic variation of kernel weight and shape-related traits under optimum, heat, and combined heat–drought conditions

MSD lines showed large phenotypic variation of all kernel traits under all three optimum (OP), heat (H) and combined heat–drought (HD) conditions (Figure 2.1). The MSD lines showed a wider range of variation under H and HD than under OP conditions, reflecting the MSD response to the stress conditions (Figure 2.1). The values of the kernel weight and shape traits were reduced under H and HD conditions, resulting in the means shifting towards the low values. The effects on kernel weight, kernel diameter, kernel size and kernel width were more potent than those on kernel length and kernel circularity (Figure 2.1).

Under OP conditions, the genotypic effect was significant for all traits except kernel width (Table 1). Differences were significant between the two seasons (S) in all traits except kernel length, and the interaction (G × S) was significant for all traits (Table 2.1). Under H and HD conditions, differences among genotypes were significant for all traits. The environment (E) affected all traits except kernel length, and G × E had no significant differences in any traits (Table 2.1). Moderate heritability (H^2) (0.42 to 0.67) was observed under OP conditions for all traits

except kernel width, which had a low heritability value (0.29). In contrast, high H^2 was observed for all the traits under the stress environments (0.88 to 0.97) (Table 2.1).

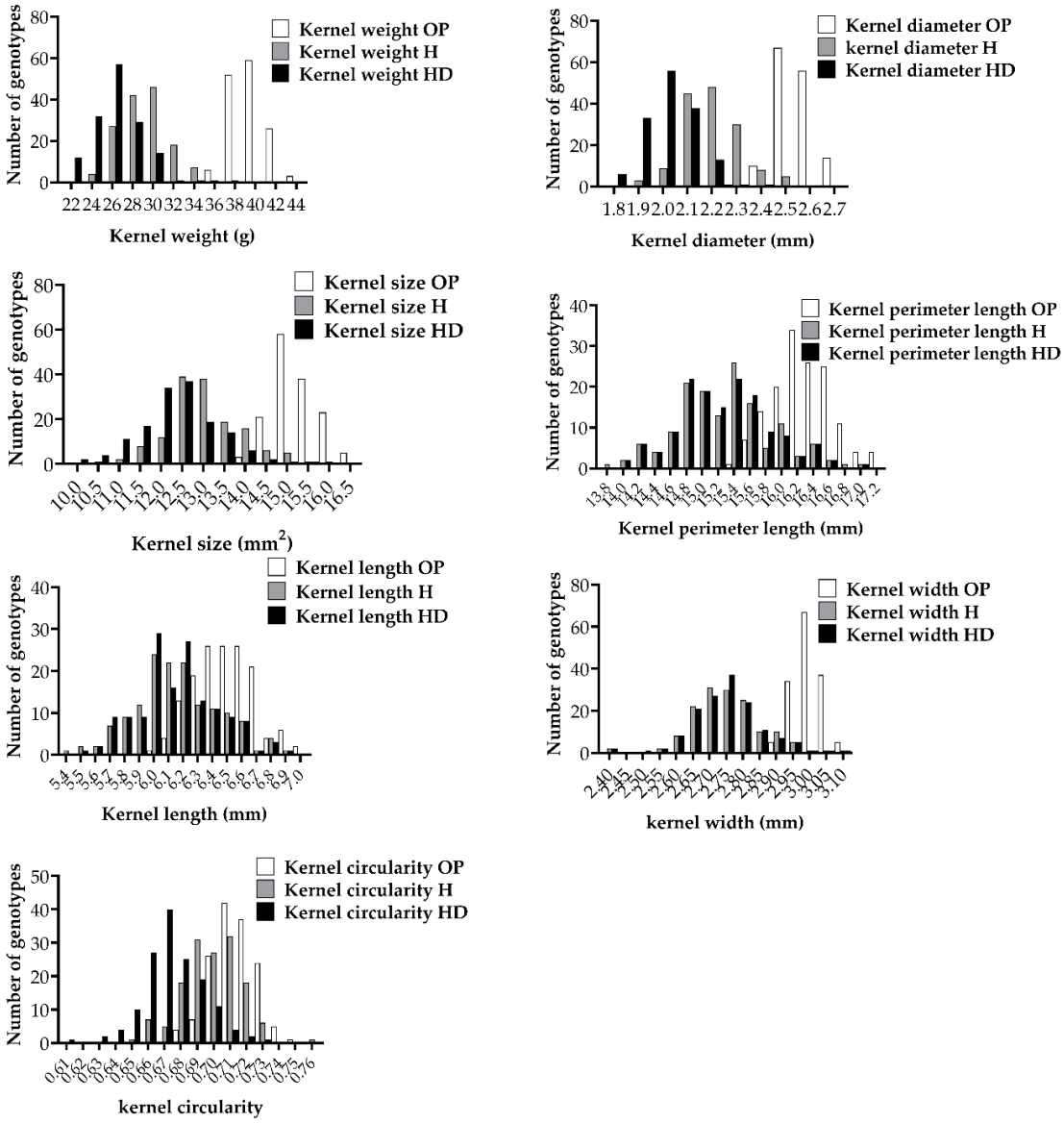


Figure 2.1. Frequency distribution of the multiple synthetic derivative lines grown under optimum (OP), heat (H) and combined heat–drought (HD) conditions. For the OP conditions, the predicted means of the values from two seasons, S1 and S2, were used.

Table 2.1. Analysis of variance and heritability of kernel weight and shape-related traits under optimum (OP), heat (H) and combined heat–drought (HD) conditions for multiple synthetic derivatives lines.

	Kernel weight (g)			**Kernel diameter (mm)**			**Kernel size (mm^2)**		
	SED±	H^2	*p*-value	SED±	H^2	*p*-value	SED±	H^2	*p*-value
C (OP)	3.33	–	***	0.13	–	***	1.94		**
S (S1 × S2)	–	–	*	–	–	***	–	–	**
G × S	3.39	0.56	***	0.13	0.64	***	1.94	0.42	**
H	1.53	–	***	0.08	–	***	0.60	–	***
HD	1.73	–	***	0.09	–	***	0.61	–	***
E	–	–	***	–	–	***	–	–	***
G × E	1.75	0.92	ns	0.07	0.88	ns	0.55	0.94	ns
	Kernel length (mm)			**Kernel width (mm)**			**Kernel circularity**		
	SED±	H^2	*p*–value	SED±	H^2	*p*–value	SED±	H^2	*p*–value
C (OP)	0.46	–	***	0.22		ns	0.02	–	***
S (S1 × S2)	–	–	ns	–	–	***	–	–	**
G × S	0.46	0.51	***	0.22	0.29	*	0.02	0.67	**
H	0.13	–	***	0.07	–	***	0.01	–	***
HD	0.18	–	***	0.08	–	***	0.01	–	***
E	–	–	ns	–	–	***	–	–	***
G × E	0.14	0.97	ns	0.07	0.88	ns	0.01	0.92	ns

C: combined analysis for the two seasons under OP conditions; SED±: standard error of differences; ns, not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

All kernel traits except kernel circularity correlated positively with kernel weight under all conditions; the correlation with kernel diameter was the strongest (Figure 2.2). Correlations were positive among kernel size, kernel perimeter length and kernel length (Figure 2.2).

To understand the performance of MSD lines under stress conditions (H and HD), we calculated the heat susceptibility index (HSI) and the combined heat–drought susceptibility index (HDSI) on the basis of the traits most strongly correlated with kernel weight, i.e., kernel diameter

and kernel size. We performed a regression analysis between the HSI and HDSI to identify any relationship between the performances of MSD lines under the different stresses (Figure 2.3). There was an association between the MSD line's performance under H and HD conditions (R^2 = 0.45, 0.33 and 0.53 for kernel weight, kernel diameter and kernel size, respectively). The HSI and HDSI of the background parent of the MSD lines 'Norin 61' and leading Sudanese cultivar Imam conditions, whereas absolute values > 26, 29 and 27 were significant at p = 0.001 under optimum, heat and combined heat–drought conditions, respectively.

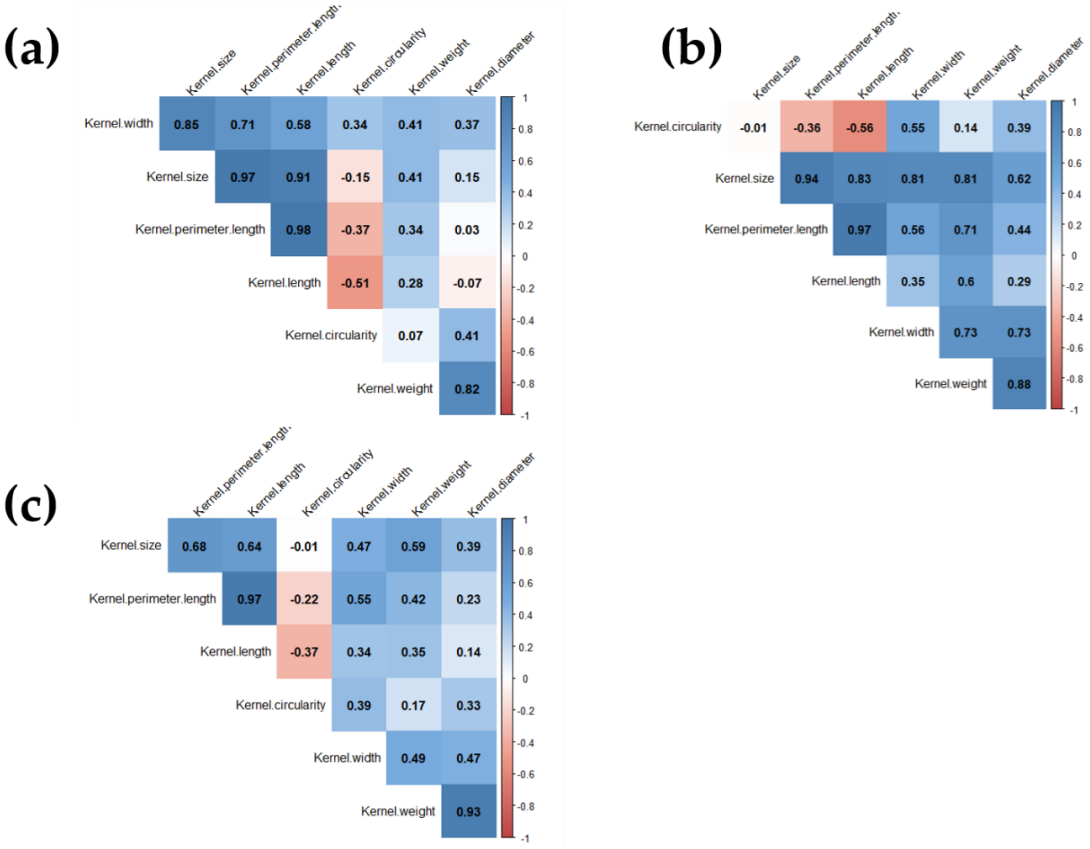


Figure 2.2. Correlation coefficient for kernel weight and shape-related traits under (a) optimum; (b) heat; and (c) combined heat–drought conditions. Absolute values > 0.15 were significant at p = 0.05; > 0.20 were significant at p = 0.01 under optimum, heat and combined heat-drought.

The HSI and HDSI of the background parent of the MSD lines ‘Norin 61’ and leading Sudanese cultivar Imam were around 1 (Figure 2.3), indicating that these genotypes showed levels of reduction similar to all genotypes studied, as seen in Figure 2.1.

Interestingly, some MSD lines (indicated with red color) showed stable performance under stress conditions; they had the highest kernel weight, kernel diameter and kernel size under H and HD stress conditions (Figure 2.3). MSD187 performed well under all conditions in all three traits, whereas MSD259 was much more strongly affected by H and HD stresses than ‘Norin 61’ and Imam (Figures 2.3 and 2.4).

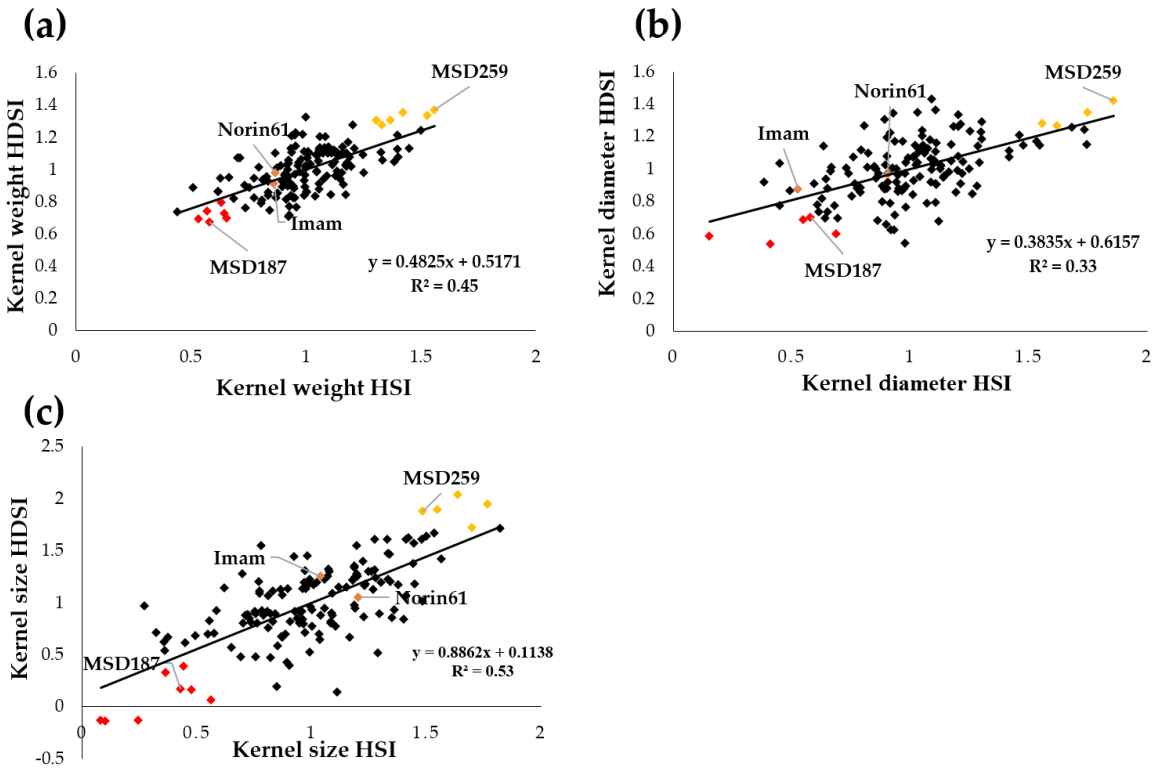


Figure 2.3. Regression analysis of the relationship between the heat susceptibility index (HSI) and the combined heat–drought susceptibility index (HDSI) for kernel **(a)** weight; **(b)** diameter; and **(c)** area size. Red color indicates tolerant lines with an index value of about 0.5 and yellow color indicates sensitive lines with an index value higher than one. The genetic background parent of the MSD lines ‘Norin 61’, the leading Sudanese cultivar Imam and two representative MSD lines showing high (MSD259) and low (MSD187) reductions are indicated.

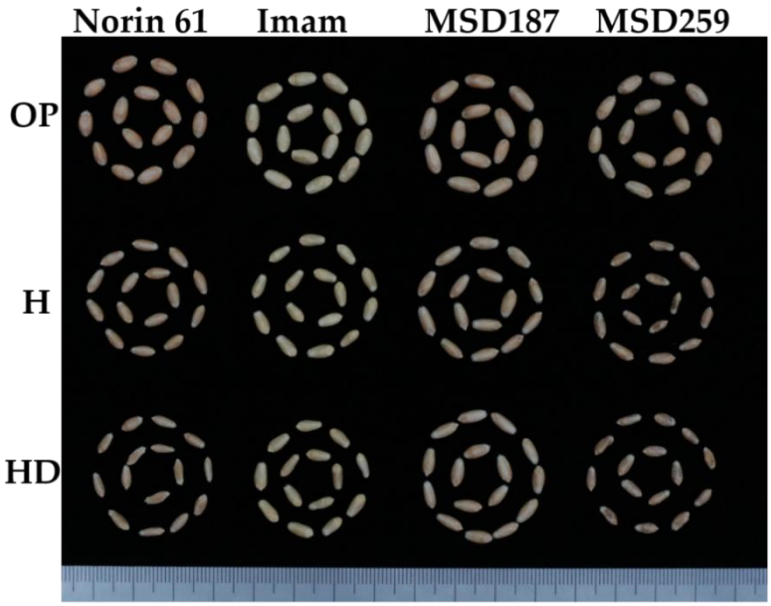


Figure 2.4. Variations in kernel weight and shape-related traits under optimum (OP), heat (H) and combined heat–drought (HD) conditions. 'Norin 61': the background of the MSD lines, showed a reduction under H and HD conditions; Imam: check cultivar known as heat tolerant, showed a small reduction; MSD187: showed a slight reduction under H and HD conditions; MSD259: highly sensitive line, showed remarkable reduction under H and HD conditions.

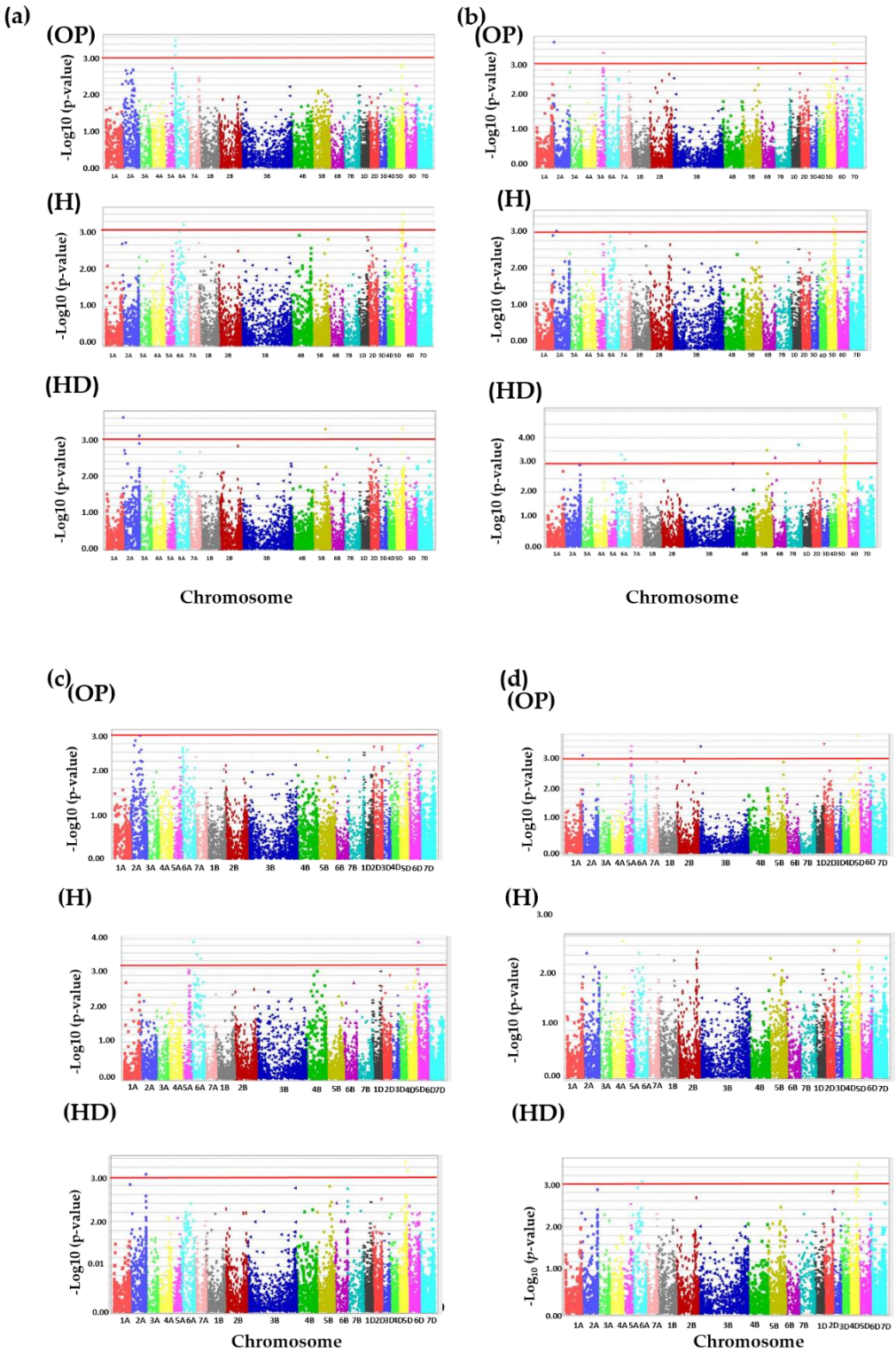
2.3.2. Marker trait associations for kernel weight and shape-related traits

We calculated the best linear unbiased predictions (BLUPs) for OP conditions with the data from two seasons, considering the significant seasonal effect in the association analysis. We identified 82 marker trait associations (MTAs) on different chromosomes under all conditions and for HSI and HDSI (Figures 2.5 and 2.6). Among the A, B and D genomes, the D genome had the highest MTA number under all conditions (Figure 2.7a).

Under OP conditions, the A, B and D genomes contributed 30%, 13% and 57%, respectively (Figure 7b). Significant markers associated with kernel weight and kernel diameter were found on chromosome 6A and those associated with kernel weight, kernel size, kernel length and kernel perimeter length were found on chromosome 5D (Figure 2.8, Table 2.2). Significant markers on chromosome 3B were associated with kernel length, kernel perimeter length and kernel circularity, while those on 7B and 7D were associated with kernel width and those on 2B and 7A with kernel length (Figure 2.8, Table 2.2). Chromosome 2D had the highest number of MTAs,

most of them associated with kernel circularity. The contribution (R^2) of markers ranged from 0.074 in marker 1113863, associated with kernel diameter, on chromosome 2A to 0.16 in marker 1088563, associated with kernel circularity, on chromosome 2D (Table 2.2). Marker 5357358, on chromosome 2A, affected kernel size and kernel perimeter length (Figure 2.9a, Table 2.2). In addition, markers 3,028,836 and 1088488|F|0-27, on chromosomes 5A and 5D, respectively, affected kernel size, kernel length and kernel perimeter length (Figure 2.9a, Table 2.2). Marker 4395641, on chromosome 6A, affected kernel diameter and kernel weight (Figure 9a, Table 2.2). In total, 30 MTAs were obtained under OP conditions (Table 2.2); some of them had pleiotropic effects, some were common between OP and HD conditions and others were associated with OP conditions (Table 2.2).

Under H conditions, 18 significant MTAs were detected (Figure 2.8, Table 2.2), i.e. about half of those detected under OP conditions (Table 2.2). All were located in the A (33%) and D genomes (67%), with no contribution from the B genome (Figure 2.7c). Chromosome 6A had markers associated with kernel weight, kernel diameter and kernel circularity, whereas chromosome 5D had markers associated with kernel weight and kernel size. Specific markers associated with kernel circularity were located on chromosomes 1A, 4A and 3D, and with kernel length on chromosome 4D (Figure 2.8, Table 2.2). R^2 ranged from 0.08 in markers 987701, 1073897|F|0-27 and 5411945, associated with kernel diameter, kernel size and kernel circularity on chromosome 4A, 5D, and 6D respectively, to 0.13 in marker 1099241|F|0-17, associated with kernel diameter, on chromosome 6D (Table 2.2). No markers except 1073897|F|0-27, associated with kernel size, on chromosome 5D were detected under OP conditions, indicating their unique association with the kernel traits under H conditions. Marker 5968258, on chromosome 6A, affected kernel weight and kernel diameter (Figure 2.9a, Table 2.2).


(a)
(OP)
(H)
(HD)
-Log10 (p-value)
Chromosome
(b)
(OP)
(H)
(HD)
-Log10 (p-value)
Chromosome
(c)
(OP)
(H)
(HD)
-Log10 (p-value)
Chromosome
(d)
(OP)
(H)
(HD)
-Log10 (p-value)
Chromosome

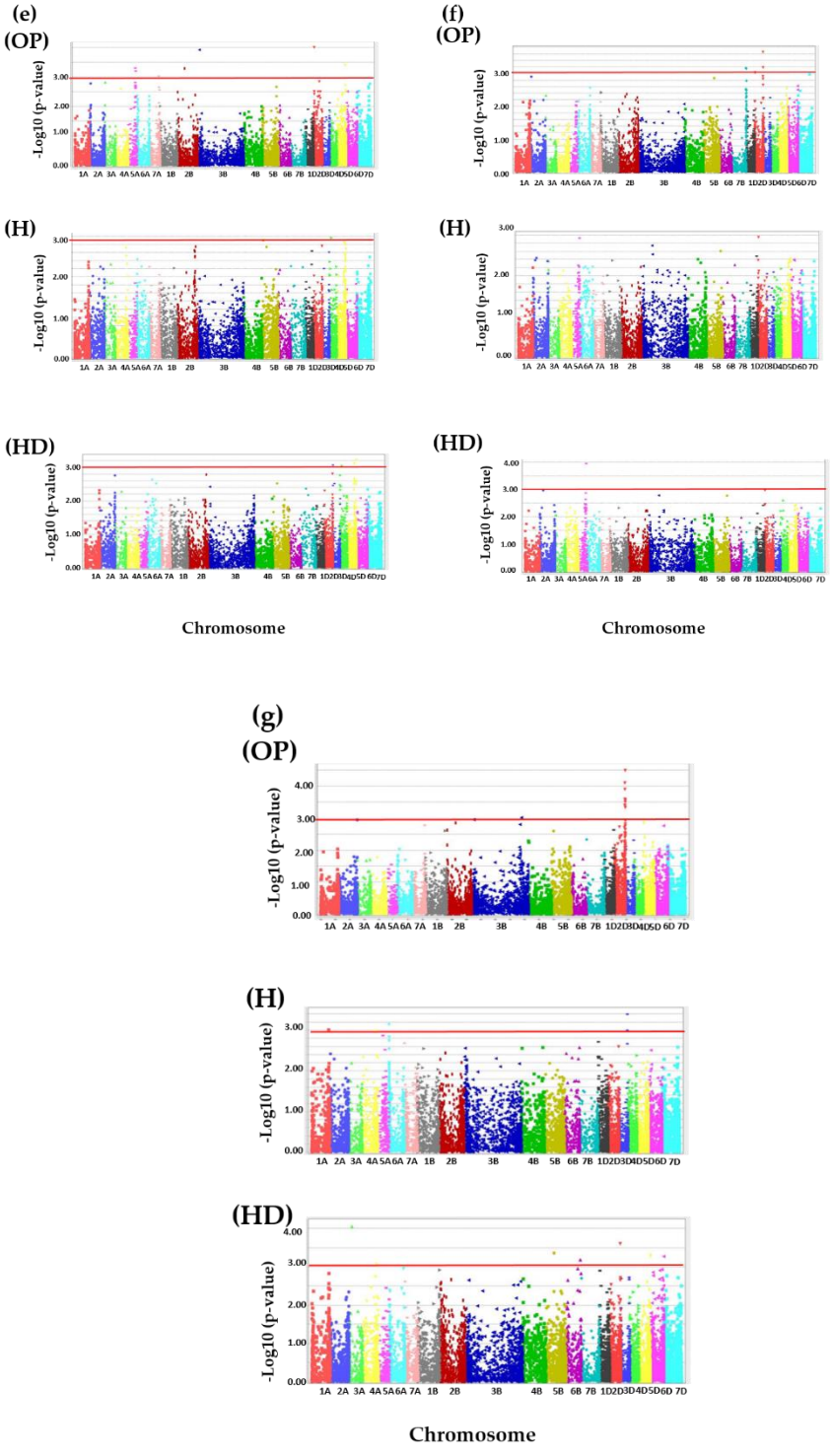


Figure 2.5. Manhattan plots for kernel weight and shape-related traits under optimum (OP), heat (H) and combined heat–drought (HD) conditions. (a) Manhattan plot for kernel weight; (b) Manhattan plot for kernel size; (c) Manhattan plot for kernel diameter; (d) Manhattan plot for kernel perimeter length; (e) Manhattan plot for kernel length; (f) Manhattan plot for kernel width; (g) Manhattan plot for kernel circularity.

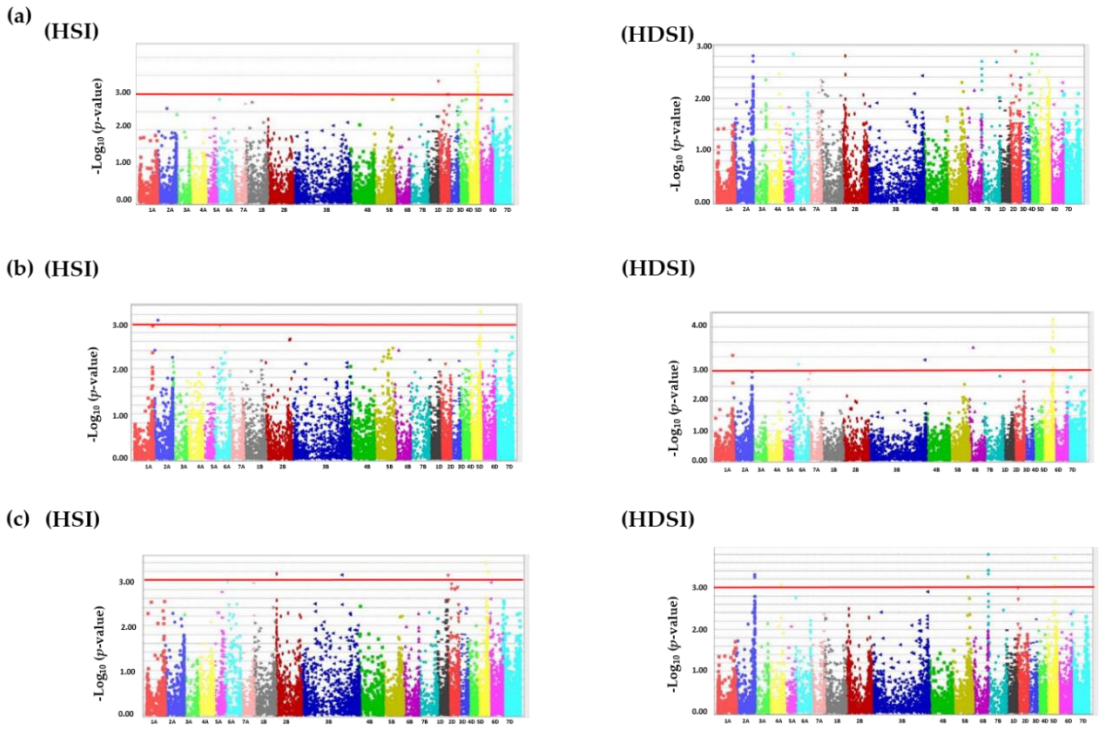


Figure 2.6. Manhattan plot for heat susceptibility index (HSI) and heat drought susceptibility index (HDSI) for **(a)** kernel weight; **(b)** kernel diameter; and **(c)** kernel size.

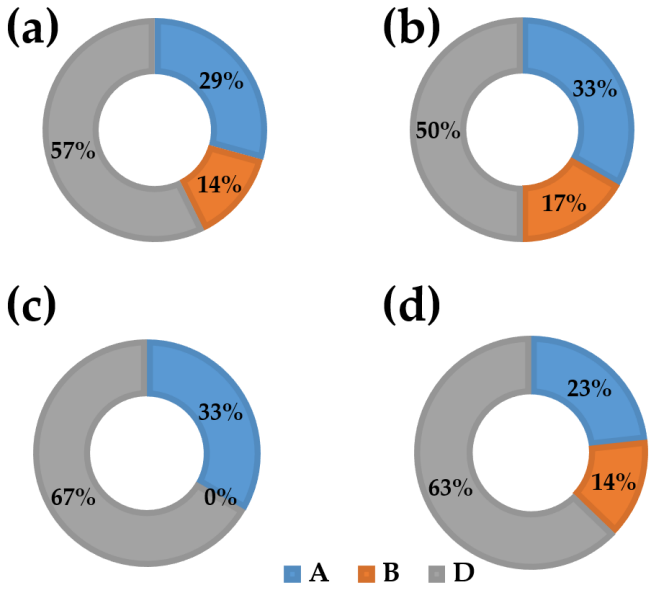


Figure 2.7. Percent contribution of marker trait associations (MTAs) in the A, B and D genomes of bread wheat. **(a)** All MTAs identified; **(b)** MTAs identified under OP conditions; **(c)** MTAs under H conditions; and **(d)** MTAs under HD conditions.

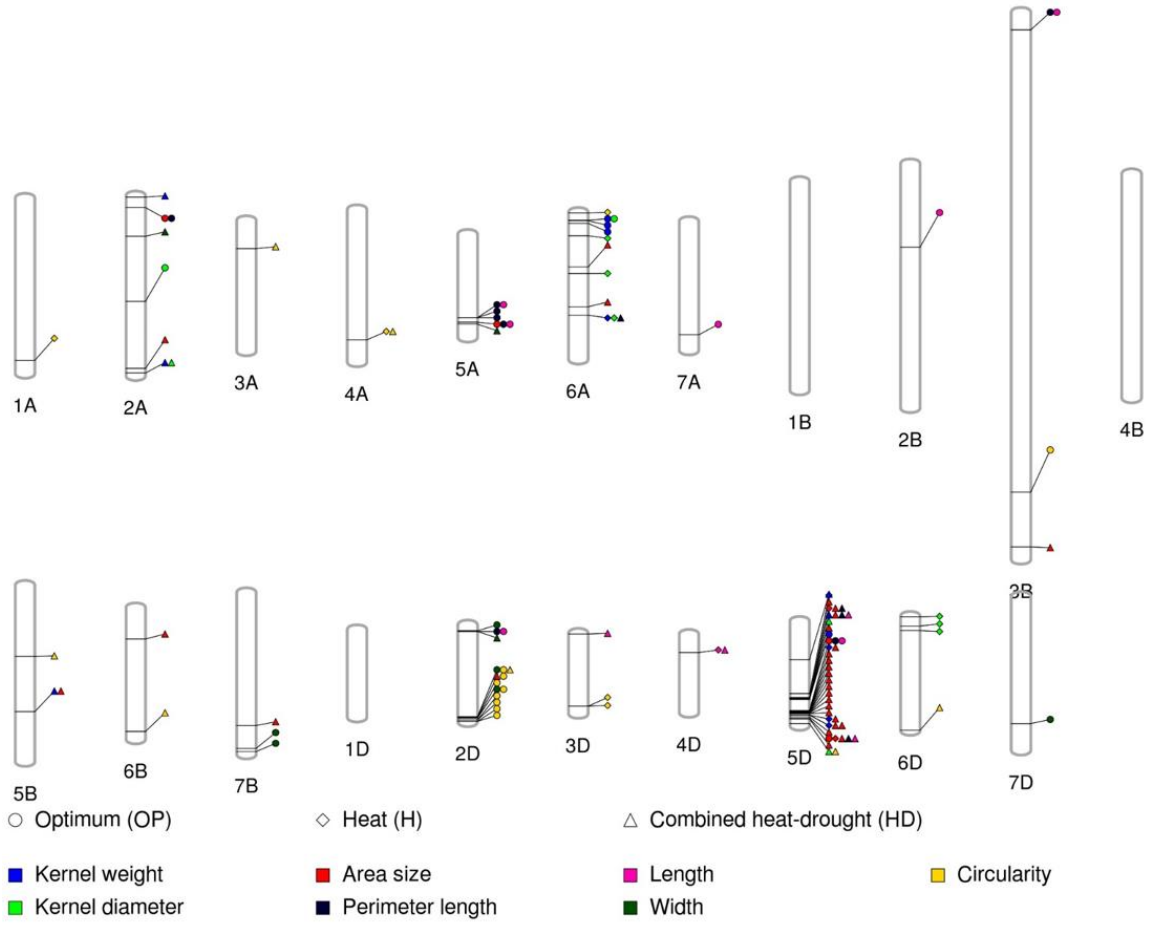


Figure 2.8. Significant marker trait associations of kernel weight and shape traits under optimum, heat and combined heat-drought conditions.

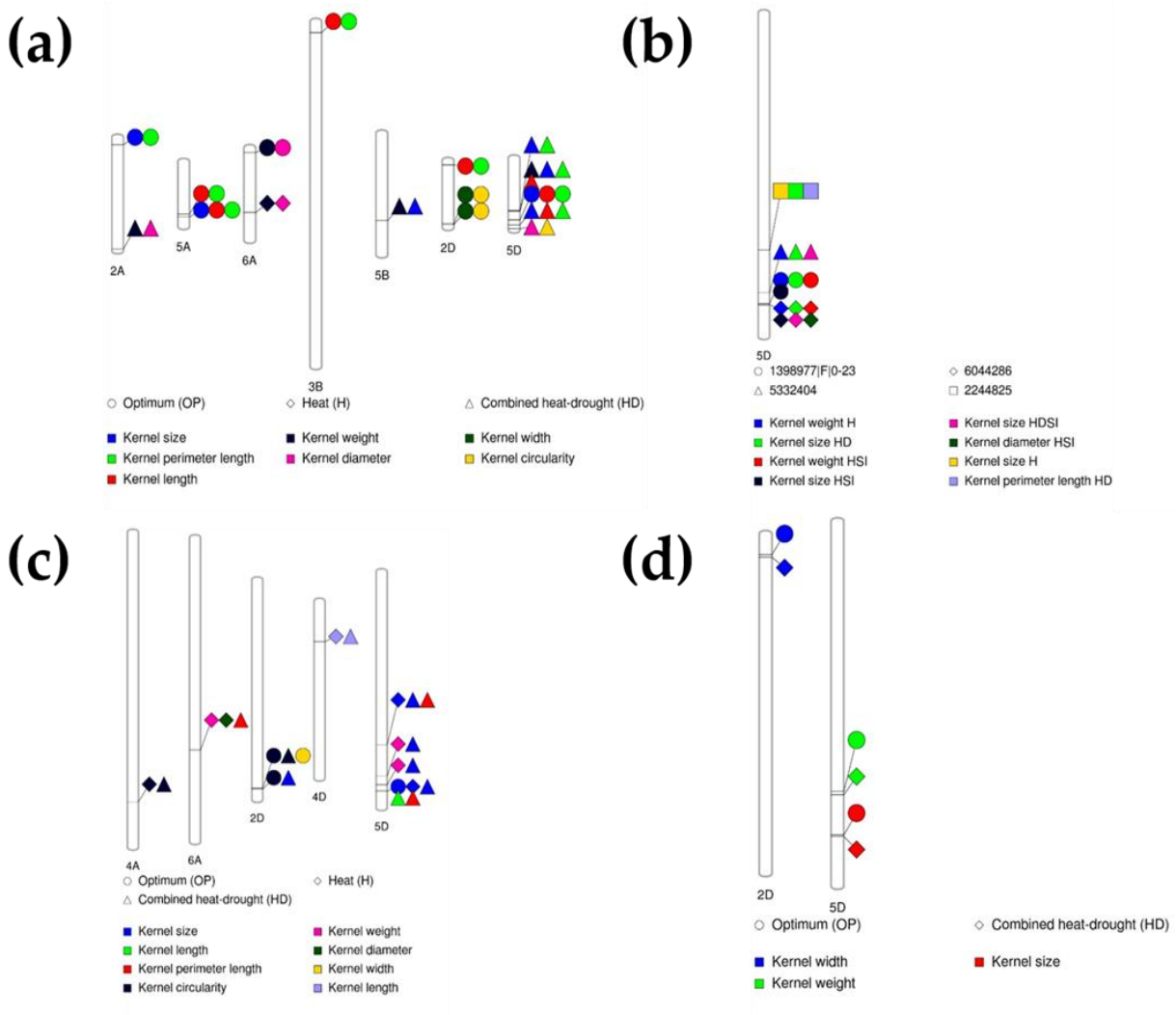


Figure 2.9. Markers for kernel weight and shape-related traits. **(a)** Pleiotropic markers under OP, H and HD conditions; **(b)** markers that showed potential for tolerance for kernel weight under H conditions and for kernel size under HD conditions and were associated with heat and heat–drought susceptibility indices; **(c)** stable markers between two or more environments; **(d)** markers that were located within distances less than 1 cM under different environments.

Table 2.2. Marker trait associations of kernel weight and shape-related traits in multiple synthetic derivative lines grown under optimum (OP), heat (H) and combined heat–drought (HD) conditions.

Chromosome	Marker Position (Bp)	Marker	Trait	Conditions	*p*-value	R^2
1A	229673410	1115856|F|0-14	Kernel circularity	H	9.75×10^{-4}	0.10
2A	1696501	3026123|F|0-8	Kernel weight	HD	2.40×10^{-4}	0.10
			Kernel size	OP	2.49×10^{-4}	0.12
	17036238	5357358	Kernel perimeter length	OP	7.51×10^{-4}	0.10
	57562452	3938604	Kernel width	HD	1.08×10^{-3}	0.09
	149553891	1113863	Kernel diameter	OP	1.67×10^{-3}	0.07
	250645685	1110845	Kernel diameter	HD	9.00×10^{-4}	0.08
			Kernel weight	HD	7.72×10^{-4}	0.08
	244243147	5010926	Kernel size	HD	1.02×10^{-3}	0.08
3A	39514910	1035262|F|0-46	Kernel circularity	HD	8.80×10^{-5}	0.11
4A	184463076	987701	Kernel circularity	H	1.01×10^{-3}	0.08
				HD	8.28×10^{-4}	0.09
			kernel length	OP	5.02×10^{-4}	0.10
5A	117490096	1079158	Kernel perimeter length	OP	5.47×10^{-4}	0.10
	117719229	1157204|F|0-51	Kernel perimeter length	OP	9.12×10^{-4}	0.10
	118132341	977527|F|0-25	Kernel perimeter length	OP	9.13×10^{-4}	0.10

	124441377	3028836	Kernel size	OP	5.09×10^{-4}	0.10
			Kernel length	OP	6.30×10^{-4}	0.09
			Kernel perimeter length	OP	3.78×10^{-4}	0.10
	127107886	3940004|F|0-21	Kernel width	HD	1.11×10^{-4}	0.19
6A	945226	2277231	Kernel circularity	H	7.23×10^{-4}	0.09
	10617494	4395641	Kernel weight	OP	2.94×10^{-4}	0.10
			Kernel diameter	OP	1.67×10^{-3}	0.08
	12184855	1115036	Kernel weight	OP	5.48×10^{-4}	0.09
	15813131	4407451	Kernel weight	OP	1.99×10^{-4}	0.10
	32701094	1019857|F|0-23	Kernel diameter	H	2.03×10^{-4}	0.10
	77372430	7327852	Kernel size	HD	4.35×10^{-4}	0.09
	86323044	3945281	Kernel diameter	H	4.39×10^{-4}	0.09
	133562282	3384829	Kernel size	HD	6.62×10^{-4}	0.09
			Kernel weight	H	7.75×10^{-4}	0.09
	145087238	5968258	Kernel diameter	H	5.89×10^{-4}	0.10
			Kernel perimeter length	HD	9.15×10^{-4}	0.08
7A	160041813	1056001	Kernel length	OP	9.34×10^{-4}	0.10
2B	118229752	7351021|F|0-10	Kernel length	OP	5.20×10^{-4}	0.09
3B	24450611	3958195	Kernel length	OP	1.21×10^{-4}	0.12

			Kernel perimeter length	OP	3.86×10^{-4}	0.10
	678386865	4396161	Kernel circularity	OP	9.64×10^{-4}	0.08
	755958103	7353565	Kernel size	HD	8.84×10^{-4}	0.09
5B	109565358	1302570	Kernel circularity	HD	4.34×10^{-4}	0.11
	196141955	2248796|F|0-42	Kernel weight	HD	5.07×10^{-4}	0.11
			Kernel size	HD	2.91×10^{-4}	0.12
6B	48765954	3020427	Kernel size	HD	5.31×10^{-4}	0.11
	191946279	993061	Kernel circularity	HD	6.60×10^{-4}	0.10
7B	205492257	5581272	Kernel size	HD	1.80×10^{-4}	0.11
	240968931	3949081	Kernel width	OP	6.80×10^{-4}	0.10
	246168466	4260892	Kernel width	OP	7.13×10^{-4}	0.10
2D	9318714	4999702	Kernel width	OP	9.34×10^{-4}	0.08
			Kernel length	OP	1.00×10^{-4}	0.14
	10253062	1000563|F|0-24	Kernel perimeter length	OP	3.28×10^{-4}	0.12
			Kernel circularity	OP	2.54×10^{-4}	0.14
	142248333	3946155	Kernel circularity	HD	2.48×10^{-4}	0.11
			Kernel width	OP	2.20×10^{-4}	0.11
	142982233	3957018	Kernel circularity	OP	7.92×10^{-5}	0.14
			Kernel size	HD	7.55×10^{-4}	0.09
	143867561	4542702	Kernel circularity	OP	1.25×10^{-4}	0.14
			Kernel circularity	OP	3.95×10^{-4}	0.14

	10453150	1076033|F|0-62	Kernel width	HD	0.00107	0.08
	143981582	3534099	Kernel width	OP	6.51×10^{-4}	0.09
	147520289	1088563	Kernel circularity	OP	3.21×10^{-5}	0.16
	147746144	4992154|F|0-9	Kernel circularity	OP	3.14×10^{-4}	0.15
	149031034	1116168|F|0-15	Kernel circularity	OP	2.72×10^{-4}	0.13
	149318699	3023828	Kernel circularity	OP	4.58×10^{-4}	0.12
3D	762269	3385313|F|0-6	Kernel length	HD	8.41×10^{-4}	0.10
	112899800	1203228|F|0-45	Kernel circularity	H	1.01×10^{-3}	0.11
	113047617	1122898|F|0-41	Kernel circularity	H	4.03×10^{-4}	0.13
4D	27756435	1001438|F|0-46	Kernel length	H	1.09×10^{-3}	0.10
			Kernel length	HD	8.93×10^{-4}	0.10
5D	59337192	1099271|F|0-23	Kernel weight	HD	9.63×10^{-4}	0.10
	112083542	1091823|F|0-41	Kernel weight	H	9.80×10^{-4}	0.10
	117708268	1072444	Kernel size	HD	8.30×10^{-4}	0.09
	118171820	2244825	Kernel size	H	6.65×10^{-4}	0.09
			Kernel size	HD	1.09×10^{-3}	0.09
			Kernel perimeter length	HD	5.62×10^{-4}	0.09
			Kernel weight	HD	4.95×10^{-4}	0.09
	119535444	991465	Kernel size	HD	1.39×10^{-5}	0.15
			Kernel length	HD	7.49×10^{-4}	0.09
			Kernel perimeter length	HD	7.08×10^{-4}	0.09

120017850	1201315|F|0-67	Kernel diameter	HD	4.98×10^{-4}	0.13
120914450	7351647|F|0-44	Kernel size	HD	4.90×10^{-4}	0.11
121111673	4910927	Kernel weight	OP	1.05×10^{-3}	0.08
138730779	1088488|F|0-27	Kernel size	OP	2.82×10^{-4}	0.13
		Kernel length	OP	3.89×10^{-4}	0.12
		Kernel perimeter length	OP	1.69×10^{-4}	0.13
139543900	5332404	Kernel weight	H	8.05×10^{-4}	0.09
		Kernel size	HD	2.38×10^{-4}	0.11
140397428	3955838|F|0-28	Kernel size	HD	6.41×10^{-4}	0.12
140551286	1101952	Kernel size	HD	2.07×10^{-4}	0.12
140719410	7350532	Kernel size	HD	5.78×10^{-4}	0.09
141061760	1087740	Kernel size	HD	2.32×10^{-4}	0.11
141600686	1215969	Kernel size	HD	6.05×10^{-5}	0.12
142135700	3941995	Kernel size	HD	4.32×10^{-4}	0.13
142429952	3946915	Kernel size	HD	1.08×10^{-3}	0.09
142680984	3954584	Kernel size	HD	6.87×10^{-5}	0.13
143115886	3026564	Kernel size	HD	7.86×10^{-5}	0.13
143728332	6041628	Kernel size	HD	6.01×10^{-5}	0.12
144829140	1398977|F|0-23	Kernel weight	H	6.45×10^{-4}	0.11
		Kernel size	HD	6.21×10^{-5}	0.17
145561848	6044286	Kernel weight	H	3.88×10^{-4}	0.11
145561848		Kernel size	HD	1.64×10^{-5}	0.17
145561848	1696241	Kernel size	HD	5.62×10^{-4}	0.09
148482930	2257612|F|0-47	Kernel size	HD	4.10×10^{-4}	0.09
150113559	1073897|F|0-27	Kernel size	OP	8.53×10^{-4}	0.08

			Kernel size	H	8.24×10^{-4}	0.08
			Kernel size	HD	2.54×10^{-4}	0.09
			Kernel length	HD	5.84×10^{-4}	0.08
			Kernel perimeter length	HD	3.56×10^{-4}	0.09
	151218934	1218899|F|0-6	Kernel size	HD	1.10×10^{-4}	0.14
	158302411	1041586|F|0-42	Kernel diameter	HD	7.44×10^{-4}	0.10
			Kernel circularity	HD	4.98×10^{-4}	0.13
6D	895318	5411945	Kernel diameter	H	1.06×10^{-3}	0.08
	15112276	1099241|F|0-17	Kernel diameter	H	2.01×10^{-4}	0.13
	22055487	1019857|F|0-52	Kernel diameter	H	2.05×10^{-4}	0.10
	176474631	1268158	Kernel circularity	HD	5.33×10^{-4}	0.09
7D	196638923	5331548	Kernel width	OP	1.05×10^{-3}	0.08

Table 2.3. Marker trait associations of heat susceptibility index and combined heat drought susceptibility index of Kernel weight and shape-related traits in multiple synthetic derivatives lines grown under optimum, heat and combined heat drought conditions

Chromosome	Position	Marker	Trait	Condition	*P*-value	R^2
1A	234706106	983365	Kernel size	HDSI	2.8×10^{-4}	0.10
2A	57562452	3938604	Kernel size	HSI	8.46×10^{-4}	0.09
	250239098	1209013			1.03×10^{-3}	0.09
	249185243	3022805	Kernel diameter	HDSI	4.91×10^{-4}	0.09
	250645685	1110845			5.61×10^{-4}	0.09
4A	169541561	7931161	Kernel diameter	HDSI	8.65×10^{-4}	0.09
6A	77372430	7327852	Kernel size	HDSI	5.87×10^{-4}	0.09
	32701094	1019857|F|0-23	Kernel diameter	HSI	1.07×10^{-3}	0.08
2B	6352113	1127429|F|0-36	Kernel diameter	HSI	7.08×10^{-4}	0.08
3B	755958103	7353565	Kernel size	HDSI	4.03×10^{-4}	0.10
	533982835	5363704	Kernel diameter	HSI	7.45×10^{-4}	0.09
5B	203991892	1106179|F|0-6	Kernel diameter	HDSI	5.57×10^{-4}	0.11
6B	48765954	3020427	Kernel size	HDSI	1.52×10^{-4}	0.13
7B	965969	3955041	Kernel diameter	HDSI	1.59×10^{-4}	0.11
	1899960	3023245			3.91×10^{-4}	0.10
	1939168	1065475			4.72×10^{-4}	0.10
	3193833	3020571			3.80×10^{-4}	0.10
2D	4635256	3031768|F|0-19	Kernel weight	HSI	4.57×10^{-4}	0.09
	4635256	3031768|F|0-19	Kernel diameter		7.77×10^{-4}	0.08
	14544740	981469		HDSI	1.03×10^{-3}	0.08
5D	112836809	4261480|F|0-22	Kernel weight	HSI	2.47×10^{-4}	0.10
	140111668	3950006			1.65×10^{-4}	0.11
	140530327	992773			3.43×10^{-4}	0.12
	140551286	1101952			8.54×10^{-4}	0.10
	144829140	1398977|F|0-23			4.87×10^{-4}	0.12
	145561848	6044286			6.78×10^{-5}	0.13
	146852419	1080556|F|0-34			6.90×10^{-4}	0.11
	119535444	991465	Kernel size	HDSI	1.56×10^{-4}	0.11
	139543900	5332404			1.96×10^{-4}	0.12
	140551286	1101952			2.53×10^{-5}	0.16

141600686	1215969			2.13×10^{-4}	0.11
142135700	3941995			8.01×10^{-4}	0.09
142680984	3954584			6.87×10^{-5}	0.13
143115886	3026564			7.09×10^{-5}	0.14
143728332	6041628			6.90×10^{-5}	0.12
144829140	1398977|F|0-23			1.88×10^{-4}	0.13
145561848	6044286		HSI	5.51×10^{-4}	0.09
145561848	1696241			1.70×10^{-5}	0.17
145561848	6044286		HDSI	4.58×10^{-5}	0.13
160742790	3022272			2.03×10^{-4}	0.11
112836809	4261480|F|0-22		HSI	4.05×10^{-4}	0.09
145561848	6044286	Kernel diameter		6.46×10^{-4}	0.09
117764881	1287670|F|0-30		HDSI	9.28×10^{-4}	0.10
120017850	1201315|F|0-67			1.94×10^{-4}	0.15

Under HD conditions, we detected 43 MTAs (Figure 2.8, Table 2.2) with the highest contribution from the D genome (Figure 2.7d). Chromosome 5D had the highest number of MTAs that were associated with all kernel traits except kernel width; most of them were associated with kernel size (Table 2.2). We found MTAs associated with kernel size, kernel width and kernel circularity on chromosome 2D and with kernel length on chromosomes 3D and 4D (Figure 2.8, Table 2.2). In the B genome, significant MTAs for kernel size were found on chromosome 3B, and for kernel weight, kernel size and kernel circularity on chromosome 5B. In the A genome, MTAs for kernel weight, kernel diameter, kernel size and kernel width were found on chromosome 2A, for kernel circularity on chromosome 4A and for kernel size and kernel perimeter length on chromosome 6A (Figure 2.8, Table 2.2). R^2 ranged from 0.08 for five markers to 0.19 in marker 3940004|F|0-21, associated with kernel width, on chromosomes 2D (Table 2.2) and 5A, respectively. Of the 43 MTAs, eight were also identified under H conditions, indicating their role in plant response to both stresses. On chromosome 2A, marker 1,110,845 affected kernel weight and kernel diameter, on chromosome 5B, marker 2248796|F|0-42 affected kernel weight and kernel size and on chromosome 5D, marker 991,465 affected kernel weight, kernel size, kernel length and kernel perimeter length (Figure 2.9a, Table 2.2).

2.3.2.1. MTAs for heat and combined heat–drought susceptibility indices

On the basis of HSI and HDSI (Table 2.3), 15 and 29 MTAs, respectively, were identified, of which the D genome contributed 61% (Table 2.S1). Among them, marker 6044286, on chromosome 5D, was associated with the HSI for kernel weight and kernel diameter, and with both the HSI and HDSI for kernel size (Table 2.3). We also identified four associations for the HSI for kernel weight and kernel size on chromosome 5D, kernel weight on chromosome 2D and kernel diameter on chromosome 5D (Table 2.3). R^2 ranged from 0.08 for marker 1019857|F|0-23, associated with the HSI for kernel diameter, to 0.15 for marker 1201315|F|0-67, associated with the HDSI for kernel diameter. Of 41 markers, five had pleiotropic effects (Table 2.3).

We identified four markers (1398977 |F|0-23, 6044286, 5332404 and 2244825) on chromosome 5D under stress conditions (H and HD) that had pleiotropic effect for kernel weight and kernel size (Figure 2.9b). Those markers were associated with the HSI and HDSI of kernel weight and kernel size (Figure 2.9b), for example, the marker 1398977|F|0-23, associated with the

kernel size HDSI; 6,044,286, associated with the kernel size HSI, kernel weight HSI and kernel size HDSI; and 5,332,404, associated with the kernel size HDSI (Figure 2.9b).

2.3.2.2. Stable MTAs for kernel weight and shape-related traits

We identified nine stable markers under two or all three conditions, of which seven were on the D genome (Figure 2.9c). Among them, the marker 1073897|F|0-27 was associated with kernel size and stable under OP and H conditions and with kernel size, kernel perimeter length and kernel length under HD conditions (Figure 2.9c). Additionally, markers associated with kernel width on chromosome 2D and with kernel weight and kernel size on chromosome 5D were located close to each other (<1 cM) and were detected under OP and HD conditions (Figure 2.9d).

2.3.2.3. Identification of putative candidate genes for kernel weight and shape-related traits

We searched for the candidate genes associated with the significant markers; we selected the candidate genes that possessed functions associated with kernel shape traits. The putative genes identified are listed in (Table 2.4). Under the three conditions, OP, H and HD, we identified genes related to stress tolerance, kernel size and yield regulation in the vicinity of the markers. Among 17 coding proteins in the region of the stable markers 1073897|F|0-27 associated with kernel size under OP, H, and HD conditions, we identified a putative RING-type *E3 ubiquitin*–protein ligase involved in kernel size and yield regulation and encoded by *TraesCS5D02G504400* (Table 2.4). The introduced SNP including T contributed to increasing the kernel area size under OP, H and HD conditions. This SNP originated from the *Ae. tauschii* accessions KU-2155 and KU-2156 collected in Iran (Figure 2.10 a-c). The lines MSD187 and MSD128 harboring this allele had higher kernel area sizes than their parent 'Norin 61' harboring the C allele under all conditions (Figure 2.10d).

The marker 5,332,404 on chromosome 5D was associated with kernel weight under H conditions, kernel size under HD conditions and the kernel size HDSI (Tables 2.2 and 2.3). There were nine coding proteins adjacent to this marker, among them the gene *TraesCS5D02G445100* which encodes for the heat stress transcription factor associated with high-temperature stress tolerance (Table 2.4). The allele A of this marker originated from eight *Ae. tauschii* accessions (IG 126387, KU-2039, KU-2124, AT 80, KU-20-10, KU-2155, KU-2156 and PI 499262) and

increased kernel weight and kernel size under H and HD but not under OP conditions (Figure 2.11e–g), indicating a special role for this allele under stress. Under stress, the MSD lines harboring the A allele had higher kernel weights and kernel sizes than ‘‘Norin 61’’ or MSD lines with the C allele (Figure 2.11d, h).

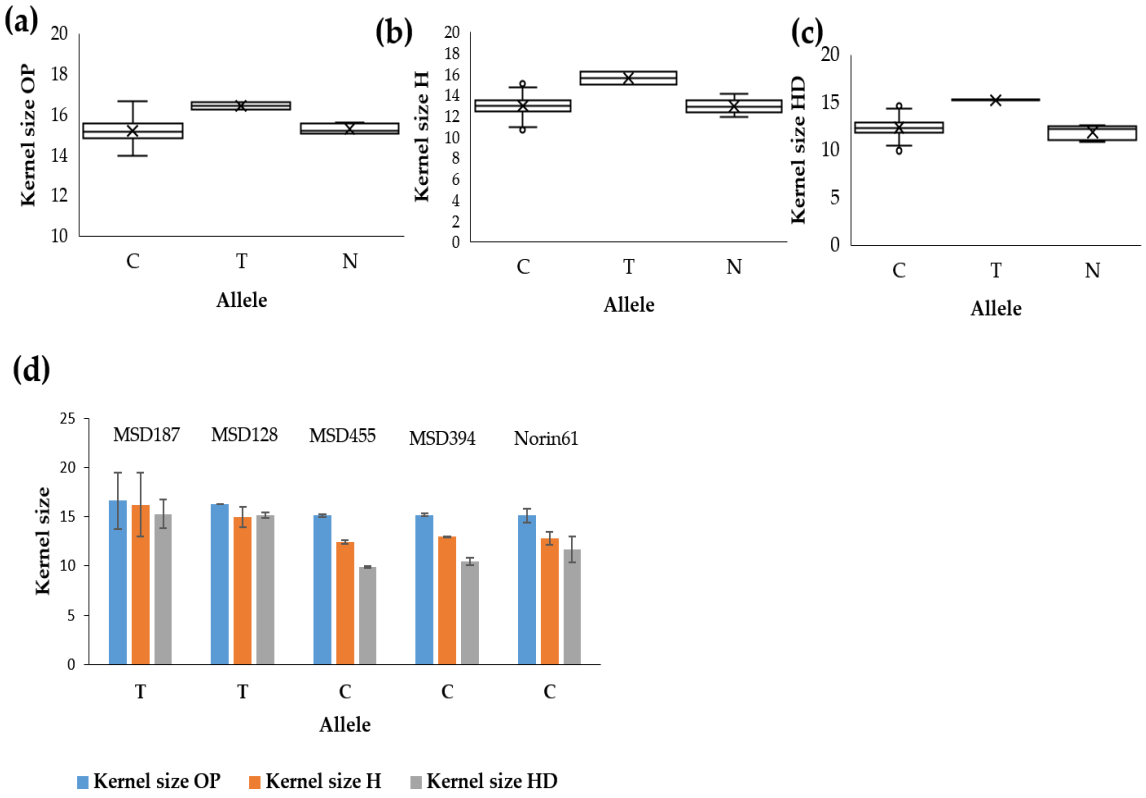


Figure 2.10. Effects of the alleles of the stable marker SNP_1073897|F|0-27 that increases kernel size under **(a)** optimum, **(b)** heat and **(c)** heat–drought conditions. **(d)** Examples of the lines harboring different alleles for SNP_1073897|F|0-27 and their parent ‘Norin 61’.

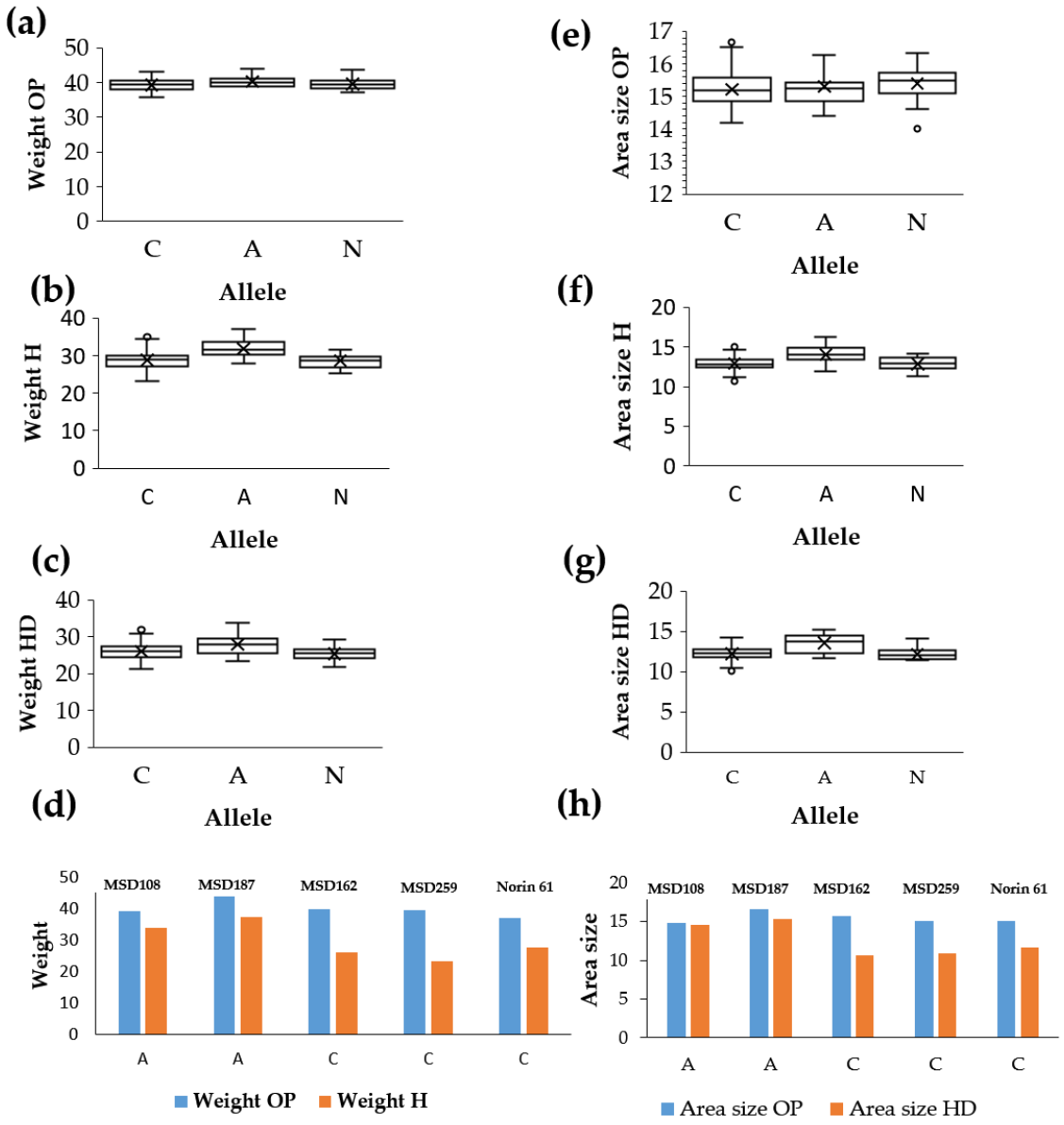


Figure 2.11. Effects of alleles of markers 5332404 associated with Weight under heat (H), area size under combined heat-drought (HD) and area size HD susceptibility index. **(a-c).** box plot of alleles effects on Weight under optimum (OP), H and HD conditions, respectively. (**e-g).** box plot of alleles effects on kernel area size under OP, H and HD conditions, respectively. **(d)** and **(h)** shows examples of the allele's effects on the Weight and area size performance, respectively, of some MSD lines and 'Norin 61' under OP, H and HD conditions.

Table 2.4. Candidate genes for kernel weight and shape-related traits under optimum, heat and combined heat–drought conditions and their putative physiological roles.

Marker	Chromosome	Trait (Environment)	R^2	Gene	Protein	Function
5357358	2A	Kernel size (OP), kernel perimeter length(OP)	0.11	*TraesCS2A02G099400*	Basic-leucine zipper (bZIP) transcription factor family protein	Regulates seed maturation
4407451	6A	Kernel weight (OP)	0.10	*TraesCS6A02G056600*	Auxin-responsive protein	Regulates auxin
4395641	6A	Kernel weight (OP), kernel diameter (OP)	0.10	*TraesCS6A02G057300*	F-box domain-containing protein	Flower development, defense response
2248796|F|0-42	5B	Kernel weight (HD), kernel size (HD)	0.11	*TraesCS5B02G302400*	Aspartic proteinase nepenthesin-1	Role in drought avoidance
1076033|F|0-62	2D	Kernel width (HD)	0.08	*TraesCS2D02G076500*	Heat shock protein Hsp20 domain-containing protein	Tolerance to biotic and abiotic stresses
5332404	5D	Kernel weight (H), kernel size (HD)	0.09	*TraesCS5D02G445100*	Heat stress transcription factor	Tolerance to environmental stress
1073897|F|0-27	5D	Kernel size (OP, H, HD), kernel length (HD), kernel perimeter length (OP, HD)	0.08	*TraesCS5D02G504400*	E3 ubiquitin–protein ligase	Regulates grain size and yield
1398977|F|0-23	5D	Kernel weight (H)	0.11	*TraesCS5D02G469900*	F-box domain-containing protein	Flower development, defense response

2.4 Discussion

Seed shape and size are the most important agronomic traits owing to their effect on grain kernel weight. Few QTLs associated with kernel traits have been identified under stress conditions in wheat through the association mapping approach. Here, we aimed to detect the effects of heat and heat–drought on kernel weight and shape-related traits using a panel of unique MSD lines harboring different D genome sources.

2.4.1. Phenotypic variation for kernel weight and shape-related traits under optimum and stress conditions

The responses to stress conditions were varied for the kernel traits, in which HD conditions severely affecting weight and shape-related traits, followed by the H conditions. We observed that kernel weight, kernel diameter and kernel size were the traits most affected by HD compared to H conditions. In addition, 'Norin 61', the backcross parent of the MSD lines, and the standard check cultivar Imam showed remarkable reductions in kernel weight and shape-related traits under HD compared to H conditions. Combined heat–drought severely affects plants due to heat stress evapotranspiration leading to severe drought stress (Lamaoui et al. 2018). Ramya et al. (2015) reported that drought and heat stress shorten the grain growth period and lead to improper grain filling, thereby reducing the kernel weight and the overall yield. Moreover, high temperature reduces the conversion of sucrose to starch due to the suppression of the soluble starch synthase enzyme, leading to shriveled kernels (Jenner 1994). Drought and heat stress accelerate leaf senescence, decrease photosystem II efficiency. As a result, this leads to the reduction of the amount of stored assimilates translocated into developing grains and reduced kernel size (Tian et al. 2018 and Telfer et al. 2018). Heat and drought affect plant growth and thereby reduce yield (Sattar et al. 2020). In this study, kernel weight was severely affected by HD compared to H conditions. Qaseem et al. (2019) also observed that combined heat–drought significantly affect kernel weight and yield. Furthermore, shape-related traits, especially kernel diameter and size, were affected by HD compared to H conditions. However, kernel length was less affected by the stresses than kernel weight and other related traits (Table 2.1). This finding is in agreement with a report that kernel length is less affected by heat than the kernel weight (Zhang et al. 2018). We identified some MSD lines, such as MSD187, that could maintain good kernel weight under stress,

unlike ‘Norin 61’ and Imam. These germplasm lines could be used in wheat breeding pro-grams for heat stress tolerance (Elbashir et al. 2017).

Most of the kernel weight and shape traits, including kernel diameter and kernel weight, exhibited moderate heritability under OP conditions, whereas high heritability under H and HD conditions was found (Table 1). Xin et al. (2020) found high heritability of kernel shape traits in four different environments. Traits with high heritability and genetic advances can be selected directly for crop improvement (Shokat et al. 2015).

The association between kernel weight, kernel diameter, kernel size, kernel width and kernel length under all conditions indicated that all these traits contribute to kernel weight, as reported in Cheng et al. (2017) and Desiderio et al. (2019) (Figure 2.2). Kernel diameter was most strongly correlated with kernel weight, with heritability ranging from 0.64 under OP conditions to 0.88 under stress. These results suggest kernel diameter as a target trait for selection in breeding programs aiming at increasing kernel weight and yield in wheat.

2.4.2. Marker trait associations for kernel weight and shape-related traits under optimum and stress conditions

More than 50% of the MTAs identified in this study were on the D genome, thus indicating its higher contribution, especially under stress conditions. This result is inconsistent with previous reports for the kernel shape that indicated a lower contribution of the D genome compared to A and B genomes (Chao et al. 2009; Wang et al. 2014; Su et al. 2018). Rasheed et al. (2014) also reported a lower contribution of the D genome, though they used synthetic hexaploid wheat. This inconsistency could be attributed to the previous studies being conducted under non-stress conditions and/or to the diversity of the D genome in the materials used being narrower than the A and B genomes; our results are in agreement with Ali et al. (2020). On the other hand, our findings reveal the uniqueness of the MSD panel as an effective and powerful platform for allele and gene mining in *Ae. tauschii*.

Thousand kernel weight is one of the yield components and QTL studies have been conducted for this trait (Gupta et al. 2020). Also, studies have identified QTLs for grain size at different chromosomes. However, further studies are necessary to understand these traits under stressed environments (Lopes et al. 2013). Here, in a genome-wide association study (GWAS) for

kernel size, we identified a high peak on the chromosome 5D under the HD environment. Similar results were described by Afzal et al. (2019), specifically, that chromosome 5D influences drought tolerance, indicating that this locus could have an important role in enhancing kernel size and yield under HD conditions. We identified markers associated with kernel weight on chromosomes 6A and 5B under H and HD conditions, respectively. Lopez et al. (2013) reported similar results under H and HD conditions for kernel weight. Under all conditions, the identified MTAs indicated that kernel diameter was most strongly associated with kernel weight. Several studies have reported the association of kernel diameter with kernel weight and other traits under stress condition (Mwadzingeni et al. 2017). These findings further support the above phenotypic correlation findings that kernel diameter, besides other traits, can be a target for selection in breeding programs to increase kernel weight and final grain yield.

Some markers had pleiotropic effects. We found a putative gene, *TraesCS6A02G057300*, for one of these markers, 4395641, on chromosomes 6A, associated with kernel weight and kernel diameter. This gene encodes an F-box domain-containing protein (Table 2.4). F-box proteins regulate leaf senescence, flower development and defense responses (Chae et al. 2008; Han and Delaney 2002). They also have a role in ethylene signaling (Binder et al. 2007). Reduction in ethylene signaling has been suggested to increase grain yield in maize and Arabidopsis (Shi et al. 2015), consistent with the critical role of kernel weight and kernel diameter in increasing wheat grain yield.

The candidate gene for the pleiotropic marker 2248796|F|0-42 for kernel weight and kernel size detected under HD conditions on chromosome 5B encodes aspartic proteinase nepenthesin-1. Yao et al. (2012) found that the overexpression of an aspartic proteinase can play a role in drought avoidance through ABA signaling. A recent study in wheat reported that an aspartic proteinase is associated with wheat stress response (Gou et al. 2020).

Among pleiotropic markers on chromosome 5D detected under H and HD conditions, 5,332,404 had the candidate gene *TraesCS5D02G445100*. This gene encodes a heat stress transcription factor, which has an important role in responses to abiotic stresses (Guo et al. 2016).

The lines MSD187 and MSD108, harboring the positive allele of this marker, performed better than ‘Norin 61’ and the check cultivar Imam, indicating the usefulness of this marker in

maintaining kernel weight under stress. After validation, this marker could be very useful in wheat breeding.

Marker 1076033|F|0-62, associated with kernel width under HD conditions, on chromosome 2D had the candidate gene *TraesCS2D02G076500*, which encodes the heat shock protein Hsp20 (Table 2.3). Heat shock proteins enhance plant immunity (Li et al. 2009). Recently, TaHSP20 genes have been shown to play an important role in abiotic stress tolerance in wheat (Muthusamy et al. 2017). Therefore, this marker and its candidate gene could be used in marker assisted selection programs to improve wheat stress tolerance.

In agreement with Ali et al. (2020), we found that kernel area size was most closely associated with kernel perimeter length and then kernel length under all conditions. The candidate gene *TraesCS2A02G099400* for marker 5,357,358 detected under OP conditions on chromosome 2A encodes a basic leucine zipper transcription factor (Table 2.4). Basic leucine zipper is a member of the transcription factor families that controls transcription of seed maturation genes and is expressed during seed development (Alonso et al. 2009).

We identified markers stable under at least two conditions. Marker 1073897|F|0-27 was associated with kernel size under all three conditions and is a good candidate for marker-assisted selection in breeding programs. Interestingly, the candidate gene for this marker, *TraesCS5D02G504400*, encodes a RING-type *E3 ubiquitin*–protein ligase, which increases grain size and yield (Bednarek et al. 2012). The ubiquitin pathway plays a crucial role in determining plant seed size (Li and Li 2014). Here, we identified a stable marker and candidate gene under OP, H and HD conditions on chromosome 5D. This study used a panel of MSD derivatives with high diversity of the D genome derived from several *Ae. tauschii* accessions; we speculate that the marker identified in our study could be related to a new gene affecting seed size in wheat. However, a detailed study is necessary to confirm this assumption.

2.5. Conclusions

In this study, we examined an MSD population with broad diversity in the D genome of bread wheat. The MSD lines were remarkably variable in the kernel traits under OP, H and HD conditions. We identified many MTAs, most of which were on the D genome, revealing the power of the MSD lines as a platform for gene mining in *Ae. tauschii*. Some MSD lines performed better

than the backcross parent ‘Norin 61’ and the check cultivar ‘Imam under H and HD stress conditions. These lines, along with the stable markers, favorable alleles and candidate genes elucidated here, represent a good resource with which to enhance wheat grain yield under stress and optimum conditions. However, more work will be necessary to validate the suitability of these markers and/or alleles. Nevertheless, our study supports the claim that *Ae. tauschii* is an important gene reservoir to breed stress-resilient bread wheat.

CHAPTER THREE

Novel Loci for Kernel Hardness Appeared as a Response to Heat and Combined Heat-Drought Conditions in Wheat Harboring *Aegilops tauschii* Diversity

3.1. Introduction

Kernel hardness is an important quality trait that greatly influences the milling and baking quality of wheat. The world wheat trade is largely carried out based on hardness grades. Wheat hardness is a quantitative trait with classes ranging from soft to hard (Muqaddasi et al. 2020). Two genes mainly determine hardness, puroindoline a and b (*Pina* and *Pinb*), which are located on the short arm of chromosome 5D (5DS), and form the molecular basis of wheat hardness (Giroux and Morris 1998) .The presence of the wild-type form of these genes is associated with the soft kernel phenotype in hexaploid wheat. The absence or mutation in either gene results in a hard kernel phenotype (Bhave and Morris 2008).

Although puroindoline genes are considered major determinants of wheat hardness, many studies have demonstrated the complex nature of this trait (Wang et al. 2012 and Kiseleva et al. 2020), and suggest that hardness is affected by several factors. These factors include abiotic stresses such as heat and drought. Heat and drought stresses pose a serious risk to agriculture (IPCC, 2014; Zandalinas et al. 2018). Several studies have demonstrated that stress from high temperatures and drought can accelerate kernel filling. These stresses compress the timing of key events during wheat kernel development, such as increasing the production of storage proteins and starch synthesis in the endosperm under stress, which together affect kernel hardness (Ashraf 2014). Furthermore, kernel hardness has been reported to be negatively associated with the thousand-kernel weight (Niu 2014 and Szabó et al. 2016), as well as with shape traits such as kernel length, width, and diameter (Niu 2014). Proteins are considered important components of wheat grains, since they govern wheat's end-use quality. Alterations of the protein fraction composition due to drought and heat stress are primarily due to changes in the total nitrogen

quantity accumulated during the seed-filling phase (Triboï et al. 2003). In turn, increasing hardness because the protein content is positively correlated with hardness (Pasha, Anjum, and Morris 2010).

In addition to the puroindoline genes, several studies have identified alleles that contribute to hardness; these were located on most of the 21 wheat chromosomes, but especially on chromosomes 1B, 2A, 4B, 5A, 5B, 5D, and 7D (Bordes et al. 2011; Wang et al. 2012; Chen et al. 2019; Muqaddasi et al. 2020). Several researchers studied hardness of plants grown under stress condition (Hernández-Espinosa et al. 2018; Tomás et al. 2020). However, the genetic factors that affected the change in hardness remained unclear. Therefore, better understanding of the change or stability in hardness under stress environment is essential because the factors or QTLs contributes to hardness stabilization or modification under stress environment will have a great value for wheat quality breeding. Therefore, we conducted this study to dissect the genetic factors that contribute to hardness under optimum, heat and combined heat-drought (HD) conditions and to investigate the association between hardness stabilization or modification and stress tolerance.

We used a panel of multiple synthetic derivatives (MSD) lines that harbor a wide diversity from a wild species, *Aegilops tauschii*. Our results revealed a significant marker trait association on chromosome 4D under heat and combined heat-drought (HD) environments. The tolerant MSD lines, with low reduction in their kernel weight, have more stable hardness than sensitive ones. The germplasm source identified and characterized in this study will be an excellent source to breed wheat stress tolerant cultivars with stable hardness.

3.2. Materials and Methods

3.2.1 Plant Materials

In this study, we used a multiple synthetic derivative (MSD) population of 400 lines developed by crossing and backcrossing 43 synthetic wheat lines with the Japanese wheat cultivar ‘Norin 61’ (Gorafi et al. 2018). Based on heading time and vernalization requirements, 140 MSD lines were selected out of the 400 original lines and tested in Sudan under heat and HD conditions. All the MSD lines were genotyped using the DArT-seq platform (Gorafi et al. 2018).

3.2.2 Field experiment

The field experiments were conducted in Japan and Sudan. We choose Japan as optimum condition, because it considered as favorable condition for wheat, while we selected Sudan for heat and HD experiment because it has been recognized as the global plat-form for heat tolerance research (Elhadi et al. 2021). In Japan, the 400 MSD lines were grown in a field at the Arid Land Research Center, Tottori University for two seasons (2015/2016 and 2018/2019), using an augmented randomized complete block design with eight blocks. We used four replicated checks in each block; 'Norin 61' (the MSD parent), 'Imam' and 'Tagana' (Sudanese heat-tolerant cultivars), and 'Safedak Ishkashim' (a Tajikistan landrace) (Elhadi et al. 2021). In Sudan, we selected 140 lines because they do not require vernalization treatment and adapt to the Sudanese conditions. Those lines were evaluated under heat and HD condition using alfa-lattice design, with two replications. The drought was imposed by withholding the irrigation after heading as described in our previous study (Elhadi et al. 2021).

3.2.3 Hardness and protein content measurements and SEM observation

Measurement of kernel hardness (index) of the MSD lines under optimum, heat and HD conditions was performed with a bulk sample of 100 clean, unbroken wheat kernels using the single-kernel characterization system (SKCS 4100, Perten Instruments, Waltham, MA, USA) at the NARO Western Region Agricultural Research Center. This ma-chine measures the force required to crush individual grains of a sample between two surfaces using (index) unit. The MSD lines were classified into hard and soft based on significant differences from the value for the background cultivar 'Norin 61'.

We measured the nitrogen content of about 50 mg of wheat kernel powder using a CN Corder (Model MT-700; Yanaco, Inc., Kyoto, Japan). We recorded the nitrogen to carbon ratio, and then calculated the nitrogen content as a percentage and converted that value to the crude protein content by multiplying the N content by the conversion factor 5.95 (Saito, Tamura, and Ogawa 2019).

To detect physical changes in response to stress, we examined the internal structure of the wheat kernels using a transverse section of the kernel under a scanning electron microscope (JSM-6610LV, JEOL, Peabody, MA, USA).

3.2.4 Statistical analysis and hardness stress indexes

We performed analysis of variance (ANOVA) for data from the Japanese experiment to detect differences between accessions using version 1.4 of the Plant Breeding Tools software (PBTools, http://bbi.Irri.org/products). We estimated the best linear unbiased predictions (BLUPs) across the two seasons and used the results for GWAS. For the Sudan experiments, we performed ANOVA using GenStat 18 (VSN International, Rothamsted Research, Harpenden, Hertfordshire, UK). Broad-sense heritability (H^2) was defined as $H^2 = VG/(VG+VE)$, where VG and VE are the genetic and environment estimation, respectively (Smith et al. 1998). We calculated relative performance for hardness under heat (*HI*) and HD conditions (*HDI*). Also, we calculated heat susceptibility index (*HSI*) and combined heat-drought susceptibility index (*HDSI*). All calculations were conducted using the following formulas:

1. *HI* = (performance in heat environment / performance in optimum environment) ×100%

2. *HDI* = (performance in HD environment / performance in optimum environment) ×100%

3. *HSI* or *HDSI* = (1–[Yh/Y])/ (1–[Xh/X])

where Yh is phenotypic means for each genotype under heat or HD condition, Y is phenotypic means for each genotype under optimum condition, Xh means for all lines under heat or HD condition, and X means for all lines under optimum condition.

3.2.5 Association analysis

We performed genome-wide association analysis for kernel hardness using a mixed linear model (MLM) with 14,355 DarT-seq markers by version 5 of the TASSEL software (Bradbury et al. 2007). Tassel MLM product were used to generate Manhattan plot using CM plot package in R. We created Manhattan plots using $p < 3\times10\text{-}3$ as the threshold to identify significant associations.

3.2.6 Bioinformatics analysis

To identify candidate genes for hardness and the related proteins, we selected the marker trait associations (MTAs) that were identified as significant under all conditions, and we BLAST them against the International Wheat Genome Sequencing Consortium (IWGS) RefSeq V.1 chromosomes, using URGI with BLAST option (https://urgi.versailles.inra.fr/blast/), accessed on

August 2020. Then, we searched for the candidate genes with high confidence in distance (± 500 kbp) for the genome region. We used version 1.0 of IWGSC_Ref_seq to search for genes with high confidence. To identify the protein function, we used version 1.1 of IWGSC_Ref_Seq_Annotations along with EnsemblPlant (https://plants.ensembl.org), accessed on August 2020. We investigated the expression levels of all candidate genes that have high contribution for hardness and compared them to the expression of the puroindoline genes using the Wheat Expression Browser expVIP (Borrill et al. 2016). This lead to understand the association between the candidate genes and hardness. This browser allows comparisons across several studies by taking an input of RNA-seq that collected from these studies. The output is viewable as browser interference with interactive filtering, sorting and export option. This allows an easy access for the researchers (Borrill et al. 2016). Visualization of the expression was realized using GENEVESTIGATOR software (https://genevestigator.com/gv/start/start.jsp). Tokyo, Japan, accessed at February 2021.

3.3. Results

3.3.1 Phenotypic variation and diversity in hardness among MSD lines under optimum and stress conditions

Under optimum environment, significant variation ($p < 0.001$) in hardness was detected among the MSD lines. No significant differences were detected between the two seasons (Table 1), while the interaction between the environment and seasons (G × S) was significant ($p < 0.05$). The MSD lines exhibited a wide range of variation for hardness (18.5 to 47.8), whereas the backcross parent (‘Norin 61’) was 29.6. We selected 140 MSD lines out of the original 400 lines based on their suitability to grow under Sudanese conditions to understand the effect of the heat and HD environments on the hardness and to detect the different factors that influence the hardness under stressed conditions. Therefore, the results of the 140 lines will be presented and discussed.

Under stress environments, significant differences were observed among the genotypes ($p < 0.05$) (Table 3.1). The environmental effect was significant, whereas the G × E effect was not significant. High heritability (> 90%) was observed under the optimum as well as heat and HD environments (Table 3.1).

Under heat and HD environments, the genotypes shifted to became harder. This appear on the frequency distribution, which ranged from 19.6 to 56.9 under heat, and from 25.5 to 60.2 under HD environments, respectively (Figure 3.1a). 'Norin 61' became harder with a hardness index of 35.7 and 35.2 under heat and HD environments, respectively, compared to 29.6 under optimum condition.

Table 3.1. Analysis of variance and heritability for hardness under optimum, heat and HD environments for MSD lines.

Source of variation	MSD range for hardness (Index)	'Norin 61'	SED (±)	*p*-value	LSD	Heritability
Optimum	18.5-47.8	29.6	-	***	-	-
S (S1 × S2)	-	-	-	ns	-	-
G × S	-	-	4.77	***	9.20	0.90
Heat	19.6-56.9	35.7	3.60	***	7.10	-
HD	25.5-60.2	35.2	6.40	***	12.70	-
E	-	-	-	**	-	-
G × E	-	-	3.59	ns	7.07	0.97

S1 and S2, seasons 1 and 2, respectively; G, genotype; E, environment (Heat and HD). SED (±), standard error of the difference. ; **, and***, significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; ns, not significant.

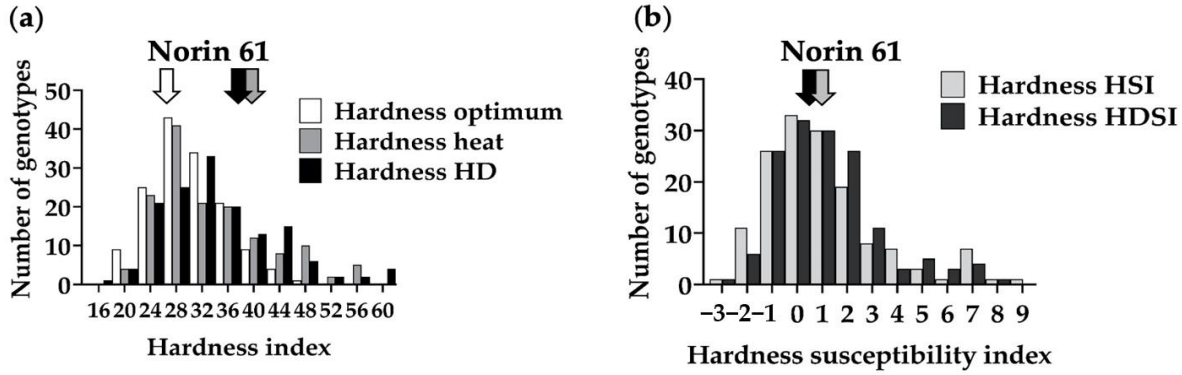


Figure 3.1. Frequency distribution of hardness and hardness indexes (**a**) Frequency distribution of hardness under optimum, heat and combined heat-drought (HD) environment and (**b**) Frequency distribution of hardness heat susceptibility index (*HSI*) and heat-drought susceptibility index (*HDSI*). 'Norin 61' is the background parent of the multiple synthetic derivative (MSD) lines.

Table 3.2. Correlation between hardness, kernel weight and shape related traits under optimum, heat and HD environments.

Trait	Environment	Hardness	kernel weight	kernel diameter	kernel size	kernel perimeter length	kernel length	kernel width	kernel circularity
Hardness	Optimum	1	-0.18**	-0.11	-0.069	-0.065	-0.057	-0.079	-0.02
kernel weight	Optimum		1	0.82***	0.41***	0.33***	0.27***	0.42***	0.08
kernel diameter	Optimum			1	0.14	0.02	-0.07	0.37***	0.47***
kernel size	Optimum				1	0.97***	0.92***	0.85***	-0.15
kernel perimeter length	Optimum					1	0.98***	0.72***	-0.37***
kernel length	Optimum						1	0.59***	-0.51***
kernel width	Optimum							1	0.33***
kernel circularity	Optimum								1
Hardness	Heat	1	-0.28***	-0.046	-0.18**	-0.25***	-0.32***	-0.013	0.22***
Kernel weight	Heat		1	0.88***	0.81***	0.70***	0.60***	0.73***	0.14
Kernel diameter	Heat			1	0.62***	0.44***	0.30***	0.73***	0.39***
Kernel size	Heat				1	0.93***	0.82***	0.81***	-0.01
Kernel perimeter length	Heat					1	0.97***	0.56***	-0.36***
Kernel length	Heat						1	0.36***	-0.55***
Kernel width	Heat							1	0.55***
Kernel circularity	Heat								1
Hardness	HD	1	-0.28***	-0.046	-0.19**	-0.23***	-0.3***	0.014	0.28
Kernel weight	HD		1	0.88***	0.66***	0.65***	0.54***	0.68***	0.02
Kernel diameter	HD			1	0.47***	0.42***	0.28***	0.69***	0.27**
Kernel size	HD				1	0.68***	0.64***	0.47***	-0.008
Kernel perimeter length	HD					1	0.97***	0.55***	-0.22**
kernel length	HD						1	0.34***	-0.37***
kernel width	HD							1	0.39***
Kernel circularity	HD								1

The frequency distribution of heat and heat-drought susceptibility indexes for hardness, HSI and HDSI (Figure 1b), showed that 51 and 49 genotypes representing about 34.4 and 33.1% of the MSD population had a negative value (less than zero) under heat and HD environment, respectively, indicating that these genotypes became softer under stressed conditions than under optimum condition. In contrast, most of MSD population (65.5 and 66.9%) changed towards harder grain under heat and HD environments, respectively (Figure 3.1b).

Hardness is one of the traits reported to correlate with kernel weight and other kernel shape traits (Niu 2014). In our previous study (Elhadi et al. 2021), we discussed the effect of the stress environments on the kernel shape traits, including the kernel weight, and we identified some tolerant genotypes under heat or HD environment based on their kernel weight reduction. The correlation between the hardness and the other kernel shape traits showed that kernel weight was significantly negatively correlated with hardness under optimum and stress conditions. However, this correlation became stronger under stress environments (Table 3.2). All of the kernel shape traits except kernel weight correlated with hardness only under stress environments (Table 3.2).

One of our objectives in the present study was to investigate whether the stabilization of kernel hardness under stress condition was associated with stress tolerance. Since kernel weight is correlated with hardness, the kernel weight susceptibility indexes, which assess the reduction under stress compared with optimum conditions. Thus, it can be used as tolerance indicators. We investigated the relationship between hardness and kernel weight for heat and heat-drought susceptibility index (*HSI* and *HDSI*) for the MSD lines (Figure 3.2). We observed a relationship between stress tolerance (a low kernel weight reduction) and hardness stabilization under stress condition. The kernel weight *HSI* ranged from 0.44 to 1.55, and the kernel weight HDSI ranged from 0.67 to 1.36. The hardness *HSI* ranged from –2.5 to 9.2, and the hardness HDSI ranged from –2.9 to 8.4. Genotypes with *HSI* and *HDSI* values less than 0.75 and 0.79, respectively (i.e., kernel weight reductions less than 20 and 27%, respectively) were considered to be tolerant, whereas the genotypes with *HSI* and *HDSI* values greater than 1.3 and 1.2, respectively (i.e., kernel weight reductions greater than 35 and 41%, respectively) were considered stress susceptible.

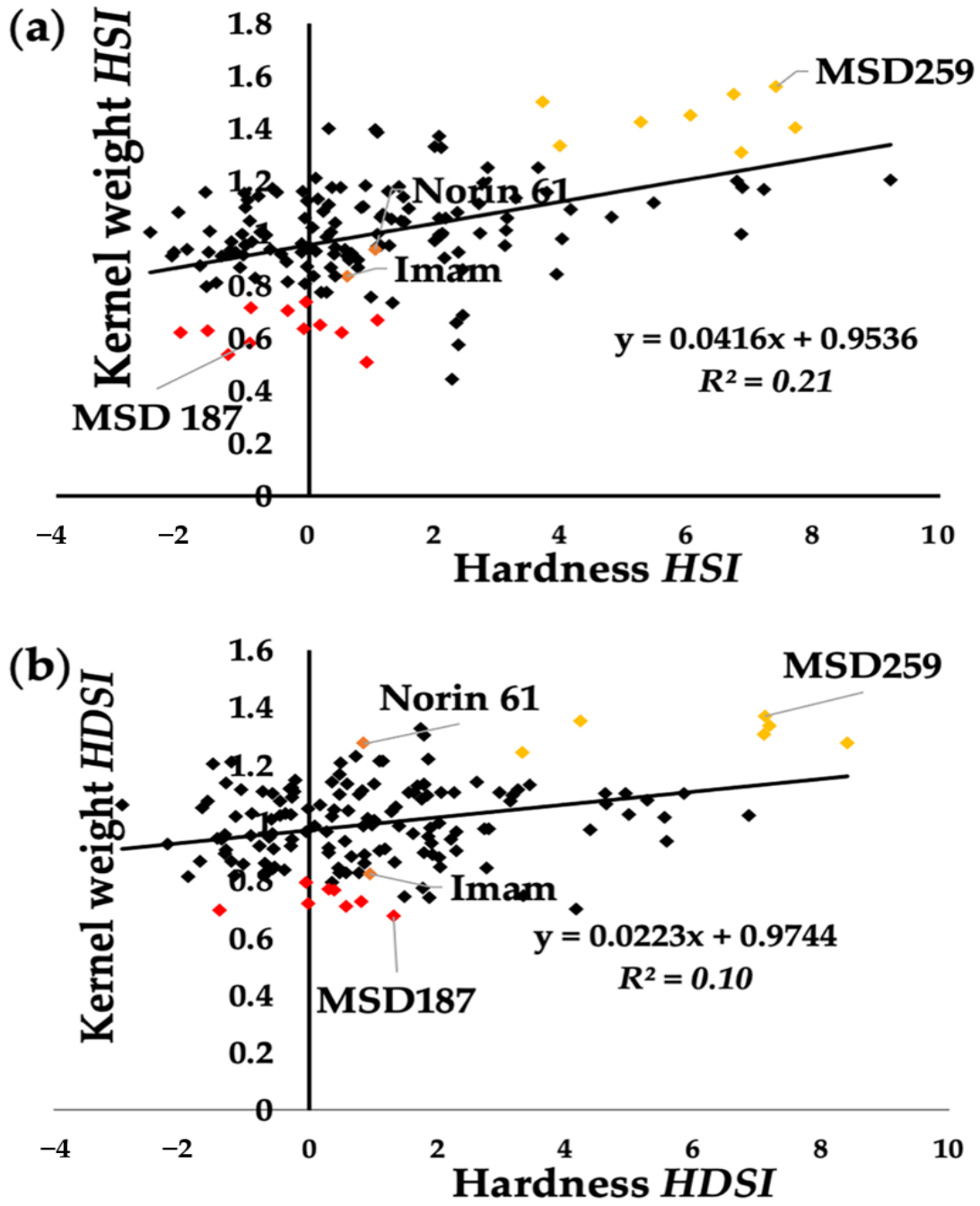


Figure 3.2. Relationship between kernel weight and hardness heat susceptibility index (*HSI*) **(a)**. And heat-drought susceptibility index (*HDSI*) **(b)**. Tolerant lines with low change in kernel hardness are indicated with red dots and sensitive lines with a high change in kernel hardness are indicated with yellow dots. 'Norin 61' is the background parent of the multiple synthetic derivatives (MSD) and 'Imam' is a heat-tolerant Sudanese cultivar.

There was a clear association between having stable hardness and tolerance to heat or HD stress. The kernel weight *HSI* showed that 17 genotypes had a low kernel weight reduction, of which 12 had only a slight change in hardness, which remained stable or became softer (as shown in red color in Figure 3.2a). Based on *HDSI*, ten genotypes had a low kernel weight reduction, of which six had a low change in hardness (as shown in red color in Figure 3.2b).

In contrast, among 14 genotypes with high kernel weight reduction, eight were largely affected in their hardness as shown in yellow color in (Figure 3.2). Of the ten genotypes that had a high reduction in kernel weight under HD, six of them had a high change in hardness (with yellow color Figure 3.2). Among these genotypes, we found five genotypes shared for both *HSI* and HDSI (Figure 3.3). The tolerant line MSD187, which we identified in our previous study (Elhadi et al. 2021), had low change in hardness under both heat and HD (Figure 3a), whereas, the sensitive line MSD259 showed a large reduction in hardness under both heat and HD (Figure 3.3b). The cultivars 'Norin 61', which is the parent of the MSD lines, and 'Imam', had relatively high reductions in kernel weight and moderate changes in hardness under heat conditions compared with MSD187 (Figure 3.3a). We further tested these lines for their internal structure and protein content to investigate the relationship between the kernel weight reduction and stabilization or modification of hardness.

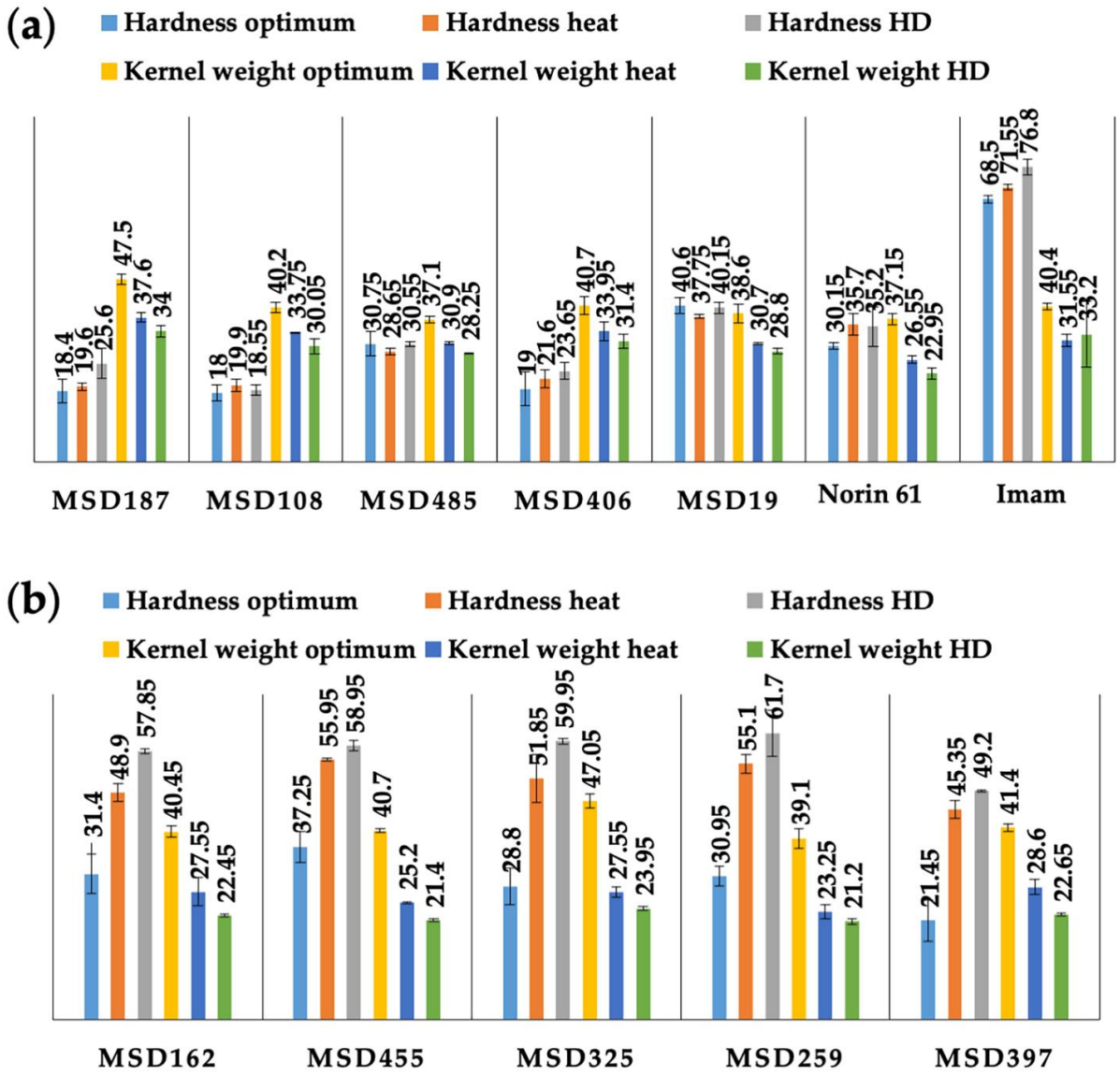


Figure 3.3. Relationship between hardness and kernel weight for the multiple synthetic derivative (MSD) lines tolerant **(a)** And sensitive **(b)** lines under optimum, heat and combined heat-drought (HD), ‘‘Norin 61’’ is the background parent and ‘Imam’ is a heat-tolerant Sudanese cultivar.

3.3.2 Internal structure and protein content

We used a scanning electron microscope (SEM) to examine the internal structure of the mature kernels of the selected tolerant (MSD187), sensitive (MSD259), 'Norin 61' accessions and of the 'Imam' (Figure 3.4a). The starch granules of 'Norin 61' were loosely packed under optimum conditions as well as heat and HD condition, which indicates a soft kernel. 'Imam', which was hard under optimum as well as heat and HD showed embedded starch granules with no gaps, which indicates hard kernels (Figure 3.4a). We observed that the surface of starch granules of the tolerant line MSD187 were separated from the protein matrix under optimum, heat and even under HD with no change due to the stresses (Figure 3.4a). In contrast, the starch granules of the sensitive line MSD259 were separated under optimum, but, under heat and specially HD starch granule were tightly attached to the protein matrix, and were embedded in the protein matrix, indicating an increase in hardness as response to the stresses.

We observed an increase in protein content under heat condition in the four tested lines comparing to optimum condition (Figure 3.4b), which could be due to the big variation between optimum in Japan and stress conditions in Sudan. However, in the Sudanese conditions, when HD and heat were compared, the protein content of the tolerant line MSD187 decreased compared to the sensitive line MSD259 and 'Norin 61' (Figure 3.4b). This indicated that decreasing in protein content associated with low change in hardness.

3.3.3 Marker traits association of hardness and hardness indexes under optimum and stress conditions

In the current study we performed a genome-wide association study (GWAS) to identify genetic loci that contributes for hardness under optimum, heat and HD conditions. We found a total of 47 statistically significant markers (Table 3.2). The Manhattan plots revealed several significant associations for hardness under all conditions (Figure 3.5). The A genome contributed to hardness only under stress conditions, in which chromosome 1A under HD, and 4A under both heat and HD environments showed significant MTAs (Figure 3.5, Table 3.2). In the B genome, only chromosome 5B contributed to hardness under optimum as well as stress condition. The D genome contributed more strongly to hardness than the A and B genomes, with the D genome explaining 91.4% of the total contribution under optimum, heat, and HD environments.

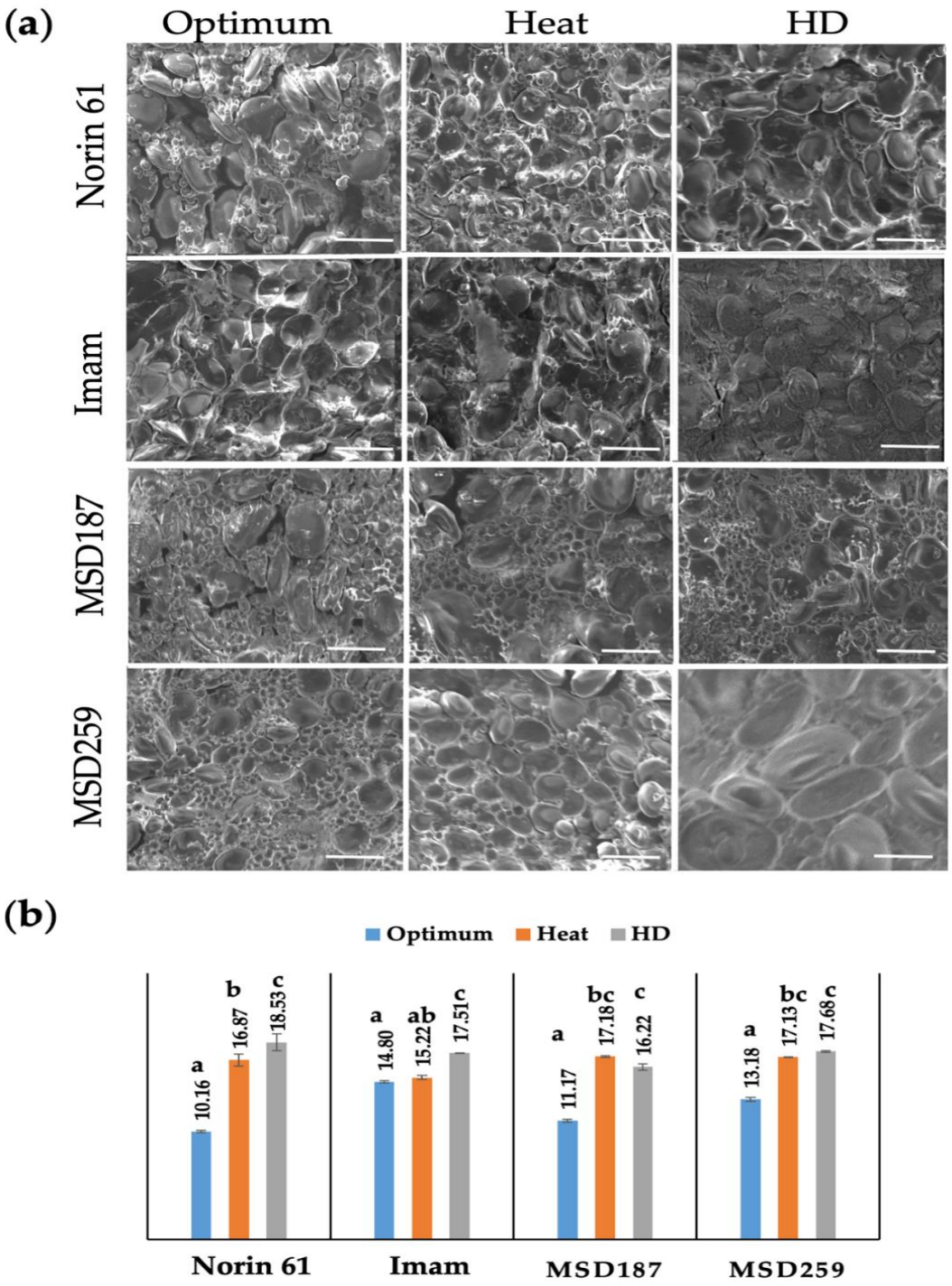


Figure 3.4. Scanning electron microscope showing the internal structure of wheat endosperm for Imam, 'Norin 61', tolerant and sensitive lines MSD187, and MSD259 respectively, under optimum, heat and combined heat-drought (HD) conditions **(a)** . Bars indicate 50 µm. Protein content (%) for the tolerant and sensitive lines, 'Norin 61' and Imam **(b)**; letters marks with different letters were significantly different at $p < 0.05$; whereas, letters marks with same letter are not significant.

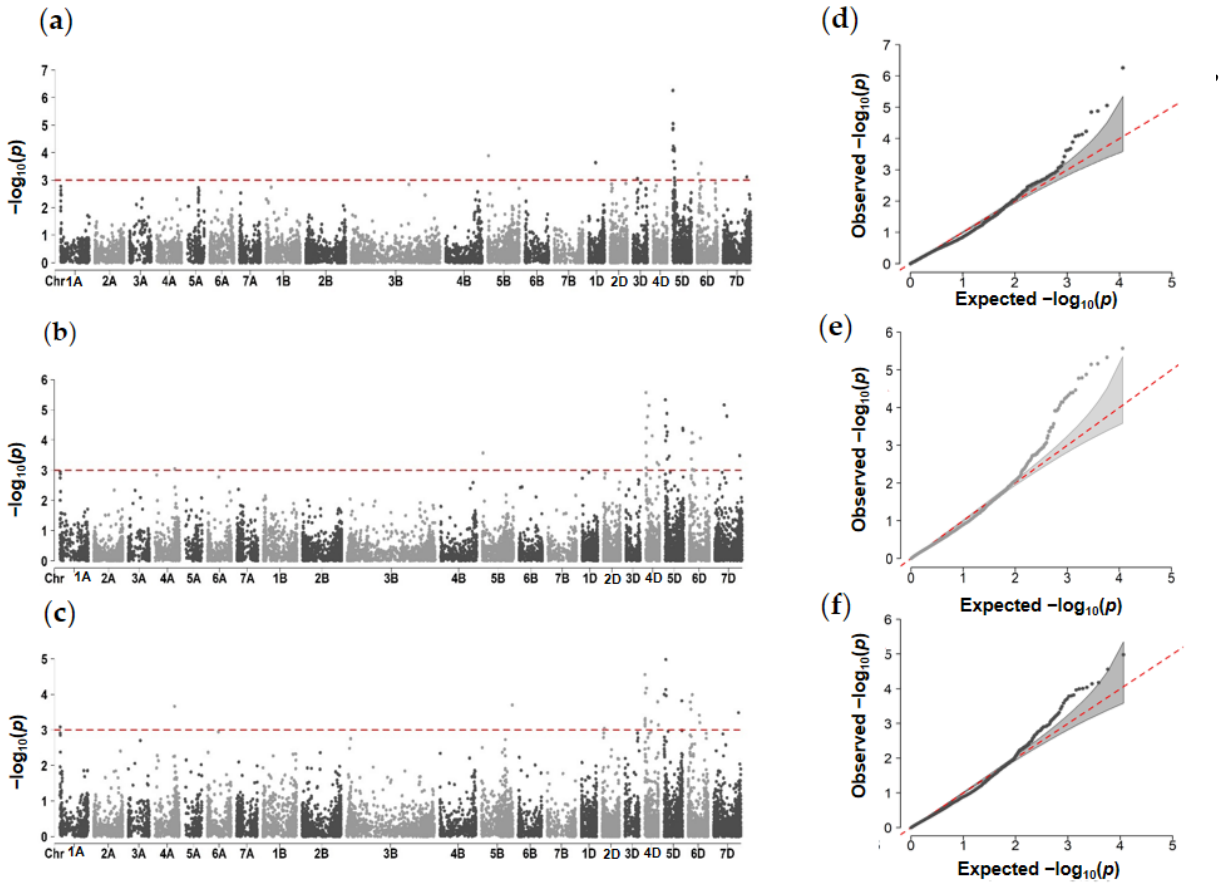


Figure 3.5. Manhattan for hardness (**a**) Under optimum environment, (**b**) Under heat environment, (**c**) Under combined heat-drought environment (HD), (**d-f**) Quantile–Quantile plots for hardness under optimum, heat, and HD.

The marker with the highest significance was 1127970 with 22.6% of the phenotypic variation explained (PVE) under optimum environment. Also, significant MTAs were observed on chromosome 4D, under heat and HD (Figure 3.5). Interestingly, these association appeared only under the stress environments. The marker with the highest significance was 1062681|F|0-26 on chromosome 4D was associated with heat and HD with PVE of 20.5 and 17.3%, respectively.

In addition, we obtained significant associations on chromosomes 6D and 7D under stress condition, but with a few significant markers under optimum conditions. Marker 1074408|F|0-12 on chromosome 6D was associated with heat and HD with PVE of 13.7 and 13.1%, respectively.

Marker 3222372|F|0-27 on chromosome 7D was associated with heat conditions, and had a PVE of 19.8%.

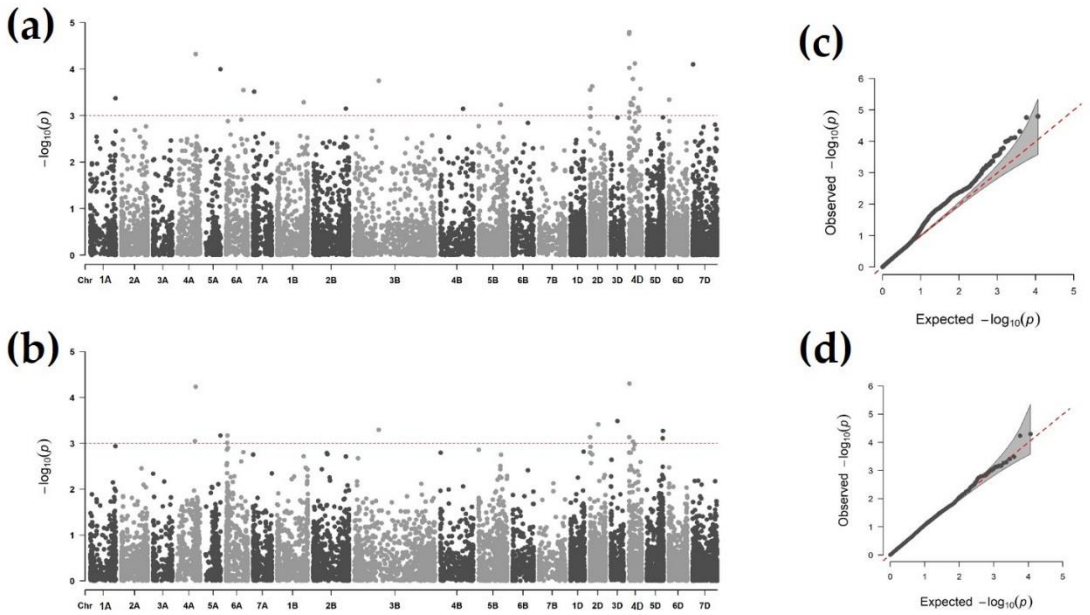


Figure 3.6. Manhattan plot and Q-Q plot for kernel hardness indices **(a)** hardness heat indices HI, **(b)** hardness drought indices HDI, **(c, d)** Q-Q plot for hardness indices.

Hardness indexes revealed 45 MTAs for hardness heat index and hardness heat drought index (*HI*, *HDI*) distributed in the A, B, and D genomes. The highest contribution was from the D genome (64%, of which 62% was located on chromosome 4D), versus 22 and 14% for the A and B genomes, respectively (Table 3.3, Figure 3.6). Marker 1062681|F|0-26 on chromosome 4D was highly significant under heat and HD environments and was associated with both hardness and the hardness indexes, indicating that it contributed only under stress condition (Table 3.3). This marker explained 20.5, 17.3 and 17.5% of the PVE of heat, *HD* and *HI*, respectively (Table 3.2 and 3.2).

3.3.4 Common and specific MTAs associated with kernel hardness under optimum and stress conditions

We identified stable markers for hardness across the three conditions, mainly in the D genome on chromosomes 5D and 6D (Figure 3.7a). We also detected significant markers in the D genome associated only with stress conditions on chromosomes 4D, 5D, 6D, and 7D (Figure 3.7b). Meanwhile, we identified markers from the A, B, and D genomes associated only with the two hardness indexes (Figure 3.7c, Table 3.3), indicating that these markers were associated with the modification of hardness in response to stress condition (Figure 3.7c). We identified markers associated with stress for hardness and the hardness indexes (Figure 3.7d). Most of the markers that we detected in the D genome were common across all conditions, whereas markers in the A and B genomes were specific to the HD condition (Figure 3.7e).

Under the optimum condition, there were significant associations with hardness only on the short arm of chromosome 5D, however, under a stress environment heat and HD there was another association on the long arm (Figure 3.5). This indicates that there are gene(s) on the chromosome 5D other than puroindoline contributing to the hardness under stress conditions.

Table 3.3. Marker trait associations of hardness grown under optimum, heat and HD conditions.

Total genome contribution %	Chromosome	Marker position	Marker	Environment	*p*-value	PVE %
A genome (4.3%)	1A	441,118	3947128	HD	8.28×10^{-04}	8.5
	4A	173,725,668	3025314	Heat	9.03×10^{-04}	8.7
				HD	2.17×10^{-04}	11.1
B genome (4.3%)	5B	4,332,688	6030814|F|0-7	Optimum	1.30×10^{-04}	13.5
				Heat	2.71×10^{-04}	12.3
		270,359,835	1082888|F|0-18	HD	1.98×10^{-04}	12.9
D genome (91.4%)	1D	59,530,872	7351912|F|0-19	Optimum	2.32×10^{-04}	15.2
	2D	17,717,364	3021443|F|0-24	HD	9.06×10^{-04}	8.3
	3D	3,6431,533	1082930	Optimum	8.81×10^{-04}	8.7
	4D	799,855	1078655	Heat	1.22×10^{-04}	13.0
		2,196,458	1062681|F|0-26	Heat	2.71×10^{-06}	20.5
				HD	2.77×10^{-05}	17.3
		3,354,073	1665831	Heat	8.53×10^{-04}	8.7
				HD	7.77×10^{-04}	8.5
		3,486,593	1126556	HD	4.85×10^{-04}	9.8
		4,063,428	4440031	HD	5.44×10^{-04}	10.5
		4,135,716	3946288	Heat	4.03×10^{-05}	15.3
				HD	6.79×10^{-04}	10.2
		4,676,059	5332499	Heat	3.39×10^{-04}	11.5
				HD	9.14×10^{-05}	12.6
		14,681,664	3574405	Heat	1.69×10^{-05}	15.6
				HD	6.69×10^{-05}	13.1

	27,756,435	1001438|F|0-46	Heat	7.18×10^{-06}	18.9
	56,518,479	998809|F|0-7	Heat	7.30×10^{-05}	14.4
			HD	5.85×10^{-04}	11.2
	98,321,462	1043872|F|0-49	Heat	5.61×10^{-04}	11.7
	113,875,447	3950661|F|0-9	Heat	6.59×10^{-04}	12.0
			HD	1.61×10^{-04}	14.6
	120,386,388	1218881	HD	7.28×10^{-04}	10.9
5D	849,701	1127970	Optimum	5.51×10^{-07}	22.6
			Heat	4.69×10^{-06}	18.9
			HD	9.94×10^{-05}	14.5
	1,268,790	3957566	Optimum	7.99×10^{-05}	14.1
	1,465,909	1090404	Optimum	1.44×10^{-05}	15.0
	2,351,222	3532985	Optimum	1.33×10^{-05}	16.1
			Heat	1.06×10^{-04}	12.1
	2,504,012	5573281|F|0-7	Optimum	8.85×10^{-06}	18.3
			Heat	3.46×10^{-05}	16.2
	3,757,073	5573405	Optimum	5.94×10^{-05}	13.0
	6,987,738	3948152	Optimum	2.14×10^{-04}	11.0
	14,328,401	4540116	Optimum	8.32×10^{-04}	8.3
	14,984,317	3944483	Optimum	7.40×10^{-05}	13.9
			Heat	1.33×10^{-05}	15.3
			HD	1.05×10^{-05}	16.3
	14,984,556	1025407	Optimum	8.54×10^{-05}	12.6
			Heat	7.17×10^{-05}	12.3
			HD	7.29×10^{-05}	12.1
	17,157,888	3021240|F|0-28	Optimum	3.77×10^{-04}	12.0
			Heat	5.40×10^{-05}	14.9

			HD	1.09×10^{-04}	13.7
	17,257,369	2242137|F|0-66	Heat	4.28×10^{-04}	12.8
	34,818,075	1093560|F|0-46	Heat	3.47×10^{-04}	12.3
	155,393,575	3950421	Heat	4.14×10^{-05}	13.6
			HD	1.52×10^{-04}	11.9
	156,123,909	3936784	Heat	4.76×10^{-05}	13.3
			HD	1.04×10^{-03}	8.4
6D	12,507,971	1102905|F|0-25	Optimum	5.84×10^{-04}	10.0
	18,973,446	3960692|F|0-32	Heat	4.25×10^{-04}	12.0
	19,795,263	1074408|F|0-12	Heat	1.23×10^{-04}	13.7
			HD	1.65×10^{-04}	13.1
	24,752,141	5357783	Heat	5.89×10^{-05}	12.8
			HD	2.62×10^{-04}	10.4
	26,576,895	1073587|F|0-24	Heat	9.59×10^{-04}	11.2
	35,991,349	2242479	Optimum	2.43×10^{-04}	12.3
			Heat	1.19×10^{-04}	12.1
			HD	1.02×10^{-04}	12.3
	99,107,045	7348732	Heat	8.68×10^{-05}	11.6
			HD	3.85×10^{-04}	9.4
	102,962,817	5992592	HD	6.21×10^{-04}	9.2
7D	80,561,020	3222372|F|0-27	Heat	6.97×10^{-06}	19.8
	105,476,798	3944062	Heat	1.63×10^{-05}	14.4
	206,383,743	1261369|F|0-13	Optimum	7.69×10^{-04}	12.5
	217,517,266	1065454|F|0-13	Heat	3.28×10^{-04}	9.5
			HD	3.27×10^{-04}	9.5

Table 3.4. Marker trait associations of hardness of multiple synthetic derivatives for heat index (*HI*) and heat-drought index (*HDI*)

Chromosome	Marker position	Marker	Index	*p*-value	PVE %
1A	242753632	5332418	HI	4.24×10^{-04}	10.1
4A	173725668	3025314	HI	4.74×10^{-05}	13.9
5A	143800150	4542455	HI	1.01×10^{-04}	12.7
6A	167205100	3024029	HI	2.84×10^{-04}	10.7
7A	11766354	5332250	HI	3.06×10^{-04}	10.4
1B	253738412	3935608	HI	5.19×10^{-04}	10.0
2B	314211442	3953978	HI	7.08×10^{-04}	10.6
3B	233106754	1099828	HI	1.78×10^{-04}	12.0
4B	218053982	3938494|F|0-20	HI	7.16×10^{-04}	10.9
5B	214881533	4394327	HI	5.87×10^{-04}	9.7
2D	6069529	3023595	HI	6.96×10^{-04}	8.8
2D	6792657	1116536|F|0-28	HI	0.00105	9.5
2D	22035717	3937638	HI	2.35×10^{-04}	11.1
2D	2936574	1101362|F|0-46	HI	2.78×10^{-04}	14.4
4D	34774472	7489093	HI	5.89×10^{-04}	9.1
4D	17740333	5331871	HI	5.82×10^{-04}	9.2
4D	63244073	5580133	HI	8.87×10^{-04}	9.5
4D	17740330	1116375	HI	5.33×10^{-04}	9.5
4D	88865945	7350544	HI	6.74×10^{-04}	9.5
4D	4135716	3946288	HI	8.39×10^{-04}	9.7
4D	110047223	7352222	HI	2.65×10^{-04}	10.1
4D	45314849	6010711	HI	4.26×10^{-04}	10.2
4D	98321462	1043872|F|0-49	HI	7.90×10^{-04}	11.6
4D	3354073	1665831	HI	9.36×10^{-05}	12.1
4D	38836536	1100833	HI	1.63×10^{-04}	12.7
4D	56518479	998809|F|0-7	HI	7.56×10^{-05}	14.5
4D	4676059	5332499	HI	1.59×10^{-05}	16.8
4D	2196458	1062681|F|0-26	HI	1.74×10^{-05}	17.5
6D	3209869	3021480	HI	4.57×10^{-04}	11.2
7D	5159347	4397755	HI	7.91×10^{-05}	15.8
4A	166472305	1114173	HDI	8.94×10^{-04}	8.6
4A	173725668	3025314	HDI	5.82×10^{-05}	13.7

5A	143800150	4542455	HDI	6.77×10^{-04}	9.4
6A	10796061	3025582	HDI	9.76×10^{-04}	8.1
6A	12539084	5372578	HDI	6.77×10^{-04}	8.7
3B	233106754	1099828	HDI	5.10×10^{-04}	10.2
2D	2936574	1101362|F|0-46	HDI	7.33×10^{-04}	12.3
2D	80816957	3939167|F|0-21	HDI	3.87×10^{-04}	14.7
3D	65283030	2244022	HDI	3.27×10^{-04}	9.7
4D	3354073	1665831	HDI	7.35×10^{-04}	8.9
4D	55141127	1032077	HDI	0.00107	8.9
4D	38836536	1100833	HDI	9.14×10^{-04}	9.3
4D	4676059	5332499	HDI	4.99×10^{-05}	13.9
5D	153796236	5350256	HDI	7.76×10^{-04}	8.9
5D	155044717	4005032|F|0-41	HDI	5.38×10^{-04}	13.2

We noticed that all the markers associated with kernel weight and shape traits on chromosome 5D under the stress environments located on the long arm (Elhadi et al. 2021). This result led us to investigate the relationships between markers for grain hardness and the kernel weight or shape traits under stress on this chromosome arm. However, we did not identify pleiotropic markers for both hardness and the kernel weight or shape traits (Figure 3.8).

3.3.5 Candidate genes for hardness and gene expression

We searched for candidate genes associated with the significant markers. We targeted the markers with a high PVE combined with a function related to hardness. The resulting candidates are listed in Table 3.4. Marker 1127970 on chromosome 5D was stable under all conditions and encodes a puroindoline gene. Marker 3532985 on chromosome 5D detected under optimum and heat environments encoded for sucrose transporter gene *SUT*.

We further investigated the *SUT* allele's contribution to hardness under optimum and heat environments (Figure 3.9a, b, and c). We found that the "C" allele and "N" allele were associated with decreased hardness, whereas, the "A" allele was associated with in-creased hardness (Figure 3.9a, b). However, when we checked a stress-tolerant line (MSD187), a sensitive line (MSD259), and their parent ''Norin 61'', we found that both the sensitive line and ''Norin 61'' harbored the "C" allele that decreased hardness, whereas the tolerant line harbored the "N" allele (Figure 3.9c).

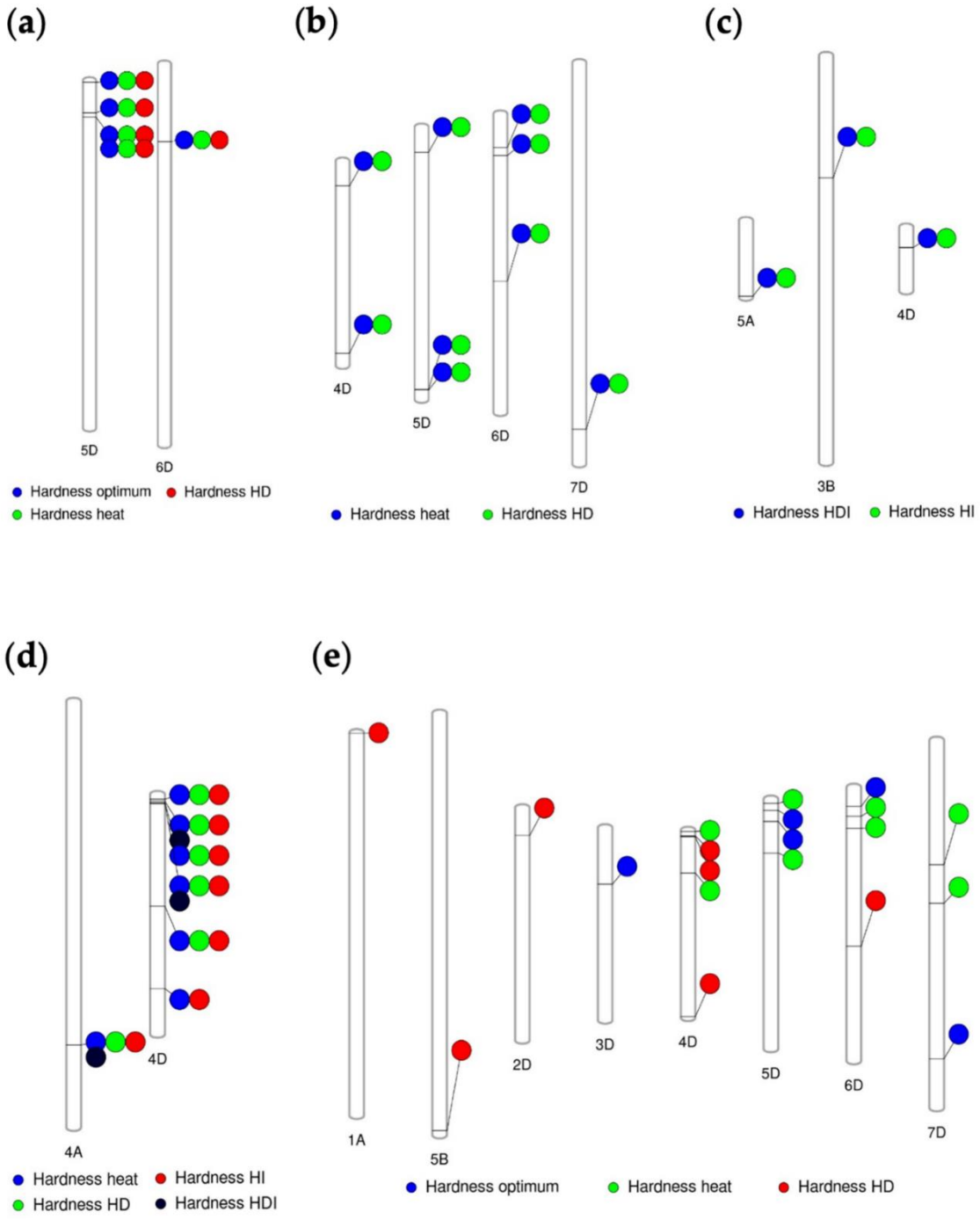


Figure 3.7. Significant marker trait associations (MTAs) for hardness that were (**a**) Stable MTAs under all conditions, (**b**) Significant markers associated with stress environment heat and HD, (**c**) Significant marker associated with hardness stress index *HI* and *HDI*, (**d**) Significant markers associated with stress condition for both hardness and hardness indexes (**e**) Significant markers associated with

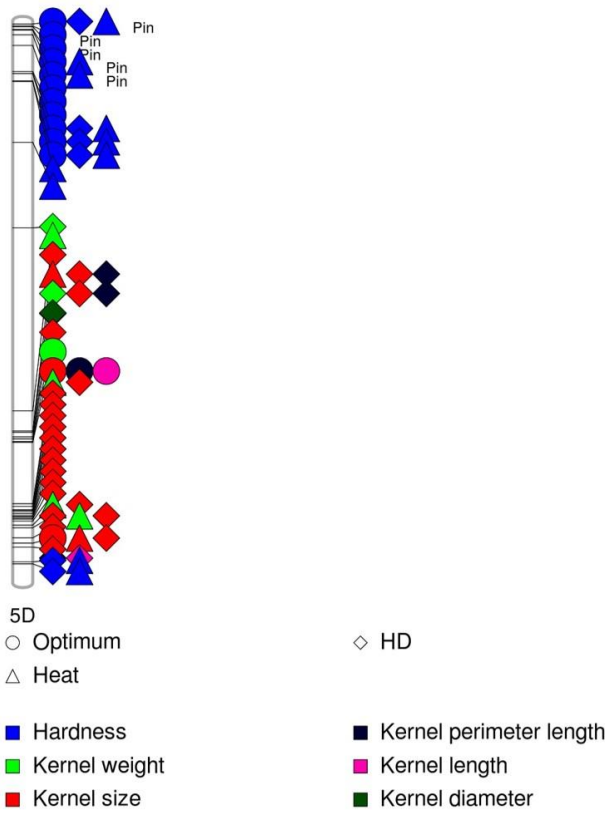


Figure 3.8. Relationship between the all markers on chromosome 5D associated with hardness and kernel weight and shape related traits.

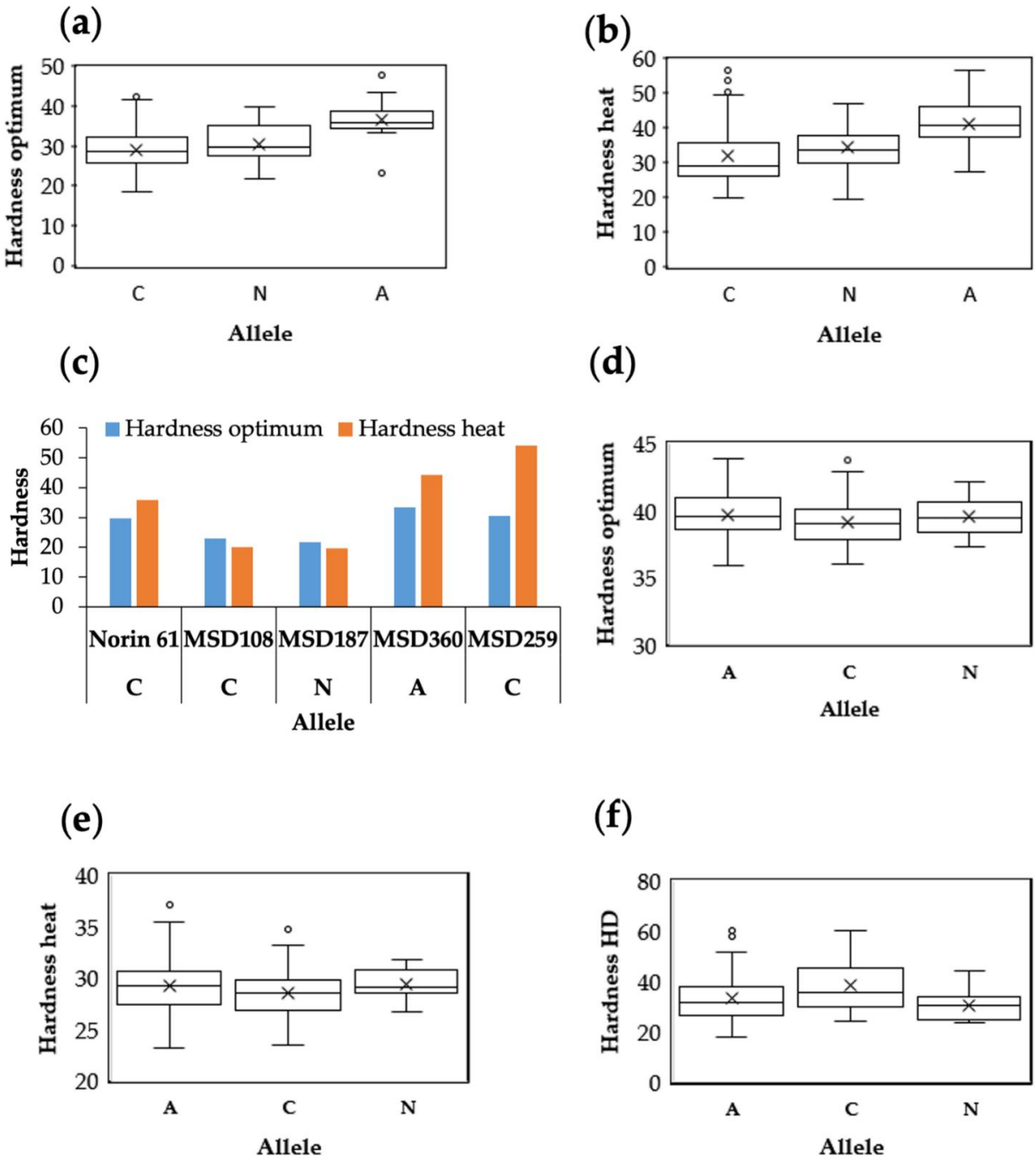


Figure 3.9. Boxplot for the marker 3532985; the marker that encodes for *SUT* gene. (**a**) Allele contributes for the marker under optimum environment, (**b**) Allele contributes for the marker under heat environment, (**c**) Allele contribution for MSD108, MSD187;the tolerant lines, and MSD162, MSD259; the sensitive lines, and ‘‘Norin 61’’ their parent, (**d-f**) Boxplot for the allele for marker 3947128 that encode for celiac disease under

Table 3.5 Candidate genes for hardness under optimum, heat, and combined heat-drought (HD) and hardness indexes (*HI*, *HDI*).

Marker	Chromosome	Trait (Environment)	PVE %	Gene	Protein	Function
3947128	1A	HD	8.5	*TraesCS1A02G008000*	Celiac disease	Glutenine sensitivity
1099828	3B	HDI	12.0	*TraesCS3B02G533100*	No apical meristem (NAM) protein domain containing protein	Increase protein content
998809|F|0-7	4D	Heat, HD, HI, HDI	14.4	*TraesCS4D02G117900*	ADP,ATP carrier protein	Nitrogen use efficiency
1062681|F|0-26	4D	Heat, HD, HI	17.3	*TraesCS4D02G047400*	Glutamine synthetase	Nitrogen use efficiency
1127970	5D	Optimum, Heat, HD	22.6	*TraesCS5D02G004100*	Puroindoline-a	Role for hardness
3532985	5D	Optimum, Heat	16.1	*TraesCS5D02G001200*	Sucrose transporter	Starch accumulation
3944483	5D	Optimum, Heat, HD	16.3	*TraesCS5D02G037000*	NB-ARC domain containing protein	Biotic stress
1025407	5D	Heat, HD	12.6	*TraesCS5D02G036300*	Zinc finger, RING-type domain containing protein	A-biotic stress
1073587|F|0-24	6D	Heat	11.2	*TraesCS6D02G105100*	NB-ARC domain containing protein	Biotic stress
5357783	6D	HD	12.8	*TraesCS6D02G105100*	NB-ARC domain containing protein	Biotic stress

Marker 3947128 on chromosome 1A was associated with hardness under HD and encodes for antigen that causes celiac disease in human, which is an autoimmune disease that triggered by wheat gluten. We investigated the allele's contributions to celiac disease (Figure 3.9d–f) and found that under optimum condition, the allele's contribution was almost the same (Figure 3.9d).

However, under HD, allele "C" contributed to increase hardness. In contrast, the allele "A" contributed to decrease hardness. Markers 3944483, 1073587|F|0-24, and 5357783 were common and stable markers under all environments and encodes for NB-ARC protein. Marker 998809|F|0-7 on chromosome 4D encodes an ATP/ADP transporter. Moreover, we found that a marker associated with a candidate no-apical-meristem (*NAM*) gene was associated with marker 1099828 on chromosome 3B.

We detected the expression for the candidate genes using the expression data from expVIP databases (Borrill, Ramirez-Gonzalez, and Uauy 2016), and compared their expression to puroindoline genes (Pina and Pinb) expression. The expression of puroindoline was high on seed part such as endo-sperm, starchy endosperm, seed coat and aleurone (Figure 3.10). The candidate gene *TraesCS5D02G001200* on the chromosome 5D showed expression on seed parts besides shoot, leaves and root. The candidate gene *TraesCS4D02G047400* on the chromosome 4D showed expression on seed part and spike (Figure 3.10). The candidate gene *TraesCS1A02G008000* on the chromosome 1A that encodes for the antigen that cause celiac disease in human showed a high expression level same as puroindoline genes (Figure 3.10).

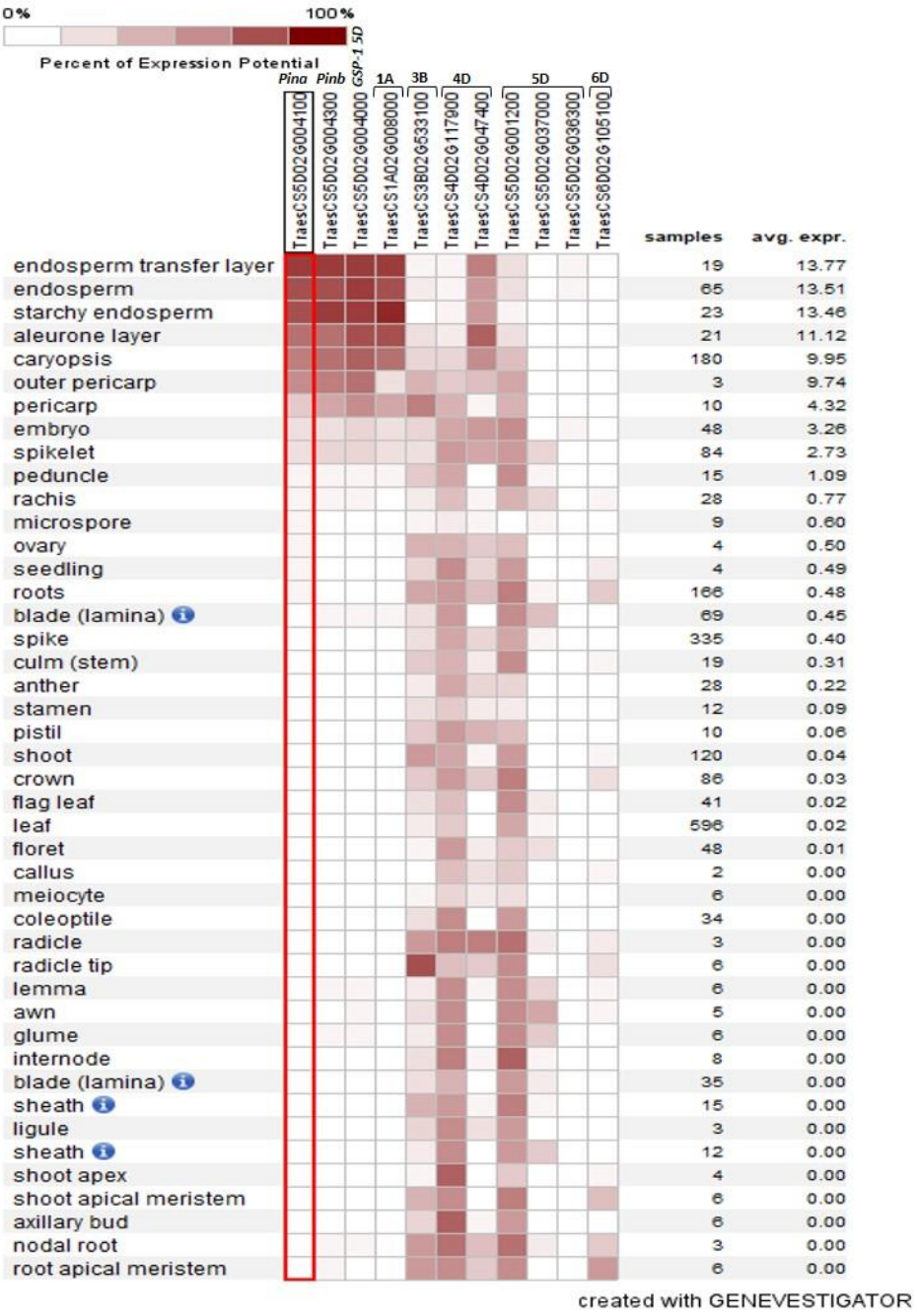


Figure 3.10. Expression of the candidate genes and *Pina*, *Pinb*, *GSP-1* in different tissues of wheat based on RNA-sequencing data collected from different experiments.

3.4. Discussion

3.4.1 Phenotypic variation and diversity for hardness

Hardness is an important milling quality trait that is known to be controlled by two puroindoline genes (*Pina* and *Pinb*) on the short arm of chromosome 5DS (Giroux and Morris 1998). In the current study, we detected the effect of heat and HD environments on hardness in the MSD population. We aimed to identify MTAs, and possible candidate genes that contributed to the stability or modification of kernel hardness under heat and combined heat-drought, and to investigate whether the stabilization of kernel hardness under stress was associated with stress tolerance genes.

The wide variation observed among the MSD genotypes indicates high diversity in this population, which includes genes derived from 43 Aegilops tauschii accessions (Gorafi et al., 2018). However, most of the genotypes had soft kernels and resembled the backcross parent, 'Norin 61'. Gedye et al. (2004) characterized several synthetic wheat lines and found that most of the population had soft kernels.

MSD population shifted to become harder under heat and HD conditions. This could be attributed to the increased protein content under stress condition (Gedye et al. 2004). In addition, stress affected the plants, causing smaller kernels; this will lead to reduced kernel weight and overall yield (Ozturk and Aydin 2004). This is due to the difference of the conversion ratio of carbohydrate and protein, i.e., the conversion ratio of carbohydrate is highly affected by the stress but not much in protein, resulting in smaller grains with a higher protein content. These seeds become harder than plump seeds with much starch (Ozturk and Aydin 2004).

Moreover, under heat and drought stress, the short kernel-filling duration results in less starch accumulation in wheat (Ramya et al. 2017). This, in turn, could also lead to increased hardness, since starch content is negatively associated with kernel hardness (Muqaddasi et al. 2020).

Climate change is adversely affecting wheat production around the world, leading to yield losses and decreased quality (IPCC 2014). Therefore, identifying varieties that have both high yield and high quality is critical for food security in the context of global climate change. These varieties will also be crucial for breeding programs. The resulting varieties will increase

satisfaction for all stakeholders in the wheat value chain (Fleitas et al. 2020). We observed a relationship between stress tolerance and stable kernel hardness or being softer under heat and HD environments. This could be attributed due to the starch accumulation that occurs under stress condition. Prathap and Tyagi (2020), found that stress tolerant rice lines can accumulate starch under drought stress due to the action of starch synthase enzymes and that this could lead to softer kernels under stress.

We confirmed our results by measuring the kernel protein content and examining the endosperm's internal structure using an SEM. One important difference between soft and hard kernels was the degree of adhesion between starch granules and the protein matrix; starch granules in soft kernels were separated from the protein matrix (Simmonds et al. 1973). Hard kernels instead had tight adhesion between starch granules and the protein matrix, and the starch granules were embedded in the protein matrix (Okada et al. 2018). The stress tolerant line MSD187 showed separated starch granules under heat and HD conditions. In contrast, the sensitive line MSD259 showed embedded starch granules with no gaps under stress condition. This indicates that the sensitive line's kernels became harder under stress condition.

Under HD condition, the protein content increased in the sensitive line, whereas the protein content for the tolerant line decreased. This result suggests that a smaller change in hardness is associated either with less change in the protein content or with decreasing protein content. Protein content is known to increase under stress condition. Peterson et al. (1992) reported that one of the factors associated with increasing kernel hardness was an increase of the protein content. All of these findings suggest that the stress-tolerant MSD lines could maintain their kernel hardness under stress conditions. These lines therefore represent an excellent resource for breeding stress-tolerant wheat genotypes with high yield and a range of end-use qualities.

3.4.2 Marker traits association for hardness under optimum and stress conditions

Our results indicated that the D genome contributed strongly to kernel hardness under all conditions, with a diverse range of D-genome markers associated with hardness. Among the MTAs in the D genome, we detected a significant association for marker 1127970 on chromosome 5D, whose associated gene encodes a puroindoline protein. This marker was stable across all conditions. We observed high expression level for puroindoline on seed parts such as endosperm,

starchy endosperm and aleurone, whereas it showed low expression levels in shoots and roots. Similar results were reported by Kiseleva et al. (2020).

In addition to the puroindoline gene, other markers associated with hardness were found on chromosome 5D under optimum as well as stress environments. Among these markers, marker 3532985 far with (1.52) Mb from *Pinb* contributed to hardness with 16.1 and 12.1% PVE under optimum and heat environments, respectively. The candidate gene *TraesCS5D02G001200* for this marker encodes the sucrose transporter *TaSUT*. Starch formation in wheat kernels requires importation of sugar in the form of sucrose via a sucrose transporter (Deol et al. 2013). *TaSUT* has five homologs, including *TaSUT2*-5D, which is located on chromosome 5D (Mukherjee et al. 2015). A recent study by Al-Sheikh Ahmed et al. (2018) showed that TaSUS1 is associated with increased kernel weight under drought stress. This may explain the association between kernel weight and hardness. However, further investigation of the alleles in the stress tolerant and sensitive lines indicate that both had alleles that decreased the hardness. This finding demonstrates that the activity of the sucrose transporter is not sufficient on its own to stabilize kernel hardness under stress. We found high expression of this gene in all wheat tissues, and this is logical since those genes are associated with sink–source relationships and starch formation.

We detected a marker 3944483 on chromosome 5D that was stable under all conditions. This marker is associated with the candidate gene *TraesCS5D02G037000*, which encodes an NB-ARC protein. The NB-ARC is a stress-resistance protein that contains a central nucleotide binding domain (Van Ooijen et al. 2008). It works as a dynamic signaling molecules per-forming reversible interaction which confer resistance to a wide variety of microbes (Belkhadir et al. 2004). Previously, Giroux et al. (2003) reviewed the role of puroindoline genes in the plant defense. These results indicated that there are factors controlling hardness associated with plant defense against pathogens. Beside this marker we also found other markers; 1073587|F|0-24, and 5357783 under both heat and HD environments have the same candicey gene *TraesCS6D02G105100*, which encodes for NB-ARC protein, indicating that this gene also affects hardness under stress condition. However, we detected a low expression level for this gene in all tissues.

We detected a significant association for both kernel weight and hardness on the long arm of chromosome 5D under a stress environment. Previously, we detected significant peak for kernel size under HD environment on the long arm of chromosome 5D (Elhadi et al. 2021). We also

found MTAs that appeared under stress and that were associated with kernel shape traits on the long arm on chromosome 5D (Elhadi et al. 2021). These results indicated the occurrence of alleles that contribute to hardness, kernel weight, and kernel shape traits on chromosome 5D under stress environment and this could explain the relationship between hardness and shape traits under these conditions. However, we couldn't identify common markers between hardness and kernel weight despite of the phenotypic correlation between them and also with the other shape traits. This may be due to the complexity of the MSD accessions, which have huge diversity resulting from the diverse D genome sources. In addition, the candidate gene *TraesCS5D02G036300* associated with marker 1025407 encodes a zinc finger RING-type domain containing protein. A recent study in wheat indicated that overexpression of this gene increases tolerance to drought and salinity (Agarwal et al. 2020).

On chromosome 1A, the marker 3947128 associated with the candidate gene *TraesCS1A02G008000* encodes a protein associated with triggering symptoms of celiac disease in humans. Celiac disease is a multisystem immune-based disease triggered by the ingestion of gluten in genetically susceptible individuals. We noticed a high expression level for this marker in the kernel, endosperm, and aleurone; same as puroindoline expression level. Ribeiro et al. (2017) found a negative but non-significant correlation between hardness and amount of toxic epitopes potentially associated with celiac disease.

We also identified MTAs that were only associated with the hardness indexes HI and HDI, which indicate an association with the change of hardness in response to stress condition. We found that all of the A, B, and D genomes contributed to the hardness change under stress environments. Among them, we found that marker 1099828 on chromosome 3B contributed to hardness, with PVE of 12 and 10% for HI and HDI, respectively. The candidate gene TraesCS3B02G533100 encodes an *NAM* gene. Uauy et al. (2006) reported that NAM-B1 underlies a high-protein-content locus (*GPC-B1*) that originated from wild emmer (durum) wheat, Triticum turgidum. This gene accelerates senescence, leading to increased protein content and nutrient content in the kernel (through a sink–source relationship). This gene shows expression on the seed coat, aleurone, and spike.

Puroindoline genes are major genes that control kernel hardness under optimum condition. However, it remains unclear whether puroindoline genes also control kernel hardness under stress

condition or whether other genetic factors are responsible. Interestingly, we identified a significant peak on chromosome 4D under heat and HD stress environments as well as hardness indexes *HI* and *HDI*. This indicates the occurrence of genetic factors on chromosome 4D that contribute to hardness maintenance under stress environment. We also identified significant peaks on chromosomes 6D and 7D under stress, indicating that the associated loci contribute to kernel hardness under stress condition. In contrast with Wilkinson et al. (2008), who found a puroindoline-like gene sequence on the long arm of a chromosome homoeologous to chromosomes 7A, 7B, and 7D, we did not identify such a gene sequence on chromosome 7D, indicating that the occurrence of other genetic factors on chromosome 7D contribute to kernel hardness.

We detected several markers on chromosome 4D. Marker 998809|F|0-7 was associated with stress condition and had a PVE of 14.4 and 11.1% under heat and HD environments, respectively. This marker encodes an ATP/ADP transporter gene. Wang et al. (2017) explained that ATP/ADP transporter genes increase the starch content in transgenic Arabidopsis. This could explain why the kernels of some genotypes became softer under stress condition.

Additionally, we found marker 1062681|F|0-26 contributes for hardness and HI as well as heat and HD environments, with PVE 20.5, 17.3 and 17.4%, respectively. The associated candidate gene *TraesCS4D02G047400* encodes glutamine synthetase. This enzyme plays an important role in nitrogen-use efficiency and in the uptake and assimilation of nitrogen (Perez et al. 2016). These findings indicate that these MTAs are associated with nitrogen-use efficiency genes under stress environment, which is an important stay-green stress-tolerance mechanism. The stay-green trait results from a balance between N demand by the kernels and the N supply during kernel filling (Borrell et al. 2001). This suggests that hard-ness resulted from a stay-green mechanism associated with glutamine synthetase. Pinto et al. (2010) showed an association of the stay-green trait in wheat with stress tolerance and identified markers on chromosomes homoeologous to 4A and 4B. Here we found the association on chromosome 4D, demonstrating that the stay-green have an effect on hardness. We observed the same expression as puroindoline in the seed in addition to the spike. Muhitch (2003) found glutamine synthetase in the developing kernel of maize plants, and it was abundant in the pedicel, pericarp, and kernel glumes.

We found marker 1001438|F|0-46 associated with heat conditions, and its contribution to hardness was high (PVE of 18.9%). The associated gene was close to the *Rht-D1* region (2.4 Mb),

indicating that dwarfing genes could be one of the factors that affects kernel hardness under heat conditions. Wang et al. (2012) studied a recombinant inbred line population derived from crosses between accessions with soft and extra soft kernels, and found a QTL on chromosome 4D that was close to the semi-dwarf gene *Rht-D1*.

Based on what is known about the above-mentioned candidate genes, we hypothesize that the expression of those genes would affect kernel hardness. For instance, the candidate sucrose transporter gene, which plays a role in transporting sucrose, promotes starch accumulation in the kernels, and the candidate ATP/ADP transporter gene also increases starch content, which leads to decreased hardness under stress. The candidate *NAM* gene, which plays a role in source–sink nutrient and protein contents, leads to increased protein content, which increases kernel hardness. Moreover, the candidate glutamate synthase gene plays a role in nitrogen-use efficiency, increasing the uptake and assimilation of nitrogen resulting in high protein content, which in turn produces harder kernels.

We hypothesize that the stress tolerant plants have a mechanism that utilizes the above mentioned genes efficiently, thereby maintaining the ratio between the starch and protein contents and leading to stabilization of kernel hardness and yield. However, further study will be needed to validate this hypothesis.

3.5. Conclusions

Heat and combined heat-drought (HD) environments increase hardness. We observed that the MSD line (MSD187) with tolerance potential to heat and HD environments had more stable hardness than the sensitive line (MSD259). We identified MTAs on chromosome 4D that associate with hardness under stress environment and hardness indexes (*HI* and *HDI*). This indicates that occurrence of MTAs contributes to the hardness changes under stress conditions. Along with dissecting candidate genes that can contributes for hardness change specially under stress environment, our findings could play an important role in understanding the factors that control the changes of the hardness under a stress environments and their relationships to the stress tolerance. This will help breeders to develop stress-tolerant cultivars that maintain high yield and stable hardness, capable of resisting the adverse effects of global warming, thereby improving food security.

CHAPTER FOUR
GENERAL DISCUSION

Wheat (*Triticum aestivum*), is one of the most important food crops that contributed significantly for human civilization (Braun et al. 2010). Although, wheat production at global level has increased significantly over the years, there is still a big gap between the demand and annual wheat production. The average annual production of wheat has been reported to be 1% while the demand for wheat increases by 1.7% annually reaching a total of 1 billion tons in 2050 (Rosegrant and Agcaoili 2010). Therefore, improving wheat yield have been an important issue for breeders. Beside improvement of yield, improving wheat quality is an important issue to satisfy consumer's desire. To fulfil this, introducing superior varieties that maintain both yield and quality is critical for human nutrition, end-use functional properties and commodity value, and could be an alternative way to face hunger issue (Nuttall et al. 2017).

Grain yield is a complex trait determined by two components; number of grains per m^2 and grain weight. Therefore, for further improvement of yield potential, grain weight is an important component to target (Quarrie et al. 2006). Grain weight in turn is determined by grain length, width, and area, which are inherited in a stable manner and show higher heritability than overall yield (Kuchel et al. 2007). In addition, kernel hardness is a key determinant for classification of wheat and end product quality (Campbell et al. 1999). Grain hardness is important for the flour industry because it has significant impacts on milling, baking and qualities of wheat (Bettge and Morris 2000).

However, one of the important factors that affect kernel weight and shape related traits and kernel hardness, in other words affects both yield and quality, was environmental factors and climate change. Demand for the food and hunger issue will become much worse, if increases in global wheat yields and grain production cannot be sustained due to the predicted increases in climate-related extremes, such as heat waves and drought (Dhankher and Foyer, 2018). Recently, a global-scale model estimated that with a 1°C increase in the mean global temperature,

there is a high probability that global wheat yields will be reduced by 4.1–6.4% by the middle of the 21st century, while at the same time the demand for wheat is expected to increase by 60% (Liu et al. 2016).

In addition, Several studies demonstrated that high temperature and drought stress accelerate kernel filling. This results in compressed key events during wheat kernel development, like increasing the storage proteins and starch synthesis in endosperm under stress conditions, which altogether affects kernel hardness (Ashraf 2014).

Therefor, in the current work, we aimed to investigate the effect of stress upon both yield and quality throught studying the effect of heat and combined heat drought stress upon kernel weight and shape related traits beside hardness.

MSD lines showed a large phenotypic variation for all kernel hardness and shape traits under optimum, heat and HD conditions. The response of MSD to stress condition was varied for hardness and kernel shape traits, in which most of the population shifted to become harder under stress condition. Meanwhile other genotypes shifted toward soft under stress condition. In addition, kernel weight, kernel diameter and area size were the most affected traits. The effect of heat and HD upon hardness and kernel shape traits attributed shortening the grain growth period and leading to improper grain filling. Thereby, reducing the weight and the overall yield (Ramya et al. 2015). Moreover, high temperature reduces the conversion of sucrose to starch due to the suppression of the enzyme soluble starch synthase leading to shriveled kernels and at the same time harder grain (Jenner 1994). However, kernel length was less affected by the stress compared to weight and other related traits. This reflecting the reduction of the accumulating starch is resulting in a decrease on the weight and size rather than length.

Under all conditions, kernel size and kernel diameter was correlated to kernel weight. However, kernel diameter the most correlated to kernel weight under all conditions. Meanwhile, the correlation between the hardness and the other kernel-shape traits showed that kernel weight was significantly negatively correlated with hardness under optimum and stress conditions. However, this correlation became stronger under stress environments. This high correlation between kernel weight and hardness and between kernel weight and diameter under all conditions, propose hardness and kernel diameter as a target traits of selection in breeding programs aiming to increase kernel weight and yield in wheat.

Climate change is adversely affecting wheat production around the world, leading to yield losses and decreased quality (IPCC 2014). Therefore, identifying varieties that have both high yield and high quality is critical for food security in the context of global climate change. These varieties will also be crucial for breeding programs. Based on this point, in the current study we aimed at identifying MSD lines that maintain both high yield and quality. We calculated HSI and HDSI based on the most correlated traits kernel weight, kernel diameter, and area size. We observed that MSD187 showed the best performance under all conditions, whereas MSD259 was highly affected by both H and HD stresses compared to their parents 'Norin 61'.

Meanwhile, since kernel weight is highly correlated with hardness under all conditions. And, since the kernel weight susceptibility indexes, can assess the reduction under stress compared with optimum conditions. Thus, it can be used as tolerance indicators. Based on this point, we aimed to investigate the relationship between hardness and kernel weight for heat and heat-drought susceptibility index (HSI and HDSI) for the MSD lines. We observed a relationship between stress tolerance (a low kernel weight reduction) and hardness stabilization under stress condition. We observed that the MSD187, had low change in hardness under both heat and HD, whereas, the sensitive line MSD259 showed a large reduction in hardness under both heat and HD. The cultivars 'Norin 61', which is the parent of the MSD lines, and 'Imam', had relatively high reductions in kernel weight and moderate changes in hardness under heat conditions compared with MSD187.

To confirm these results, we further analyzed these lines for their internal structure using SEM and protein content. MSD187 showed separated starch granules under heat and HD conditions. In contrast, the sensitive line MSD259 showed embedded starch granules with no gaps under stress condition. This indicates that the sensitive line's kernels became harder under stress condition because starch granules in soft kernels were separated from the protein matrix, whereas, hard types have tight adhesion between starch granules and protein matrix (Okada et al. 2018). Under HD condition, the protein content increased in the sensitive line, whereas the protein content for the tolerant line decreased. This result suggests that a smaller change in hardness is associated either with less change in the protein content or with decreasing protein content. All of these findings suggest that the stress-tolerant MSD lines could maintain their kernel hardness under stress conditions. These lines therefore represent an excellent resource for breeding stress-tolerant wheat genotypes with high yield and a range of end-use qualities.

Our results indicated that the D genome contributed strongly to kernel hardness and kernel shape traits under all conditions. This finding indicated that, D genome have higher contribution for hardness and kernel related traits, especially under stress conditions. I observed that chromosome 5D has significant association for kernel hardness and kernel weight and shape traits. This association was appeared on the long arm of chromosome 5D under stress environments. These results indicated the occurrence of alleles that contribute to hardness, kernel weight, and kernel shape traits on chromosome 5D under stress environment, and this could explain the relationship between hardness and shape traits under these conditions. However, we couldn't identify common markers between hardness and kernel weight despite of the phenotypic correlation between them and also with the other shape traits. This may be due to the complexity of the MSD accessions, which have huge diversity resulting from the diverse D genome sources.

Among D genome markers, we identified markers stable under all conditions Marker 1073897|F|0-27. Interestingly, the candidate gene for this marker, *TraesCS5D02G504400*, encodes a RING-type *E3 ubiquitin*–protein ligase. The ubiquitin pathway plays a crucial role in determining plant seed size (Bednarek *et al*. 2012). The wild allele T contributed to increase the kernel area size under OP, H and HD conditions and was originated from the *Ae. tauschii*. The line MSD187 harboring this allele had higher kernel area size than their parent 'Norin 61' harboring the (C) allele under all conditions.

In addition, the candidate gene sucrose transporter, which have role in transporting sucrose results on abundant amount of starch in kernel, which leads to change hardness to softer under stress environment. The candidate gene no epical meristem (*NAM*), which plays a role in source-sink nutrient and protein content, leads to increased protein content, hence to a change in kernel texture to harder. Moreover, the candidate genes ADP/ATP transporter and glutamate synthase play a role in nitrogen use efficiency, increasing macro and micro nutrients ratio and resulting in soft texture or low nutrient and high protein content and in turn hard texture. We speculated that the tolerant plant has a mechanism that utilizes those genes efficiently, which results in the maintaining ratio between the starch and protein content, leading to stabilization on hardness and productivity. However, further study is needed to validate our hypothesis.

CHAPTER FIVE
SUMMARY OF THE STUDY IN ENGLISH

Kernel hardness of wheat is one of the most important characteristics for milling and baking quality. It is defined as the force needed to crush the kernels. Wheat grain has three major components; those are starch, protein and lipid. Interactions between these three components determine the quality composition of the wheat grain and its suitability. Wheat endosperm texture ranges from very soft to hard. Soft wheat kernels are easy to be fractured, which results in production of large number of intact starch granules, whereas flour that produced by hard wheat, having broken granules and higher levels of starch damage. Hard wheat is more suitable for bread while it is good to use flour of soft wheat for cookies, cakes and pastries due to less protein and starch damage. The flour of bread wheat is used to make bread, chapatti, biscuits, and pastry products.

Kernel hardness is genetically controlled by two puroindoline genes, *Pina* and *Pinb*, which are 60% identical, which are located at the distal end of the short arm of chromosome 5D. Kernel hardness has several related traits, among them kernel weight and shape traits. Kernel weight is considered to be an important approach for further improving yield potential. Also it considered as the most heritable trait among yield components. In addition, milling yield could be increased by optimizing kernel weight and size. Kernel weight is closely associated with kernel size traits, such as kernel length, kernel width, and kernel diameter. Therefore, improving kernel weight and size is a prime breeding target for wheat yield potential and end use quality.

Beside puroindoline genes which was considered major determinants of wheat hardness, many studies have demonstrated the complex nature of this trait and suggest that hardness is affected by several factors. These factors include abiotic stresses such as heat and drought. Several studies have demonstrated that stress from high temperatures and drought can accelerate kernel filling. These stresses compress the timing of key events during wheat kernel development, such as increasing the production of storage proteins and starch synthesis in the endosperm under stress, which together affect kernel hardness beside kernel weight and shape related traits, leading to yield losses and decreased quality. Therefore, identifying varieties that have both high yield and high

quality is critical for food security in the context of global climate change. These varieties will also be crucial for breeding programs.

Several studies described kernel hardness and shape related traits under normal conditions, but extensive studies under stress conditions have not conducted. In addition, the genetic factors that affected the change in hardness remained unclear. Hence, better understanding of the change or stability in hardness under stress environment is essential. Thus, to improve wheat genotypes that maintain high yield and quality even under stress condition, knowledge of genotypic and environment interaction is necessary. Therefore, the current study aimed at investigating the effect of heat and combined heat-drought upon hardness and shape related traits and to explore the genetic loci for kernel hardness and shape related traits. I evaluated hardness, weight and shape-related traits and applied genome-wide association analysis to a panel of wheat multiple synthetic derivative (MSD) lines harboring genomic fragments from *Aegilops tauschii*, grown under optimum conditions in Japan and under heat and combined heat-drought conditions in Sudan. My results revealed promising markers and alleles that will contribute to enhance and maintain high yield and quality under stressed condition and they could be used in wheat breeding after validation.

Chapter one outlines the objectives of this study, providing the hardness and shape related traits overview, importance, and measurements, along with providing the literature review for the relative studies. Also, it elucidates the impact of abiotic stress upon yield and quality beside its impact on hardness and shape related traits.

In chapter two, we studied the effect of heat and combined-heat drought upon kernel weight and shape related traits along with performing genome wide association to a panel of 160 MSD lines. We aimed to explore the genetic loci for weight and shape related traits acts under stress conditions that can be useful for enhancing yield under stress conditions. We identified tolerant line MSD187 that has a good performance under optimum as well as stress condition. We identified stable marker under all condition on chromosome 5D which associated with a candidate gene encoding a RING-type *E3 ubiquitin*-protein ligase and originated from *Aegilops tauschii*. This marker contributes to increase the kernel size under stress condition and hence yield. The tolerant line MSD187 harbor the positive allele for this marker, which contributes to increase kernel size.

In chapter three, we investigated the impact of heat and combined heat-drought upon kernel hardness. We aimed to identify the genetic loci that contributes for hardness under optimum condition in Japan, and heat and combined heat-drought conditions in Sudan, and to investigate the association between hardness stabilization and stress tolerance. We observed that less reduction of kernel weight is associated with either low change or stable kernel hardness. Also, we found a significant association with hardness under stress on chromosome 4D, along with dissecting several candidate genes associated with the change of hardness under stress.

Chapter four, outlines the general discussion for the study along with providing and elucidating the general understanding for the two studies.

The current work, aimed at investigating the effect of stress upon both yield and quality throught studying the effect of heat and combined heat drought stress upon kernel weight and shape related traits beside hardness. The MSD tolerant lines that identified in this study, which performed good under heat and heat drought stress conditions. These lines, along with the stable markers, favorable alleles and candidate genes elucidated here, represent a good resource with which to enhance wheat grain yield under stress and optimum conditions after validation. Among the tolerant lines, (MSD187) which have good performance under all conditions. In addition, the (MSD187) with tolerance potential to heat and heat drought conditions had more stable hardness than the sensitive line (MSD259). Moreover, the significant peak on chromosome 4D, that observed for hardness under stress conditions. This indicates that occurrence of MTAs contributes to the hardness changes under stress conditions. In addition, the MTAs and candidate genes that obtained for hardness could play an important role in understanding the factors that control the changes of the hardness under a stress conditions and their relationships to the stress tolerance. This will help breeders to develop stress-tolerant cultivars that maintain high yield and stable hardness, capable of resisting the adverse effects of global warming, thereby improving food security.

SUMARY OF THE STUDY IN JAPANESE

コムギの穀粒の硬度は、製粉や製パンの品質に最も重要な特性の一つである。硬度は穀粒を押しつぶすのに必要な力として定義される。コムギの胚乳の品質には非常に柔らかいものから硬いものまで様々である。軟質コムギの穀粒は破砕されやすく、その結果、多数の無傷のデンプン顆粒が生成されるが、硬質コムギから生成される小麦粉は、顆粒が破壊され、デンプンの損傷レベルが高い。硬質コムギはパンに適するが、軟質小麦の小麦粉はタンパク質やデンプンの損傷が少ないため、クッキーやケーキ、ペストリーへの使用に適している。

穀粒の硬さは、5D 染色体短腕末端に位置する 2 つのピューロインドリン遺伝子、*Pina* および *Pinb* によって遺伝的に制御されている。穀粒硬度には、いくつかの関連する形質があり、その中には穀粒の重量と形質がある。穀粒重量は、収量を向上させるために重要な形質と考えられている。また、穀粒重量は収量成分の中で最も遺伝率の高い形質であると考えられている。さらに、穀粒重量と形状を最適化することで、製粉歩留を向上させることができる。穀粒重量は、穀粒長、穀粒幅、穀粒径などの穀粒の大きさに関する形質と密接に関連している。したがって、穀粒重量と大きさを改善することは、コムギの収量性と最終用途品質の向上のための重要な育種目標である。

コムギの硬度の主要な決定因子と考えられているピューロインドリン遺伝子以外にも、多くの研究がこの形質の複雑な性質を明らかにし、硬度がいくつかの要因によって影響を受けることを示している。これらの要因には、高温や干ばつなどの非生物ストレスが含まれる。いくつかの研究では、高温や干ばつによるストレスが、穀粒の登熟を早め、ストレス下での胚乳での貯蔵タンパク質の生成やデンプン合成の増加など、コムギの穀粒形成中において重要な事象が起こるタイミングが早まり、その結果、穀粒重量や形状関連形質の他、穀粒硬度にも影響を与えて、収量の低下や品質の低下につながっていることを示している。したがって、世界的な気候変動の中での食糧安全保障を実現するためには、高収量と高品質の両方を兼ね備えた品種を開発することが重要である。また、これらの品種は、育種プログラムにとっても重要である。

いくつかの研究では、通常条件下での穀粒硬度と形状に関連する特性について説明しているが、ストレス条件下での広範な研究は実施されていない。さらに、硬度の変化に影響を与える遺伝的要因は不明のままである。したがって、ストレス環境下での硬度の変化または安定性をよりよく理解することが不可欠である。ストレス条件下でも高い収量と品質を維持するコムギの遺伝子型を改良するには、遺伝子型と環境の相互作用の知見が必要である。したがって、今回の研究は、高温と高温・乾燥複合条件が、硬度と形状に関連する形質に及ぼす影響を調査し、これらに関連する遺伝子座を調査することを目的としている。私は、硬度、重量、形状に関連する特性を評価し、日本での最適条件下および、スーダンの高温および高温・乾燥の複合条件下で栽培された、タルホコムギからのゲノム断片を含むコムギの多重合成コムギ派生系統 (MSD)のパネルにゲノムワ

イド関連解析を適用した。私の結果は、ストレス条件下で高い収量と品質向上に維持する有望なマーカーと対立遺伝子を明らかにし、検証後にコムギの育種に使用できるようになると思われる。

第 1 章では、本研究の目的を概説し、硬度と形状関連形質の概要、重要性、測定方法を示し、関連研究の文献レビューを行った。また、硬度と形状関連形質への影響に加えて、収量と品質に対する非生物的ストレスの影響を明らかにした。

第 2 章では、MSD の 160 系統からなるパネルを用いてゲノムワイド関連分析を行い、高温および高温・乾燥複合ストレスが穀粒重量および形状関連形質に及ぼす影響を調査した。その結果、ストレス条件下での収量増加に有効な、穀粒重量および形状関連形質に作用する遺伝子座を探索することができた。MSD187 系統は、最適条件とストレス条件の両方で良好な成績を示す系統であることが明らかになった。このマーカーは、タルホコムギに由来する RING 型 E3 ユビキチン-プロテインリガーゼをコードする候補遺伝子に関連するものであり、ストレス条件下での穀粒の大きさの増加、さらには収量の増加に寄与する。耐性系統である MSD187 はこのマーカーの対立遺伝子をもっていた。

第 3 章では、高温と高温乾燥複合ストレスが穀粒硬度に与える影響を調査した。日本の最適条件、スーダンの高温および高温・乾燥複合条件で、硬度に関与する遺伝子座を特定し、硬度の安定化とストレス耐性との関連性を調べることを目的とした。その結果、穀粒重量が減少が少ないほど、穀粒硬度の変化が少なく安定していることが確認された。また、4D 染色体にストレス下での硬さとの有意な関連を見出し、さらにストレス下での硬さの変化に関連するいくつかの候補遺伝子を解析した。

第 4 章では、研究に関する総合的な考察を行い、2 つの研究結果を総括した。

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LIST OF PUBLICATIONS

Elhadi, G. M. I., Kamal, N. M., Gorafi, Y. S. A., Yamasaki, Y., Takata, K., Tahir, I. S. A., Itam, M. O., Tanaka, H. and Tsujimoto, H. 2021 Exploitation of tolerance of wheat kernel weight and shape-related traits from *Aegilops tauschii* under heat and combined heat-drought stresses. International Journal of Molecular Sciences 22: 1830. doi.org/10.3390/ijms22041830 (Chapter Two).

Elhadi, G. M. I., Kamal, N. M., Gorafi, Y. S. A., Yamasaki, Y., Ban, Y., Kato, K., Tahir, I. S. A., Ishii, T., Tanaka, H. and Tsujimoto, H. 2021 Novel loci for kernel hardness appeared as a response to heat and combined heat-drought conditions in wheat harboring *Aegilops tauschii* diversity. Agronomy 11: 1061. doi.org/10.3390/agronomy11061061 (Chapter Three).