Utilization of Alien Genetic Diversity for Improving Drought Tolerance in Bread Wheat

By

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Islamabad, Pakistan

(2019)

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Dedication

I dedicate all my research work to my father **Mr. Abdul Rashid Khalid** and my mother **Mrs. Nasreen Akhtar** for the understanding and encouragement they provided during this course of study. They have supported me all the way since the beginning of my studies. They have never failed to give me financial and moral support, providing for all of my needs during this time and for teaching me that even the biggest task can be accomplished if it is done one step at a time.

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List of Abbreviations

Abscisic acid	ABA
Analysis of variance	ANOVA
Amplified fragment length polymorphism	AFLPs
Biomass	BM
Broad sense heritability	H
Bacterial artificial chromosome	BAC
Barley yellow dwarf virus	BYDV
Cleaved amplified polymorphic marker	CAPS
Cytokinin oxidase/dehydrogenase	OsCKX
Cell wall invertase	*CWI*
Complementary DNA	cDNA
Cytoplasmic invertase	CYT-INV
Cetyl trimethyl ammonium bromide	CTAB
Composite interval mapping	CIM
Days from emergence to maturity	DEM
Dry mass	DM
Dry root weight	DRW
Dry shoot weight	DSW
Double haploid	DH
Diversity array technology	DArT
Days to heading	DH
Days to maturity	DM
Ear length	EL
Expressed sequence tags	ESTs
Fresh leaf mass	FM
Functional markers	FMs
Fusarium root rot	FRR
Fresh root weight	FRW
Fresh root weight under well-watered condition	FRW_{WW}
Fresh root weight under water-limited condition	FRW_{WL}
Fresh shoot weight	FSW
Fresh shoot weight under well-water condition	FSW_{WW}
Fresh shoot weight under water-limited condition	FSW_{WL}
Genomic estimated breeding values	GEBVs
Genotype by sequencing	GBS
Genetic variance	σ^2g
Grain yield	GY
Grains per spike	GpS
Gram	g

Granule-bound starch synthase 1	GBSS
Gross Domestic Product	GDP
Harvest index	HI
International Wheat and Maize Improvement Center	CIMMYT
Induced systematic resistance	ISR
Insertions and deletions	Indels
International Triticeae Mapping Initiative	ITMI
Interval mapping	IM
Kompetitive allele specific PCR	KASP
Khyber Pakhtunkhwa	KPK
Linkage disequilibrium	LD
Logarithm of odd	LOD
Least significant tests	LSD
Marker assisted selection	MAS
Multiparent advance generation intercrosses	MAGIC
Minor allele frequency	MAF
Multiple interval mapping	MIM
Million years ago	MYA
National Agriculture Research Stations	NARS
National Agriculture Research Center	NARC
Next generation sequencing	NGS
Nested association mapping	NAM
Polyphenol oxidase	PPO
Phenotypic variance	σ^2p
Phenotypic variance explained	PVE
Photoperiod	PpD
Protein variation effect analyzer	PROVEAN
Plant height	PH
Quantitative trait loci	QTL
Reactive oxidation species	ROS
Relative cell injury percentage	RCI%
Recombinant inbred lines	RILs
Random amplification of polymorphic DNA	RAPD
Root length	RL
Relative water content	RWC
Restricted maximum likelihood	REMLMM
Relative water content	RWC
Root length under well-watered condition	RL_{WW}
Root length under water-limited condition	RL_{WL}
Spikelets per spike	SpPS
Superoxide dismutase	SOD

Shoot length	SL
Single nucleotide polymorphisms	SNPs
Synthetic derived lines	SDLs
Synthetic derivatives	SYN-DER
Sucrose synthase	SuSy
Systematic acquired resistance	SAR
Synthetic hexaploid wheat	SHW
Synthetic/bread wheat derivative	SH/BW
Spike length	SL
Shoot length under well-watered condition	SL_{WW}
Shoot length under water-limited condition	SL_{WL}
Thousand grain weight	TGW
Thousand kernel weight	TKW
Tiller numbers	TN
Turgid mass	TM
Thousand-grain weight	TGW
Vacuolar invertase	VAC_INV
Vernalization	Vrn
Water soluble carbohydrates	WSC
Well-watered	WW
Water-limited	WL

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Abstract

Drought stress at all stages affects physiological and morphological characteristics important for wheat growth and development. Two types of population, i.e., International Triticeae Mapping Initiative (ITMI) and Synthetic derivatives (SYN-DER) were utilized to study the effects of drought stress in wheat. 209 recombinant inbred lines of ITMI, derived from synthetic wheat (W7984) x Opata, were evaluated at seedling stage for quantitative trait loci (QTL) identification. Our results indicated moderate to high broad sense heritability (H) among the RILs population with significant differences ($p<0.01$) revealed by analysis of variance (ANOVA). A high-density linkage map was constructed with 2639 genotyping-by-sequencing (GBS) markers that covered 5047 cM with an average marker density of 1.9 cM/marker. 16 QTLs were identified by composite interval mapping (CIM), distributed over nine chromosomes, out of which 10 QTLs were identified under water-limited (WL) conditions and 6 were identified under well-watered (WW) conditions with 4 to 59% of the phenotypic variance. In addition, 216 accessions of synthetic wheat germplasm (SYN-DER) were evaluated using 124 Kompetitive allele specific PCR (KASP) functional assays on 87 functional genes. KASP genotyping results indicated that beneficial alleles for genes underpinning flowering time (*Ppd-D1* and *Vrn-D3*), thousand-grain weight (TGW) (*TaCKX-D1, TaTGW6-A1, TaSus1-7B* and *TaCwi-D1*), water-soluble carbohydrates (*TaSST-A1*), yellow-pigment content (*Psy-B1* and *Zds-D1*), and root lesion nematodes (*Rlnn1*) were fixed in diversity panel with frequency ranging from 96.4-100%. The association analysis of functional genes with agronomic phenotypes in WW and WL conditions revealed that 21 marker-trait associations (MTAs) were consistently associated with agronomic traits in both conditions. *Vrn-A1, Rht-D1,* and

Ppd-B1 exhibited confounding effect on several agronomic traits including plant height, TGW and grain yield in both WW and WL conditions. The accumulation of favorable alleles for grain size and weight genes additively enhanced TGW in diversity panel. *CWI* gene has a conserved WECPDF domain. The result showed that 123 accessions have Hap-4A-C haplotype at *TaCwi-A1*, which is significantly associated with TGW and other agronomic traits under both WW and WL conditions. The non-synonymous substitutions observed in *TaCwi-B1* in the conserved domain (WECPDF) were Glu372Lys, Glu372Gly, Pro374Gln, Asp375Thr, while Phe376Leu, Tyr377Thr, Val379Cys variants. *In silico* analysis revealed that these point mutations were sequentially and structurally influencing the biological function of the *TaCwi-B1* protein.

INTRODUCTION AND LITERATURE REVIEW

1.1. Taxonomy and Genome of Wheat

Wheat (*Triticum aestivum*) belongs to Phylum Angiospermatophyta, Class: Monocotyledonopsida, Order: *Poales*, family: *Poaceae* (*Gramineae*) and Genus: *Triticum* (Bálint *et al*., 2000). *Poaceae* is amongst one of the largest families of flowering plants, that includes major crops like wheat, maize, rye, rice and oat (Kellogg, 2001). Grasses are mostly polyploid, which consists of autopolyploid, allopolyploid and segmental allopolyploid. It belongs to tribe *Triticeae* which is considered as a significant group of this family. It comprises of both annual and perennial types with 15 genera and 300 species characterizing compressed spikelets, compound spike, and simple starch grain. It belongs to genus Triticum (Finot *et al*., 2011). Its most widely grown genera are *Aegilops, Hordeum, Secale, Agropyron,* and *Triticum.* These wild relatives of *Triticum* are useful in transferring genes for major traits in order to attain resistance against both biotic and abiotic stresses (Al-Saghir, 2016; Bálint *et al.*,2000). Wheat is classified into three main categories based on their ploidy level; these are diploid wheat 2×=14, tetraploid wheat 4×=28 and hexaploid wheat 6×=42 known as bread wheat (Kilian *et al.,* 2009). Bread wheat is allohexaploid with the largest genome among crop species. It contains 70% transposable elements that are not always associated with gene segments (Breen *et al*., 2010).

1.2. Origin and Cultivation of Wheat

Wheat cultivation began approximately 10,000 years ago during the 'Neolithic period' when people started to change their basic mode of living. They gradually transitioned from hunting and gathering style of living to more settled lifestyle, which, in turn, resulted in promotion and recognition of agriculture, amongst the world masses. At that time, the cultivated wheat mostly comprised of diploid (AA) and tetraploid (BBAA) genomes. The cultivation of wheat has its roots in the land of Turkey. Nevertheless, some of its remains are traced in Jorden, Syria and Eastern Iraq. In 5000 B.C., landraces were the earliest cultivated form of wheat, which later spread to Near East in 9000 B.C.

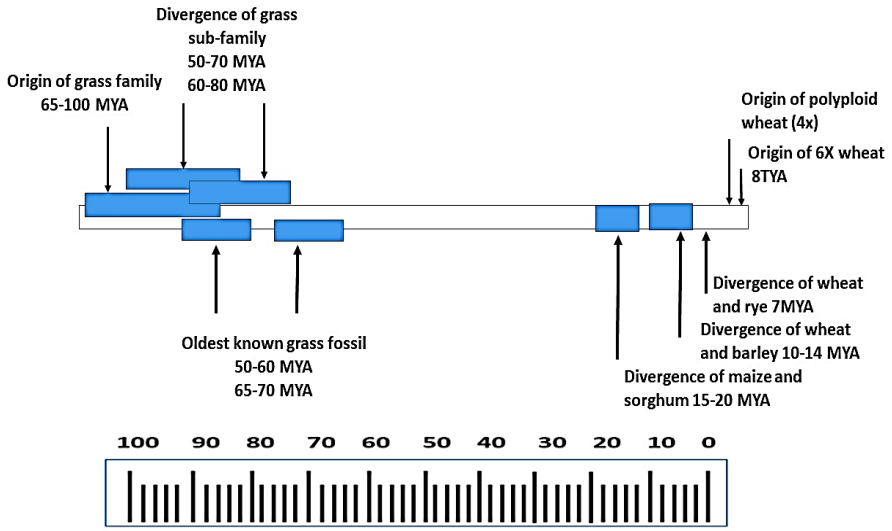

Fig 1.1: Evolutionary history of grasses; TYA= Thousand years ago; MYA= million years ago; 100 million years of grass family evolution. Triticum-Aegilops evolved around 3MYA, polyploid wheat originated during 0.5 MYA (Gill *et al.*, 2004).

Diploid, tetraploid and hexaploid wheat varieties are the cultivated forms of wheat, which have tough rachis (Snir *et al*., 2015). Domestication of natural population resulted in the development of emmer and einkorn. An early domesticated form of emmer, spelt and einkorn was hulled due to the reason that glumes tightly adhere to the grain. Farmers hybridized emmer wheat with wild grass of *Aegilops tauschii* several times to produce bread wheat from which they selected bread wheat with superior quality (Shewry, 2009). It is contemplated that bread wheat was instigated near Nile valley and diversified in Indus and Euphrates valley in England by 2000 B.C., China by 2500 B.C. and 4000 B.C. (Feldman *et al.*, 2001). Nowadays, wheat is cultivated in more land area than any other commercial crop. Wheat was naturally classified into three taxonomic groups, i.e., dinkel, emmer and einkorn. These taxonomic groups are known as ancient wheats. Emmer wheat is tetraploid (2n=4×=28) whereas, dinkel wheat is hexaploid (2n=6×=42), but they have the same basic chromosome number ×=7 (McFadden & Sears, 1946).

1.2.1. Origin of Einkorn, Emmer, Dinkel, and Kamut

The origin of einkorn was reported to have been in the upper region of Fertile Crescent of Near East. It consists of A-genome. An early evidence for the domestication of einkorn wheat dates from around 16000-15000 B.C. (Zohary & Hopf, 1993). Wild einkorn (*Triticum boeticum*), which includes *T. thaodar*, and *T. urartu* was named as *Triticum monococcum* were the first grain to be domesticated as early as 12000 B.C. It remained popular up till early 20th century. Nowadays, it is mostly grown in Yugoslavia, Turkey and Italy (Harlan, 1972). Emmer wheat *(T. dicoccoides)* was the second ancient wheat that emerged about 10,000 B.C. in Egypt. Origin of emmer wheat is traced near the regions of

Near East (Nevo, 1988). Cultivated emmer (*T. dicoccum*) was used in the late Mesolithic ages and early Neolithic Ages around 10,000 B.C. It consists of B-genome (Harlan, 1981; Zohary & Hopf, 1993). Dinkel wheat was grown around 7000 B.C. However, its origin was contentious based on two hypotheses. One hypothesis suggested the origin of dinkel wheat was in the Iranian region and Southeastern European region. Other hypothesis suggested that its origin can be traced to the mid-late Neolithic period 6000-5000 B.C. (Zohary & Hopf, 1993). It consists of three distinct genomes (BBAADD). The D-genome was donated by wild grass *Ae. tauschii* resulting in adaptation to wider environmental conditions. Kamut is considered as an old relative of modern durum wheat. Its scientific name is *Triticum turgidum,* ssp. *Polonicum.* Cultivation of kamut began in the Fertile Crescent region, which then further spread to Egypt and Tigris Euphrates valley (Khlestkina *et al.,* 2006).

1.3. Hybridization of BBAADD component of Bread Wheat

Around 8000 years ago, two distantly related wild kinds of grass were naturally hybridized i.e., *Aegilops speltoides* (BB or SS) and *Triticum urartu* (AA). These grasses were self-fertilized (2n=2×=14, AB) around 6000 B.C ago ultimately giving rise to wild emmer with BBAA composition (McFadden & Sears, 1946). Then, wild emmer and *Ae. tauschii* (donor of D-genome) were naturally hybridized resulting in hexaploid wheat known as bread wheat i.e., *Triticum aestivum* with 2n=6×=42, BBAADD (Jiang *et al.,* 1994). Wheat is the world leading cereal crop that provides nearly 20% of global calories (Gutierrez, 2017). Wheat has the greatest ratio among cereals that plays a significant role in the world economy in terms of food supply and cultivated land. It also has essential

nutritional value as it bestows food to 40% of the world's population (Gupta *et al.*, 2005). It includes gluten which allows the dough to rise upon fermentation (Hanson *et al.*, 1982). It is also rich in lipids, fats, minerals, and carbohydrates and is high in fiber content as compared to meat diet (Johnson *et al.*, 1985). USDA estimates total wheat production of 739.53 million metric tons worldwide. If compared to previous years, wheat production showed a decline of 14.57 million tons. The total wheat production in China alone is 131,000,000 metric tons according to the recent survey (https://www.statistica.com/). Wheat has also been significant in various religious and cultural traditions. Flattened bread named as 'matzo' is utilized in Judeo Christian tradition to signify a host as a Christian Eucharist. Subsequently, the Roman Catholic Church makes use of an unleavened wafer. In some Islamic countries like Kyrgyzstan and Uzbekistan, round bread is treated with respect after being stamped, they are neither thrown on the ground, nor in public (Shewry, 2009). Wheat is a Rabi crop of Pakistan, sown in October and harvested in April. It is a staple crop that grows almost all over Pakistan. Major growing areas of wheat are Punjab, followed by Sindh, Khyber Pakhtunkhwa (KPK), and Baluchistan. Total 80% of farmers grow wheat in nine million hectares i.e., ~40% of the total cultivated land, which contributed 10% value in agriculture and 2% in gross domestic product (GDP) in 2016. In 2017, total wheat production in Pakistan was recorded to be 26,200,000 metric tons. There is a marginal decrease in the wheat area in Pakistan due to poor soil moisture and dry conditions and because some farmers have substituted wheat cultivation with sugarcane and corn. It is foreseen that global wheat production will slightly increase in Russia and European Unions. Wheat import will be elevated in Iraq, Ethiopia, Mexico and Brazil and slightly decrease in Jordan, Vietnam and India.

1.4. Stresses of Wheat

Wheat has successfully evolved to survive in various types of environment, under a multitude of stresses. Various biotic and abiotic factors affect its gross yield. Thus, sustainable wheat production has been constantly pretentious by various stresses compounded by increasing effects of climatic change (Pandey *et al*., 2017). Abiotic stresses such as drought, water logging, salinity, high and low temperature, flood, different types of pollution and radiations are the major stresses that affect crop yield and productivity (Lawlor & Cornic, 2002; Mariani & Ferrante, 2017). Biotic stress is caused by plant-pathogen interaction. Its effect depends on the environment that varies from region to region (Angessa & Li, 2016). Plants and pathogens interact with variety of organisms throughout their lifecycles either by mutualism, predation, protocooperation, commensalism, amensalism or parasitism along with environmental stimuli such as temperature, light, physical stress or gravity (Abdullah *et al*., 2017; Bonfante & Genre, 2010; Redman, *et al*., 2001). Biotic stresses such as rust, Karnal bunt, powdery mildew, barley yellow dwarf virus (BYDV) and spot blotch negatively affect crop productivity (Kazi *et al*., 2013). Developing high yielding wheat varieties, resistant to both biotic and abiotic stresses is the only practical solution to meet the ever-increasing demand for wheat and wheat products (Braun, *et al*., 2010).

1.5. Drought Stress in Wheat

It is estimated that 1.8 billion people will be suffering from drought stress by 2025 (Nezhadahmadi *et al*., 2013). It is the most significant environmental stress of wheat, which

affects about 50% of wheat production. With continuous global climate change, the uncertainty in precipitation has resulted in continuous decline of wheat production (Daryanto *et al.*, 2016). Pakistan is also susceptible to massive climatic changes threatening food security because the change in rainfall pattern can cause damage to wheat production at the time of harvest (Ali *et al*., 2017). Extreme drought stress results in morphological, physiological and metabolic changes in a plant resulting in considerable yield losses. Drought negatively influences vegetative as well as the reproductive growth of wheat but severity depends on the growth stage of wheat. It negatively influences root, shoot, tiller number, spike length, grain numbers per spike, 1000-grain weight, awn length and peduncle length (KiliÇ & Tacettin, 2010). The extent of yield loss is determined by the duration of drought stress. The principle behind yield loss is a reduction in photosynthetic rate due to metabolic limitation, stomatal closure and oxidative damage to chloroplast leading to shriveled grain development (Farooq *et al*., 2014).

1.5.1. Effect of Drought Stress at Seedling Stage

Seed germination and seedling growth are the most crucial growth stages when the plant stumbles upon drought stress. It typically delays seed germination and suppresses growth as soil moisture is the principal source of seed germination (Mickky & Aldesuquy, 2017). Thus, it affects dry root weight, root/shoot ratio and harvestable yield of wheat (Ahmadi *et al*., 2012). At the cellular level, electrolyte leakage through cell membrane affects the physiology and biochemistry of seeds. The drought tolerant genotypes have relatively stable cell membrane to ensure minimum leakage through the membrane. This

is known as cell membrane thermostability or relative cell injury percentage (RCI%). This can be used as a standard to screen drought tolerant genotypes (Ahmad *et al*., 2015).

1.5.2. Effect of Drought Stress at Flowering Stage

Drought stress at flowering stage reduces pollination leading to relatively fewer number of grains per spike ultimately reducing plant yield (Akram, 2011). When plant senses drought stress, photosynthesis ceases to occur but respiration continues. The plant utilizes stored carbohydrates until the resumption of photosynthesis (Hammad *et al*., 2014). In case, photosynthesis remains ceased for an extended period of times, shortage of carbohydrates occurs that sustains parental respiration but florets get deprived of it. Ultimately, it affects the grain setting and grain yield (Ruan *et al*., 2010).

1.5.3. Effect of Drought Stress at Seed Development Stage

Sugar is translocated in the form of sucrose. As higher plants develop from an embryo, they are dependent on storage products like starch, produced as a consequence of heterotrophic nutrition. When vibrantly photosynthetic tissues are developed, the products of photosynthesis are exported to organs that are potentially less vibrant in photosynthesis, such as seeds or fruits. The mechanism of translocation can either be symplastic or apoplastic (Gupta & Kaur, 2005). In the apoplastic pathway, cell wall invertase plays an important role in transporting sucrose by converting into glucose and fructose via hexose transporters situated in the plasma membrane (Bush, 1999). In the cytoplasm, in addition

to invertase enzyme, sucrose synthase (SuSy) also catalyzes the hydrolysis of sucrose (Bush, 1999). Drought stress affects carbohydrates translocation due to a significant reduction in photosynthesis in leaves that ultimately reduces the exportation of assimilates to fruits and seeds.

1.6. Drought Tolerance in Wheat

1.6.1. Genes Conferring Drought Tolerance in Wheat

Traits controlling drought stress are polygenic in nature. Hence it is complex to understand both physiological and molecular mechanisms of drought tolerance. There are different types of regulator genes and enzymes involved in regulating drought response such as late embryo abundant proteins (LEA), vacuolar acid invertase, Abscisic acid (ABA) genes expression, Glutathione-S-transferase (GST), invertase genes (INV), dehydration responsive element binding proteins (DREB), ethylene responsive factors (ERF), zinc finger proteins (ZFP) and WRKY (Kulkarni *et al*., 2017; Nezhadahmadi *et al*., 2013). Invertase (β-D-fructofuranoside fructanohydrolase) is an enzyme engaged in the transportation of sucrose from source to sink for plant growth and development (Hari *et al*., 1985). There are three types of invertase enzymes classified on the basis of subcellular localization, solubility, and isoelectric point. These enzymes include cell wall invertase (CW-INV), vacuolar invertase (VAC_INV) and cytoplasmic invertase (CYT-INV) (Roitsch & Gonzalez, 2004). The cell wall invertase enzyme plays an important role in

pollen development and plant metabolism. The storage of carbohydrates becomes different under drought conditions as compared to well-watered conditions (Ji *et al*., 2010).

1.6.1.1. Role of Cell Wall Invertase gene in Drought

Carbohydrate is an important source of sucrose that is transported from source to sink tissue by phloem in plants. It also has a role in plant reproduction. Expression analysis in anthers of tobacco plant has revealed its role in microspore development as well and repression in *CWI* expression resulted in male sterility (Goetz *et al*., 2001). Similar findings were reported in some other crops such as tomato and rice (Oliver *et al.*, 2001; Zanor *et al.*, 2009). There is also evidence that cell wall invertase contributes towards seed development. In plants, pollination subsequent to double fertilization results in embryo formation and a fusion of male gamete with central cell forms a triploid zygote that develops into endosperm. Cell wall invertase controls cell division in the endosperm (Weber *et al.*, 1996). Cell wall invertase gene has a function in a source-sink relationship that changes in response to different biotic and abiotic stresses and during developmental stages (Albacete *et al.*,2014). During abiotic stress such as drought, carbon supply is limited owing to competition between physiological processes and sink organs resulting in a reduction in strength of sink tissues, ultimately reducing crop yield (Cuartero & Fernández-Muñoz, 1998). It has also been reported that water deficit downregulates cell wall invertase CW-IVR1 but not IVR3 and IVR5 (Koonjul *et al.*, 2005). IVR1 enzymes play an important role in both transcriptional and post-transcriptional mechanisms (Rausch & Greiner, 2004). CWI-INVs inhibition under stress condition reduces enzymatic activity

by ensuring that each microspore receives a small number of carbohydrates for fertilization (Jin *et al.*,2009).

1.6.2. Breeding Wheat for Drought Tolerance

The main purpose of plant breeding is to change the heredity characteristics of a crop plant in a way that can improve the overall plant performance such as yield and quality. The Increase in wheat yield results in the increased food supply and decreases the overall cost of food products for the consumer. There are two methods of wheat yield improvement I) Improving cultural environment II) Improving the genetic potential of the cultivar. International Centre for Wheat and Maize Improvement (CIMMYT) has played an important role in improving wheat yield by its breeding program.

1.7. Role of CIMMYT in Wheat Production

Evolution of modern wheat is associated with 'Green revolution' (Hanson, 1982). Rockefeller foundation research program developed Mexican semi-dwarf varieties that showed better results in reducing lodging and increasing disease resistance, subsequently increasing grain yield in a short period of time (Hanson, 1982). The initial seed transfer was done by CIMMYT, established in Mexico in 1966. Over the last 50 years, CIMMYT has developed wheat germplasm with high grain yield potential by addressing biotic and abiotic stress resistance and tolerance. This germplasm is then distributed to various National Agriculture Research Stations (NARS) of developing countries leading to the development of wheat improvement system internationally (Rajaram, 1995; Heisey *et al*., 2002). In addition, wheat genetic resources preservation was also started in 1968. The

germplasm collection includes wild species, landraces, breeding lines and modern cultivars (Hoisington *et al*., 1999).

1.7.1. International Triticeae Mapping Initiative (ITMI)

In the 1980s, CIMMYT and Cornell University collaborated to check polymorphisms among a large number of diverse wheat collection (Anderson *et al*., 1993). It was reported that synthetic wheat is highly polymorphic with a vast amount of genetic diversity in it. They selected synthetic wheat W7984 and bread wheat cultivar Opata to make a mapping population. Total 150 F_1 seeds from the cross between synthetic wheat W7984 (Altar84/*Aegilops tauschii* (219) CIGM86.940) and Opata M85 lines were selected and advanced by single seed descent method (SSD) up to F_7 generation in the greenhouse. This population was then distributed to different collaborators all over the world and is referred as ITMI population. It is used as a reference population for genetic mapping (Sorrells *et al*., 2011). Later on, the same population was produced again by two methods with more precaution. The two types of population; double haploid (DH) and recombinant inbred lines (RILs) were generated with 215 F_1 derived populations (DH) and 2043 F_6 recombinant inbred lines (RILs) (Sorrells *et al*., 2011).

This is because the traditional farmers' varieties were replaced by modern varieties by continuous selection resulting in narrowing genetic diversity. On the other hand, mutations in viral, bacterial, fungal and insects' population is quite rapid. This became a scientific premonition during 1970s when southern corn leaf blight was burst. First signs of narrowing genetic base germplasm emerged as a consequence of shoot fly and Karnal bunt epidemics (Dalrymple, 1985).

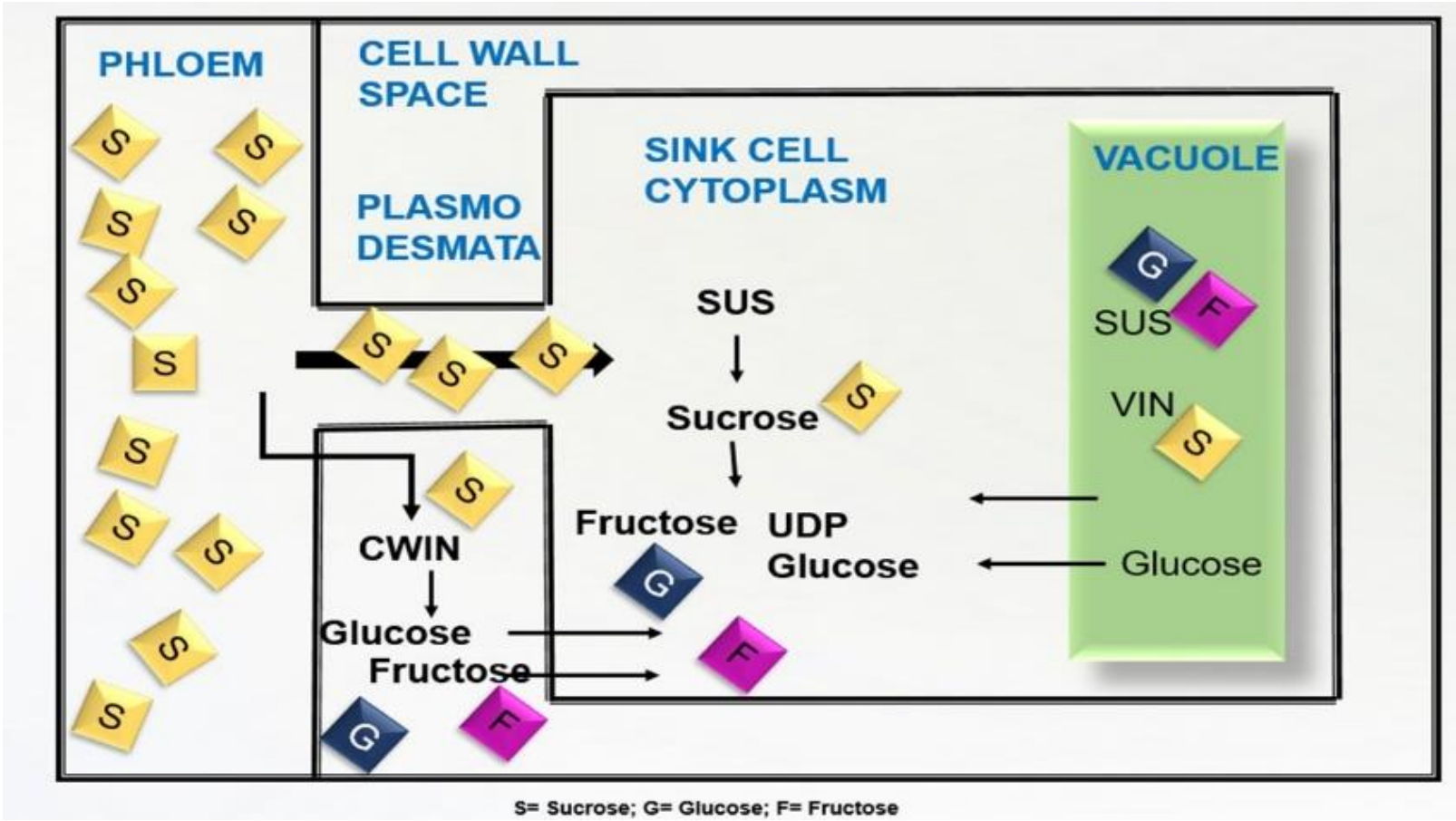

Fig 1.2: Role of invertase in sink tissue; S, sucrose; F, fructose; G, Glucose; invertase hydrolyzes sucrose into glucose and fructose (Roitsch & González, 2004)

Nevertheless, conventional plant breeding techniques aimed at improving crops by widening the genetic base by incorporating new materials and by exchanging diverse germplasm between breeding programs.

The introgression of alien genetic diversity by intergeneric hybridization or non-conventional techniques like gene transformations were exploited to increase genetic diversity (Mujeeb-Kazi, 1996). Wheat-rye translocation also represents introgression of alien genetic diversity. Translocation of rye 1R (short arm) either in a form of 1AL.1RS or 1BL.1RS translocation contains useful fungal and insect resistance genes such as aphid's infestation, powdery mildew and rusts.

This introgression leads to better yield but with poor end-use quality (Villareal *et al.*, 1998a). The first synthetic wheat was developed in 1946 by McFadden and Sears. During the 1990s, CIMMYT focused on interspecific hybridization between durum wheat and *Aegilops tauschii* to generate synthetic hexaploid wheat (SHW). This new variation was then transferred to elite breeding lines of CIMMYT (Mujeeb-Kazi *et al*., 1996).

The artificial hybridization of tetraploid wheat and *Aegilops tauschii* resulted in synthetic wheat. In the production of synthetic wheats, durum wheat was utilized as a female parent and *Ae. tauschii* as a male parent (Valkoun, 2001). Colchicine was employed for artificial doubling of chromosomes, which inhibits chromosome segregation in haploid plants (Blakeslee & Avery, 1937). *Aegilops tauschii* was considered as a valuable parent for incorporating alien resistance in wheat (Mujeeb-Kazi *et al*., 2008b) whereas durum wheat provided good agronomic traits (Luo *et al.*, 2007).

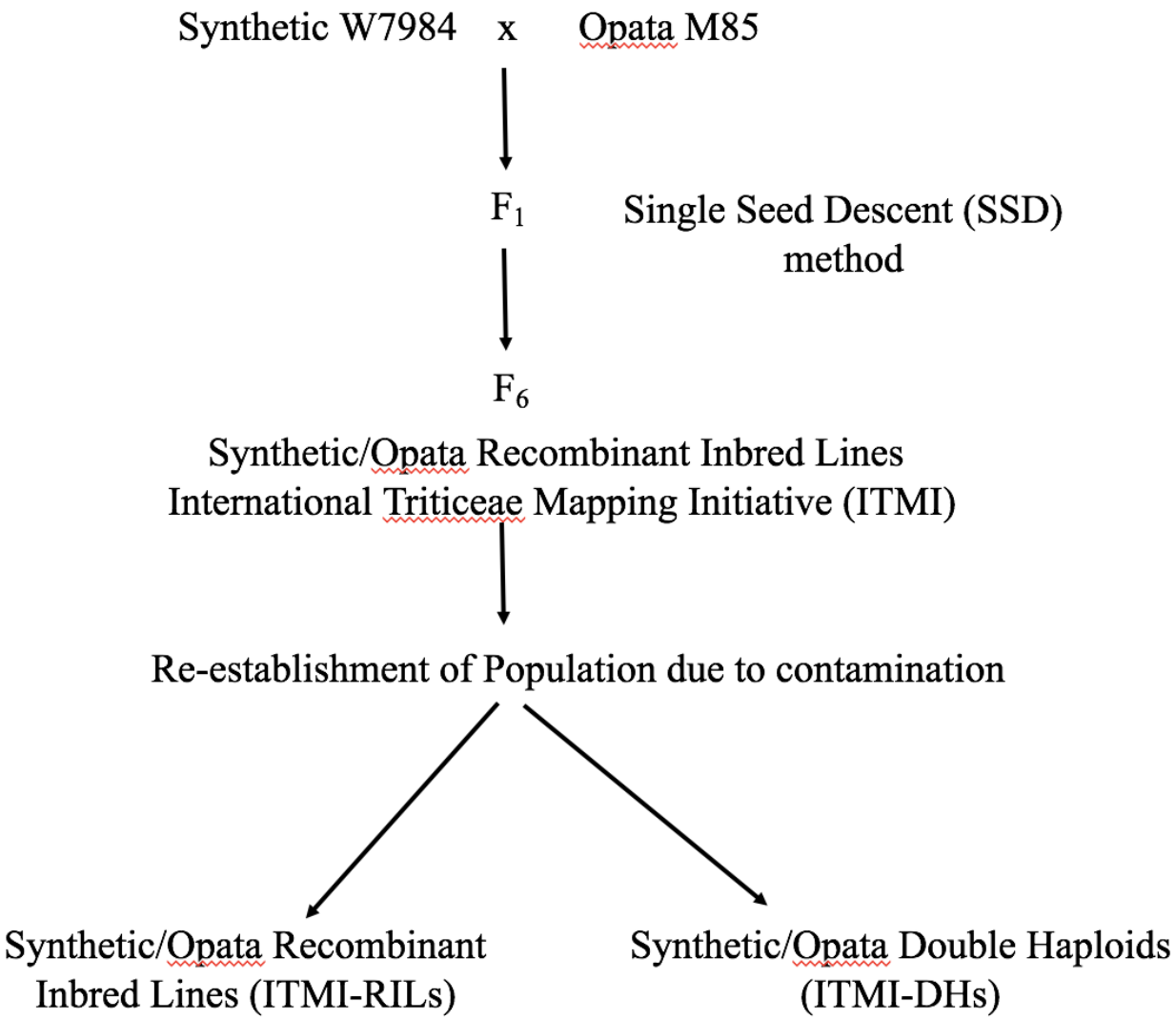

Fig 1.3: Schematic diagram of ITMI population; Hybridization of drought tolerant (Synthetic W7984) and drought susceptible (Opata M85) to form recombinant inbred lines (RILs) and double haploid (DHs) populations (Sorrells *et al*., 2011)

1.7.2 Synthetic Hexaploid Wheat

1.7.2.1 History and Production of Synthetic/Bread Wheat derived Population

Almost four decades back, the scientific community was alarmed by the impending threats of 'genetic erosion'. Genetic erosion can arise as a result of crop improvement and narrowing germplasm (Harlan, 1972). This is because the traditional farmers' varieties were replaced by modern varieties by continuous selection resulting in narrowing genetic diversity. On the other hand, mutations in viral, bacterial, fungal and insects' population is quite rapid. This became a scientific premonition during 1970s when southern corn leaf blight was burst. First signs of narrowing genetic base germplasm emerged as a consequence of shoot fly and Karnal bunt epidemics (Dalrymple, 1985). Nevertheless, conventional plant breeding techniques aimed at improving crops by widening the genetic base by incorporating new materials and by exchanging diverse germplasm between breeding programs.

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Primary synthetic hexaploid wheat was formed with poor quality and quantity (Kirigiwi *et al*., 2009). The first synthetics were used to make Karnal bunt resistant germplasm. Following the success of these attempts, breeders realized the importance of genetic diversity and genetic potential in this germplasm. Total 1200 synthetic lines were made by crossing different durum wheats with various *Ae. tauschii* accessions (Ortiz *et al*., 2008). *Ae. tauschii* accessions were initially screened for morphological, biotic and abiotic stresses before random hybridization (Mujeeb-Kazi *et al.*, 2000). The genetic diversity of synthetic lines was exploited by CIMMYT and other breeding programs by incorporating resistance to biotic stresses such as leaf and stem rusts (Warburton *et al*., 2006; Friesen *et al*., 2008). In addition to biotic stresses, SHW also exhibited better abiotic stress tolerance**,** such as drought, due to increased root mass, root length and deeper proliferation in soil that allowed it to extract more water from deeper soil layers (Trethowan & Mujeeb-Kazi, 2008; Reynolds & Trethowan, 2007).

1.7.2.2. Synthetic/Bread Wheat Derivatives (SYN-DER)

The genetic pool of wheat was enriched by crossing SHW with elite bread wheat cultivars. Primary synthetic hexaploid wheat showed high variation for various abiotic and biotic resistances (Lage *et al*., 2006). They showed poor agronomic quality such as difficulty in threshing, poor yield and unusual tallness (Mujeeb-Kazi *et al*., 2008). In the past 16 years, various crosses have been made between synthetic wheat and advanced lines to make synthetic/bread wheat derivatives. National Agriculture Research Centre (NARC), Islamabad, Pakistan developed synthetic wheat derivatives by crossing SHW with elite bread wheat cultivars of Pakistan (Mujeeb-Kazi *et al.,* 2009). Synthetic hexaploid wheat (SHW) was characterized initially for favorable traits and the selections were later made on the basis of desired traits. This approach provided transfer of targeted traits from donor SHW to adapted germplasm thereby widening the genetic diversity of bread wheat (Trethowan & Ginkel, 2009).

1.7.3. The Contribution of SHW in Drought Tolerance

Synthetic hexaploid wheat (SHW) has a great potential for improving tolerance to abiotic stresses. There are various studies that reported better performance of SHW under drought stress. In one study, 34 SHWs and some bread wheat were evaluated under well-watered (WW) and water-limited conditions (WL). SHWs showed an increase in plant height, increase biomass, longer spikes and increase flag leaf area (Liu *et al*., 2012). In another study, six synthetic wheat varieties and some winter wheat varieties were evaluated in plastic tubes under drought stress. Root biomass, root morphology, plant water relation

and stomatal attributes showed significant correlation with drought tolerance index and showed high stomatal density and smaller stomatal aperture (Becker *et al*., 2016). In another study, synthetic wheats were crossed with bread wheat by using single seed descent method. Individuals were selected for short stature, flowering, early heading, minimal lodging and free-threshing and yield trials were conducted under drought, heat and irrigated conditions. Genomic estimated breeding values (GEBVs) increased drought tolerance with 0.42 genomic predictions for yield (Jafarzadeh *et al*., 2016). It was also reported that synthetically derived lines (SDLs) increase drought tolerance by early flowering thereby increasing water use efficiency at the anthesis stage. Furthermore, grain yield was increased up to 26% by higher root mass under terminal drought. Therefore, the crossing of synthetic wheat with modern wheat cultivars may produce more productive cultivars (Lopes & Reynolds, 2011). The yield potential of synthetic by bread wheat derivatives (SH/BW) lines was studied in a rainfed environment of Australia that showed better yield by 8 to 30% (Ogbonnaya *et al*., 2008). Synthetic lines were also hybridized with winter wheat and evaluated for yield and yield-related traits. It was reported that yield was improved due to increase in grains per head and heads per unit area (Cooper *et al*., 2012). In China, four synthetic derived cultivars were released and widely grown by farmers i.e., Chaunmai 47, Chaunmai 43, Chaunmai 42 and Chaunmai 38. Of all these, Chaunmai 42 was also resistant to biotic stresses i.e., Karnal bunt and stripe rust (Yang *et al*., 2009).

1.8. Measures of Genetic Diversity

Genetic diversity on the basis of molecular markers has been studied for almost three decades (Hamrick & Godt, 1990). DNA marker development is a laborious task in wheat,

owing to the immense size of its genome (16 x 10^9), hexaploid nature and low level of polymorphism compared to other cereals crops, particularly D-genome (Chalmers *et al*., 2001). DNA markers are of two types i.e., neutral markers and functional markers. Neutral markers are conventional markers that are phenotypically neutral and are derived from genomic regions closely linked to the gene of interest. These neutral markers can be amplified fragment length polymorphism (AFLPs), randomly amplified polymorphic DNA (RAPD) and simple sequence repeats (SSRs). In recent times, diversity array technology (DArT) and single nucleotide polymorphisms (SNPs) have been widely employed for scanning QTLs on the genomic level in many crop plants. Nowadays, SNP markers are becoming markers of choice for the construction of high resolution genetic maps, genomic selection, genome-wide association mapping and evolution history studies (Zhao *et al*., 2007; Fleury *et al*., 2010). The main drawback of neutral markers is, however, that they are dependent on the linkage between markers and locus.

1.9. Non-neutral Markers/Functional Markers

Functional markers (FMs) are developed within functional genes. Due to the momentum gained with regards to gene discovery and advancement in sequence technology, large sets of functional sequence data are available in the form of bacterial artificial chromosome (BAC) clones, expressed sequence tags (ESTs), complementary DNA (cDNA) clones and many *in silico* experiments are available in various databases. Molecular markers are developed from this sequence information in intergenic regions. Functional markers are correlated directly with gene function and developed from insertions and deletions (Indels) and single nucleotide polymorphisms (SNPs) in different

alleles of functional genes (Andersen & Lubberstedt, 2003). It was reported that 30 wheat loci have been cloned so far, 97 functional markers (FMs) have been developed for the total of 93 alleles for various biotic, abiotic, agronomic and qualitative traits (Liu *et al*., 2012).

These molecular markers were developed for marker-assisted selection (MAS) that increases genetic improvement efficiency. But, these methods are labor-intensive and time-consuming (Yang *et al.,* 2012). Mutations within a gene are either in the form of single nucleotide polymorphisms (SNP**s**) or in the form of insertions and deletions (Indels). These Indels and SNPs contribute towards various phenotypic variations. Functional markers were made from these polymorphisms. Polymorphisms are synonymous or non-synonymous in nature. Usually, non-synonymous mutations lead to phenotypic variation. There are many successful examples of functional markers developed in cereal crops (Kage *et al*., 2015).

Functional markers were developed for sucrose synthase gene (*TaSUS2-2B*) to screen wheat genotypes for thousand grain weight (TGW) (Jiang *et al*., 2011). Cleaved amplified polymorphic marker (CAPS) was developed for grain weight gene (*TaGW2*).

It was based on two SNPs that are used for screening of grain weight and grain width (Su *et al.*, 2011). The allele-specific marker was developed for grain weight gene (*TaGW2*) on the basis of SNP detected on the eighth exon of this gene in wheat. These SNPs screened genotypes for kernel width and 1000-grain weight (Yang *et al.,* 2012). A novel indel marker has been developed for rice orthologue rice cytokinin oxidase/dehydrogenase gene (*OsCKX*). It regulates cytokinin level and affects 1000-grain weight (Zhang *et al*., 2012). CAPS marker for pre-harvest sprouting gene was developed for *TaSDr* gene to screen the

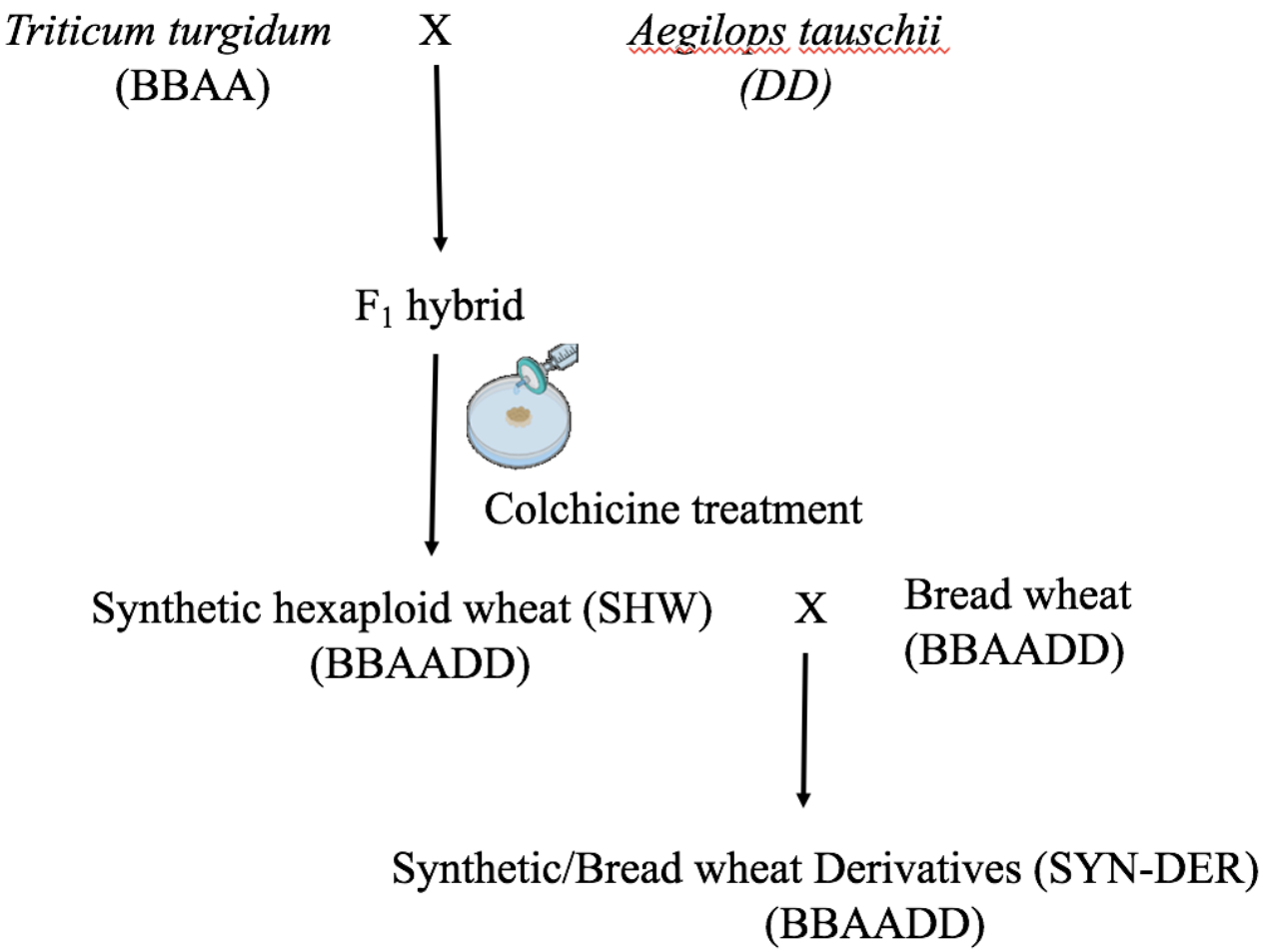


Fig 1.4: Schematic diagram of synthetic hexaploid production by hybridizing *Triticum turgidum* with *Aegilops tauschii* and their cross with bread wheat to form synthetics/bread wheat derivatives (Cox *et al*., 2017)

CAPS marker for pre-harvest sprouting gene was developed for *TaSDr* gene to screen those genes that are low in-spike germination indices as pre-harvest sprouting (PHS) gene, resulting in poor yield and low quality of wheat (Zhang *et al.,* 2014). Functional markers for two semi-dwarf genes *Rht-B1b (Rht1)* and *Rht-D1b (Rht2)* were developed to screen semi-dwarf varieties of wheat (Ellis *et al*., 2002). The co-dominant marker was developed for yellow pigment contents (*YP7A*) to screen genotypes for yellow pigment contents of wheat that increases the quality of pasta and other wheat products (He *et al*., 2008). Indel markers were developed for polyphenol oxidase (*PPO*) to screen wheat genotypes for better end product qualities such as noodles and pasta (He *et al*., 2007). Another indel marker was developed for UMN19 gene for the screening of better quality wheat genotypes and high molecular weight glutenins (Liu *et al*., 2008). The allele-specific marker was developed for *Glu-B3* gene for screening wheat genotypes for better quality end-use products of wheat (Wang *et al*., 2009). Functional markers were also developed for stripe rust/leaf rust/yellow rust and powdery mildew (*Lr34/Yr18/Pm38*) (Lagudah *et al.*, 2009). In addition, functional markers were developed for powdery mildew (*Pm3*) that showed resistance to *Blumeria graminis.* These markers were used to screen genotypes having resistant alleles in winter wheat (Mohler *et al.,* 2011). The allele-specific marker was developed for *TaMYB2* gene for wheat to screen genotypes for dehydration tolerance (Garg *et al.,* 2012). The allele-specific marker was also developed for *Dreb1* gene based on two SNPs to screen wheat cultivars for drought stress (Wei *et al.*, 2009).

Functional markers development is rapidly increasing due to the sequencing of the reference genome which results in gene discovery for economic traits. Despite the

availability of a large number of functional markers, there are certain drawbacks attached to it**.** First, screening large progenies is expensive and secondly, optimizing conventional PCR conditions and gel electrophoresis is both laborious and time-consuming. The alternative method for mapping known as 'Next generation sequencing (NGS)' is in fashion these days as it can sequence millions of short reads of DNA sequences and provides many folds' coverage of whole genome (Skovgaard *et al*., 2011) conveniently. NGS is more powerful than DNA fingerprinting and effective for molecular breeding (Yang *et al.,* 2012). Many NGS-based technologies discovered SNPs that allowed the development of molecular markers or functional markers. Therefore, Kompetitive allele-specific PCR (KASP) is used as it increases the efficiency in wheat breeding programs (Rasheed *et al.*,2016). It is a fluorescent-based genotyping technique that detects insertion-deletion, cleaved amplified polymorphic sequences (CAPS) and indels (He *et al*., 2014). Alleles are detected on the basis of fluorescent dye. In this assay, two primers and two allele-specific probes are used that can detect indels, SNPs, and CAPS site. The fluorescent dye is attached with the probe along with quencher. The function of quencher is to suppress dye fluorescence. Taq polymerase enzyme cleaves the probe when the probe binds to a targeted site that results in separation of dye from quencher which ultimately leads to fluorescence (Bernardo *et al*., 2015; Iannucci, 2017). More than 100 FMs are developed so far and converted into KASP assays in wheat (Rasheed *et al.*, 2016). But, there is a drawback associated with KASP assay i.e., it is uniplexed (Bernardo *et al*., 2015).

Alternatively, the multiplexed genotyping technique was developed by combining multiple samples and multiplexed PCR was done by using barcodes. Genotype by

sequencing (GBS) allowed multiplexed genotyping of markers linked to important QTLs. Among various different NGS technologies, genotype by sequencing (GBS) has been proved to be the most effective in plant breeding. GBS has been used to develop the high-density map of about 20,000 SNPs in wheat (Poland *et al.,* 2012). It is quite useful for the discovery of marker if reference genome is available as short reads are mapped back to reference genome to discover SNPs. It is also important for genomic selection as it reduces the duration of selection for perennial crops. In wheat, thousands of QTLs are linked to SNPs which can be CAPS or indel based markers. Hence, multiplexed assays facilitate the effective use of marker-assisted selection (MAS) (Bernardo *et al*., 2015). A successful GBS map was constructed with 416,856 markers (Ray *et al.,* 2014). There are two types of strategies that have been developed for genotype by sequencing, one is restriction enzyme digestion and the other is multiplex enrichment PCR. Restriction enzyme digestion takes place by digesting DNA with enzymes and adapters are used for ligation. This method is ideal for new markers discovery. The second strategy uses the PCR primers for the amplification of DNA. GBS was basically developed for association studies of maize (He *et al.,* 2014). Nowadays, it is being used for various complex genomes. By using these methodologies, a large number of molecular markers is discovered. Besides, a high density of genetic map can be constructed by using a large number of molecular markers which can also be used to serve as a reference genome for physical and linkage mapping. GBS can be utilized in studying polyploidy but it is challenging as it is difficult to distinguish between inter and intragenomic variations (Chung *et al.,* 2017).

1.10. Genetic Mapping and Drought Tolerance

Plants evolved a series of mechanisms to overcome drought stress at physiological, biochemical and morphological level. These mechanisms are drought escape, drought avoidance, drought tolerance and drought recovery. Drought escape is the plant's ability to complete their lifecycle before drought season begins (Khan & Iqbal, 2011). Drought avoidance is the maintenance of water potential by high water use efficiency, minimizing water loss during drought stress condition. In this mechanism, plant growth becomes slow followed by the stomatal closure and reduced photosynthetic and metabolic rate (Shavrukov *et al.,* 2017). Drought tolerance is plant's ability to tolerate water deficiency through manipulating physiological and biochemical conditions thus avoiding injurious drought effects. It is a complex trait controlled by polygenes and various environmental elements (Khan & Iqbal, 2011). It may include remobilization of water-soluble carbohydrates (WSC) in stem and accumulation of protectants for proper functionality under drought stress conditions (Farooq *et al*., 2014). Drought recovery depends on various factors including growing region and weather conditions. The prominent mechanisms include drought tolerance and drought avoidance (Fang & Xiong, 2014).

Genetic mapping identifies QTLs responsible for phenotypic variation. Two types of genetic mapping are applied in plants i.e., linkage mapping and association mapping. Linkage mapping is the process of arranging markers in order along individual chromosomes on the basis of recombination occurring between markers for particular mapping population. Different mapping populations exist in complexed crossing schemes. Recombinant inbred lines are developed by crossing two individuals with diverse

genotypes differing in the phenotypic trait of interest. These two individuals having contrasting genotypes are then hybridized and F_1 population is then homogenized by self-pollination. Another method is to produce double haploids (DHs) in which F_1 population is subjected to chromosome doubling to form a completely homozygous individual. These RILs and DHs are tested in multiple environments over many years. This conventional mapping technique has been successfully used in various crop species. However, this method has a few disadvantages i.e., low mapping resolution, low speed and low allelic richness. It is also known as family-based population such as biparental and multi parental mapping. Biparental population includes recombinant inbred lines, backcross, double haploids and near-isogenic lines (Chen *et al*., 2014). QTL mapping by using biparental population explores complex traits to identify new genetic resources for breeding purposes (Chen *et al*., 2014). QTL mapping depends on population size, marker type and marker density used for gene mapping (Chen *et al*., 2014). The limitation of the biparental population is low genetic diversity due to a fewer recombinant events. The multi-parental population made with high phenotypic diversity leads to higher resolution for QTL mapping. These populations include nested association mapping (NAM) and multiparent advance generation intercrosses (MAGIC). Enormous efforts have been made to analyze complex traits by using QTL mapping approach. For higher resolution, high-density linkage mapping based on high density of markers like SNPs is necessary (Huang *et al*., 2009).

1.10.1. Genetic Linkage Map Construction

Linkage groups are made by grouping markers together that are on the same chromosome. This can be performed by applying statistics such as logarithm of odds (LOD) between marker pairs. Recombination frequencies are calculated by dividing the number of recombined gametes between two loci onto the total number of gametes. Difference between recombinant and non-recombinant individuals is that; a non-recombinant individual would have the same allele as alleles of parental lines for two markers, whereas, recombinant allele would have different allele at one of the two markers loci when compared with parental genotypes. LOD scores are calculated as:

$$LOD\ scores = \log 10 \frac{Probability\ that\ two\ loci\ are\ linked}{Probability\ that\ two\ loci\ are\ unlinked}$$

If LOD score is three or greater than three, it means there is a linkage between two markers. This is because three means likelihood of linkage is 1000 times greater than the likelihood of no linkage (Xu, 2017). Many studies on linkage mapping have been reported. Later, a method was devised to transform recombination fractions into interpretable map distances (Haldane, 1919). This mapping function is based on the Poisson model in which it is assumed that there is no crossing over occurring along the chromosome (Zhao & Speed, 1996). Then, an alternative method was reported, in which Poisson model was replaced by Kosambi mapping function where it is assumed that there is more crossing over with the increase in genetic marker density (Kosambi, 2011).

1.10.2. QTL Mapping

Quantitative trait loci (QTL) mapping identifies genomic regions under the control of phenotypic evaluation. Analysis of variance (ANOVA) is the simplest method to link QTL and phenotype with the help of a genetic marker. Marker regression was proposed for QTL mapping in which populations were split into groups on the basis of single marker genotype and then compared genotypic grouping with associated phenotypes by considering that phenotype is a qualitative trait and is discrete rather than continuous. ANOVA is best suited for marker regression where the phenotype is qualitative. Marker regression is simplistic in nature. However, dense marker coverage is needed in order to ensure that little recombination takes place between best markers identified to enable an accurate estimate of QTL effect (Soller *et al*., 1976).

Later on, interval mapping (IM) method was proposed that involves LOD scores and evaluate flanking markers that hopefully contain QTL. It provides evidence for a QTL that may be present between flanking markers. Location of QTL is better explained by interval mapping. If QTL location is well-defined, it provides an estimation of the QTL effect on phenotype (Lander *et al.*, 1989). Composite interval mapping utilizes interval mapping that also includes a selection of covariates. Covariates are described as other markers that are found in the linkage group. It allows more refined interval scan across the genome (Jansen, 1993). Multiple interval mapping was also proposed that is similar to composite interval mapping but it uses genomic intervals rather than covariates. It also does not use the assumption that only single QTL is involved when searching for marker-trait associations (Kao *et al*., 1999). The simulated datasets can be used to elucidate appropriate threshold

values for reducing Type 1 error to an acceptable level in order to find a real QTL. The proper LOD score threshold can be set by considering factors such as number of individuals evaluated, type of cross involved and genome size (Lander & Botstein, 1989).

In 2007, 127 recombinant inbred lines were made by crossing drought tolerant (Dharwar dry) and drought susceptible (Sitta) to identify possible QTLs for grain yield and other components after the evaluation of these lines under two field conditions with different soil moistures. A QTL on 4AL was found with a significant impact under reduced moisture conditions (Kirigwi *et al.,* 2007). In another study, recombinant inbred spring wheat was grown in six different drought stress environments. In the final multi QTL model, one QTL associated to yield was consistent across all environments (Mathews *et al.*, 2008).

Later, a high-density linkage map was made by using genotype by sequencing (GBS) consisting of 364M1 markers spanned across 1959 cM with an average distance of 1.8 cM between markers. A linkage map was constructed for QTL mapping of flag leaf width, length, and the area in four different environments. Two major QTLs were identified for flag leaf length and two for flag leaf width (Hussain *et al.,* 2017). In addition, novel high yield consensus wheat genetic map consisted of 1127 marker loci was obtained on the basis of three RILs populations and the important chromosomal region was specified for yield and yield-related traits (Cui *et al*., 2014). Furthermore, interval mapping population of RILs population of the cross between c306 and HUW206 was analyzed for drought-related components such as flag leaf temperature, chlorophyll content and grain yield under stress

conditions. Five QTLs were identified for flag leaf, four for chlorophyll content and five QTLs were identified for low flag leaf temperature under stress (Kumar *et al.,* 2012).

Wheat grain yield is a product of several developmental processes that occur throughout the growth period. In order to achieve necessary yield productivity, it is necessary to explore wheat genetic resources to identify genes useful for stress tolerance. To efficiently explore genetic resources, high-throughput genotyping techniques and computational analysis can be helpful in combining favorable traits and breeding approach. In this study, our focus has been on exploring genetic diversity in two different germplasm sets using high-throughput genotyping techniques and to determine the effect of non-synonymous mutation on cell wall invertase gene.

1.11. Aims and Objectives

Following are the aims and objectives of this study:

1. Characterization of wheat cell wall invertase genes associated with drought tolerance in a diversity panel derived from synthetic/bread wheat derivatives.
2. Genome-wide linkage mapping for seedling morphology under drought stress in a synthetic wheat (W7984)/Opata population (ITMI).
3. Allelic effects of major genes controlling developmental traits and drought tolerance in synthetic/bread wheat derivatives.

2. MATERIALS AND METHODS

2.1. Mutations in Wheat Cell Wall Invertase gene (*TaCwi-B1*) underpin Drought Tolerance in Synthetic/Bread Wheat Derivatives (SYN-DER)

2.1.1. Germplasm Collection of Synthetic/Bread Wheat Derivatives

Two hundred and nine advanced lines derived from synthetic hexaploid wheat and elite bread wheat cultivars were used in the study (Annex 7.1) (Afzal *et al.,* 2017). Synthetic/bread wheat derivatives (SYN-DERs) were developed by crossing primary synthetic hexaploid wheats with advanced lines and elite cultivars from CIMMYT and Pakistan. The initial collection was 800 that was further reduced to 171 by continuous selection in National Agriculture Research Centre (NARC), Islamabad, Pakistan. Additionally, the SHW and bread wheat parents of these advanced lines and durum parents of the SHW were also used to identify the source of alleles.

2.1.2. Phenotyping of Synthetic/Bread Wheat Derivatives in Glasshouse Condition

All the accessions were evaluated under polyethylene glycol (PEG)-induced osmotic stress condition in a controlled laboratory environment. Wheat seeds were sterilized by using 1% solution of mercuric chloride for 5 min and then washing thoroughly with double distilled deionized (dd) water. Seeds were germinated for two days on wet filter papers in petri plates. Seedlings were then transferred to Jiffys® containing peat moss for further growth at room temperature. After six days, uniform height seedlings were then transplanted to hydroponic cultured boxes (8 × 8 × 12 cm) containing Hoagland's nutrient

solution (Annex 7.3) for 10 days at an irradiance of 130 µmol $m^{-2}s^{-1}$, 22–25°C air temperature and 12-h photoperiod. The hydroponic solution was supplemented with a fresh medium each day and was replaced completely with a fresh nutrient solution after 6 days. After sixteen days, seedlings were subjected to osmotic stress by gradually adding polyethylene glycol (PEG 6000). The hydroponic solution was aerated by air pumps (Tetra Blacksburg, 115 V). In case of control condition, roots were maintained in nutrient solution only for comparison purpose. After stress imposition, relative water content (RWC) was measured according to Turner (1981) using the following equation:

$$RWC = \frac{FM-DM}{(SM-DM)}$$

where FM is the fresh mass of leaf, DM is the dry mass after drying leaves at 75°C for 2 h and TM is the turgid mass. Shoot length from the base of the plant to the tip of the main tiller and root length data were recorded.

2.1.3. Phenotyping of Synthetic/Bread Wheat Derivatives in Field Condition

A field experiment was also set up with an alpha lattice design in two environmental conditions, i.e., well-watered (WW) and water-limited (WL). The details of the experiments are provided in Afzal *et al.*, (2017). Accessions were screened during 2014-2015 and 2015-2016 in Barani Agriculture Research Institute (BARI), Chakwal, Pakistan, which is a rainfed area (575 m above sea level). The soil is well-drained with moderately fine particles, pH 7.6, non-saline and slightly calcareous with 0.32 dS/m electrical conductivity (Islam *et al.*, 2013). In WW condition, all genotypes were planted with two replications in the field. Three irrigations were provided to WW plants and soil moisture

was maintained at 100 % field capacity till harvesting. In WL conditions, genotypes were planted in polyethylene tunnel with two replications. The tunnel was supported by an iron frame to protect from precipitation. Irrigation was provided till tillering stage. All genotypes were sown in 2m length row with 30 cm row spacing in both conditions. Physiological, agronomic and biochemical traits were measured accordingly.

2.1.4. DNA Extraction of Synthetic/Bread Wheat Derivatives

DNA extraction was performed by cetyl trimethyl ammonium bromide (CTAB) method (Doyle *et al*., 1987). The seedlings were germinated in a growth chamber. Approximately 0.1-1.5g of leaf samples were collected without a necrotic tissue in 2ml microtube with clean steel ball and placed in liquid nitrogen for few minutes. The leaf samples were crushed meticulously in a tissue lyser (Qiagen Tissue Lyser). 900µl CTAB (2% Cetyl trimethyl ammonium bromide, 1.4M NaCl, 20mM EDTA, 100mM Tris-HCl) was preheated at 65^{o}C and then added into each microtube. The mixture was well mixed and incubated in water bath at 65^{o}C for 30 minutes. All samples were permitted to cool for 3 minutes. Total 900µl of phenol-chloroform: isoamyl alcohol (25:24:1) was supplemented to each microtube and the mixture was centrifuged for 10 minutes at 12000 rpm. The upper aqueous layer was removed with the help of pipette and transferred to a new 2ml microtube. 3-5 µl RNase A was then added to remove RNA. After this, an equal amount of chloroform and isoamyl alcohol (24:1) was supplemented for DNA purification. The samples were centrifuged for 10 minutes at 12000 rpm and the supernatant was transferred to another microtube. An equal amount of isopropyl alcohol was added and samples were then kept

in the refrigerator for 1- 3 hours followed by centrifugation at 12000 rpm for 10 minutes and the supernatant discarded. The samples were washed with 500µl of 70% ethanol and then dried overnight. DNA of all samples were diluted by adding 400µl T.E buffer. The concentration of DNA was checked using a spectrophotometer and was stored at -20^{o}C.

2.1.5. Primer Designing and PCR Amplification of Synthetic/Bread Wheat Derivatives

Out of total 196 SYN-DER accessions (Annex 7.1), 10 accessions were selected with varying RWC data for cloning and sequencing. Primers were designed by taking a reference sequence (AF030240.1) from Webster *et al*, (2011). It was then BLAST to wheat genome survey sequence database specific for chromosome 5B (http://i.versailles.inra.fr/blast/blast.php). The resulting contig (4492773) with the highest similarity and lowest error value was downloaded, translated and aligned with reference sequence using Geneious Pro software (version 4.8.3). The aligned contig sequence was verified by observing WECPDF domain which is a characteristic of cell wall invertase gene. A gene-specific primer pair (CWI-B1-F2/TACWI-B1-R) was designed to clone 1200bp of *TaCwi-B1* gene flanking the WECPDF domain. The forward primers were chosen by using software Primer 3 (http//primer3.ut.ee). The reverse primer was made by using website www.cellbiol.com. The primers were checked by using amplification of nullitetrasomic line (CS-N5B) as shown in figure 2.3. These primers sequences were sent to Beijing TransGen Biotech, Beijing, China for primer preparation. The primer stock solution was diluted by adding 50 µl T.E buffer and 450 µl ddH_2O.

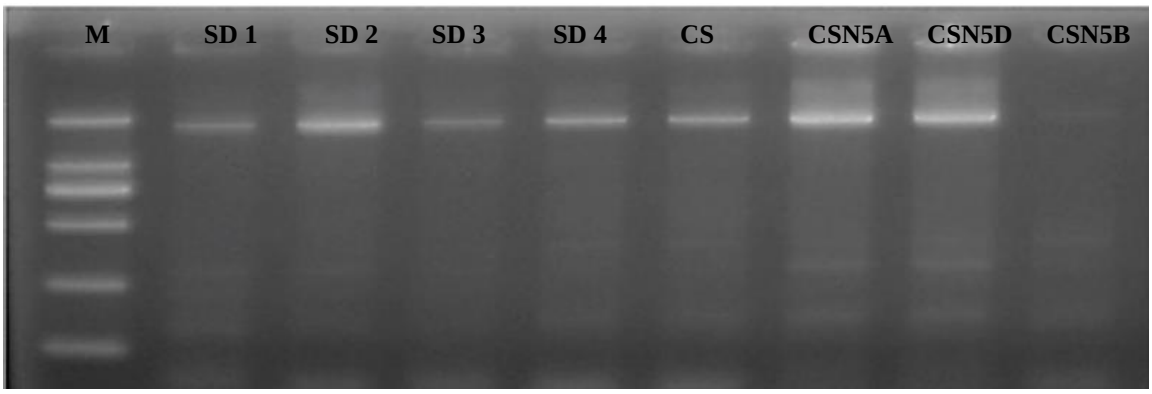


Figure 2.1: Amplification of *TaCwi-B1* (*Triticum aestivum*, Cell wall invertase on chromosome 5B) gene in synthetic/bread wheat derivatives (SD) and Chinese Spring nullitetrasomic genetic stocks (CSN5A, CSN5D, CSN5B). Arrow indicates no amplification in Chinese Spring nullitetrasomic 5B (CSN5B)

A total volume of 20µl was prepared including 1µl forward and reverse primer each, 6.5 µl ddH_2O and 10 µl PCR mixture. After master mix preparation, the mixture was centrifuged at 10,000 rpm and 1.5 µl DNA was then added. The mixture was then vortexed and 20 µl of mineral oil was added. PCR reactions were placed in Dual Block PCR machine (ESCO, Swift. Max. Pro.) and PCR reaction was set for amplification of specified regions as shown in figure 2.4.

2.1.6. Gel Electrophoreses and Gel Elution of PCR Product

The PCR product was loaded on 2% agarose gel, which was prepared by adding 4g of agarose in 200ml TBE buffer. The gel was poured in casting tray, the comb inserted and after15-20 minutes, PCR product (7-8 µl) was run on it at 250V for 25 minutes. The gel

was then placed in ethidium bromide gel tray for 10-15 minutes. Image analysis was performed using software Quantity one 4.6 (http://quantity-one.software.informer.com/4.6/).

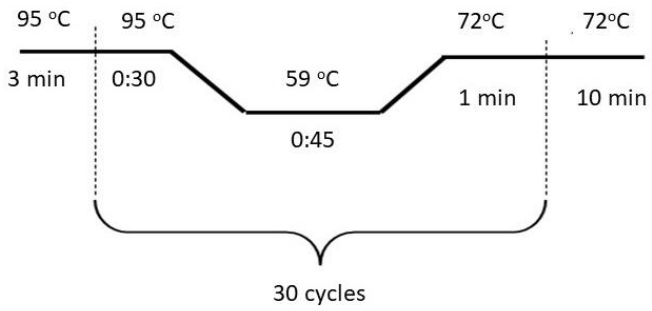


Figure 2.2: PCR condition for amplification of *TaCwi-B1*

The DNA band was purified from agarose gel using silica bead DNA gel elution Kit according to manufacturer protocol. The amplified PCR product of the desired size was cut from 1% agarose gel with the help of clean switchblade and shifted to pre-weighed 1.5ml microtube. The binding buffer of 3:1 volume was supplemented into the microtube, mixed thoroughly and incubated for 15 minutes at 55ºC. Total 7ul of silica powder was added in the microtube and incubated for 5 minutes at 55ºC. The reaction mixture was blended at the speed of 10625xg for 30 seconds to get a pellet. The supernatant was discarded and 500ul of silica washing buffer was added to the pellet. The pellet was resuspended and centrifuged for 1 min at the speed of 10625xg. The supernatant was discarded and 20ul of TE buffer was added to dilute the pellet. It was then incubated for 5 minutes at 55ºC. After

centrifuging it at the speed of 10625xg for 1 minute, it was transferred to a fresh tube for ligation.

2.1.7. Cloning of *TaCwi-B1*

pEASY-T1 cloning vector (1-1.5 µl) was added to the PCR tube containing 4µl purified DNA fragments and incubated for 20 minutes at 25^{o}C. The ligation mixture was incubated on ice for 10 minutes. It was then added to 50µl competent cells following the manufacturer's protocol. *Trnas*1-T1 phage resistant chemically competent cells (CD501, TrnasGene Biotech, Beijing, China) were prepared as outlined in the manual. The mixture was incubated in ice for 30 minutes. Heat shock was given for 30 seconds to 1 minute at 42^{o}C in water bath before incubation on ice for 2 minutes. L.B media (600 µl) was added and mixed with the help of pipette. The sample was incubated in a shaker at 37^{o}C with 200 rpm. After one hour, all mixture was spread on plates with antibiotic (Ampicillin1ml/L), LB media (annex 7.4) added and incubated in dark at 37^{o}C for 16 h. Sterile pipette tips were used to pick single bacterial colonies and placed into five different microtubes with 500 µL LB media (with 1ml/L Ampicillin). Samples were incubated at 37^{o}C at 250 rpm for 7h to ensure cell dispersal and growth. Colony PCR was performed by taking 2µL of the sample as PCR template and used same conditions for PCR amplification as described in section 2.9.3 to confirm the relevant gene. After confirmation, three replicates of each sample were sent for sequencing to Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

2.1.8. Nucleotide Sequences Retrieval and Alignment of *TaCwi-B1*

First, the nucleotide sequences were analyzed for possible indels. All sequences were aligned with the reference sequence (AF030240.1) and potential substitutions were noted. The CLUSTAL-X was used for aligning the sequences (Larkin *et al*., 2007).

2.1.9. Analysis of DNA indels with novoSNP of *TaCwi-B1*

The novoSNP was used for analyzing the variations observed in nucleotide sequences. Novo SNP uses reference sequence and trace files as input and generates the probable variations with a score that filters the non-synonymous substitutions with the synonymous ones. The tool novoSNP is available online (Weckx *et al*., 2005).

2.1.10. Protein Sequence Retrieval and Alignment of *TaCwi-B1*

All the nucleotide sequences were translated into protein sequences by using Clustal X. Full-length protein sequences were aligned to the reference sequence *TaCwi-B1* with default BLOSUM scoring matrices. The aligned sequences were properly trimmed in order to avoid unnecessary gaps in the alignment and to preserve the conservation of functionally important motifs (Librado & Rozas, 2009).

2.1.11. Protein Variation Effect Analyzer (PROVEAN) of *TaCwi-B1*

The variants extracted from multiple sequence alignment were first checked through PROVEAN tool (Choi & Chan, 2015). This tool predicted the non-synonymous

substitutions that were affecting the biological function of the protein. Further, these mutations were selected to carry out the rest of the analysis.

2.1.12. Protein Structure Prediction of *TaCwi-B1*

To predict the 3D structure of the protein, the first step is to look for homologous proteins that provide a significant hit over a fair length of target protein. For *TaCwi-B1* no such templates were available hence ab-initio threading methods were used for the structure prediction by using Phyre2 (Kelley *et al*., 2015) and I-TASSER (Zhang, 2008) webservers.

2.1.13. Prediction of Disordered Regions of *TaCwi-B1*

The intrinsic unstructured regions that were not predicted as flexible regions are defined as disordered regions. Disordered regions possess an important biological function that includes post-translational modification and binding to other proteins. Many studies have reported how mutations affect the folding and stability of proteins (Doss *et al*., 2012). In this analysis, two tools were included i.e., IUPRED (Dosztányi *et al*., 2005) and MetaPrDOS (Ishida & Kinoshita, 2008). The threshold above the value 0.5 was considered as disordered.

2.1.14. Prediction of Protein Stability Changes of *TaCwi-B1*

The non-synonymous variations can cause stability changes in the protein structure. In order to analyze this factor, I-mutant was utilized which is provided in Capriotti *et al*,

(2005). This tool takes in mutant residue and its position and output the stability change value that labels the mutations as either stabilizing or destabilizing (Capriotti *et al.*,2005).

2.1.15. Analyzing Point Mutations by Hope Server in *TaCwi-B1*

Hope is a web server that analyzes the structural effects of the mutations from a protein sequence. Selected mutations were provided to the Hope server and results were collected (Venselaar *et al.*, 2010).

2.1.16. Phylogenetic Analysis of *TaCwi-B1*

To examine the relationship of *CWI* proteins and to investigate the evolutionary history of this protein among different species of grass family, phylogenetic analysis was constructed by using Geneious 11.0.4. by neighbor-joining (N-J) method. The phylogenetic tree was drawn using 100 bootstrap value. Protein sequences of *Aegilops tauschii, Hordeum vulgare, Brachypodium distachyon, Triticum urartu and Zea mays* were retrieved and nine sequences of wheat including reference sequence were subjected to phylogenetic analysis.

2.2 Genome-wide Linkage Mapping for Seedling Morphology under Drought Stress in ITMI

2.2.1. Germplasm Collection and Phenotyping of ITMI Population through Cigar Roll Method

Two hundred and nine RILs from ITMI mapping population (Annex 7.2) including both parents were used for this study. These lines constitute a subset of new ITMI

population consisting of 2043 F_6 RILs (Sorrells *et al.*, 2011). All lines were phenotyped at seedling stage under control and drought stress conditions by using a cigar roll method (Zhu *et al.,* 2005). Seeds were first surface sterilized with 10% bleach (1-part bleach for every 9 parts of sterilized water) followed by 70% ethanol (70ml of 100% ethanol dissolved in 30ml of sterilized water). It was then rinsed three to five times with distilled water. Sterilized seeds were placed in petri plates containing filter paper and placed in a dark place for germination as shown in figure 2.1. After 5 days, five uniformly germinated seeds were placed in 1cm below the top center of germination paper which was then rolled to a final dimension of 2 cm in diameter and 35-38 cm in height supported vertically by a metal mesh with 2 cm x 2 cm holes. The rolled papers were placed in 1 L beakers as shown in figure 2.2. For well-watered conditions, beakers were filled with 100 ml of tap water. For water-limited conditions, 50 ml of tap water was supplied. Throughout the experiment, the constant water level was maintained. Seedlings were grown in a controlled environment with temperature 25/27^{o}C and 11.5/12.5h day/night. After 7 to 10 days, root length (RL), shoot length (SL), fresh root weight (FRW), fresh shoot weight (FSW), dry root weight (DRW) and dry shoot weight (DSW) were measured both in well-watered (WW) and water-limited (WL) condition. All data for each trait in all replications and both treatments were collected on the same day. All trait abbreviations were suffixed by condition names, e.g. RL in WW and WL were expressed as RL_{WW} and RL_{WL}, respectively. For RL, roots were separated from the root-shoot junction without any breakage in the root system and laid on a flat surface and stretched to measure its length and weight using a sophisticated weighing balance. It was then dried in an incubator at 60^{o}C for 24 hours to measure DRW.

The same procedure was done for shoots of all genotypes. Three technical replicates were performed. All data was collected and the mean values were calculated.

2.2.2. Genotyping by Sequencing of ITMI Population

DNA was extracted by using the protocol described in section 2.1.4. A GBS library was constructed according to protocol proposed by Poland *et al,* (2012) with two restriction enzymes, one is rare-cutting enzyme: *PstI* (CTGCAG) and the other is common-cutting enzyme *MspI* (CCGG). The adapters were ordered in the form of normal oligonucleotides. Two oligonucleotides were ordered which were complementary pairs for each adapter. These oligonucleotides were annealed to form double-stranded adapters so that they are stable enough to be stored at -20^{o}C for a long period of time. The annealing of adapters was carried out by heating at 95^{o}C and gradually cooling at the rate of 1^{o}C per minute to end temperature 30^{o}C.

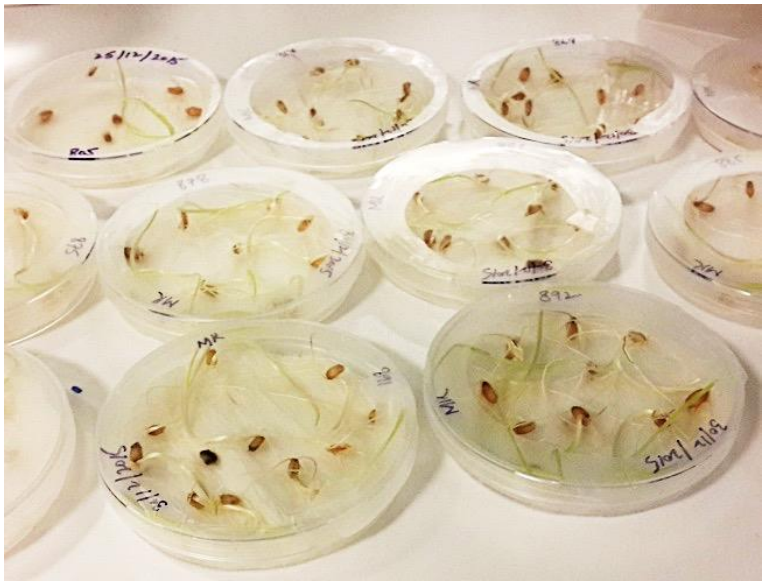


Figure 2.3: Germinated seedling in petri plates

Figure 2.4: Seedlings grown by cigar roll method

This process was carried out in a thermal cycler with PCR cycle programmed at 95^{o}C for 1 minute and decreasing the temperature by 1^{o}C up to 30^{o}C for 65 cycles. The barcoded adapters were ordered in plate and quantification was carried out after annealing completed in a plate format. The quantification of adapters was carried out in order to check the correct concentration. Uniform number of reads is directly proportional to the uniform concentration of adapters when sequencing the multiplexed library. The concentration of DNA was critical for producing even numbers of sequenced tags which were required for each sample. For this purpose, DNA quantification was carried out by using Pico Green, a fluorescent-based quantification method. Two enzymes *PstI* and *MspI* were used as a

double digest for this protocol. Overhang *PstI* is complementary to adapter 1 which was barcoded and the *MspI* corresponds to common adapter 2 (Y-adapter). Sequencing reaction was carried out from adapter 1 and proceeds through the barcode. For the optical working of double-digest restriction enzyme, NEB buffer 4 was used.

Digestion and ligation were carried out on the same plate having NEB 4 buffer with the addition of NEB T4 DNA ligase number M0202 and ATP. For optimal ligation of all fragments, a very high concentration of T4 was used. Adapter 1 concentration depends on the species under study. For wheat, 0.1 pmol is the optimal amount for 200ng of genomic DNA. Inactivation of ligation should be carried out at 65^{o}C for 20 minutes after the ligation is completed, prior to multiplexing the samples. The multiplexing was carried out in a 96-well plate and the products were sequenced on a single lane of Illumina Hiseq. The PCR amplification of multiplex library was carried out using a short extension time. This short extension time will enrich the fragments of 200-500 bp range which is suitable for bridge amplification. Only fragments with *PstI* and *MspI* cut side were amplified. Y adapter was designed as a common reverse adapter which is an exact match to reverse primer but not complimentary in order to avoid *MspI-MspI* amplification. Multiple PCR reactions were performed for each library in order to reduce overly amplified amplification that might occur during a single reaction. It was then pooled by plate into libraries and amplified by PCR. The library was constructed in 95-plex using P38-4A adapter set and sequenced to 100bp on a single lane of Illumina Hiseq 2000 at Kansas State University, Kansas, USA.

2.2.3. Statistical Analysis of Phenotypic and Genotypic Data of ITMI Population

Genotypic and phenotypic variances were calculated by the following formulas (Afzal *et al*., 2017).

$$Phenotypic\ variation\ (\sigma 2p):\ \sigma 2 + \sigma 2err$$

$$Genetic\ variance\ (\sigma 2g):\ \frac{(MSf - MSft)}{rt}$$

$$Genetic\ x\ treatment\ variance\ (\sigma 2gt):\ \frac{MSft - MSe}{r}$$

Where r = number of replications, t = number of treatments, MSg = mean square for genotype, e = environment. MS_f = genotype mean square, MS_{ft} = genotype x treatment interaction mean square. σ^2 error = MS_e = Error mean square. All phenotypic data were analyzed by SAS 9.2 software. Broad-sense heritability (H) was estimated by:

$$Broad\ sense\ heritability\ (H):\ \frac{\sigma 2g}{\sigma 2g + \frac{\sigma 2gt}{r} + \frac{\sigma 2\varepsilon}{rt}}$$

Where σ^2g = genetic variance, σ^2gt = genotype by treatment interaction, r = replication, t = treatments and σ^2p = phenotypic variation. Statistical differences were estimated by threshold probability of $P \leq 0.05$.

2.2.4. Linkage Map Construction and Linkage Analysis

GBS markers were grouped using IciMapping 4.0 software (http://www.isbreeding.net). Linkage analysis was performed using JoinMap 4.0 (Stam, 2005). Following are the three steps for linkage analysis using JM program:

1. Data reading by JM program
2. Calculation of LOD scores and pairwise recombination frequencies for the pairs with complete data
3. Development of Linkage group

The linkage map was then constructed by using R package 'synbreed' (Wimmer *et al*., 2012). Map distances between markers were calculated with the Kosambi mapping function. QTL analysis was performed using inclusive composite interval mapping (ICIM) with IciMapping 4.0 software (Meng *et al.,* 2015). Phenotypic values of all RILs in both WW and WL treatments were used for QTL detection. Missing phenotypic data was deleted using "Deletion" command. The walking speed chosen for all QTLs was 1.0 cM, with P= 0.001 in step-wise regression. Based on 2000 permutations at a probability level of 0.01, the LOD scores to declare significant QTLs for all traits ranged from 2.0 to 2.5. Thus, the LOD threshold of 2.5 was chosen for a declaration of putative QTLs. The phenotypic variance explained (PVE) was estimated using step-wise regression. QTLs were named according to the rules of International Rules of Genetic Nomenclature (http://wheat.pw.usda.gov/ggpages/wgc/98/Intro.htm).

2.3. Allelic Effects of Major Genes controlling Developmental Traits and Drought Tolerance in a Diversity Panel derived from Synthetic/Bread Wheat Derivatives

2.3.1. Genotyping of SYN-DER using KASP Assays

DNA was extracted from all genotypes using CTAB method described in section 2.3.1. Allele-specific KASP markers for 87 different loci were used. KASP assay to genotype targeted SNP in SYN-DER was developed by following standard KASP guidelines. KASP reaction consisted of three components i.e., DNA of interest, KASP assay mix, and KASP master mix. KASP assay mix consisted of two competitive alleles specific forward primer with two universal fluorescent resonance energy transfer (FRET) and one common reverse primer. KASP master mix consisted of FRET cassettes including FAM (5′ GAAGGTGACCAAGTTCATGCT 3′) and HEX (5′ GAAGGTCGGAGTCAACGGATT 3′). Primer mixture included 46 µl ddH_2O, 30 µl common primer (100 µM) and 12 µl of each tailed primer (100 µM) (LGC Genomics, 2013). Assays were tested in 384-well format and set up as a 5 µl reaction [2.2 µl DNA (10-20 ng/µl), 2.5µl of 2 × KASP master mixture and 0.056 µl primer mixture]. PCR cycling was performed using the protocol as shown in figure 2.3.

Table 2.1: Ingredients used in KASP assay

Ingredients	Volume
ddH_2O	46 µl
Common reverse primer	30 µl (100 µM)
FAM, HEX forward primer	12 µl (100µM)
DNA	2.2 µl (10-20 ng/µl)

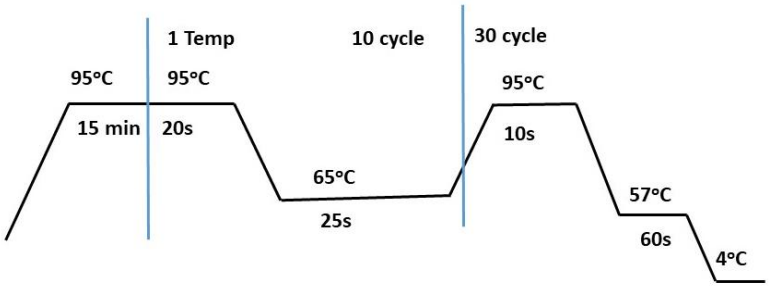


Figure 2.5: PCR condition for KASP assay

The hot start was kept at 95°C for 15 min, followed by ten touchdown cycles (95°C for 20s; touchdown 65°C-1°C per cycle 25s), further followed by 30 cycles of amplification (95°C for 10s; 57°C for 60s). The extension step is unnecessary as amplicon is less than 120 bp. The plate was read in BioTek H1 system and data analysis was performed manually using Klustercaller software (version 2.22.0.5; LGC Hoddesdon, UK).

2.3.2. Development of KASP Assays for *TaSST* genes

Two KASP assays were developed for *TaSST-D1* and *TaSST-A1* for genes to underpin water-soluble carbohydrates (Dong *et al.,* 2016). One KASP marker was developed for SNP A/G at position 1093 corresponding to CAPS marker WSC7D of *TaSST-D1*. Second KASP marker was developed for two neighboring SNP GTT/ATA at position 438 and 440 in TaSST-A2 gene. The results were compared to wheat cultivars in Dong *et al.,* (2016).

2.3.3. Statistical analysis using Tassel software

Restricted Maximum Likelihood (REMLMM) was used to measure gene allelic effects on plant traits. Genotypes served as random effect and gene served as a fixed effect. Least significant tests (LSD) were used to compare means with threshold probability $P<0.05$ for multiple haplotypes in a given trait. For statistical significance, a t-test was used to check the allelic effect of single KASP marker. Tassel software version 5 was used to investigate the relationship between genotypes and phenotypes and analysis of linkage disequilibrium. (https://bitbucket.org/tasseladmin/tassel-5-source/overview)

General linear model (GLM) was used to check the association between marker, trait and phenotypic values. GLM accounts for only population structure in association analysis. Population membership is considered independent of population structure. Hence, it does not account kinship for a genotype-phenotype relationship. Three files were made to upload into tassel software; I) Phenotype file II) Genotype file III) structure file. The phenotypic file was generated by using a special format and file was saved as mdp_ phenotype.txt, same as in case of genotype file. Alleles were coded as 'AA' or 'TT' and trait was written as' <Trait>'. Genotype file was saved as mdp_genotype.txt. Tassel software was installed from website https://bitbucket.org/tasseladmin/tassel-5-source/overview. All three files were uploaded as hap map file. Files were then U join to join all three files. GLM analysis was performed and 1000 permutations were specified. Output file gave the estimated values of traits associated with the marker data.

3. RESULTS

3.1. Mutations in Cell Wall Invertase gene (*TaCwi-B1*) underpin Drought Tolerance in Synthetic/Bread Wheat Derivatives

3.1.1. Allelic Variation at *TaCwi-A1, TaCwi-B1, TaCwi-D1* and their Effects on Phenotypes

In diversity panel, 58% accessions had Hap-4A-C haplotype which is favorable to yield components (Figure 3.1 & Table 3.1). *TaCwi-A1*was associated with days to heading (DH), grains per spike (GpS), plant height (PH), proline and thousand grain weight (TGW) in both well-watered and water-limited condition. The KASP marker *CWI-5D-312* was used to identify haplotypes at *TaCwi-D1* and results indicated that *Hap-5D-C* haplotype was fixed in diversity panel and none of the accession had contrasting *Hap-5D-G* haplotype. Previously, no sequence variation was observed at *TaCwi-B1* in bread wheat (Jiang *et al*., 2014), therefore this gene was sequenced in diversity panel to identify any new variations probably contributed by synthetic hexaploid wheat.

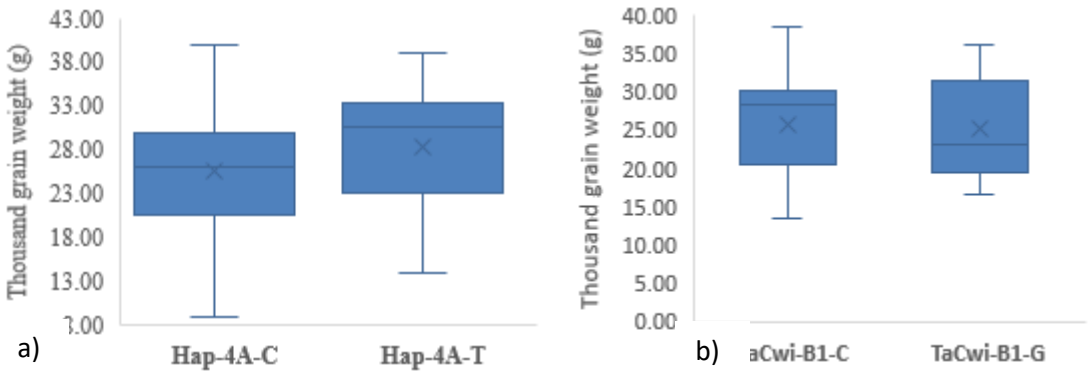


Figure 3.1: Allelic effects and haplotypes of *TaCwi-A1* and *TaCwi-B1* genes on thousand grain weight. The x-axis represents the accession group with specific alleles and the y-axis represents the phenotypic value in units for relevant traits

Table 3.1: Phenotypic validation of KASP assays for *TaCwi-A1* under well-watered and water-limited conditions

		Well Water Condition			**Water Limited Condition**		
Gene	**Trait[a]**	**P [b]**	**R^2 [c]**	**Estimate effect**	**P**	**R^2**	**Estimate effect**
TaCwi-A1	DH	0.03287	0.02212	-0.91968	0.000132	0.06932	11.1087
	GpS	0.03281	0.02214	3.90401			
	PH	0.000259	0.06257	4.79532			
	Proline	0.01348	0.02913	-26.499			
	TGW	0.01502	0.02823	-2.3943	0.02869	0.02325	-2.1635

[a]DH: days to heading; GpS: grains per spike; PH: plant height; TGW: thousand grain weight
[b] Significance at p>0.05
[c] R^2: Phenotypic variance explained by the marker

3.1.2. Analysis of DNA Indels in *TaCwi-B1* with novoSNP

Two variations were observed at the positions 153 (C153del) and 320 (G320C). Both were analyzed by novoSNP. In 54% SYN-DERs the variant G320C was observed significantly. The scores generated by this tool identifies the variation at position 320 (G320C) as a deleterious one. However, variation at position 153 C153del was predicted as neutral. Wild-type (TaCwi-B1-G) showed high thousand grain weight (TGW) as compared to mutated (TaCwi-B1-C).

3.1.3. Mutations Analysis by PROVEAN of *TaCwi-B1*

The highly conserved motif in the B genome was previously defined as WECPDF. The variants identified in this conserved region and in the surroundings were Glu372Lys, Pro374Gln, Asp375Thr, Tyr377Thr, Val379Cys, Ala380Val, Phe376Leu, and Trpp371del. Few other substitutions observed in the sequence were Ser107Thr, Arg108Thr,

Val111Ile, Ile115Leu, Gly121Ser, Gln132Pro. All these substitutions were analyzed through PROVEAN tool. The results predicted all the mutations of the conserved domain as deleterious while other substitutions were predicted as neutral. The results are tabulated in table 3.2.

3.1.4. Protein Structure Prediction of *TaCwi-B1*

We predicted the 3D structure of *TaCwi-B1* by using Phyre2. Phyre2 modeled 324 residues with 100% confidence covering 38% of the whole sequence based on the template available. As the structure predicted by Phyre2 covered less percentage of the whole target sequence so I-TASSER web server was used for *TaCwi-B1* protein structure prediction.

I-TASSER uses top ten threading templates that cover the larger percentage of the entire target sequence. The second model predicted by I-TASSER was picked up as it provides the highest C-score and covers 60% of the entire sequence with 2.99 Z-score. The predicted model was also analyzed by the values generated as normalized B factor from I-TASSER. The negative value means the residues are more stable. We analyzed the WECPDF domain and found that all these residues were more stable in the structure. Hence mutations in this region can cause serious damage to the structure and function of the *TaCwi-B1* protein. Structures are shown in figure 3.2.

Table 3.2: Variants predicted from PROVEAN in the sequence of wheat cell wall invertase gene (*TaCwi-B1*) and effect on stability upon mutation of cell wall invertase (*TaCwi-B1*) predicted from I-Mutant in synthetic/bread wheat derivatives

Variant	PROVEAN Score	Prediction	Stability Change (I-Mutant)	Effect
Glu372Lys	-3.908	Deleterious	ΔΔG = -0.87367226	Destabilizing
Glu372Gly	-6.830	Deleterious	ΔΔG = -1.0654503	Destabilizing
Pro374Gln	-7.225	Deleterious	ΔΔG= -0.96078728	Destabilizing
Asp375Thr	-5.984	Deleterious	ΔΔG= -0.44249054	Destabilizing
Tyr377Thr	-6.147	Deleterious	ΔΔG= -1.5475124	Destabilizing
Val379Cys	-4.144	Deleterious	ΔΔG= -0.82590609	Destabilizing
Ala380Val	-1.972	Neutral	ΔΔG= -0.39137295	Destabilizing
Ser107Thr	0.355	Neutral	ΔΔG= -0.325	Destabilizing
Val111Ile	-0.270	Neutral	ΔΔG= -0.265	Neutral
Ile115Leu	0.234	Neutral	ΔΔG= -0.365	Neutral
Gly121Ser	-0.575	Neutral	ΔΔG= -0.245	Neutral
Gln132Pro	1.270	Neutral	ΔΔG= -0.367	Neutral
Trp371del	-19.920	Deleterious	ΔΔG= -0.435	Destabilizing
Phe376Leu	-5.439	Deleterious	ΔΔG = -0.98579368	Destabilizing
Arg108Thr	0.378	Neutral	ΔΔG =-1.0654539	Destabilizing

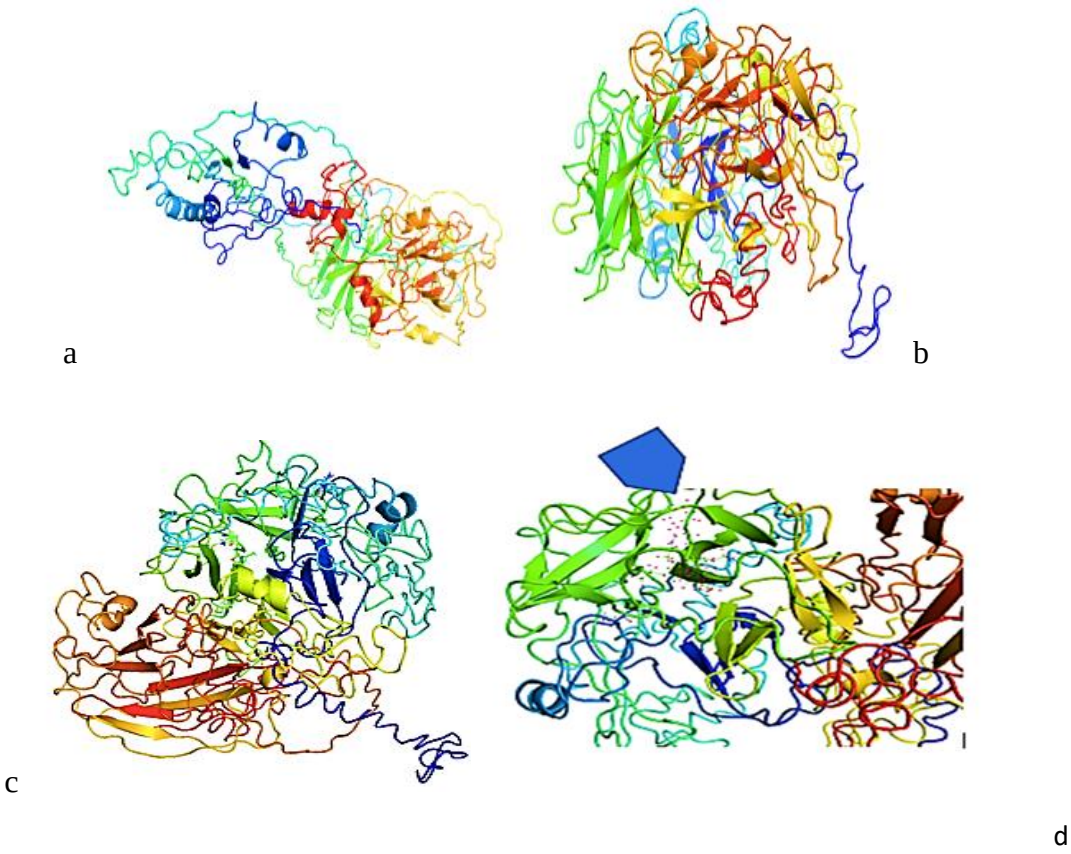


Figure 3.2: The *TaCwi-B1* protein structures predicted by (a) Phyre2, (b) I-TASSER, (c) mutated structure generated by PyMOL (d) zoomed view of the mutations in the *TaCwi-B1* protein structure

3.1.5. Disorder Prediction of *TaCwi-B1*

To identify the disordered regions, two tools were employed and the results were compared. The variation in consideration occurring in the conserved domain did not lie in the disordered region as predicted by both software. Figure 3.3 shows the disordered regions graphically.

3.1.6. Protein Stability Changes upon Mutation in *TaCwi-B1*

The stability of the protein structure was analyzed by I-Mutant tool for all of the mutations identified in the *TaCwi-B1* protein sequence. I-Mutant showed that all the

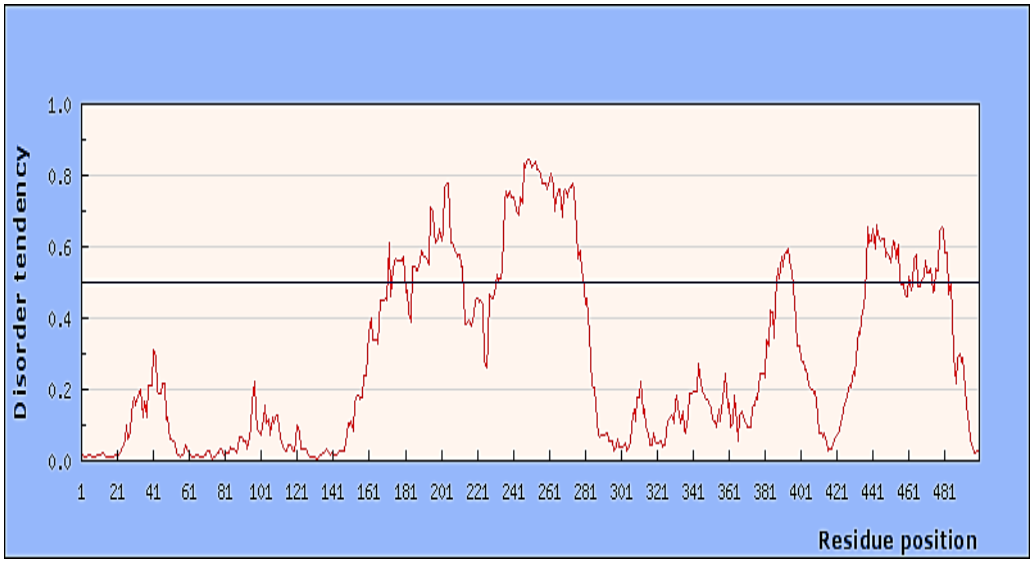
deleterious substitutions in WECPDF domain were also destabilizing the structure of the protein while some neutral variants were also found in sequence. The results are tabulated in table 3.2. Also, the mutated structure was generated through PyMOL.

3.1.7. Point Mutation Analysis by Hope server in *TaCwi-B1*

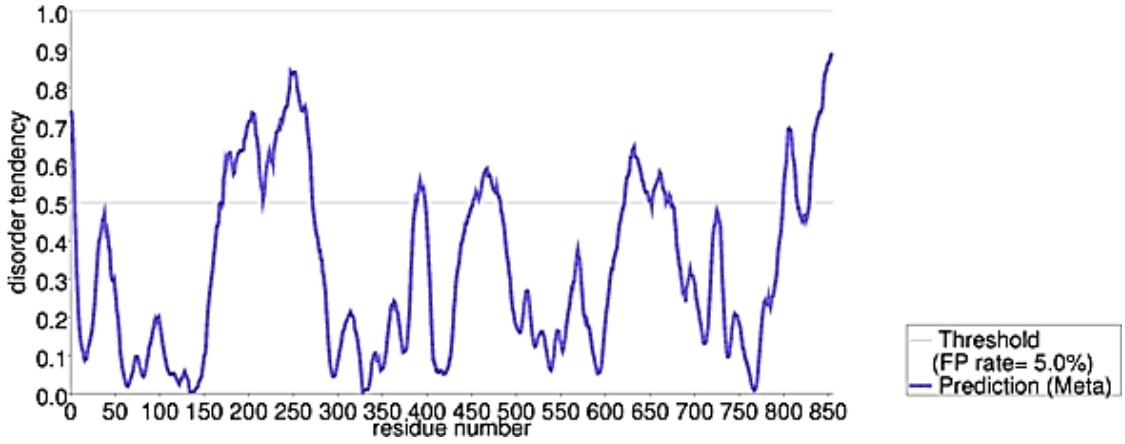
Selected point mutations were analyzed through Hope server to find the impact of these mutations on the structure of the protein. All identified variants were predicted as affecting the protein structure by disrupting the protein-protein interactions. If a small-sized amino acid substituted by a large-sized amino acid or vice versa, it can cause drastic conformational changes in the protein. Hence, these features should be considered for analyzing the structural effect upon mutations induced.

3.1.8. Phylogenetic Analysis of *TaCwi-B1*

The tree is divided into two major clades showing reference protein sequence (*TaCwi-B1*) and other wheat sequences in one major clade whereas *Brachypodium distachyon*, *Aegilops tauschii*, Z*ea mays*, *Triticum urartu* and *Hordeum vulgare* in another clade. Branch labels show bootstrap values as shown in Figure 3.4.



a



b

Figure 3.3: Intrinsic disordered regions a) The results generated from IUPred b) The graph generated from MetaPrDOS. The grey line is the threshold. Our domain lies below the line**.**

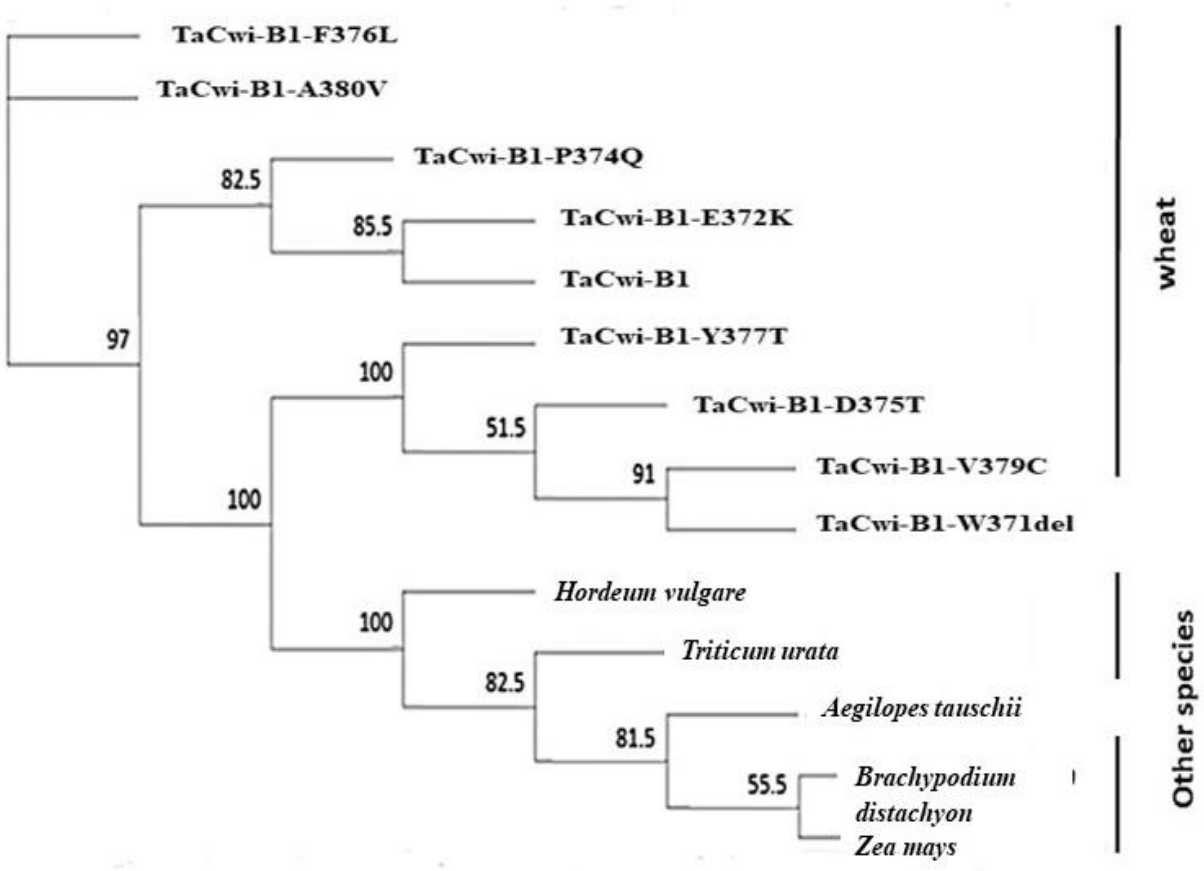


Figure 3.4: Phylogenetic analysis of *TaCwi-B1* gene of *Triticum aestivum* (wild-type and mutated), *Brachypodium distachyon*, *Aegilops tauschii*, *Zea mays*, *Triticum urartu* and *Hordeum vulgare*

3.2 Genome-Wide Linkage Mapping for Seedling Morphology Under Drought Stress in ITMI

3.2.1. Phenotypic Variation and Coefficient of Correlation

The average, maximum and minimum values of each trait varied among different lines in both treatments (Table 3.3). Estimate of variance components showed significant differences ($P < 0.05$) among genotypes, between treatments and genotype by treatment interaction (Table 3.4).

Table 3.3: Maximum, minimum and average values of agronomic traits at the early seedling stage of RILs and their parents under well-watered and water-limited conditions

		Parents		RILs		
Treatment	**Traits**	**Synthetic (W7984)**	**Opata**	**Min**	**Max**	**Average**
Well-watered	RL (cm)	10.100	20.2	0.400	21.50	12.110
	SL (cm)	12.967	14.30	2.600	17.33	10.680
	FRW (g)	0.040	0.09	0.002	0.099	0.043
	DRW (g)	0.008	0.006	0.001	0.048	0.057
	FSW (g)	0.089	0.135	0.009	0.150	0.074
	DSW (g)	0.011	0.009	0.001	0.063	0.009
Water-limited	RL (cm)	10.300	6.500	1.700	19.25	9.910
	SL (cm)	10.250	8.300	1.200	21.00	8.711
	FRW (g)	0.039	0.028	0.004	0.089	0.027
	DRW (g)	0.007	0.006	0.001	0.585	0.004
	FSW (g)	0.099	0.055	0.005	0.164	0.057
	DSW (g)	0.012	0.006	0.001	0.082	0.007

[a]RL, root length; SL, shoot length; FRW, fresh root weight; DRW, dry root weight; FSW, fresh shoot weight; DSW, dry shoot weight

Both RL and SL were the least affected and were reduced by 18% in WL condition, followed by DSW (19.9%). DRW was most affected and reduced by 91% followed by

FRW (37.7%) and FSW (23%) in WL treatment. Broad-sense heritability ranged from 0.51 (DSW) to 0.96 (FRW).

Table 3.4: Mean square from combining analysis of variance (ANOVA) for agronomic traits at early seedling stage evaluated in a glass house under well-watered and water-limited conditions

Source	DF	RL	SL	FRW	DRW	FSW	DSW
Genotype	201	25.2	11.2	4.50E-04	2.85E-05	6.66E-04	3.93E-05
Treatment	1	490	395	2.68E-02	1.17E-04	2.98E-02	3.37E-04
G × T	202	7.5	3.1	1.63E-04	2.35E-05	2.23E-04	3.81E-05
Error	407	7.6	3.1	1.61E-04	2.33E-05	2.24E-04	3.78E-05
Heritability		0.75	0.79	0.96	0.54	0.71	0.51

G × T: genotype × treatment, DF: the degree of freedom, RL: root length, SL: shoot length, FRW: fresh root weight, FSW: fresh shoot weight, DSW: dry shoot weight

All seedling traits showed moderate to strong positive correlation with each other in both treatments (Table 3.5). The highest positive correlation was observed between SL and FSW (r = 0.74) in WL treatment. In WW treatment, RL showed highly positive correlation with SL (r = 0.68). Similarly, FRW showed significant correlation with RL (r = 0.70). FSW showed high correlation with SL (r = 0.72). On the other hand, DRW was not strongly correlated with any of the traits.

Table 3.5: Pearson's coefficient of correlation for six seedling traits. Upper diagonal represents values under water-limited treatment, and lower diagonal represents correlation under well-watered treatment

	RL	SL	FRW	DRW	FSWW	DSW
RL		0.68*	0.66*	0.17*	0.59*	0.37*
SL	0.71*		0.54*	0.18*	0.74*	0.43*
FRW	0.70*	0.64*		0.31*	0.67*	0.35*
DRW	0.24*	0.28*	0.29*		0.08	0.08
FSW	0.56*	0.72*	0.69*	0.26*		0.49*
DSW	0.17*	0.21*	0.16*	0.06	0.09	

All * values are significant at $p<0.05$; RL: root length, SL: shoot length, FRW: fresh root weight, DRW: dry root weight, FSW: fresh shoot weight, DSW: dry shoot weight

3.2.2. Linkage Mapping and QTL Analysis

In total, 16 QTLs for root and shoot traits were identified explaining 4.7% (*QSL.nust-7B*) to 59% (*QFSW.nust-7B*) of phenotypic variation (Table 3.6). Ten QTLs were identified in WL for RL, SL, DSW and six in WW treatment for FRW and FSW. No QTL was identified for DRW under both conditions. The LOD values ranged from 2.51 (*QFSW.nust-6D*) to 14.27 (*QFSW.nust-7B*) (Table 3.6). QTLs were distributed on 9 chromosomes, significant at P = 0.05. Out of which, seven QTLs were mapped to chromosome 7B, two were mapped on 3A and one on each of the chromosomes 3B, 3D, 4A, 4D, 6D, 7A, and 7D. The additive effects of 12 QTLs were from synthetic 'W9874' allele and the remaining 4 QTLs were from Opata allele. Three QTL for RL_{WL} were identified on chromosomes 3A (209 cM), 4D (37 cM) and 7B (122 cM) as shown in Table 3.6. The phenotypic variation ranged from 6.98% (*QRL.nust-7B*) to 12.57% (*QRL.nust-3A*). The positive additive effect for *QRL.nust-3A* and *QRL.nust-7B* was observed from synthetic allele and negative additive effect of *QRL.nust-4D* from Opata allele.

Six QTLs for SL_{WL} were detected on 3A, 4A, 7B (3) and 7D chromosomes. The phenotypic variation ranged from 4.7 (*QSL.nust-7B*) to 14.6% (*QSL.nust-7B*). Three QTLs for SL_{WL}; *QSL.nust-7B, QSL.nust-7B* and *QSL.nust-7B* identified at 64 cM, 104 cM and 221 cM are likely to be different QTLs on the same chromosome. The additive effect of four QTLs; *QSL.nust-3A, QSL.nust-7B, QSL.nust 7B* and *QSL.nust-7D* were contributed by the synthetic allele. Four QTLs for FRW_{WW} were mapped on chromosomes 3B (at 54 cM), 7A (at 38 cM) and two QTLs on 7B (at 124 and 128 cM). These QTLs explained

7.92% (*QFRW.nust-3B*) to 10.06% (*QFRW.nust-7B)* of the phenotypic variation and the additive effect was contributed by synthetic allele. Two QTLs for FSW_{WW} were detected on 6D and 7B. *QFSW.nust-7B* was major QTL and explained 59% of the phenotypic variance whereas *QFSW.nust-6D* 9.56%. Both were contributed by the synthetic parent. A major QTL for DSW_{WL} was also detected on chromosome 3D at 63 cM, explaining 14.1% of the phenotypic variance with additive effect from synthetic allele. Two epistatic QTLs were identified for RL_{WL} and SL_{WL} on chromosome 3A. These QTL were identified by the same markers at 209 cM (*QRL.nust-3A, QSL.nust-3A*). Seven QTLs were identified on chromosome 7B, of which one is for RL_{WL} (*QRL.nust-7B*), three for SL_{WL} (*QSL.nust-7B*), two for FRW_{WW} (*QFRW.nust-7B*) and one for FSW_{WW} (*QFSW.nust-7B*) (Table 3.6).

Table 3.6: Summary of QTL detected in ITMI population using GBS markers

Trait	QTL	Position (cM)[a]	Left Marker	Right Marker	LOD[b]	PVE (%)[c]	Add[d]	References
RL$_{WL}$	*QRL.nust-3A*	209	chr3A_21408488	chr3A_20841712	4.39	12.57	1.40	Ayalew *et al.*, 2017
	QRL.nust-4D	37	chr4D_11509632	chr4D_16607007	3.39	9.67	-1.23	Czyczyło-Mysza *et al.*, 2014
	QRL.nust-7B	122	chr7B_611967289	chr7B_610782563	2.54	6.99	1.07	Zhang *et al*., 2013
SL$_{WL}$	*QSL.nust-3A*	209	chr3A_21408488	chr3A_20841712	4.64	9.19	0.79	
	QSL.nust-4A	130	chr4A_3383160	chr4A_3351311	4.69	8.93	-0.78	Zhang *et al*., 2013
	QSL.nust-7B	64	chr7B_739128842	chr7B_732570779	2.61	4.79	-0.57	
	QSL.nust-7B	104	chr7B_659612030	chr7B_657034962	7.32	14.60	1.00	
	QSL.nust 7B	221	chr7B_5156956	chr7B_1384484	3.03	5.96	0.63	
	QSL.nust-7D	295	chr7D_5365880	chr7D_4175447	4.38	8.36	0.75	
FRW$_{WW}$	*QFRW.nust-3B*	54	chr3B_69622431	chr3B_64066119	2.58	7.92	0.01	
	QFRW.nust-7A	38	chr7A_723652273	chr7A_721520274	3.52	10.77	-0.01	
	QFRW.nust-7B	128	chr7B_597213328	chr7B_594383475	4.04	12.42	0.01	

	QFRW.nust-7B	124	chr7B_609103054	chr7B_606740729	2.73	10.06	0.01	
FSW$_{WW}$	*QFSW.nust-6D*	153	chr6D_27859416	chr6D_27356480	2.51	9.56	0.01	Xinyuan *et al*. 2017
	QFSW.nust-7B	129	chr7B_587492916	chr7B_580857474	14.27	59.02	0.01	Zhang *et al*. 2013
DSW$_{WL}$	*QDSW.nust-3D*	63	chr3D_363865612	chr3D_554158392	2.73	14.13	0.00	

RL_{WL}= Root length and water limited condition; SL_{WL} = Shoot length at water limited condition; FRW_{WW} = Fresh root weight at well water condition; $FSW_{WW=}$ Fresh shoot weight at well water condition; $DSW_{WL=}$ Dry shoot weight at water limited condition. [a] Position of QTL located on chromosome as cM distance from the top of each map, [b] A LOD threshold of 2.5 was used for declaration of QTL, based on 2000 permutations at a significance level of 0.01, [c] Phenotypic variance explained by QTL, [d] Positive "additive effect" indicates an increasing effect from Synthetic (W9874); negative "additive effect" indicates an increasing effect from Opata

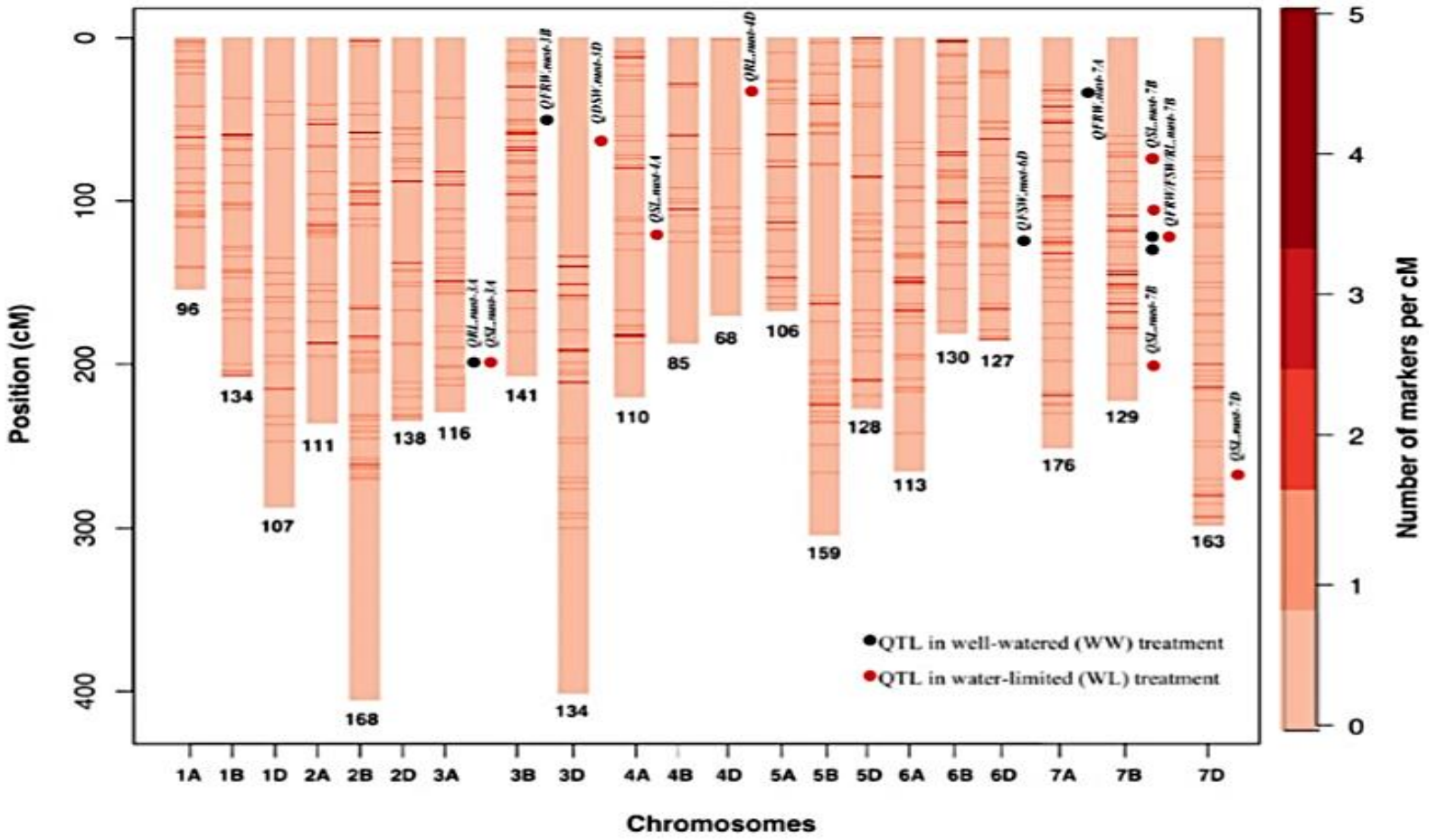


Figure 3.5: Linkage map of Synthetic (W7984) and Opata RILs population (ITMI) using 2639 GBS markers

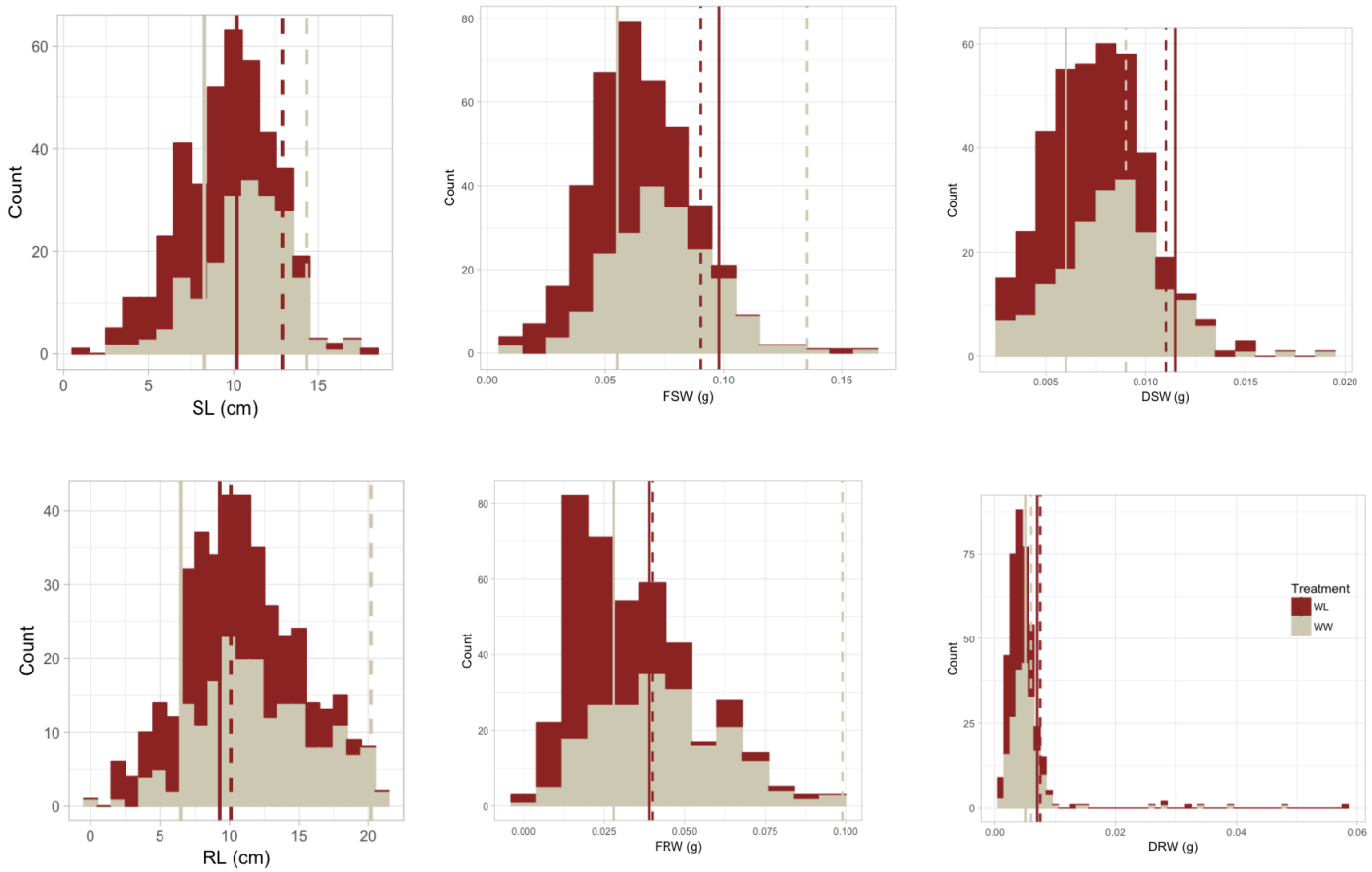


Figure 3.6: Graphical representation of the phenotypic variation of RL, SL, DRW, FRW, DSW, FSW under well-watered and water-limited conditions. RL: root length, SL: shoot length, FRW: fresh root weight, DSW: Dry shoot weight, FSW: Fresh shoot weight

3.3. Allelic Effects of Major Genes controlling Developmental Traits and Drought Tolerance in a Diversity Panel derived from Synthetic/Bread Wheat Derivatives

In total, 124 KASP markers were used to identify alleles at 84 loci in SYN-DER diversity panel (Annex 7.7). The results described here are presented for the alleles or haplotypes at each locus, instead of presenting results for individual markers. Because, in some cases, several KASP assays were used to identify multiple alleles of functional genes. In total, alleles at 30 loci were fixed with relatively high frequency ranging from 96-100% in diversity panel (Table 3.7). Such markers were not used for association analysis. In total, 58 loci showed allelic variation with minor allele frequency >5% as mentioned in (Table 3.8). These include two loci for plant stature, nine loci for flowering time, ten loci for grain size and weight, five loci for drought adaptability, eighteen loci for end-use quality, seven loci for grain color and dormancy and six loci for disease resistance.

3.3.1. Allele Fixation at Loci of Breeding interest in SYN-DER

The alleles present in more than 95% of the accessions were referred to the fixed or highly selected alleles and are mentioned in Table 3.7. Alleles were referred to 'favorable alleles' if positively associated with the improved phenotype in literature and referred to 'unfavorable alleles' if the allelic effect is not favorable for the improved phenotype. At *Ppd-D1* locus, the photo-period insensitive allele, *Ppd-D1a*, was fixed with the frequency of 99.6%. Similarly, a Jagger-type allele at *Vrn-D3* gene was present in 99.04% accessions, which is associated with the short-vernalization requirement. The Cadenza-type and wild-type alleles associated with delayed flowering were identified at *TaElf3-B1* and *TaElf3-D1*

respectively. The unfavorable alleles associated with low TGW were identified at *TaSus2-2B, TaGW2-6A,* and *TaTGW6-4A*, while Hap-L associated with low grain numbers per spike was identified at *TaMoc1-A1*.

Table 3.7. Alleles of functional genes completely or pre-dominantly fixed in diversity panel derived from synthetic/bread wheat

Group	Gene	Allele	Number	Frequency	Allelic effect of major allele
Flowering time	*Ppd-D1*	*Ppd-D1a*	203	97.60	Early flowering
	Vrn-B3	*vrn-B3 (CS)*	203	97.60	Delay flowering
	Vrn-D3	Jagger-type	206	99.04	Short vernalization requirement
	TaELF3-B1	Cadenza-type	194	93.27	Delay flowering
	TaELF3-D1	Wild-type	203	0.96	Delay flowering
Yield compo-nents	*TaSus2-2B*	Hap-L	208	100.00	Low TGW
	TaGW2-6A	Hap-6A-G	202	97.12	Low TGW
	TaCKX-D1	*TaCKX-D1a*	186	89.42	High TGW
	TaTGW6-A1	*TaTGW6-A1a*	204	98.08	High TGW
	TaMoc-A1	Hap-L	204	98.08	Low grain number
	TaSus1-7B	Hap-T	195	93.75	High TGW
	TaTGW6-4A	*TaTGW6-b*	201	96.63	Low TGW
	TaGS2-B1	A	187	89.90	NA
	TaCwi-D1	Hap-5D-C	206	99.04	High TGW
Drought adaptability	*AWN_BWc8266_5AL*	Presence	204	98.08	AWN present
	TaSST-A1	A2a	196	94.23	High WSC

End-use quality	*PPOA2b_230*	A2a,c	206	99.04	NA
	Psy-B1	*Psy-B1a or b*	204	98.08	Low YPC
	Zds-D1	*TaZds-D1b*	205	98.56	Low YPC
	Lox-B1	*Lox-B1a*	188	90.38	High LOX
	PPO-A2	*PPO-A2a*	206	99.52	NA
Disease resistance	*Fhb1*	*Fhb1-*	205	98.56	FHB Susceptible allele
	Sr36/Pm6	*Sr36-*	208	100.00	Stem rust susceptible allele
	Lr37	*Lr37-*	208	100.00	Leaf rust susceptible allele
	Lr9	*Lr9-*	208	100.00	Leaf rust susceptible allele
	Lr67	*Lr67-*	208	100.00	Leaf rust susceptible allele
	Lr68	*Lr68+*	208	100.00	Leaf rust resistance allele
	Lr47	*Lr47-*	191	91.83	Leaf rust susceptible allele
	Rlnn1	*Rlnn1+*	202	97.12	Root lesion nematode resistance allele
	CU8	*CU8-*	208	100.00	Eye spot susceptible allele

The favorable alleles associated with low yellow-pigment contents (YPC) were identified at *Psy-B1* and *Zds-D1*. Similarly, none of the accessions carried resistance alleles for Fusarium head blight gene (*Fhb1*), stem rust resistance gene (*Sr36/Pm6*), leaf rust resistance gene (*Lr37, Lr9, Lr67,* and *Lr47*) and eye-spot resistance gene (*CU8*). However, resistance allele for root lesion nematode resistance gene (*Rlnn1*) was present in 97.1 accessions (Table 3.7).

3.3.2. Allelic Variation at Loci of Breeding interest in SYN-DER

Several genes had more than two alleles, and only those genes are described here which have at least two alleles with more than 5% frequency. The minor allele frequency at 58 loci ranged between 5% (several alleles) to 47.6% (*1fehw3*). Allelic variation at these genes is described below according to their phenotypic associations (Table 3.8).

3.3.2.1. Wheat Adaptability and Development-related Genes

In total, 11 loci were categorized in this group. At both *Rht* genes, wild-type alleles, *Rht-B1a* and *Rht-D1a,* were most frequently present in 50.9% and 77.4% accessions. 1BL.1RS translocation was present in 55 (26.4%) accessions. In case of both photoperiod responses related genes, the photo-period insensitive alleles, *Ppd-A1a* and *Ppd-B1a*, were present in 61.1 and 75.5% accessions (Table 3.8). Almost 16 (7.7%) accessions have GS-105 type *Ppd-A1* allele which has 1117 bp deletion in *Ppd-A1* and is likely to be transferred from the durum parent of synthetic hexaploid wheat. Across three vernalization genes, the spring-type alleles had a higher frequency at *Vrn-A1* (45.2%), *Vrn-B1* (86.1%) and *Vrn-D1* (88.9%). The KASP assay TaBradi2g14790 was used to identify deletion of *Elf3-D1* gene associated with early flowering and 46.1% of the accessions have gene deletion (Table 3.8). Similarly, two paralogs of *Ppd1* gene; *PRR73-A1*, and *PRR73-B1* were genotyped, and Hap-I was frequent (57.7%, 94.3% respectively) at both loci. Hap-I at *PRR73-A1* is

associated with early flowering, while Hap-I at *PRR73-B1* is associated with delayed flowering (Zhang *et al.*, 2016b).

3.3.2.2. Grain Size and Weight-related Genes

In this category, 10 functional genes showed allelic variation, out of which two genes (*TaSus1-7A* and *TEF-7A*) showed more than two alleles. At *TaGS-D1*, the favorable allele, *TaGS-D1a*, associated with high TGW was most frequent (82.2%). Similarly, higher frequency of favorable alleles was observed at *TaCwi-A1* (84.1%), *TaSus2-2A* (55.3%), *TaSus1-7A* (86.5%), *TaGS5-A1* (79.8%), *TaGW2-6B* (61.5%), *TaGS2-B1* (63.9%) and *TaGS1a* (58.1%). The unfavorable alleles associated with lower TGW were observed at *TaGASR* (88.9) and *TEF-7A* (65.8%).

3.3.2.3. Drought Adaptability-related Genes

Five genes related to drought adaptability showed higher allelic variation in SYN-DER. The favorable alleles, Hap-4A-C and *TaDreb-B1b*, showed higher frequency at *TaCwi-4A* and *TaDreb1* genes. Similarly, the favorable allele *COMT-3Ba* associated with high lignin content under water-limited conditions was identified in 55.2% accessions. Almost balanced allele frequency was observed at *1fehw3* gene related to water-soluble carbohydrate (WSC) content in SYN-DER diversity panel.

3.3.2.4. Pre-harvest Sprouting and Grain Color-related Genes

Seven genes related to grain color and dormancy showed a higher allelic variation, which also includes three homeologous genes *TaMyb10* at A-, B- and D-genome (Figure

3.7). `The alleles encoding white grain color were predominant at *TaMyb10-A1* and *TaMyb10-D1* genes, while red grain color encoding allele was predominant at *TaMyb10-B1*. The *Vp1-B1c* allele at *Vp1-B1* (69.2%), a Rio-Balnco-type allele at *Phs1* (75.9%) and a Zen-type allele at *TaMFT-A1* (85%) associated with pre-harvest sprouting tolerance were present with higher frequency. At *TaSdr-B1*, the pre-harvest sprouting susceptibility allele, *TaSdr-B1b* was observed at high frequency.

Table 3.8: The allele frequency of functional genes in the diversity panel derived from synthetic hexaploid wheat

Group	Gene	Mode[a]	Mode frequency	Alleles/haplotypes	Number	Frequency (%)
Adaptability	*Rht-B1*	*Rht-B1a*	106	*Rht-B1a*	106	50.96
				Rht-B1b	97	46.63
	Rht-D1	*Rht-D1a*	161	*Rht-D1a*	161	77.40
				Rht-D1b	45	21.63
	1BL.1RS	*1B1R-*	136	1B1R-	136	65.38
				1B1R+	55	26.44
Flowering time	*Ppd-A1*	*Ppd-A1a*	127	*Ppd-A1a*	127	61.06
				Ppd-A1a (1027)	1	0.48
				Ppd-A1a (1117)	16	7.69
				Ppd-A1b	64	30.77
	Ppd-B1	*Ppd-B1a*	157	*Ppd-B1a*	157	75.48
				Ppd-B1a(Chayenne-type)	10	4.81
				Ppd-B1a(Sonoro)	41	19.71
	Vrn-A1 (Exon7)	Claire-type	155	Claire-type	155	74.52
				Hereward-type	39	18.75
	Vrn-A1	*Vrn-A1w*(Jagger)	91	*Vrn-A1a*	6	2.88
				Vrn-A1s	47	22.60
				Vrn-A1w	18	8.65
				Vrn-A1w(2147)	46	22.12
				Vrn-A1w(Jagger)	91	43.75

	Vrn-B1	*Vrn-B1a*	179	*Vrn-B1a*	179	86.06
				Vrn-B1b	11	5.29
				Vrn-B1c	2	0.96
				vrn-B1	4	1.92
	Vrn-D1	*Vrn-D1*(S)	185	*Vrn-D1*(S)	185	88.94
				vrn-D1	23	11.06
	TaBradi2g 14790	T	96	A	86	41.35
				T	96	46.15
	TaMOT1-D1	Wild-type	164	Ria-type	8	3.85
				Wild-type	164	78.85
	PRR73-A1	Hap-I	120	Hap-I	120	57.69
				Hap-II	77	37.02
	PRR73-B1	Hap-I	196	Hap-I	196	94.23
				Hap-II	10	4.81
Yield components	*TaGS-D1*	*TaGS-D1a*	171	*TaGD-D1b*	29	13.94
				TaGS-D1a	171	82.21
	TaCwi-A1	*TaCwi-A1a*	175	*TaCwi-A1a*	175	84.13
				TaCwi-A1b	30	14.42
	TaGASR-A1	H1g	185	H1c	17	8.17
				H1g	185	88.94
	TaSus2-2A	Hap-A	115	Hap-A	115	55.29
				Hap-G	85	40.87
	TaSus1-7A	CTCG	180	CTCC	17	8.17
				CTCG	180	86.54
				TTCC	6	2.88
				TTCG	2	0.96
	TaGS-A1	A1a	166	A1a	166	79.81
				A1b	40	19.23
	TaGW2-6B	CGA	128	CGA	128	61.54
				CGC	80	38.46
	TaGS2-A1	*TaGS2-A1b*	133	*TaGS2-A1a*	61	29.33
				TaGS2-A1b	133	63.94
	TEF-7A	TC	137	CA	4	1.92
				CC	46	22.12
				TA	20	9.62
				TC	137	65.87
	TaGS1a	Hap-II	121	Hap-I	75	36.06

				Hap-II	121	58.17
Drought adaptability	*1fehw3*	Kauz-type	104	Kauz-type	104	50.00
				Westonia-type	99	47.60
	TaCwi-A1	Hap-4A-C	123	Hap-4A-C	123	59.13
				Hap-4A-T	68	32.69
	COMT3B	3Ba	115	3Bb	86	41.35
				3Ba	115	55.29
	TaSST-4D	4Db	113	4Da	80	38.46
				4Db	113	54.33
	Dreb_SNP	C	104	A	86	41.35
				C	104	50.00
Grain color and dormancy	*TaSdr-B1*	TaSdr-B1b	177	*TaSdr-B1a*	23	11.06
				TaSdr-B1b	177	85.10
	Vp1-B1	Vp1-B1c	144	*Vp1-B1a*	62	29.81
				Vp1-B1b	2	0.96
				Vp1-B1c	144	69.23
	PHS1	RioBlanceo type	158	NW97S186 type	38	18.27
				RioBlanceo type	158	75.96
	TaMFT-A1	Zen-type	172	CCG	177	85.00
				TCG	31	15.00
	Tamby10-A1	R-A1a	108	*R-A1a*	108	51.92
				R-A1a(Norin)	2	0.96
				R-A1b	76	36.54
	Tamyb10-B1	*R-B1b*	195	*R-B1a*	12	5.77
				R-B1b	195	93.75
	Tamyb10-D1	*R-D1a*	178	NaN	2	0.96
				R-D1a	178	85.58
				R-D1b	28	13.46
End-use quality	*Glu-A1*	2*	84	1	57	27.40
				2*	84	40.38
				Null	67	32.21
	Glu-D1	5+10	105	2+12	94	45.19
				5+10	105	50.48
	Pina-D1	*Pina-D1b*	170	*Pina-D1a*	32	15.38
				Pina-D1b	170	84.62
	Pinb-D1	*Pinb-D1b*	132	*Pinb-D1a*	58	27.88

				Pinb-D1b	132	63.46
	Pinb2	*Pinb-B2a*	119	*Pinb-B2a*	119	57.21
				Pinb-B2b	83	39.90
	PPO-A1	*Ppo-A1a*	83	*Ppo-A1a*	75	36.06
				Ppo-A1b	40	19.23
	PPO-D1	*Ppo-D1b*	105	*Ppo-D1a*	93	44.71
				Ppo-D1b	105	50.48
	Ppo-B2	B2c	177	B2a,b	24	11.54
				B2c	177	85.10
	PPO-D2	D2b	188	D2a	19	9.13
				D2b	188	90.38
	Psy-A1	*Psy-A1a*	123	*Psy-A1a*	123	59.13
				Psy-A1b	78	37.50
	Psy-D1	*Psy-D1a*	197	*Psy-D1a*	197	94.71
				Psy-D1g	2	0.96
	Wx-B1	*Wx-B1a*	145	*Wx-B1a*	145	69.71
				Wx-B1b	63	30.29
	Pds-B1	*TaPds-B1a*	190	*TaPds-B1a*	190	91.35
				TaPds-B1b	11	5.29
	Pod-A1	*TaPod-A1a*	203	*TaPod-A1a*	203	97.60
				TaPod-A1b	1	0.48
	LYC-B1	*TaLyc-B1b*	132	*TaLyc-B1a*	59	28.37
				TaLyc-B1b	132	63.46
	TaZds-A1	*TaZds-A1a*	112	*TaZds-A1a*	112	53.85
				TaZds-A1b	73	35.10
	WBM	wbm-	170	wbm+	34	16.35
				wbm-	170	81.73
	NAM-A1	*NAM-A1c*	106	*NAM-A1a*	102	49.04
				NAM-A1c	106	50.96
Disease resistance	*Lr34*	Lr34+	113	Lr34+	113	54.33
				Lr34-	90	43.27
	Lr14a	Lr14a-	135	Lr14a	71	34.13
				Lr14a-	135	64.90
	Sr2	Sr2-	201	Sr2+	7	3.37
				Sr2-	201	96.63
	Lr21	Lr21-	198	Lr21+	10	4.81
				Lr21-	198	95.19
	SbmP	Sbmp-	188	Sbmp+	20	9.62

				Sbmp-	188	90.38
	Cre8	Cre8-	146	Cre8+	47	22.60
				Cre8-	146	70.19

[a] Mode: Major allele/allele with the highest frequency

3.3.2.5. End-use Quality-related Genes

Two loci encoding high-molecular-weight glutenins subunits had a relatively high frequency of Ax1 (27.4%) and Ax2* (40.3%) at *Glu-A1* and Dx5+Dx10 (50.4%) at *Glu-D1* which are associated with strong gluten contents and superior bread-making quality attributes. *WBM* is another newly identified bread-making quality gene, however, only 34 (16.3%) of the accessions have this favorable allele. Three major loci underpinning grain texture had a high frequency of alleles associated with hard grain texture at *Pina-D1* (84.6%) and *Pinb-D1* (63.4%), while soft grain texture at *Pinb-B2* (57.2%). Low YPC is a desirable trait and the alleles associated with YPC included *Psy-A1b, Psy-D1a*, and *Zds-A1a* were identified in 37.5%, 94.7% and 53.8% accessions.

3.3.2.6. Biotic Stress Resistance Genes

The frequency of adult plant resistance gene *Lr34/Yr18* and *Sr2* was 54.3% and 3.3% respectively. Similarly, two other leaf rust resistance genes *Lr14a* and *Lr21* were observed in 34.1% and 4.8% accessions, respectively. The alleles associated with virus resistance, *SbmP*, and soil born disease, *Cre8*, were observed in 9.6% and 22.6% of the accessions of diversity panel, respectively (Table 3.8, Figure 3.7)

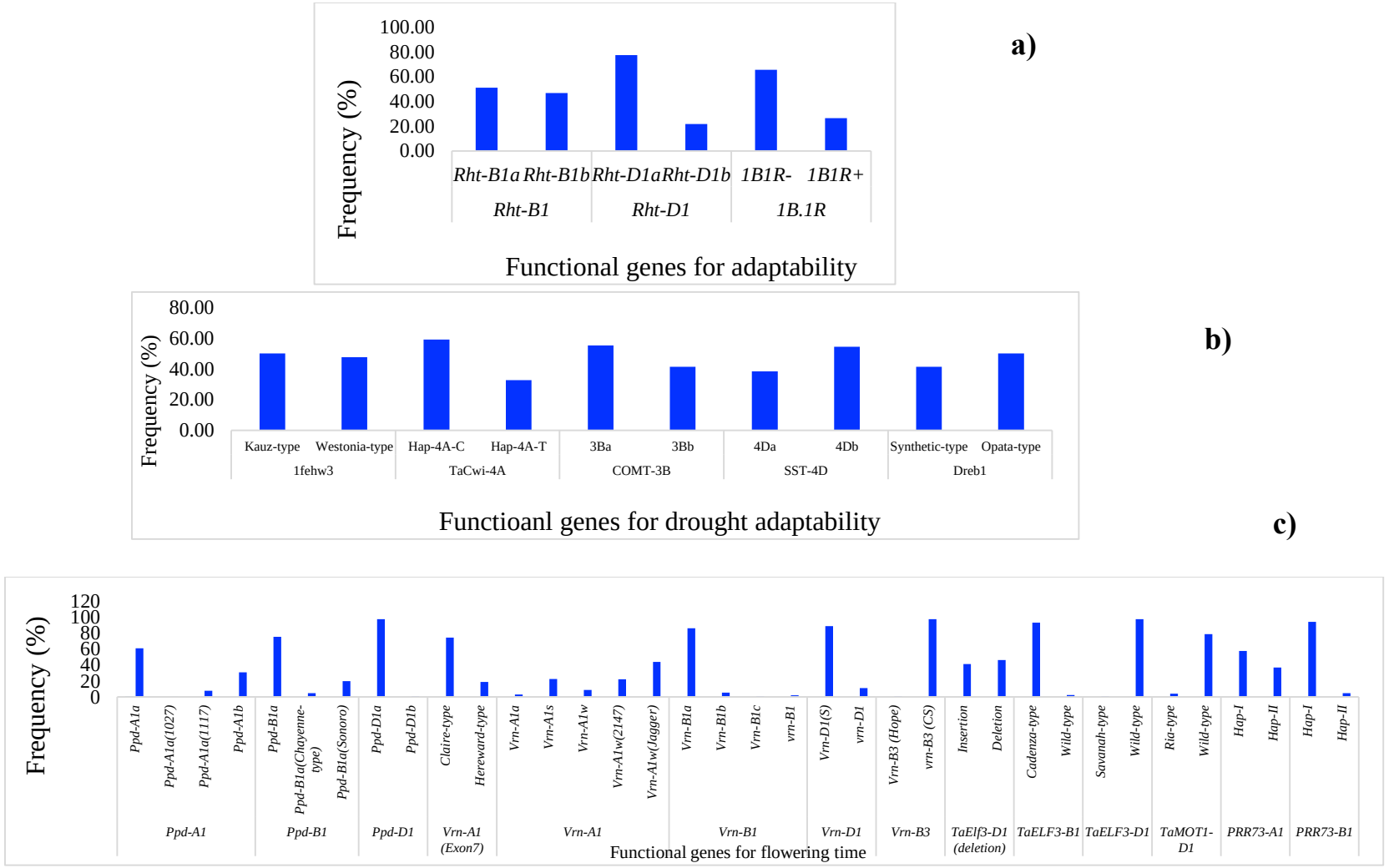


Fig 3.7: Allelic frequency of functional genes in diversity panel: a) Functional genes for adaptability; b) Functional genes for drought adaptability; c) Functional genes for flowering time

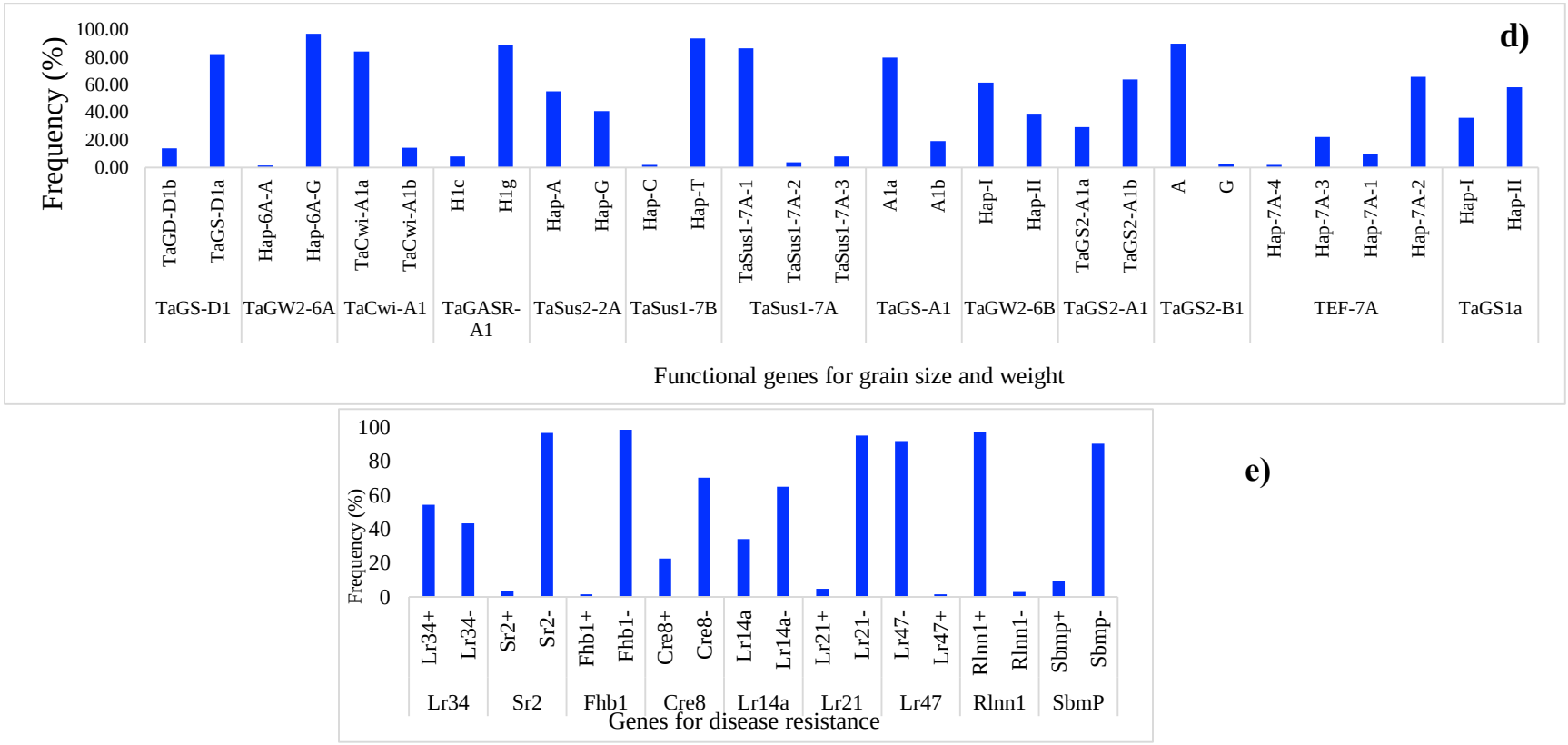


Fig 3.7: Allelic frequency of functional genes in diversity panel; d) Functional genes for grain size and weight e) functional genes for disease resistance

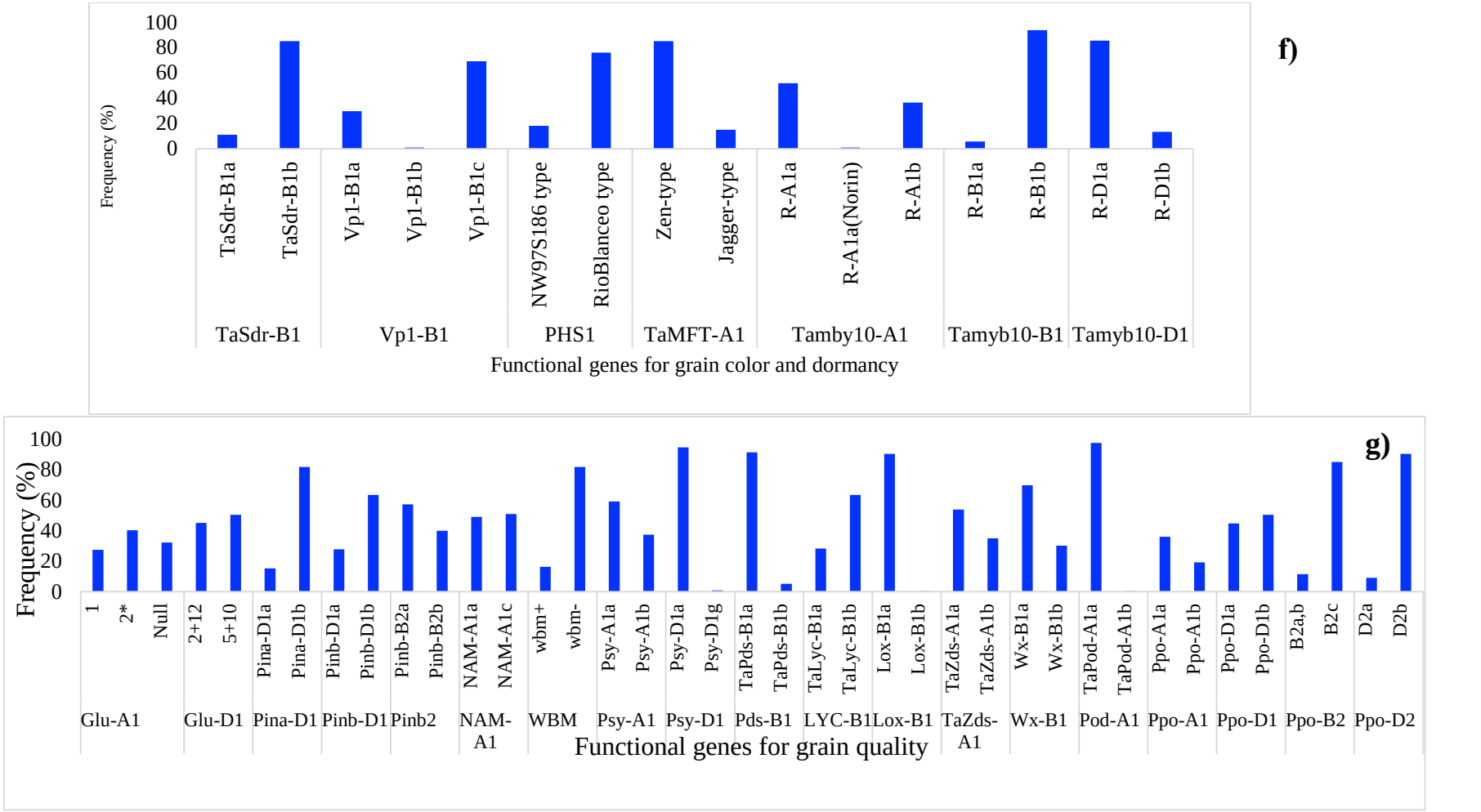


Fig 3.7: Allelic frequency of functional genes in diversity panel; f) allelic frequency of functional genes for grain color and dormancy; g) allelic frequency of functional genes for grain quality

3.3.3. Genetic Diversity in Diversity Panel based on Functional Genes

Genetic diversity was estimated in the diversity panel based on the functional marker data (Figure 3.8a). The accessions were categorized into synthetic-derivatives (those having *Ae. squarrosa* in their pedigree) and bread wheat advanced lines. The first two principal components explained 8.8 and 6.3% of the total variability, respectively. Most of the bread wheat accessions were separated on the PC2, and some were admixture within SYN-DER clusters. The phylogenetic tree corroborated the PCA analysis (Figure 3.8b).

3.3.4. Allelic Effects of Functional Genes in SYN-DER

All the KASP assays showing minor allele frequency >5% were used for marker-trait associations (MTAs) in diversity panel using agronomic and biochemical traits under WW and WL conditions. To avoid false associations, population structure matrix based on 100 unlinked SNP markers was used as co-variate (Fakiha Afzal, personal communication). However, a relaxed criterion based on $p<0.05$ was used to declare MTAs. Based on this criterion, 132 MTAs were observed, out of which 51 were associated with traits under WL conditions, 96 were associated with traits under WW conditions and 21 were associated across WW and WL conditions (Table 3.9, Figures 3.9, 3.10, 3.11). However, under the stricter criterion of $p<0.01$, the number of MTAs reduced to 26 under WL, 39 in WW and 5 across both water conditions. These include COMT-3B associated with soluble sugars, *TaCwi-A1* with PH, and *Rht-D1*, *TaGS1a, Ppd-B1* and *Vrn-A1* with TGW.

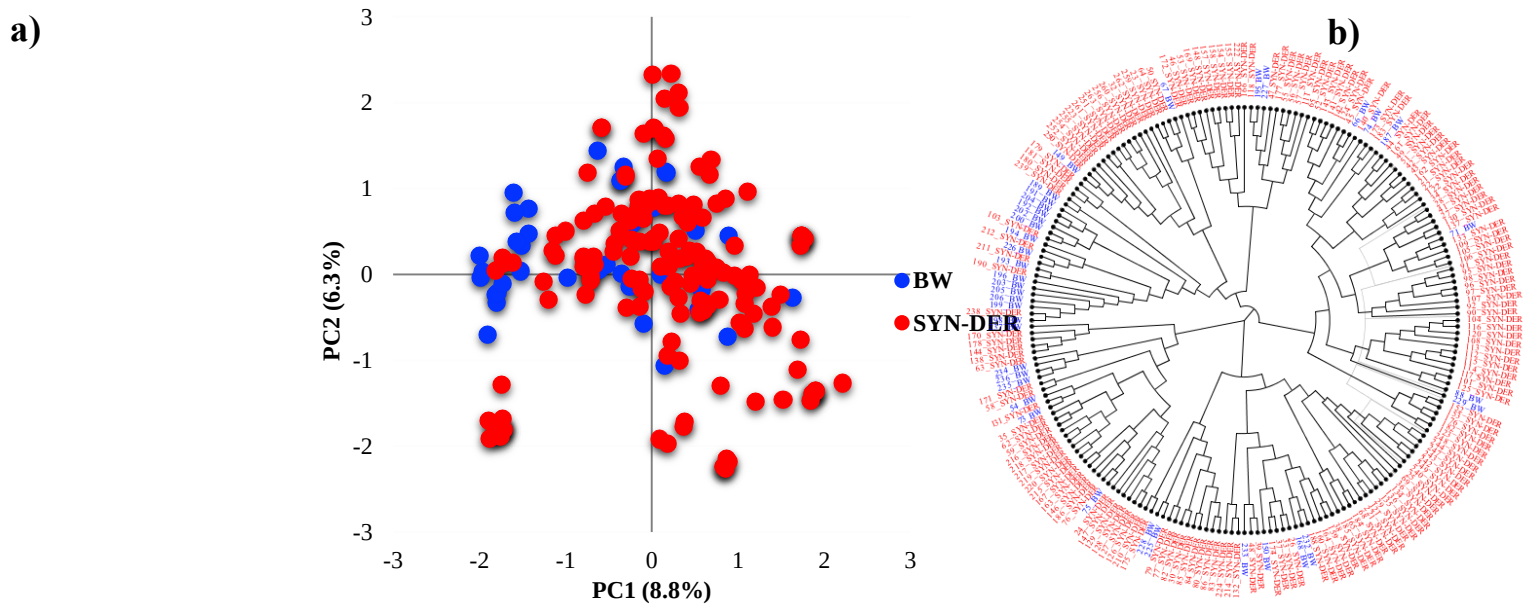


Figure 3.8: a) Genome-wide SNP marker density on each bread wheat chromosome b) phylogenetic analysis of diversity panel including bread wheat (red) and SYN-DER (blue) genotypes.

Five KASP assays for *Ppd1* homeologous genes were significantly associated with days to emergence (DEM), days to heading (DH), grain yield (GY), relative water content (RWC), spike length (SL) and thousand-grain weight (TGW). For *Ppd-A1* gene, GS105-type *Ppd-A1a* was associated with SL under WL condition and GY and spikelets per spike (SpPS) under WW conditions. The paralogue of *Ppd1* gene, *PRR73A1* was associated with DH and DM under WL conditions, GpS under WW condition, and tiller numbers (TN) under both conditions (Table 3.9). The newly identified *Elf3-D1* and *TaMOT-D1* genes were associated with DEM, DH, DM and HI under WL condition, while *TaMOT-D1* was also associated with DH and DM under WL condition. The KASP assays for *TaSus1-7A* were associated with TGW and GY under WW condition, and SL under both conditions (Figure 3.7). Wheat cell wall invertase gene, *TaCwi-4A* was associated with DH and TGW under both water conditions, and GpS, PH, and proline under WW conditions. Drought-related gene such as *1fehw3* was associated with GY, PH, and SL under WW conditions. *Dreb1* was associated with CT leaf and GpS under WW condition and HI under WL condition. However, surprisingly 1BL.1RS representing wheat-rye translocation was associated with GpS, GY, SL, SOD, SpPS, and TGW under WW condition, however, no MTA was identified under WL conditions. *Rht-B1* was associated with GY, PH, and SL under WW condition and GpS under both water conditions. *Rht-D1* was associated with CT, GpS, RWC and SOD under WW conditions, DEM and EL under WL conditions, and GY, PH and TGW under both water conditions. The KASP assay for *TaSST-4D* developed in this study was associated with biomass (BM) and TGW under WL conditions.

Table 3.9: Marker-trait associations in diversity panel derived from synthetic hexaploid wheat under well-watered and water-limited conditions

				Well-watered			**Water-limited**	
Trait	**Marker**	**MAF%**	**P**	**R2**	**Estimate**	**P**	**R2**	**Estimate**
DH	*TaCwi-4A*	34	0.03287	0.02212	-9.20E-01	1.32E-04	0.06932	11.1087
DH	*Vrn-A1*	2.88	0.04634	0.01932	0.99058	4.06E-04	0.0596	-11.918
GpS	*Rht-B1*	46.63	0.0367	0.02122	3.60554	0.02302	0.02507	-1.4765
GY	*Vrn-A1*	2.88	0.01673	0.02774	16.48309	0.0064	0.03536	1.30053
GY	*TaGASR-A1*	8.17	0.03661	0.02093	-6.13E-01	0.01286	0.02952	-1.5195
GY	*Rht-D1*	21.63	0.0398	0.02026	0.47975	0.02049	0.02604	16.11085
PH	*TaCwi-A1*	14.42	0.00139	0.04897	-3.65E+00	0.03649	0.02096	-3.7085
PH	*Rht-D1*	21.63	0.01849	0.0269	-2.37E+00	7.23E-04	0.05462	4.59233
PH	*TaSST-4D*	38.46	0.01628	0.02756	2.99226	0.00585	0.03611	4.07361
SL	*TaSus1-7A*	8.17	0.03354	0.02196	-5.53E-01	0.02516	0.02433	-1.0799
SOD	*TaSus1-7B*	6.25	0.01563	0.0279	-1.02E+00	0.0051	0.03728	-1.6676
Soluble Sugar	*TaCwi-A1*	32.69	0.03978	0.02026	33.00629	0.00342	0.04065	24.98329
Soluble Sugar	*COMT-3B*	41.95	0.00952	0.03203	-2.96E+01	0.03399	0.02153	-12.956
TGW	*Vrn-A1*	2.88	2.32E-04	0.06353	4.12727	4.41E-04	0.05889	3.98449
TGW	*Rht-D1*	21.63	0.00539	0.0368	3.19611	8.32E-04	0.05341	3.8277
TGW	*TaGS1a*		0.00264	0.04346	-2.67E+00	5.07E-04	0.05767	-3.2395
TGW	*TaCwi-A1*		0.03511	0.02127	3.04749	0.00553	0.03712	3.69649
TGW	*TaSST-4D*		0.01654	0.02743	-2.22E+00	0.02073	0.02594	-2.7171
TGW	*Ppd-B1*		0.00573	0.03629	-3.20E+00	0.02722	0.02368	-2.4347
TGW	*TaCwi-4A*		0.01502	0.02823	-2.39E+00	0.02869	0.02325	-2.1635
TN	*PRR73-A1*		0.02115	0.02541	0.38799	0.0115	0.0309	-0.77298

MAF: Minor allele frequency; P: P-values; R2: Phenotypic variation explained by marker; Estimate: Allelic effect of minor allele, DH, days to heading; GpS, grains per spike; GY, grain yield; PH, plant height, SL, spike length; TGW, thousand grain weight; TN, tiller number; SOD, superoxide dismutase

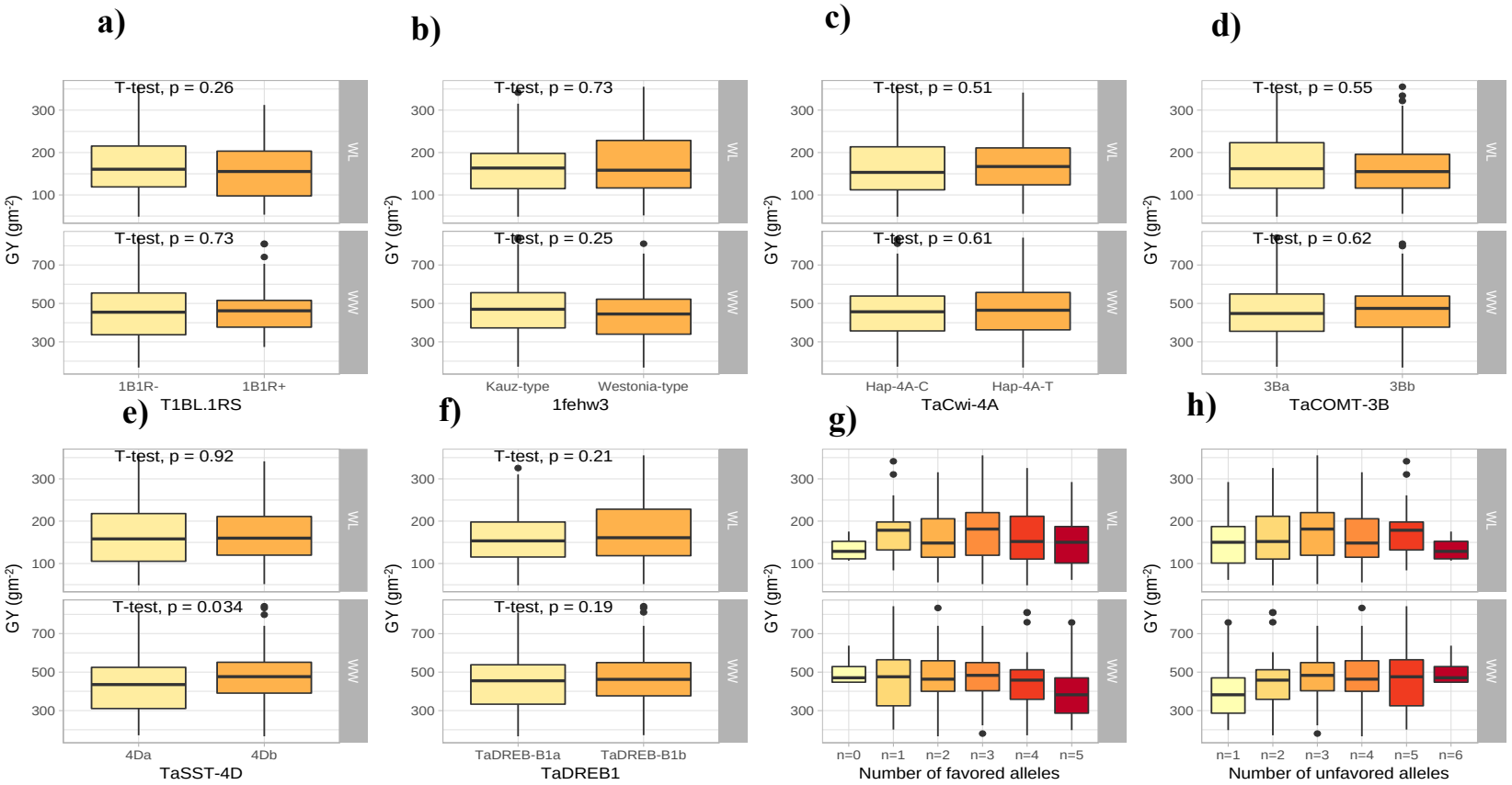


Figure 3.9: Allelic effects of SNPs on grain yield (GY) across two water regimes, a) T1BL.1RS; b) 1fehw3; c) TaCwi-4A; d) TaCOMT-3B; e) TaSST-4D;f) TaDREB1; g) number of favored alleles; h) number of un-favored alleles (all information is annotated).

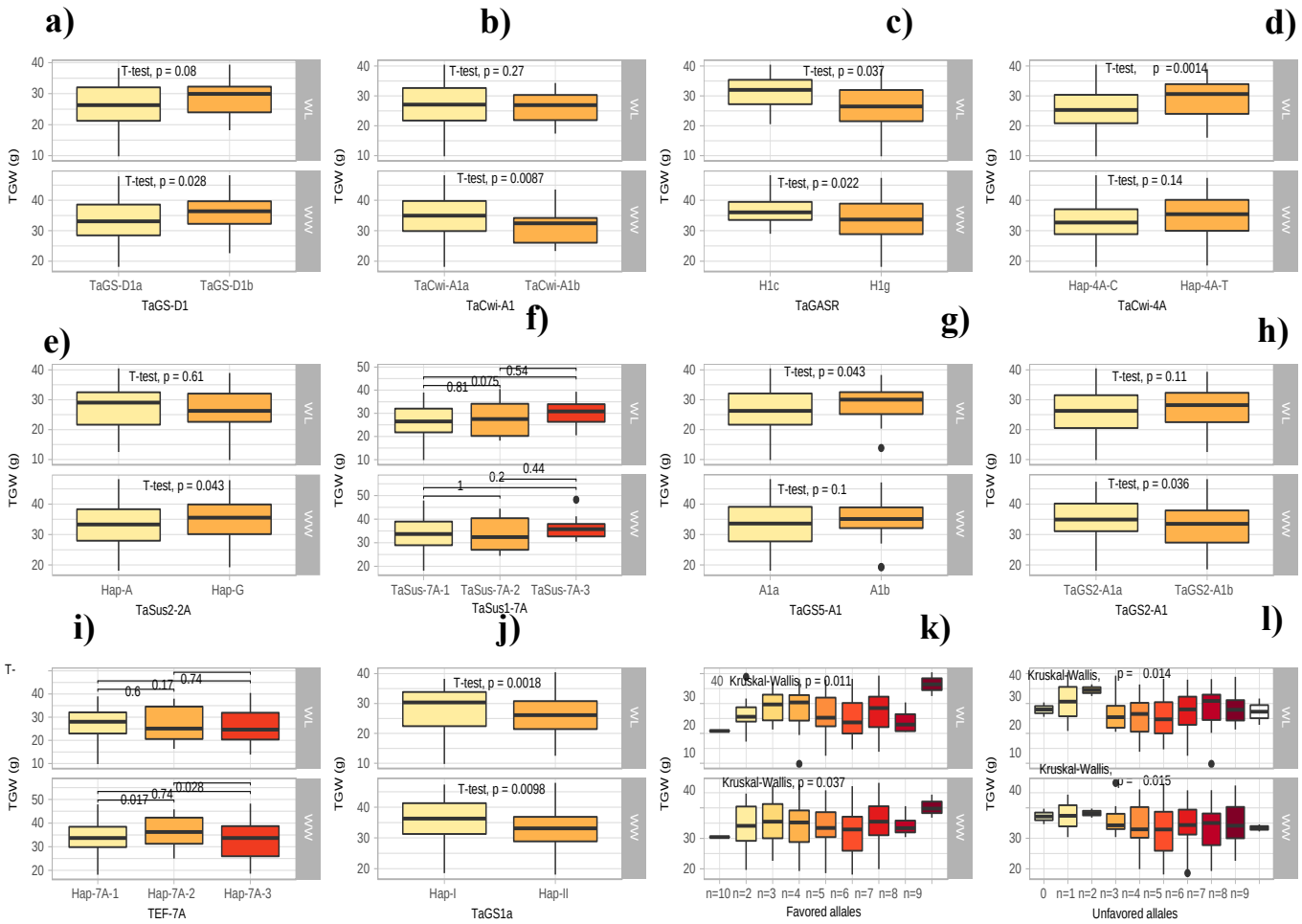


Figure 3.10: Allelic effects of SNPs on thousand grain weight (TGW) across two water regimes, a) TaGS-D1; b) TaCwi-A1; c) TaGASR; d) TaCwi-4A; e) TaSus-2A; f) TaSus-7A; g) TaGS5-A1; h) TaGS2-A1; i) TEF-7A; j) TaGS1a; k) favored alleles; l) un-favored alleles (all information is annotated).

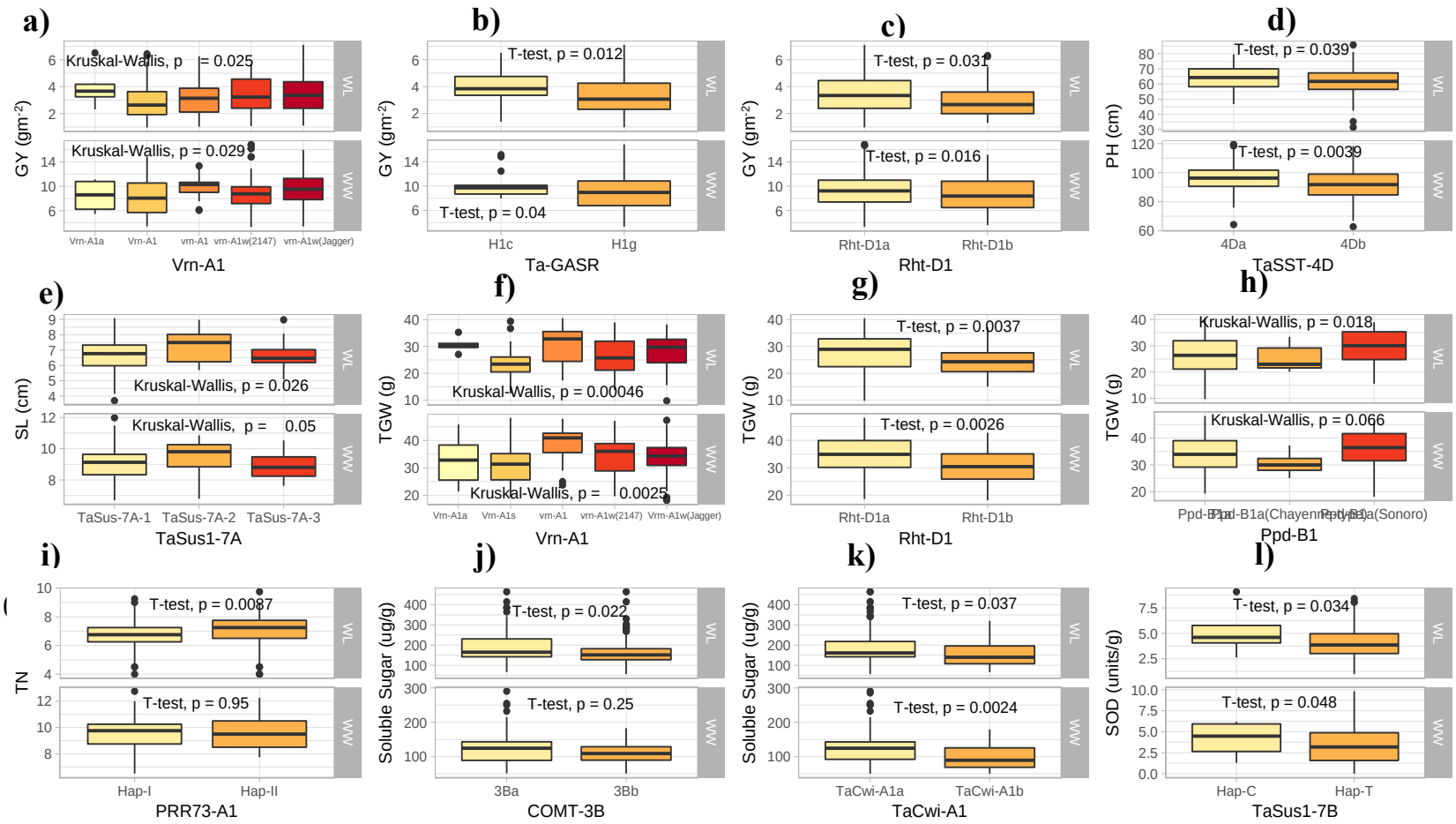


Figure 3.11: Allelic effects of SNPs on grain yield (GY), thousand grain weight (TGW), spike length (SL), tiller number (TN), soluble sugar and Superoxide dismutase (SOD) across two water regimes; a) Vrn-A1; b) Ta-GASR; c) Rht-D1; d) TaSST-4D; e) TaSus1-7A; f) Vrn-A1; g) Rht-D1; h) Ppd-B1; i) PRR73-A1; j) COMT-3B; k) TaCwi-A1; l) TaSus1-7B (all information is annotated).

4. DISCUSSION

One of the potential threats for global food security is climate change. Variable and ever-increasing temperatures and drought spells are impacting weather patterns around the globe. Under such threatening weather conditions, food systems are continuously under stress. Many vulnerable areas around the world for food insecurity are already facing severe climatic changes leaving large populations malnourished (Godfray *et al.*, 2010). Challenges related to food security is one of the greatest concerns of our time. The significant effect on farmers' purchasing power and lack of quality food lead to malnourished population. Several problems related to food security have been addressed in the recent years and substantial improvements are also noticed. However, various surveys indicate that two billion people still suffer from macronutrient deficiencies, leading to underweight children with stunted growth. Furthermore, future prediction indicates that this pressure will continuously increase on global food system. For example, a 50% increase in the current food demand is expected by 2030 due to global population increase. All these factors demand sustainable intensification of agricultural systems (Moss *et al.*, 2010).

Various risk factors are inter-related to climatic change and food security causing uncertainties to societies and ecologies. According to the United Nation (UN) Food and Agriculture Organization (FAO), food security is illustrated as 1) Availability of sufficient quantities of food of appropriate quality supplied through domestic production or imports. II) Access of individuals to appropriate quality and quantity of food for a nutritious diet.

III) Utilization of this available food and clean water supplies with appropriate sanitary measures and health care in order to fulfill physiological needs. IV) Stability in the reasonable supply of quality and quantity of food for sustainable livelihood (White *et al.,* 2011). According to FAO standards of food security, around two billion people, which is more than 25% of the global population is food insecure because it falls short of one or several FAO's illustrations of food security. This holds particularly true for countries situated in Sub-Saharan Africa and South Asia (Rotter *et al.,* 2011). According to the global distribution of hunger as quantified by 2012 global index, India and Pakistan are particularly scaled as 'alarming' and 'serious' among the South Asian countries.

Carbon dioxide in the atmosphere has increased from about 284 ppm in 1832 to 397 ppm in 2013. Greenhouse gases like carbon dioxide is a major cause of global warming. It is mainly produced by the burning of fossil fuels extensively utilized by human population as a non-renewable energy source. Thus, climate change is expected to bring changes in temperature, rainfall frequency and patterns and drought. It is predicted that by the end of this century, the global mean temperature could rise to 1.8- ^{o}C as compared to the previous century (Meehl *et al*., 2007). South Asia is particularly affected by extreme weather conditions particularly drought stress. Due to ever-increasing population and climate change, drought effect is mounting and reducing the global productivity of wheat. Various efforts have been made to mitigate its effect, however, drought is a complex quantitative trait that is dependent on the environment as well.

Wheat has a large and complex genome. Several breeding efforts have been made to make drought-tolerant varieties. However, continuous selection for desired traits in the breeding programs leads to a decrease in genetic diversity, which increases the chances of diseases affecting crop yield and quality (Asseng *et al*., 2013). There are many reverse genetic approaches to find the importance and functions of genes for wheat improvement. In addition, various forward genetic approaches can also be utilized for the identification and characterization of genes. One of those is mutation analysis of genes under stress conditions. We have analyzed the mutation analysis of cell wall invertase (*CWI*) gene in synthetic/bread wheat derivatives in this study.

4.1 Mutations in Wheat (*TaCwi-B1*) underpin Drought Tolerance in Synthetic/Bread Wheat Derivatives

The plant cell undergoes various genetic or environmental changes, which can affect the process of cell division. Some of the changes are repaired by plants internally, those, which are not repaired are termed as variations or mutations. In advanced genomics study, the mutation is defined as a change in DNA sequence, which damages the structure and function of gene. Most of the mutations occur randomly as a result of some changes emerging in a plant cell but a few are stimulated by environmental factors such as abiotic or biotic stresses (Livanos *et al*., 2012). The spontaneous mutation can be triggered either by deamination which is the conversion of Cytosine (C) to Uracil (U) in DNA or by copy number error during DNA replication. If the mutation occurs in somatic cell, it isn't transferred to the next generation but if it occurs in the reproductive cells, it is transferred to the next generation and is termed as lethal mutations (Lipka *et al*., 2015). Gene mutations either causes a change in a single base pair or results in deletion or insertion of a segment

of base pairs at certain residues. The switching of one base pair with another one is termed as a point mutation. The point mutation can be synonymous if the mutation is not disrupting the protein sequence and non-synonymous if the variation is altering the protein sequence. Similar protein sequences often project similarity in the structure and function of the protein. This leads to pairwise alignment if the comparison is between two proteins and multiple sequence alignment if the sequences are exceeding more than two. Further, if the sequences are showing similarity this will facilitate in finding the structure-function relationship. In proteins, usually, the structurally and functionally important sites are confined into domains. Since a domain reflects the highly similar regions in protein sequence, it results in the clear identification of the structure and function of protein (Chen *et al*., 2016).

Non-synonymous mutations have a greater impact as the missing, replaced or deleted nucleotide results in frameshift mutation, which disrupts the whole reading frame of the amino acid by altering the codons, resulting in a different protein. The high risk associated with non-synonymous mutations is dependent on the location of the mutation. For instance, if it's occurring in the beginning of the sequence, it can modify the whole protein resulting in lethal mutation. Missense variants have the ability to alter protein stability, protein-protein interactions and also protein folding. The bioinformatics tools take the amino acid sequence and predict the effect of variants by analyzing the homologous sequences. In the field of proteomics and genomics, amino acid variation affecting the protein stability is the major obstacle faced by the biologists. Many computational efforts have been reported to analyze these variations. Filtering the high-risk mutations from the large datasets is a

challenging task. Several *in-silico* tools are available that facilitates categorizing the missense variants with the neutral ones. These tools utilize the sequence features like evolutionary conservation of amino acid residues, the physiochemical descriptors, the disordered regions and structural features including the 3D structure of the protein, protein stability changes and protein folding (Seifi *et al*., 2018). Among all, the missense variants affecting the protein stability are in the limelight of current research. Accurately predicting protein stability changes serves as a valuable tool in understanding the protein structure and function relationship as protein stability has a direct connection with the improper functioning of the protein. The missense variant affects the conformational changes of the proteins, often leading to protein misfolding. This results in either improper functioning of protein or total loss of its function (Steinbrecher *et al*., 2016).

There are various drought tolerance genes in wheat such as *DREB*, *CWI* and *1-fehw3*, flowering genes such as photoperiod genes (*Ppd*), vernalization genes (*VRN1*), early flowering genes (*ELF3*) and grain yield-related genes (*TaGS1, TaGW2*), which play an important role in improving growth and development. As wheat is hexaploid in nature, a very high similarity among wheat homologs mask the effect of recessive alleles (Uauy, 2017). Such limitations reduce the chances of selecting favorable mutation in a natural population (Krasileva *et al*., 2017). Wheat genomes have a high level of sequence identity among sub-genomes as it masks the recessive allele effect. This results in a reduction in the probability of selecting the favorable mutations during natural selection. The non-synonymous missense mutations occurring in the coding region of the genome are the most significant genetic variations (Krasileva *et al*., 2017). In this study, cell wall invertase

enzyme was selected for carrying out the mutation analysis as it has an important role in sugar transportation from source to sink in a different form (sucrose) to get utilized by plants in a different form (glucose) (Sherson *et al*., 2003). Invertase is a hydrolase enzyme that converts sucrose into fructose and glucose. Cell wall invertase is localized in cell wall, which plays an important role in catalyzing sucrose, carbon partitioning, pollen development and sink development (Jiang *et al*., 2014). Although we only identified haplotypes and allelic distribution in *TaCwi-A1*, polymorphism may exist within *TaCwi-D1* in synthetic derivative as it has been shown in recombinant inbred lines RILs (Rasheed *et al*., 2016). It is also reported that by constitutive expression of *CWI* driven by cauliflower mosaic virus (CaMV) 35 S promoter, the grain yield was increased up to 145.3% as compared to wild-type in maize (Li *et al*., 2013). In this study, drought stress was imposed and agronomic traits were observed in genotypes in comparison with control conditions. These findings are in accordance with Jiang et al (2014).

WECPDF is the most conserved domain in *TaCwi-B1* genome (Webster *et al*., 2012). The multiple alignments both at the nucleotide level and protein level of B-genome sequences of SYN-DERs was carried out using bioinformatics tool CLUSTAL X. The results showed few non-synonymous substitutions at certain residues. These variants were further subjected to structure and function analysis by exploiting both sequence and structure derived features. Most of the deleterious mutations were observed in the conserved domain WECPDF starting from position 370 to 376, whereas a few mutations were confined in the neighborhood of this domain. For the mutation, Glu372Gly wild-type and mutant residue differ in size as Glycine is smaller in size than Glutamic acid. Further,

the Glycine is a flexible amino acid hence can disturb the rigidity of the protein at position 372. The effect of the mutations was also evaluated on the contacts made by the mutant. The wild-type residue makes a hydrogen bond and salt bridge with Lysine at position 318. As the residue gets mutated into Glycine, it affects hydrogen bond formation and ionic interactions made by the wild-type. For the second mutation Pro374Gln, the mutant residue is bigger in size than the wild-type. The hydrophobicity measure is also different in the two residues. The wild-type residue is buried in the core of the protein as identified in the structure. As the size of mutant residue is big, it can disrupt the structure and function of protein.

In the mutation Asp375Thr, the mutated amino acid is smaller in size and has a neutral charge than the wild-type which is negatively charged. As the wild-type residue forms a salt bridge with Lysine at position 411, the difference in charge introduced by mutation will disturb the ionic interaction. Loss of charge after mutation will also disturb the interactions with other residues in other domains. The mutation Phe376Leu causes an empty space in the core of the protein as in this case a large-sized amino acid is mutated into a smaller size with the difference in hydrophobicity measures. In the case of Ala380Val, Valine is bigger in size than Alanine. The wild-type residue is located on the surface of the protein hence it can clearly disrupt protein-protein interactions on the local and global level. The mutations Arg108Thr, Tyr377Thr, and Val379Cys are expected to disrupt the interactions of the protein as in both cases the mutated amino acids are smaller in size and will cause conformational changes in the protein. In Arg108Thr the mutation introduces the hydrophobic residue which can lead to the loss of hydrogen bond and disrupt

the protein folding. The mutation was predicted as neutral by PROVEAN but it is decreasing the stability of the protein as predicted. Hence this substitution needs further verification in order to define it as a high-risk mutation.

Taken together all the results, it has been inferred that all high-risk SNPs identified in this study were either affecting the folding of the protein or disrupting the stability of the folded protein. For a protein to perform its function properly, it has to attain proper conformation at first. The destabilization of the native structure will result in loss of function in most cases. Hence, in this case, mutated *TaCwi-B1*would no longer be able to perform its function. Up to the best of our knowledge, no study has ever been conducted previously that identifies the missense mutations in *TaCwi* gene, crucial for the structure and function of protein. Furthermore, phylogenetic analysis revealed that these proteins have a high level of conservation during evolution and performed similar functions. Hence, further phenotypic experiments and expression analysis experiments can reveal the association of this mutation with drought and explore susceptibility and resistance of water stress.

4.2 Genetic Mapping in Linkage Population

The field of genetics has developed exponentially from the 1950s to the present era started with the discovery of the double helix of DNA to whole genome sequencing by passing through various milestones. As a result, the understanding of genetics has been extended from molecular to functional genomic level. This progress has led to the

development of different sequencing techniques, screening methods, gene discovery for the selection of superior genotypes and incorporating genes to make superior genotypes as well as improved breeding strategies. Genetic mapping is one of the examples of progress in molecular studies to determine the position of QTL into the relative position of a chromosome which can lead to gene discovery (Semagn *et al*., 2007).

Genetic mapping is also known as 'linkage mapping' or 'meiotic mapping' that determines various positions of QTLs and measure distances between them along a chromosome. During meiosis, chromosomes assort into gametes following Mendel's second law of inheritance '*Segregation of alleles of one gene is independent of another allele*'. But, this law is true for the genes that are located on different chromosomes. When genes are close to each other on the same chromosome, the law of independence does not hold true. These genes are linked to each other and transmitted from parents to progeny together. When the genes are located on different chromosomes, there is a 50% chance that they can crossover with each other during meiosis but, when they are on the same chromosome, there are less than 50% chances of crossing over. Crossing over between two genes on the same chromosome depends on the distance between two genes (Bartholomé *et al*., 2016).

QTL mapping is the correlation of markers and traits in a mapping population. The agronomic and yield-related traits are complex traits that are controlled by many genes. These traits are known as quantitative traits or multifactorial traits. The loci or region containing genes, linked to particular traits is known as quantitative trait loci. The

identification and characterization of quantitative trait loci take place with the help of molecular markers. The molecular markers are utilized for the construction of genetic maps. Genetic map construction requires molecular markers to identify chromosomal regions containing genes that control particular quantitative trait. This process is known as QTL mapping or genetic mapping (Collard *et al.,* 2005). Genetic mapping can be done by selecting those molecular markers that are linked with a particular phenotypic trait. These markers can then be utilized for marker-assisted selection (MAS). Marker-assisted selection can be done on the basis of absence or presence of marker for selecting particular phenotypic trait.

Molecular markers are nucleotide sequence differences among individuals. Molecular markers are located at the close proximity of genes and act as a sequence tags. These markers can be insertion-deletion or point mutation. The markers have improved conventional plant breeding efficiency. These markers can be dominant or codominant. Dominant markers are those that differentiate between homozygous and heterozygous individuals. Whereas, co-dominant markers may have different alleles (Collard *et al.,* 2005). There are several molecular markers that have been developed and utilized in plant breeding programs. With the advancement of genomic studies, single nucleotide polymorphism (SNPs) are nowadays being utilized to score variations among genotypes. Genotyping by sequencing is the advanced next-generation sequence technology that generates a large number of SNPs for genotyping and genetic analysis. The linkage analysis is then performed to find the recombination frequencies, linkage groups, and map distances by using different statistical programs.

Mapping population can be made by selecting two divergent genotypes for one trait of interest in order to make the population genetically divergent. The minimum size of population for genetic mapping ranges from 50 to 250 genotypes. For QTL mapping, it is necessary that population should be phenotypically evaluated (Collard *et al.,* 2005). Backcross (BC), recombinant inbred lines (RILs), double haploid (DHs) and near-isogenic lines (NILs) are used to make mapping populations in self- pollinated crop. The main advantage of these population is that they are easy to construct. The disadvantage of RILs populations is that it requires six to eight generations but the main advantage of RILs and DHs population is that they produce homozygous lines. Many studies have been conducted in wheat for drought stress and a large amount of QTLs have already been reported for different traits. Sorrells *et al* (2011) developed the RILs and DHs wheat population known as ITMI population, which is used as a reference for the genetic mapping. In this study, the genome-wide linkage analysis of ITMI population was analyzed at early seedling stage under well-watered and water-limited conditions.

4.2.1. Phenotypic Variation in ITMI Population

There are various methods to evaluate morphological traits for genetic analysis under water-limited conditions (Iannucci *et al*., 2017). In genome-wide linkage mapping study, ITMI population showed transgressive segregation for root length (RL) and dry root weight (DRW) as compared to other traits under well-watered and water-limited conditions. The reason for extreme phenotypes for root traits might be the loss of turgidity, small leaf size and reduced transpiration (Bibi *et al*., 2012). Phenotypic results in this study were in agreement with (Ayalew *et al.*, 2017). In addition, low heritability has been found in all

traits under water-limited conditions. This might be because drought adaptability is a complex genetic trait. Higher dry root weight has been reported if drought stress is given at seedling stage. This can be utilized as a selection criterion for drought tolerance at seedling stage (Bibi *et al.*, 2012). Hence, phenotypic variation in this population indicates that valuable alleles might be present for all these traits which can be used for drought tolerance.

4.2.2. QTL identified for Seedling Traits in ITMI Mapping Population

There are many studies reported for association mapping and linkage mapping in bread wheat for drought tolerance (Liu *et al*., 2005). These studies have identified chromosomal regions related to drought tolerance that have shown phenotypic variability. Moreover, these mapping studies have identified new haplotypes, which can help in identifying genes for drought tolerance (Iannucci *et al*., 2017). High marker density is important for efficient genotyping technologies. Besides, cost efficiency is also important for generating genotyping data (Beissinger *et al*., 2013). Sequence-based approaches based on SNP include next-generation sequencing technologies (NGS) useful for gene discovery such as GBS (Rasheed *et al*., 2017). ITMI population is a valuable genetic resource to discover new drought-related genes. In this study, the GBS platform was used that included 2639 SNPs for QTL, which facilitated the development of the high-density map. Total 16 QTLs were distributed on nine chromosomes for all traits under well-watered and water-limited conditions except DRW. Phenotypic variation for these QTLs ranged from 4.7- 59%. Out of these, five QTLs explained more than 10% phenotypic variation and were considered as major QTLs. Among these five QTLs, *QFSW.nust-7B* explained the highest phenotypic

variation (59%) that may harbor an important gene. Seven identified QTLs have already been reported in previous studies (Ayalew *et al*., 2017; Czyczyło-Mysza *et al*., 2014; Kabir *et al*., 2015; Luo *et al*., 2016; Ma *et al*., 2012; Ogbonnaya *et al*., 2008) by using different types of markers for QTL mapping. In the present study, some new QTLs are also identified on the same ITMI population.

4.2.3. QTL Identification under Stress Conditions

In the present study, QTL identified for RL on 3A chromosome under stress condition was in agreement with Ayalew *et al.* (2017). In addition to that, QTLs on chromosome 4D and 7B were also reported in the previous studies as *QRlengthPC.csdh-4D, QRL.WL.7B.1, and QRL.WL.7B.2*. However, *QRlengthPC.csdh-4D* and *QRL.WL.7B.2* were identified under controlled conditions whereas *QRL.WL.7B.1* was reported under both conditions (Czyczyło-Mysza *et al*., 2014; Zhang *et al.* 2013). In the case of SL_{WL}, *QSL.nust-4A* identified on chromosome 4A under stress condition was reported in the previous study as Zhang *et al.* (2013). To the best of our knowledge, all the remaining QTLs identified in the current study have not been reported before and are novel QTLs. Similarly, QTL for DSW_{WL} (*QDSW.nust-3D*) is also novel.

4.2.4. QTL Identification under Control Conditions

Under well-watered conditions, four QTLs were identified on 3B, 7A, and 7B (2) chromosomes for FRW. QTL on 3B was previously reported as *QRFW.WL.3B* (Zhang *et al.*, 2013). Similarly, QTLs on chromosomes 6D and 7B for FSW have also been

previously reported as *QPFWR-WJ-6D* on chromosome 6D (Xin *et al.,* 2017) and *QSFW.WY.7B* on chromosome 7B (Zhang *et al.,* 2013). There were many QTL clusters reported in the previous studies (Kabir *et al*., 2015; Luo *et al*., 2016; Ma *et al*., 2012). In this study, one co-located QTL cluster was identified on chromosome 7B. These clusters were identified for SL (3), FRW (2), RL, and FSW. As *QFSW.nust-7B* has high phenotypic variance, it may harbor a major gene for shoot biomass (Zhang *et al*., 2013). Co-localization indicated the closer association of traits that are highly correlated. These traits may be controlled by the same gene. The positive alleles of these traits are from synthetic parent W7985 which could be traced back to its durum parent i.e., 'Altar 84'.

4.3 Allelic Effects of Major Genes Controlling Developmental Traits and Drought Tolerance in a Diversity Panel Derived from Synthetic/Bread Wheat Derivatives

Scientists are trying to regain the genetic diversity through various conventional and non-conventional methods. CIMMYT made synthetic wheats by hybridization of durum wheat with *Aegilops tauschii*. Synthetic hexaploid wheat is a diverse population with high genetic diversity to improve traits like yield and biotic and abiotic stress resistance and tolerance.

Molecular genetic data was usually obtained from neutral markers such as SSR, RFLP, AFLP, and DArT. A large number of previous studies are based on neutral molecular markers. These research studies have, however, provided partial information on genetic diversity, taxonomic designation, and evolutionary potential. However, recent sequencing techniques i.e., next-generation sequencing and various genomic studies are improving

wheat by providing the information to develop non-neutral markers also known as functional markers (Bassi *et al.*,2016). These markers can directly influence the fitness and overcome inbreeding depression (Kirk *et al*., 2011). High throughput genotyping assays like Kompetitive allele-specific PCR (KASP) for genomic studies have made it possible to genotype large populations at numerous loci within a very short time (Rasheed *et al*., 2016). In this study, our objective was to investigate the allelic variation of functional genes in SYN-DER panel to underpin developmental, agronomic, drought tolerance and grain yield-related genes.

4.3.1. Allelic Effects of KASP assays for Developmental Traits

In the current study, use of high-throughput KASP markers for functional loci in wheat provided valuable insight into the genetic architecture of synthetic-derived wheat. This also enabled us to identify the favorable and unfavorable alleles underpinning important breeding traits that are exhaustively selected. It had been challenging to practice molecular breeding in wheat despite the discovery and knowledge of a huge array of functional genes for a range of important breeding traits in wheat (Liu *et al.*, 2012b). It was largely due to the absence of a high-throughput genotyping platform that can align with the breeding program without compromising flexibility (Rasheed *et al.*, 2017). Several recent studies have used KASP markers to identify the allelic variation of functional genes in wheat cultivars from China (Rasheed *et al.*, 2016b), US (Grogan *et al.*, 2016) and Canada (Perez-Lara *et al.*, 2017).

Flowering time is one of the most important developmental traits for wheat adaptability in diverse environments. It is controlled by vernalization (*VRN1*), photoperiod (*Ppd1*) and early flowering (*Elf3*) genes. Vernalization is the main flowering signal, which remains repressed until it is cold-induced (Dubcovsky *et al.,* 2006). It is repressed by the dominant allele of *VRN1,* which results in spring growth habit whereas the presence of recessive alleles results in winter growth habit (Langer *et al.,* 2014). The large deletion in intron 1 of *Vrn-B1* and *Vrn- D1* results in spring growth habit (Fu *et al.,* 2005). The diversity panel largely consisted of spring-type *VRN1* alleles which were expected due to the selection of these accession in Pakistan. Previously, frequency of *Vrn-A1* allele was reported in germplasm from China (25%), USA (44%), Argentina (56%), Canada (85%), Pakistan (36%), USA (82%), Russia (92%), CIMMYT (41%) and India (57%) (Rasheed *et al.,* 2016c). In Pakistan, bread wheat contains the most frequent spring wheat habit *Vrn-B1* in 64% and *Vrn-D1* in 61% cultivars (Iqbal *et al.,* 2011). It was reported that 26 spring wheat cultivars contain 61% *Vrn-B1a* allele and 23% *Vrn-B1c* allele (Shcherban *et al.,* 2012). There is an association of *VRN1* alleles with developmental traits like DH, DM, DEM and yield-related traits like ear length EL, GpS, GY, SL, SpPS, TGW and TN in different water treatments. These results are in agreement with previous studies that flowering time also plays an important role in grain number and quality (Zikhali & Griffiths, 2015).

After fulfillment of vernalization requirement, flowering time is then modified by mutations in *Ppd1* genes (Shaw *et al.,* 2013). *Ppd1* belongs to a pseudo-response regulator family (*PRR*); orthologue of *Ppd-H1* gene of barley which has CCT domain,

that shows similarity to Arabidopsis PRR7 that has the function of circadian clock (Turner *et al.,* 2005). *Ppd1* genes are localized on chromosome 2A (*Ppd-A1*), 2B (*Ppd-B1*) and 2D (*Ppd-D1*). According to a response to daylight period in wheat, photoperiod is classified into two groups 'photoperiod sensitive' and 'photoperiod insensitive'. In case of a short period of vernalization (10 hours' light or less), those wheat varieties that are insensitive to photoperiod flower early as compared to photoperiod sensitive varieties (Zhou *et al.,* 2016; Dubcovsky *et al.,* 2006). Photoperiod sensitivity is controlled by *Ppd-D1* and copy number variants at *Ppd-B1*, however, it is less affected by *Ppd-A1* (Grogan *et al.,* 2016). The most important findings were that GS105-type *Ppd-A1a* alleles were retained in SYN-DER diversity panel at a relatively high frequency. These specific alleles are only present in durum wheat cultivars and are likely to have a significantly higher expression for photoperiod insensitivity. Since they are present in durum parents of synthetic hexaploid wheat, therefore this allele represents a novel and potentially useful source of earliness in bread wheat. Because the diversity panel is fixed for major photoperiod insensitive allele, *Ppd-D1a*, therefore these new variations from the durum source could help fine-tune the flowering time of bread wheat. *Ppd-1* genes were found to be associated with other agronomic traits like GY, SL, SpPS, TGW, and TN in diversity panel and these results are in agreement with previous findings (Boden *et al.,* 2015; Rasheed *et al.,* 2016c). *PRR73* is a paralog of *Ppd-D1* in bread wheat, and it was reported that accessions having Hap-I at *PRR73-A1* and Hap-II at *PRR73-B1* were earlier in heading and taller under long day conditions than accessions having contrasting haplotype (Zhang *et al.,* 2016a). The association of *PRR73-B1* with PH and DH in SYN-DER diversity panel confirmed these findings.

Earliness per se regulates flowering when vernalization and photoperiod are satisfied (Zikhali & Griffiths, 2015). It is reported that Molybdenum transporter gene (*MOT*) is completely linked to Eps phenotype (*Eps-A^{m}1*) in *T. monococcum* genetic map (Faricelli, Valárik, & Dubcovsky, 2010). They are also likely candidate for the 1DL chromosome. Our germplasm has wild-type alleles. In our study, *MOT-D1* is found to be associated with days to heading (DH), days to maturity (DM) and days from emergence to maturity (DEM). On the other hand, *ELF3-D1* is also a likely candidate for *Eps-D1*. Its function is to repress flowering time (Zikhali *et al.,* 2014).

4.3.2. Allelic Effects of KASP assays for Drought-related Traits

Dehydration responsive element binding proteins *DREB 1* are induced by water stress, low temperature and salinity (Shen *et al.,* 2003). Moderately low frequency (43%) of our germplasm contains tolerant allele. It is linked with spike length (SL), grains per spike (GPS) and harvest index (HI). These genes were validated with grain yield in previous studies (Rasheed *et al.,* 2016a).

Fructans 1 exohydrolase (*1-FEH*) is ABA insensitive gene which is responsible for stable membrane and remobilizing water-soluble carbohydrates (WSC) including fructan along with glucose and sucrose from stem to develop grains (Zhang *et al.,* 2009). Rasheed *et al* (2016a) developed the KASP marker for *1fehw3* gene. The diversity panel showed 48% of the accessions have a favorable allele of *1fehw3* and it is associated with GY, SL and PH. The association of *1fehw3* with TGW is reported in a previous study by Zhang *et al.,* (2015).

4.3.3. Allelic Effects of KASP assays for Agronomic Traits

Grain size and weight are an important component of yield in wheat and recently more than 15 genes have been cloned in wheat related to grain size and weight. Most of the genes genotyped in this study have been positively selected, for example, *TaGS-D1a* (85%) and *TaCwi-A1* (84.1%). 1BL.1RS translocation also has a significant yield advantage and has a positive impact on canopy water status (Howell *et al.,* 2014). This translocation is widely used in breeding programs because it gives resistance to biotic and abiotic stresses (Rasheed *et al.,* 2016c). Our germplasm showed a low frequency (38%) of this translocation. 1BL.1RS translocation is present in 45%, 22%, 4%, 44% and 21% in germplasm from CIMMYT, Turkey, China, Iran and U.S (Rasheed *et al.,* 2016c).

Cell wall invertase is an enzyme, which hydrolyzes sucrose into fructose and glucose, which are important for pollen development. It plays an important role in crop yield (Jiang *et al.,* 2011). Functional markers were developed for *CWI* gene by Hou *et al.,* (2014b). In this study, this gene is present in high frequency (85%) and is found to be associated with plant height. *CWI* is also linked with thousand grain weight (TGW) and days to heading (DH).

PH is an important trait largely controlled by *Rht-B1* and *Rht-D1* genes. Its important alleles; *Rht-B1b* and *Rht-D1b* significantly reduce PH by 14 –17 %, decrease lodging and increase harvest index (Rasheed *et al.,* 2016a). *Rht-B1b* allele is present in 45% of our germplasm. The moderately high frequency of germplasm containing wild-type *Rht-D1a* allele is likely due to the fact that it is derived from synthetic hexaploid wheat and mostly

selected under drought conditions. The association of *Rht* genes with many adaptive traits like CT, DH, EL, GpS, GY, PH, RWC, SL, SOD and TGW indicate the pleiotropic effect of these two genes.

4.3.4. Allelic Effects of KASP assays for Wheat Quality

Wheat has a unique flour quality as compared to other cereal crops. Wheat flour can be processed into multiple food products such as noodles, bread and cakes because of its good dough quality. Its dough quality is controlled by gluten proteins.

Gluten proteins belong to a gene family named Prolamin. Prolamin gene family is important for being a member of multigene families present on each of the three genomes of wheat, i.e., A-, B- and D-genomes. Gluten consists of two groups, i.e., glutenin and gliadins. Glutenin proteins consist of two types, i.e., high molecular weight glutenin subunit (HMW- GSs) and low molecular weight glutenin subunit (LMW-GSs) (Dong *et al.*, 2017). The HMW-GSs contains two copies in each genome, i.e., x-type and y-type (Payne *et al.*, 1987). These subunits have the largest contribution in wheat quality. The HMW-GSs include *Glu-A1, Glu-B1* and *Glu-D1*. These genes are located on the long arm of the group 1 chromosomes (*Glu-1*), i.e., 1A, 1B, and 1D (Payne *et al.*, 1984). *Glu A1, Glu B1* and *Glu D1* contain multiple alleles that control the end-use quality of wheat. It has been reported that *Glu-A1a, Glu-A1b* are the favorable alleles in bread making quality. Owing to the consequences of gene silencing, some of the alleles do not express which result in low-quality scoring such as *Glu- A1c* and y-type of *Glu-A1* genes. The HMW-GSs subunits were identified according to the numbering system described by Payne and

Lawrence (1984). This numbering system compares the varieties under study with reference varieties having a known composition such as Solitaire (2*, 5,17, 18, 10) (Tahir *et al.,* 1995). It is reported that if *Glu-A1* gene contains 1 and 2* subunits, it has positive effects on bread making quality and if it contains null allele, it exerts negative effects on bread-making quality (Liu *et al.,* 2008). In the current study, ~67% of germplasm contains favorable allele (Ax1 and Ax 2*). In case of *Glu- D1,* the subunits 5+10 are considered as favorable as it has positive effects on wheat quality. The composition of HMW-GSs are generally of good quality in SYN-DERs with approximately 50% of genotypes containing favorable subunits i.e., 5+10 in case of *Glu-D1*.

One of the important end-use quality traits is kernel hardness which is defined as the softness or hardness of endosperm. It plays a vital role in flour making quality as hard texture results in coarse flour plus more water is absorbed as compared to soft endosperm resulting in less flour yield and small particle size (Morris & Behave, 2008). Hard grains are best for making bread while soft grains are best for making cookies and biscuits (Morris & Rose, 1996). Kernel hardness is controlled by 'Purioindaline a' gene (*Pina-D1*) and 'Purioindaline b' gene (*Pinb-D1*). Kernel hardness also depends on environmental factors such as humidity, precipitation, irrigation, temperature and soil type. The *Pina-D1* and *Pinb-D1* genes are located on chromosome 5D. These genes contain wild-type and mutated alleles. The wild-type alleles are designated as 'a' and mutated alleles are designated as 'b, c, and d' respectively. It is reported that softly textured varieties carry wild-type alleles whereas hard textured varieties carry a mutated allele. The mutated allele results in altering protein structure and complete loss of function resulting in kernel hardness (Muhammadi

et al., 2014). In the current study, the maximum proportion of germplasm contains hard texture wheat quality i.e., ~84% and 63% of germplasm contain *Pina-D1* and *Pinb-D1*. This is because synthetic varieties are derived from durum wheat as tetraploid wheat presents the hardest kernel among all types of wheat cultivars. Nonetheless, the hard texture has more proteins as compared to soft textured wheat. In spite of this, hard texture wheat requires more water absorption compared to soft texture wheat as it contains arabinoxylan (AX) that is present in cell wall of wheat endosperm. Hence, the type of puroindolines genes provide the basis for deciding which type of food can be processed i.e., biscuits or pasta depending on the softness or hardness of wheat.

Polyphenol oxidase is an enzyme that catalyzes the phenol oxidation into o-quinones which undergoes further reactions to give dark brownish colors which are undesirable in any processed food (Anderson & Morris, 2001). It catalyzes two types of reactions; one is o-diphenol formation and the other is *o*-quinone formation. The *o*-quinone undergoes self-polymerization or react with amines and give dark brown color to food products. Although this color in bran is mostly removed during the milling process, contamination during milling process can cause discoloration (Massa *et al*., 2007). PPO genes in wheat are classified into two groups, i.e., kernel group and non-kernel group. These group of genes is located on each of the homeologous chromosome of group 2 (Sun *et al*., 2011). The first gene family named '*Ppo-1'* contains *Ppo-A1, Ppo-B1*, and *Ppo-D1*. The second gene family named '*Ppo-2*' contains *Ppo-A2, Ppo-B2,* and *Ppo-D2*. In group 1, *Ppo-A1,* and *Ppo-D1* have a vital role in PPO activity. The allelic variation of *Ppo-A1* and *Ppo-D1* is associated with kernel group PPO activity. Sun *et al* (2011) reported that *Ppo-A1a* allele is

associated with high PPO activity and *Ppo-A1b* is associated with low PPO activity. Three more variants were detected by He *et al* (2007) i.e., *Ppo-A1e, Ppo-A1f,* and *Ppo-A1g* in which *Ppo-A1e* and *Ppo-A1g* were associated with low PPO activity and *Ppo-A1* was associated with high PPO activity. Similarly, Ppo-D1 contains *Ppo-D1a* and *Ppo-D1b* alleles in which *Ppo-D1a* was associated with low PPO activity and *Ppo-D1b* with high PPO activity (Hystad *et al*., 2015). In group 2, *Ppo-2* genes are located at a 10cM distance from *Ppo-1* (Beecher & Skinner, 2011). *Ppo-A2* contains three types of alleles i.e., a, b, and c. *Ppo-D2* contains two types of alleles i.e., *Ppo-D2a* and *Ppo-D2b* in which *Ppo-D2a* is found to be associated with low PPO activity and *Ppo-D2b* is associated with high PPO activity. *Ppo-B2* has three allelic variants, i.e., a, b, and c in which *Ppo-B2a* and *Ppo-B2c* are found to be associated with high PPO activity and *Ppo-B2b* with low PPO activity (Taranto *et al.*, 2015). In our germplasm, ~33% and ~44% contain *Ppo-A1a* and *Ppo-D1b*, associated with high PPO activity. In group 2, ~50% and ~85% of germplasm contain *Ppo-B2c* and *Ppo-D2b*, associated with high PPO activity.

Another important quality protein is waxy protein; also known as granule-bound starch synthase 1 (GBSS). This enzyme plays an important role in amylose synthesis in endosperm tissue (Saito *et al.,* 2009). The first waxy wheat was made by hybridization between ‘BaiHuo’ which is a D1 single null line and ‘Kanto’; a *wx-A1/wx-B1* double null line. It resulted in progenies that lacked waxy proteins. The gene for waxy protein *wx-B1*, when carrying deletion is designated as *wx-B1a* and wild-type is designated as *wx-B1b*. Our ~60% germplasm contains *wx-B1a*.

The yellow color pigment formation in wheat is controlled by an enzyme; Phytoene synthase (*Psy*) that synthesizes carotenoids (Stepanenko *et al.,* 2017). Carotenoids are an important component in the production of yellowing pigment in flour. There are three groups of phytoene synthase i.e., *Psy1, Psy2* and *Psy3* located on chromosomes 7 and 5. The *Psy-A1a* is associated with high grain carotenoid content and *Psy-A1b* is associated with low grain carotenoid content. Four allelic variants have been reported in *Psy-B1* gene i.e., a, b, c, and d. *The Psy-B1a* and *Psy-B1b* are associated with low grain carotenoid content and *Psy-B1c* is associated with high grain carotenoid content (He *et al*., 2009). There are seven allelic variants in *Psy-D1* gene i.e., a, b, c, d, e, f, and g respectively. *Psy-D1a* is associated with the high carotenoid content. In our study, a large percentage of SYN-DER is associated with high carotenoid content.

4.3.5. Allelic Effects of KASP assays for Biotic Stresses of Wheat

Leaf rust, caused by *Puccinia triticina,* is one of the most important foliar diseases of bread wheat. Several genes resistant to leaf rust have been identified so far, but most of these genes are race specific (Kithiri *et al*., 2018). Some slow resistance genes, such as *Lr34,* have also been identified, which is characterized by slow disease progress. In this study, seedling rust resistant genes i.e., *Lr14, Lr34* and *Lr21* were studied. 54% of our SYN-DER germplasm contains *Lr34* resistant genes whereas 34% and ~4% contain *Lr14* and *Lr21* resistance genes respectively. *Sr2* is a stem rust resistance gene, located on chromosome 3B. It is a recessive gene that confers partial resistance when present in homozygous form (Malik *et al.,* 2013). In this study, it has been found that a total of 3% of our germplasm contains *Sr2* resistance gene. Wheat yield loss is also caused by soil-

borne pathogens such as cereal cyst nematodes. It is a global problem that causes damage to cereal crops, particularly under drought stress. One common specie of nematode is *Heterodera avenae*, which causes significant yield loss by damaging roots. Several resistance genes have been mapped for cereal cyst nematode. *Cre8* is one of the resistance genes located on chromosome 6B (Dimanthi *et al.*, 2015). In this study, ~24% of our germplasm contains *Cre8* resistance gene.

In conclusion, the diversity panel was highly divergent for several functional genes, which provided an opportunity to manipulate important genes to further fine-tune the expression of several agronomic traits. A high percentage of the favorable alleles was fixed in this cultivar. This is good to be used as a selection criterion for these traits in wheat breeding programs. Several accessions were selected based on the combination of favorable alleles such as SD 89 with 30 favorable alleles and SD36 having 27 favorable alleles for important traits.

CONCLUSION AND FUTURE PROSPECTS

This study revealed that SYN-DER and ITMI populations have great potential for drought tolerance. QTL mapping using GBS markers on ITMI population identified 16 QTLs for seedling morphology. Seven QTLs detected on chromosome 7B positioned from 64 to 221cM, could harbor important genes for drought adaptability. On the other hand, functional genes analysis by using KASP assay revealed high genetic diversity in synthetic hexaploid wheat (SHW) indicating 21 selective loci associated with agronomic traits under both moisture conditions. *Vrn-A1, Rht-D1,* and *Ppd-B1* showed the confounding effect on several agronomic traits including plant height, TGW and grain yield under both WW and WL conditions. The accumulation of favorable alleles for grain size and weight genes additively enhanced TGW in diversity panel. These results improved our knowledge on the deployment of favorable and unfavorable alleles of functional genes through unconscious selection, identified the genetic improvement bottleneck and provided opportunities to deploy alleles with beneficial effects in wheat breeding in future. However, mutations in functional genes could affect the structure and biological function of genes. Non-Synonymous mutations were found in *TaCwi-B1* particularly on the conserved domain i.e., WECPDF that affected the proper functioning of *CWI* gene due to improper hydrolysis of sucrose distressing the translocation of nutrients from source to sink, that ultimately affects the development of seeds and grain yield. Further studies need to explore the important drought-related genes that are still hidden on the 7B chromosome and expression analysis of *CWI* gene is required to analyze the effect of mutations on the function of *CWI* gene.

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ANNEXURES

7.1 List of accessions of SYN-DER used in the current study

No.	Pedigree	Background
SH/BW 1	MAYOOR//TKSN1081/AE.SQUARROSA(222)/3/OPATA/6/68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA(878)	SYN-DER
SH/BW 2	MAYOOR//TKSN1081/AE.SQUARROSA(222)/3/OPATA/6/68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA(878)	SYN-DER
SH/BW 3	68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA (878)/6/CETA/5/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 4	MAYOOR//TKSN1081/AE.SQUARROSA(222)/3/OPATA/4/ CETA/AE.SQUARROSA(895)	SYN-DER
SH/BW 5	TURACO/5/CHIR3/4/SIREN//ALTAR 84/AE.SQUARROSA(205)/3/3*BUC/6/FCT/6/DOY1/AE.SQUARROSA(458)	SYN-DER
SH/BW 6	OPATA//CETA/AE.SQUARROSA(895)	SYN-DER
SH/BW 7	68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA (878)/6/CETA/6/CETA/AE.SQUARROSA(895)	SYN-DER
SH/BW 8	OPATA//DOY1/AE.SQUARROSA(372)	SYN-DER
SH/BW 9	CHAPIO/INQALAB 91/6/68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA (878)	SYN-DER
SH/BW 10	D67.2/P66.270//AE.SQUARROSA(223)3/ARLIN_1/T.MONOCOCCUM(95)	SYN-DER
SH/BW 11	TURACO/5/CHIR3/4/SIREN//ALTAR84/AE.SQUARROSA(205)/3/3*BUC/6/CNO/7/ CROC_1/AE.SQUARROSA(444)	SYN-DER
SH/BW 12	MAYOOR//TKSN1081/AE.SQUARROSA(222)/3/OPATA/4/ DOY1/AE.SQUARROSA(372)	SYN-DER
SH/BW 13	MAYOOR//TKSN1081/AE.SQUARROSA(222)/3/OPATA/6/68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA(878)	SYN-DER
SH/BW 14	D67.2/P66.270//AE.SQUARROSA(223)/3/ARLIN_1/T.MONOCOCCUM(95)	SYN-DER
SH/BW 15	URES/PRL//BAV92/3/YAV_2/TEZ//AE.SQUARROSA(249)	SYN-DER
SH/BW 16	GAN/AE.SQUARROSA (897)//OPATA/3/D67.2/P66.270//AE.SQUARROSA(223)	SYN-DER
SH/BW 17	OPATA//CETA/AE.SQUARROSA(1031)	SYN-DER
SH/BW 18	OPATA//DOY 1/AE.SQUARROSA(255)	SYN-DER
SH/BW 20	OPATA//CETA/AE.SQUARROSA(1031)	SYN-DER

SH/BW 21	OPATA//ROK/KML// AE.SQUARROSA(214)	SYN-DER
SH/BW 22	OPATA//DOY 1/AE.SQUARROSA(517)	SYN-DER
SH/BW 23	OPATA//DOY 1/AE.SQUARROSA(517)	SYN-DER
SH/BW 24	OPATA//ALTAR 84.AE.SQUARROSA(J BANGOR)	SYN-DER
SH/BW 25	OPATA//68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA(878)	SYN-DER
SH/BW 26	OPATA//68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA(878)	SYN-DER
SH/BW 27	OPATA//AE.SQUARROSA(1026)/DOY 1)	SYN-DER
SH/BW 28	OPATA//68.112/WARD//AE.SQUARROSA(369)	SYN-DER
SH/BW 29	OPATA//68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 30	GANAE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB91/5/ BKH-94	SYN-DER
SH/BW 31	BKH-93/6/CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA(208)/5/OAPTA	SYN-DER
SH/BW 32	PBW-343/6/YAV_3/SCO//JO69/CRA/3/YAV79/4/AE.SQUARROSA (498)/5/OPATA	SYN-DER
SH/BW 33	CHIR3/CBRD//OPATA	Bread wheat
SH/BW 34	SAAR/INQALAB 91/4/MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/CBRD	SYN-DER
SH/BW 35	SAAR/INQALAB 91/4/MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/CBRD	SYN-DER
SH/BW 36	SAAR/INQALAB 91/4/MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/CBRD	SYN-DER
SH/BW 37	MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/PASTOR/4/SARSABZ	SYN-DER
SH/BW 38	MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/CBRD/4/KAMBARA	SYN-DER
SH/BW 39	PBW343/6/YAV_3/SCO//JO69/CRA/3/YAV79/4/AE.SQUARROSA(498)/5/OPATA	SYN-DER
SH/BW 40	CHIR3/CBRD/3/GAN/AE.SQUARROSA (897)//OPATA	SYN-DER
SH/BW 41	MAYOOR//TK SN1081/AE.SQUARROSA (222)/4/SABUF/3/BCN// CETA/AE.SQUARROSA(895)/5/GAN/AE.SQUARROSA (897)//OPATA	SYN-DER
SH/BW 42	KAUZ/3/MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/CBRD	SYN-DER
SH/BW 43	BKH-93/BORLOUG M95	Bread Wheat
SH/BW 44	BKH-93/BORLOUG M95	Bread Wheat

SH/BW 45	FUS/BW-595-(ALTAR 84/AE.SQUARROSA (224)//2*YACO/7/OPATA/ 6/68.111RGB-U//WARD/3/FGO/4/…) x INQ-120-(162 SAAR/INQALAB 91)	SYN-DER
SH/BW 46	FUS/BW-586-(ALTAR 84/AE.SQUARROSA (224)//2*YACO/3/MAYOOR //TK SN1081/AE.SQUARROSA (222)/4/KUKUN) x SH/BW.R.KB-1-(ALTAR 84/AE.SQUARROSA (221)//YACO)	SYN-DER
SH/BW 47	SARSABZ//CHIR3/CBRD	Bread Wheat
SH/BW 48	OPATA/PASTOR	Bread Wheat
SH/BW 49	PBW-343*2/CHAPIO/3/D67.2/P66.270//T.BOEOTICUM(66)	SYN-DER
SH/BW 50	PBW-343*2/CHAPIO/3/D67.2/P66.270//T.BOEOTICUM(66)	SYN-DER
SH/BW 51	OPATA/5/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA (1038)	SYN-DER
SH/BW 52	ALTAR 84/AE.SQUARROSA(224)//2*YACO/3/MAYOOR/TK SN1081 /AE.SQUARROSA(222)/4/KUKUN/5/GAN/AE.SQUARROSA(248)	SYN-DER
SH/BW 53	ALTAR 84/AE.SQUARROSA(224)//2*YACO/3/MAYOOR/TK SN1081 /AE.SQUARROSA(222)/4/KUKUN/5/GAN/AE.SQUARROSA(248)	SYN-DER
SH/BW 54	ALTAR 84/AE.SQUARROSA (224)//2*YACO/3/MAYOOR/TK SN1081 /AE.SQUARROSA(222)/4/KUKUN/5/GAN/AE.SQUARROSA(248)	SYN-DER
SH/BW 55	ALTAR 84/AE.SQUARROSA(224)//2*YACO/3/MAYOOR/TK SN1081 /AE.SQUARROSA(222)/4/KUKUN/5/GAN/AE.SQUARROSA(248)	SYN-DER
SH/BW 56	ALTAR 84/AE.SQUARROSA(224)//2*YACO/3/MAYOOR/TK SN1081 /AE.SQUARROSA(222)/4/KUKUN/5/GAN/AE.SQUARROSA(248)	SYN-DER
SH/BW 57	ALTAR 84/AE.SQUARROSA(224)//2*YACO/3/MAYOOR/TK SN1081 /AE.SQUARROSA(222)/4/KUKUN/5/GAN/AE.SQUARROSA)(248)	SYN-DER
SH/BW 58	ALTAR 84/AE.SQUARROSA(224)//2*YACO/3/MAYOOR/TK SN1081 /AE.SQUARROSA(222)/4/KUKUN/5/GAN/AE.SQUARROSA(248)	SYN-DER
SH/BW 59	ALTAR 84/AE.SQUARROSA(224)//2*YACO/3/MAYOOR/TK SN1081 /AE.SQUARROSA(222)/4/KUKUN/5/GAN/AE.SQUARROSA(248)	SYN-DER
SH/BW 60	MH 97/2/D67.2/P66.270//T.BOEOTICUM(66)	SYN-DER
SH/BW 61	TURACO/5/CHIR3/4/SIREN//ALTAR84/AE.SQUARROSA(205)/3/3*BUC/6/CNO/7/ CPI/GEDIZ/3/GOO//JO/CRA/4/AE.SQUARROSA(227)	SYN-DER
SH/BW 62	TURACO/5/CHIR3/4/SIREN//ALTAR84/AE.SQUARROSA(205)/3/3*BUC/6/CNO/7/ CPI/GEDIZ/3/GOO//JO/CRA/4/AE.SQUARROSA(227)	SYN-DER
SH/BW 63	TURACO/5/CHIR3/4/SIREN//ALTAR84/AE.SQUARROSA(205)/3/3*BUC/6/CNO/7/ CPI/GEDIZ/3/GOO//JO/CRA/4/AE.SQUARROSA(227)	SYN-DER
SH/BW 64	RABE/2*MO88/5/68.111/RGB-U//WARD RESEL/3/STIL/4/AE. SQUARROSA(1038)	SYN-DER
SH/BW 65	RABE/2*MO88/5/68.111/RGB-U//WARD RESEL/3/STIL/4 /AE.SQUARROSA(1038)	SYN-DER
SH/BW 66	RABE/2*MO88/5/68.111/RGB-U//WARD RESEL/3/STIL/4/ AE.SQUARROSA(1038)	SYN-DER
SH/BW 67	MAYOOR//TKSN1081/AE.SQUARROSA(222)/3/OPATA/6/68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA(878)	SYN-DER

SH/BW 68	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA(208)/5/OAPTA/6/68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA(878)	SYN-DER
SH/BW 69	MAYOOR//TKSN1081/AE.SQUARROSA(222)/3/OPATA/6/68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA(878)	SYN-DER
SH/BW 70	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA (208)/5/OAPTA/6/ 68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 71	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA(208)/5/OAPTA/6/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 72	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA(208)/5/OAPTA/6/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 73	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA(208)/5/OAPTA/6/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 74	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA(208)/5/OAPTA/6/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 75	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA(208)/5/OPATA/6/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 76	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA(208)/5/OPATA/6/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 77	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA(208)/5/OPATA/6/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 78	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA(208)/5/OPATA/6/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 79	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA(208)/5/OPATA/6/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 80	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA(208)/5/OPATA/6/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 81	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA(208)/5/OPATA/6/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 82	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA(208)/5/OPATA/6/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 83	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA(208)/5/OPATA/6/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(783)	SYN-DER
SH/BW 84	CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA (208)/5/OPATA/6/68.111/RGB-U//WARD RESEL/3/STIL/4 /AE.SQUARROSA(783)	SYN-DER
SH/BW 85	TURACO/5/CHIR3/4/SIREN//ALTAR84/AE.SQUARROSA(205)/3/3*BUC/6/CNO/7/ CPI/GEDIZ/3/GOO//JO/CRA/4/AE.SQUARROSA(273)	SYN-DER
SH/BW 86	OPATA/6/68.111/RGB-U//WARD/3/FGO/4/RABI/5/ AE.SQUARROSA(878)	SYN-DER
SH/BW 87	INQALAB 91/TSAPKI//SCA/AE.SQUARROSA(518)	SYN-DER
SH/BW 88	ALTAR 84/AE.SQUARROSA(221)//YACO/3/INQALAB 91/4/D67.2/ P66.270//T.BOEOTICUM(66)	SYN-DER
SH/BW 89	CNDO/R143//ENTE/MEXI_2/3/AE.SQUARROSA(TAUS)/4/WEAVER/5/2*KAUZ/6/ DOY1/AE.SQUARROSA) (458)	SYN-DER
SH/BW 90	CNDO/R143//ENTE/MEXI_2/3/AE.SQUARROSA(TAUS)/4/WEAVER/5/2*KAUZ/6/ DOY1/AE.SQUARROSA) (458)	SYN-DER

SH/BW 91	CNDO/R143//ENTE/MEXI_2/3/AE.SQUARROSA(TAUS)/4/WEAVER/5/2*KAUZ/6/ DOY1/AE.SQUARROSA) (458)	SYN-DER
SH/BW 92	CNDO/R143//ENTE/MEXI_2/3/AE.SQUARROSA(TAUS)/4/WEAVER/5/2*KAUZ/6/ DOY1/AE.SQUARROSA) (458)	SYN-DER
SH/BW 93	CNDO/R143//ENTE/MEXI_2/3/AE.SQUARROSA(TAUS)/4/WEAVER/5/2*KAUZ/6/DOY1/AE.SQUARROSA(458)	SYN-DER
SH/BW 94	SERI/5/68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA(392)	SYN-DER
SH/BW 95	TURACO/5/CHIR3/4/SIREN//ALTAR84/AE.SQUARROSA(205)/3/3*BUC/6/OPATA/7/ SCA/AE.SQUARROSA(518)	SYN-DER
SH/BW 96	MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/CBRD/4/ ARLIN_1/T.MONOCOCCUM(95)	SYN-DER
SH/BW 97	MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/CBRD/4/ ARLIN_1/T.MONOCOCCUM(95)	SYN-DER
SH/BW 98	CHAPIO/INQALAB 91/4/68.111/RGB-U//WARD/3/AE.SQUARROSA (452)	SYN-DER
SH/BW 99	CHAPIO/INQALAB 91/4/68.111/RGB-U//WARD/3/AE.SQUARROSA (452)	SYN-DER
SH/BW 100	CROC-1/AE.SQUARROSA(224)//KAUZ/3/CETA/AE.SQUARROSA(895)	SYN-DER
SH/BW 101	CROC-1/AE.SQUARROSA(224)//KAUZ/3/CETA/AE.SQUARROSA(895)	SYN-DER
SH/BW 102	OPATA/PAS//DOY1/AE.SQUARROSA(1024)	SYN-DER
SH/BW 103	MAYOOR//TKSN1081/AE.SQUARROSA(222)/3/OPATA/4/ DOY1/ AE.SQUARROSA(515)	SYN-DER
SH/BW 104	DOYI/AE.SQUARROSA(1018)/6/CPI/GEDIZ/3/GOO//JO69/CRA/4/AE.SQUARROSA (208)/5/OPATA	SYN-DER
SH/BW 105	DOYI/AE.SQUARROSA(1018) x CPI/GEDIZ/3/GOO//JO69/ CRA/4/AE.SQUARROSA (208)/5/OPATA	SYN-DER
SH/BW 106	DOYI/AE.SQUARROSA(1018) x CPI/GEDIZ/3/GOO//JO69/ CRA/4/AE.SQUARROSA (208)/5/OPATA	SYN-DER
SH/BW 107	MAYOOR/TK SN1081/AE.SQUARROSA (222)/3/PASTOR/4/CROC_1 /AE.SQUARROSA(444)	SYN-DER
SH/BW 108	MAYOOR/TK SN1081/AE.SQUARROSA(222)/3/PASTOR/4/ CROC_1/AE.SQUARROSA(444)	SYN-DER
SH/BW 109	PBW-343//DOY1/AE.SQUARROSA(188)	SYN-DER
SH/BW 110	OPATA//CETA/AE.SQUARROSA(895)	SYN-DER
SH/BW 111	OPATA//INQALAB 91/AC8528	Bread wheat
SH/BW 112	OPATA//INQALAB 91/AC8528	Bread wheat
SH/BW 113	OPATA//GAN/AE.SQUARROSA(408)	SYN-DER
SH/BW 114	OPATA//DOY1/AE.SQUARROSA(458)	SYN-DER

SH/BW 115	OPATA//DVERD_2/AE.SQUARROSA(333)	SYN-DER
SH/BW 116	OPATA//DVERD_2/AE.SQUARROSA(333)	SYN-DER
SH/BW 117	OPATA//DVERD_2/AE.SQUARROSA(333)	SYN-DER
SH/BW 118	OPATA//DVERD_2/AE.SQUARROSA(333)	SYN-DER
SH/BW 119	OPATA/6/68.111/RGB-U//WARD/3/FGO/4/RABI/5/ AE.SQUARROSA(629)	SYN-DER
SH/BW 120	OPATA/6/x 68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA(629)	SYN-DER
SH/BW 121	OPATA//ALTAR 84/AE.SQUARROSA(J BANGOR)	SYN-DER
SH/BW 122	OPATA//ALTAR 84/AE.SQUARROSA(J BANGOR)	SYN-DER
SH/BW 123	OPATA//ALTAR 84/AE.SQUARROSA(J BANGOR)	SYN-DER
SH/BW 124	OPATA//ALTAR 84/AE.SQUARROSA(J BANGOR)	SYN-DER
SH/BW 125	OPATA//ALTAR 84/AE.SQUARROSA(J BANGOR)	SYN-DER
SH/BW 126	OPATA//CETA/AE.SQUARROSA(1027)	SYN-DER
SH/BW 127	OPATA//ALTAR 84/AE.SQUARROSA(205)	SYN-DER
SH/BW 128	OPATA//INQALAB 91/TSAPKI	Bread Wheat
SH/BW 129	OPATA//DOY 1/AE.SQUARROSA(1026)	SYN-DER
SH/BW 130	MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/PASTOR/4/MH-97	SYN-DER
SH/BW 131	PAS/4/MAYOOR//TK SN1081/AE.SQUARROSA)(222)/3/FCT	SYN-DER
SH/BW 132	PAS/4/MAYOOR//TK SN1081/AE.SQUARROSA)(222)/3/FCT	SYN-DER
SH/BW 133	MAYOOR//TK SN1081/AE.SQUARROSA)(222)/3/FCT/6/ YAV_3/SCO //JO69/CRA/3/YAV/79/4/AE.SQUARROSA(498)/5/OPATA	SYN-DER
SH/BW 134	MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/FCT /6/YAV_3/SCO //JO69/CRA/3/YAV/79/4/AE.SQUARROSA(498)/5/OPATA	SYN-DER
SH/BW 135	KAUZ/4/MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/CBRD	SYN-DER
SH/BW 136	KAUZ/4/MAYOOR//TK SN1081/AE.SQUARROSA (222)/3/CBRD	SYN-DER
SH/BW 137	MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/PASTOR/4/SARSABZ	SYN-DER
SH/BW 138	BKH-93/4/MAYOOR//TK SN1081/AE.SQUARROSA)(222)/3/FCT	SYN-DER

SH/B W 139	PASTOR/4/MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/CBRD	SYN-DER
SH/B W 140	PASTOR/4/MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/CBRD	SYN-DER
SH/B W 141	PASTOR/4/MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/CBRD	SYN-DER
SH/B W 142	BKH-93/4/MAYOOR//TK SN1081/AE.SQUARROSA)(222)/3/FCT	SYN-DER
SH/B W 143	BAV/4/MAYOOR//TK SN1081/AE.SQUARROSA(222)/3/CBRD	SYN-DER
SH/B W 144	BKH-94/4/D67.2//P66.270//AE.SQUARROSA(257)/3/OPATA	SYN-DER
SH/B W 145	BKH-94/4/D67.2//P66.270//AE.SQUARROSA(257)/3/OPATA	SYN-DER
SH/B W 146	CROC-1/AE.SQUARROSA(205)//BORL95/3/ALTAR84	SYN-DER
SH/B W 147	KAMBARA/INQALAB	Bread wheat
SH/B W 148	INQALAB/7/68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA (878)/6/CETA	SYN-DER
SH/B W 149	INQILAB 91/RABI//INQALAB	Bread wheat
SH/B W 150	INQALAB/7/68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA (878)/6/CETA/	SYN-DER
SH/B W 151	CHAPIO/INQALAB 91/4/PICUS/3/KAUZ*2/BOW//KAUZ)	Bread Wheat
SH/B W 152	CHAPIO/INQALAB 91/4/PICUS/3/KAUZ*2/BOW//KAUZ)	Bread Wheat
SH/B W 153	CHAPIO/INQALAB 91/4/PICUS/3/KAUZ*2/BOW//KAUZ	Bread Wheat
SH/B W 154	CHAPIO/INQALAB 91/4/PICUS/3/KAUZ*2/BOW//KAUZ	Bread Wheat
SH/B W 155	CHAPIO/INQALAB 91/4/PICUS/3/KAUZ*2/BOW//KAUZ	Bread Wheat
SH/B W 156	CHAPIO/INQALAB 91/4/PICUS/3/KAUZ*2/BOW//KAUZ	Bread Wheat
SH/B W 157	CHAPIO/INQALAB 91/4/PICUS/3/KAUZ*2/BOW//KAUZ	Bread Wheat
SH/B W 158	CHAPIO/INQALAB 91/4/PICUS/3/KAUZ*2/BOW//KAUZ	Bread Wheat
SH/B W 159	CHAPIO/INQALAB 91/4/PICUS/3/KAUZ*2/BOW//KAUZ	Bread Wheat
SH/B W 160	CHAPIO/INQALAB 91/4/PICUS/3/KAUZ*2/BOW//KAUZ	Bread Wheat
SH/B W 161	CHAPIO/INQALAB 91/4/PICUS/3/KAUZ*2/BOW//KAUZ	Bread Wheat
SH/B W 162	CHAPIO/INQALAB 91/4/PICUS/3/KAUZ*2/BOW//KAUZ	Bread Wheat

SH/BW 163	CHAPIO/INQALAB 91/4/PICUS/3/KAUZ*2/BOW//KAUZ	Bread Wheat
SH/BW 164	CHAPIO/INQALAB 91/4/PICUS/3/KAUZ*2/BOW//KAUZ	Bread Wheat
SH/BW 165	MAYOOR//TK SN1081/AE.SQUARROSA)(222)/3/BCN/6/ 68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA (878)/6/CETA/	SYN-DER
SH/BW 166	TURACO/5/CHIR3/4/SIREN//ALTAR 84/AE.SQUARROSA(205) /3/3*BUC/6/CNO/7/ 68.111/RGB-U//WARD/3/FGO /4/RABI/5/ AE.SQUARROSA (878)/6/CETA/	SYN-DER
SH/BW 167	TURACO/5/CHIR3/4/SIREN//ALTAR 84/AE.SQUARROSA(205)/3/3* BUC/6/CNO/7/ 68.111/RGB-U//WARD/3/FGO/4/RABI /5/AE.SQUARROSA (878)/6/CETA/	SYN-DER
SH/BW 168	TURACO/5/CHIR3/4/SIREN//ALTAR 84/AE.SQUARROSA(205)/3/3* BUC/6/CNO/7/ 68.111/RGB-U//WARD/3/FGO/4/RABI /5/AE.SQUARROSA (878)/6/CETA/	SYN-DER
SH/BW 169	TURACO/5/CHIR3/4/SIREN//ALTAR 84/AE.SQUARROSA(205) /3/3*BUC/6/CNO/7/ 68.111/RGB-U//WARD/3/FGO/ 4/RABI/5/AE.SQUARROSA (878)/6/CETA/	SYN-DER
SH/BW 170	MAYOOR//TK SN1081/AE.SQUARROSA)(222)/3/FCT/4/PASTOR	SYN-DER
SH/BW 171	MAYOOR//TK SN1081/AE.SQUARROSA)(222)/3/FCT/4/PASTOR	SYN-DER
SH/BW 172	MAYOOR//TK SN1081/AE.SQUARROSA)(222)/3/FCT/4/PASTOR	SYN-DER
SH/BW 173	MAYOOR//TK SN1081/AE.SQUARROSA)(222)/3/FCT/4/PASTOR	SYN-DER
SH/BW 174	MAYOOR//TK SN1081/AE.SQUARROSA)(222)/3/FCT/4/PASTOR	SYN-DER
SH/BW 175	MAYOOR//TK SN1081/AE.SQUARROSA)(222)/3/FCT/4/PASTOR	SYN-DER
SH/BW 176	MAYOOR//TK SN1081/AE.SQUARROSA)(222)/3/BCN/4//6/68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA (878)/6/CETA/	SYN-DER
SH/BW 177	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/ PBW-343	SYN-DER
SH/BW 178	MAYOOR//TK SN1081/AE.SQUARROSA)(222)/3/FCT/4/PASTOR	SYN-DER
SH/BW 179	MAYOOR/PASTOR	Bread wheat
SH/BW 180	MAYOOR/PASTOR	Bread wheat
SH/BW 181	MAYOOR/PASTOR	Bread wheat
SH/BW 182	MAYOOR/PASTOR	Bread wheat
SH/BW 183	MAYOOR/PASTOR	Bread wheat
SH/BW 184	CHIRYA/PBW-343	Bread wheat

SH/B W 185	CHIRYA/PBW-343	Bread wheat
SH/B W 186	CHIRYA/PASTOR	Bread wheat
SH/B W 187	CHIRYA/Weebill-1	Bread wheat
SH/B W 188	CHIRYA/Weebill-1	Bread wheat
SH/B W 189	CHIRYA/BKH-94	Bread wheat
SH/B W 190	DOY1/AE.SQUARROSA(224)//HANS/PRL	SYN-DER
SH/B W 191	ALTAR 84/AE.SQUARROSA(193)//PASTOR	SYN-DER
SH/B W 192	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/ BKH-94	SYN-DER
SH/B W 193	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/BKH-94	SYN-DER
SH/B W 194	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/BKH-94	SYN-DER
SH/B W 195	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/BKH-94	SYN-DER
SH/B W 196	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/BKH-94	SYN-DER
SH/B W 197	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/ PBW-343	SYN-DER
SH/B W 198	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/PBW-343	SYN-DER
SH/B W 199	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/ PBW-343	SYN-DER
SH/B W 200	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/PBW-343	SYN-DER
SH/B W 201	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/PBW-343	SYN-DER
SH/B W 202	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/PBW-343	SYN-DER
SH/B W 203	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/PBW-343	SYN-DER
SH/B W 204	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/PBW-343	SYN-DER
SH/B W 205	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/PBW-343	SYN-DER
SH/B W 206	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/PBW-343	SYN-DER
SH/B W 207	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/PBW-343	SYN-DER
SH/B W 208	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/PBW-343	SYN-DER

SH/BW 209	GAN/AE.SQUARROSA(236)//CETA/AE.SQUARROSA(895)/3/MAIZ/4/INQALAB 91/5/PBW-343	SYN-DER
CC501	Inquilab-91	Bread wheat
CC502	Seher 2006	Bread wheat
CC503	Chakwal 50	Bread wheat
CC504	Wafaq 2001	Bread wheat

7.2 List of accessions of ITMI mapping population

S. No.	Germplasm	S. No.	Germplasm	S. No.	Germplasm
1	OPATA	41	ITMI-1221	81	ITMI-217
2	SYNTHETIC	42	ITMI-1233	82	ITMI-219
3	ITMI-10	43	ITMI-1235	83	ITMI-229
4	ITMI-1006	44	ITMI-1239	84	ITMI-251
5	ITMI-1008	45	ITMI-1258	85	ITMI-266
6	ITMI-102	46	ITMI-1259	86	ITMI-278
7	ITMI-1033	47	ITMI-126	87	ITMI-286
8	ITMI-1054	48	ITMI-1279	88	ITMI-287
9	ITMI-1065	49	ITMI-128	89	ITMI-289
10	ITMI-107	50	ITMI-1288	90	ITMI-305
11	ITMI-1070	51	ITMI-1294	91	ITMI-32
12	ITMI-1080	52	ITMI-1303	92	ITMI-324
13	ITMI-1083	53	ITMI-1329	93	ITMI-338
14	ITMI-109	54	ITMI-133	94	ITMI-34
15	ITMI-1095	55	ITMI-1338	95	ITMI-344
16	ITMI-110	56	ITMI-1339	96	ITMI-346
17	ITMI-1105	57	ITMI-134	97	ITMI-348
18	ITMI-1106	58	ITMI-1340	98	ITMI-382
19	ITMI-1112	59	ITMI-135	99	ITMI-383
20	ITMI-112	60	ITMI-1353	100	ITMI-390
21	ITMI-113	61	ITMI-137	101	ITMI-391
22	ITMI-1134	62	ITMI-138	102	ITMI-392
23	ITMI-1147	63	ITMI-146	103	ITMI-394
24	ITMI-1152	64	ITMI-1478	104	ITMI-395
25	ITMI-1155	65	ITMI-1496	105	ITMI-401
26	ITMI-1156	66	ITMI-152	106	ITMI-404
27	ITMI-1158	67	ITMI-154	107	ITMI-405

28	ITMI-1163	68	ITMI-1550	108	ITMI-408
29	ITMI-1164	69	ITMI-160	109	ITMI-416
30	ITMI-1165	70	ITMI-1662	110	ITMI-418
31	ITMI-1168	71	ITMI-169	111	ITMI-419
32	ITMI-1169	72	ITMI-170	112	ITMI-425
33	ITMI-1172	73	ITMI-171	113	ITMI-426
34	ITMI-1180	74	ITMI-172	114	ITMI-428
35	ITMI-1187	75	ITMI-173	115	ITMI-430
36	ITMI-1189	76	ITMI-174	116	ITMI-431
37	ITMI-1196	77	ITMI-177	117	ITMI-433
38	ITMI-1205	78	ITMI-185	118	ITMI-434
39	ITMI-1214	79	ITMI-190	119	ITMI-437
40	ITMI-122	80	ITMI-206	120	ITMI-438
121	ITMI-443	165	ITMI-768	208	ITMI-998
122	ITMI-447	166	ITMI-783	209	ITMI911A
123	ITMI-448	167	ITMI-805	210	ITMI911B
124	ITMI-449	168	ITMI-806		
125	ITMI-455	169	ITMI-813		
126	ITMI-456	170	ITMI-818		
127	ITMI-459	170	ITMI-818		
128	ITMI-464	171	ITMI-86		
129	ITMI-466	172	ITMI-869		
130	ITMI-467	173	ITMI-87		
131	ITMI-470	174	ITMI-870		
132	ITMI-471	175	ITMI-874		
133	ITMI-474	176	ITMI-875		
134	ITMI-475	177	ITMI-878		
135	ITMI-48	178	ITMI-881		
136	ITMI-531	179	ITMI-885		
137	ITMI-54	180	ITMI-887		
138	ITMI-544	181	ITMI-890		
139	ITMI-546	182	ITMI-891		
140	ITMI-550	183	ITMI-892		
141	ITMI-557	184	ITMI-895		
142	ITMI-559	185	ITMI-903		
143	ITMI-563	186	ITMI-907		
144	ITMI-564	187	ITMI-908		
145	ITMI-58	188	ITMI-913		
146	ITMI-62	190	ITMI-916		
147	ITMI-621	191	ITMI-918		
148	ITMI-626	192	ITMI-919		
149	ITMI-632	193	ITMI-924		
150	ITMI-639	194	ITMI-929		
151	ITMI-640	195	ITMI-931		
152	ITMI-643	196	ITMI-94		

153	ITMI-654	197	ITMI-943		
154	ITMI-66	198	ITMI-960		
156	ITMI-696	199	ITMI-962		
157	ITMI-707	200	ITMI-964		
158	ITMI-73	201	ITMI-968		
159	ITMI-735	202	ITMI-969		
160	ITMI-741	203	ITMI-97		
161	ITMI-748	204	ITMI-976		
162	ITMI-751	205	ITMI-981		
163	ITMI-760	206	ITMI-982		
164	ITMI-764	207	ITMI-990		

7.3 Hoagland's complete nutrient solution

Stock solutions to prepare 1 liter of nutrient solution:
1. 1.00 M $NH_4H_2PO_4$ use 1 ml/L of nutrient solution
2. 1.00 M KNO_3 use 6 ml/L of nutrient solution
3. 1.00 M Ca $(NO_3)_2$ use 4 ml/L of nutrient solution
4. 1.00 M $MgSO_4$ use 2 ml/L of nutrient solution

Micronutrient stocks
The following amount of salt was combined in a total volume of one liter of water, then 1 ml/L was used of this stock mixture to make a total of 1 L of nutrient solution.

5. 2.86 gm H_3BO_3
1.81 g $MnCl_2.4H_2O$
0.22 g $ZnSO_4$.$7H_2O$
0.08 g $CuSO_4.5H_2O$
0.02 g $H_2MoO_4.H_2O$
(Assaying 85% MoO_3)

Note: Hoagland's recipe called for 1 ml of 0.5% iron tartrate stock per liter of the nutrient solution but we use the above substitution.

7.4 L. B media preparation

Tryptophan	2.5g
Yeast	1.25g
NaCl	2.5g
ddH_2O	250ml

7.5 L.B Agar Preparation

Tryptophan	2.5g
Yeast	1.25g
NaCl	2.5 g
Agar	3.75g
ddH_2O	250ml

7.6 Primer sequences and allelic information of individual KASP assays

Primer name	Primer sequence 5' to 3'	Purpose
CWI-B1-F2	F: GAGTGTGGTAAATTGCAAGTTG R: GTTCGACATCAACGGTTGCT	Purpose to resequence *TaCwi-B1*
CWI-4A-1523	F1: GAAGGTGACCAAGTTCATGCTTTTATTTAAAATTTGATGAACTTTTCATAAAC F2: GAAGGTCGGAGTCAACGGATTTTTATTTAAAATTTGATGAACTTTTCACAAAT R: CATCGAATTGAAGAAAAGTTCACGC	KASP assay for SNP at 1523 bp at TaCwi-A1
CWI-5D-312	F1: GAAGGTGACCAAGTTCATGCTTAGAGGAGATCAAGTCATTGCGTGC F2: GAAGGTCGGAGTCAACGGATTTAGAGGAGATCAAGTCATTGCGTGG R: GGCCTTGTTGCTGACATTGACGTGCT	KASP assay for SNP at 312 bp in TaCwi-D1

7.7: KASP markers used in the study to identify alleles of functional genes in diversity panel derived from synthetic hexaploid wheat along with allele information and their effect on phenotypes

				FAM-allele			HEX-allele		
Trait	**Gene**	**Polymorphism**	**Marker name**	**Call**	**Allele**	**Associated phenotype**	**Call**	**Allele**	**Associated phenotype**
Reduced height	*Rht-B1*	C/T	Rht-B1_SNP	C	*Rht-B1a*	Wild type	T	*Rht-B1b*	Dwarf
	Rht-B1	160 bp InDel	Rht-B1_160IND	Ins	Rht-B1a+160 bp	Reduced height	Del	*Rht-B1a*	Wild type
	Rht-B1	197 bp InDel	Rht-B1_197IND	Ins	Rht-B1a+197 bp	Reduced height	Del	*Rht-B1a*	Wild type
	Rht-D1	G/T	Rht-D1_SNP	G	*Rht-D1a*	Wild type	T	*Rht-D1b*	Dwarf
Photoperiod response	*Ppd-A1*	InDel	GS100-1027IND	Ins	Wildtype	Sesitive	Del	GS-100 type	Insensitive
	Ppd-A1	InDel	GS105-1117IND	Ins	Wildtype	Sensitive	Del	GS-105 type	Insensitive
	Ppd-A1	InDel	Cdex5-6ID	Ins	Insertio 5-6 exon	insensitive	Del	Cappelle-Desprez type	sensitive
	Ppd-A1	InDel	CSInt5TE	Del	non CS-type		Ins	CS-insertion-neutral	
	Ppd-B1	RT InDel	TaPpdBJ001	Del	Non-truncated	sensitive	Ins	truncated copy	CS-type-insensitive
	Ppd-B1	Null/A	*TaPpdBJ003*	Null	Wild-type	sensitive	A	Sonoro-64-type	insensitive
	Ppd-B1	A/G	*TaSNPppdB1-10*	A	Others	sensitive	G	CS-type	insensitive
	Ppd-B1	C/T	*TaSNPppdB1-4*	C	Chayenne type	Sensitive	T	Paragon-type	Insensitive
	Ppd-D1	2089 bp InDel	TaPpdDD001	C	Wildtype	Sensitive	T	Deletion	Insensitive
	Ppd-D1	Intron 1 InDel	TaPpdDI001	G	Insertion	Sensitive (Mercia)	C	Wildtype	Insensitive
	Ppd-D1	5 bp InDel	TaPpdDD002	G	Wildtype	Sensitive	C	Deletion	Insensitive
Vernalization	*Vrn-A1*	A/G	Vrn-A1_9K 0001	A	*vrn-A1*	Winter	G	*Vrn-A1*	Spring
	Vrn-A1	C/A	Vrn-A1b-Marq	C	*Vrn-A1b*	Winter	A	*Vrn-A1a*	Spring
	Vrn-A1	C/T	Exon7_C/T_Vrn-A1	C	Calire-type	Early flower	T	Hereward-type	Late flower
	Vrn-A1	T/C	*Vrn-A1.Y.14418*	T	2147-type	long-vern	C	Jagger-type	short-vern
	Vrn-B1	T/A	Vrn-B1_A	T	*vrn-B1, Vrn-B1c*	Winter	A	*Vrn-B1a,b*	Spring
	Vrn-B1	G/C	Vrn-B1_B	G	Null	Winter	C	*Vrn-B1b*	Spring
	Vrn-B1	InDel	Vrn-B1_C	Del	Del	winter	Ins	*Vrn-B1c*	spring
	Vrn-D1	G/C	Vrn-D1-D1a_A	G	Null	Winter	C	*Vrn-D1a*	Spring
	Vrn-B3	G/T	VrnB3_5300IND	G	*vrn-B3 (CS)*	Late flower	T	*Vrn-B3 (Hope)*	Early flower
	Vrn-D3	G/C	Vrn-D3_SNP	C	Jagger	Short Vern	G	2174	Long Vern
Flowering time	TaELF3-B1	A/G	TaELF3-B1	A	Cadenza-type	Late flower	G	Wild-type	Early flower
	TaElf3-D1	T/A	TaBradi2g14790	T	GeneDeletion	Early flower	A	Insertion	Late flower
	TaElf3-D1	A/G	TaELF3-D1-1	A	Savanah-type	Early flower	G	Wild-type	Late flower
	TaElf3-D1	T/C	TaELF3-D1-2	T	Savanah-type	Early flower	C	Wild-type	Late flower
	TaMOT1-D1	C/G	TaMOT1-D1	C	Wild-type	Early flower	G	Ria-type	Late flower
	TaPRR73-A1	9 bp InDel	PRR73A1-9IND	Del	*Hap-I*	Early flower	Ins	*Hap-II*	Late flower
	TaPRR73-B1	A/G	PRR73B1-4558	A	*Hap-I*	Late flower	G	*Hap-II*	Early flower
Yield	*TaSus 2-2B*	T/C	TaSus2-2B_SNP	T	Hap-L	Low TGW	C	Hap-H	High TGW
	TaGS-D1	G/T	TaGS-D1_SNP	G	*TaGS-D1a*	High TGW	T	*TaGD-D1b*	Low TGW
	TaCKX-D1	18 bp InDel	CKX-D1_IND	Ins	*TaCKX-D1b*	Low TGW	Del	*TaCKX-D1a*	High TGW
	TaGASR-A1	2kb InDel	TaGASR_IND	Ins	H1g	Low TGW	Del	H1c	High TGW
	TaCwi-A1	C/A	CWI-CIMMYT	C	TaCwi-A1b	low TGW	A	TaCwi-A1a	hight TGW
	TaCwi-4A	C/T	CWI4A_SNP	C	Hap-4A-C	Favored	T	Hap-4A-T	Non-Favored
	TaCwi-5D	C/G	CWI5D_SNP	C	Hap-5D-C	Favored	G	Hap-5D-G	Non-Favored
	TaMoc-7A	G/A	MOC	G	Hap-H	High grain number	A	Hap-L	Low grain number
	TaGW2-6A	C/A	GW2-CIMMYT	C	TaTGW2-A1b	low TGW	A	TaTGW2-A1a	high TGW
	TaGW2-6A	A/G	GW2-CAAS	A	Hap-6A-G	low TGW	G	Hap-6A-A	high TGW
	TaGW2-6B	C/T	GW2-6B-83S NP	C	Hap1,2	Higher TGW	T	Hap3,4	Lower TGW
	TaGW2-6B	G/A	GW2-6B-721S NP	G	Hap1, 2, 3	Higher TGW	A	Hap-4	Lowest TGW
	TaGW2-6B	A/C	GW2-6B-1709S NP	A	Hap-1	Higher TGW	C	Hap2, 3, 4	Lower TGW
	TaSus 2-2A	G/A	Sus2-2A-20S NP	G	Hap-G	Lower TGW	A	Hap-A	Higher TGW
	TaSus 1-7B	6bp InDel	Sus1-7B-2932IND	Ins	Hap-T	Higher TGW	Del	Hap-C	Lower TGW
	TaSus 1-7A	C/T	Sus1-7A-1185S NP	C	Hap1, 3, 5		T	Hap2, 4,	
	TaSus 1-7A	TC/AA	Sus1-7A-1599S NP	AA	Hap4, 5		TC	Hap1, 2, 3	
	TaSus 1-7A	G/C	Sus1-7A-3544S NP	G	Hap1, 5		C	Hap2, 3, 4	
	TaTGW6-A1	G/A	TaTGW6_SNP	G	*TaTGW6-A1a*	High TGW	A	*TaTGW6-A1b*	Low TGW
	TEF-7A	C/T	TEF7A_547_SNP	C	Hap-7A-3	High TGW	T	Hap-7A-1, 2	Low TGW
	TEF-7A	1bp InDel	TEF7A_1IND_F	C	Hap-7A-2, 3		G	Hap-7A-1	
	TGW6-4A	C/G	TGW6-4A	C	TaTGW6-b	Low TGW	G	TaTGW6-a	High TGW
	TaGS5-A1	G/T	GS5-2334-S NP	G	TaGS5-A1a	Low TGW	T	TaGS5-A1b	High TGW
Pre-harvest sprouting	*TaPHS1*	G/A	PHS_646	G	RioBlanceo type	Low PHS	A	NW97S186 type	High PHS
	TaPHS1	T/A	PHS_666	T	RioBlanceo type	Low PHS	A	NW97S186 type	High PHS
	TaSdr-A1	C/T	SDRA1_643	C	TaSdr-A1a	Low PHS	T	TaSdr-A1b	High PHS
	TaSdr-B1	A/G	SDR_SNP	A	*TaSdr-B1a*	Low germination	G	*TaSdr-B1b*	High germination
	TaMFT-A1	A/G	TaMFT-721J	CG	Jagger-type	PHS resistance	GC	Others	PHS susceptible
	TaMFT-A1	G/C	TaMFT-1617Z	T	CS-type, Jagger	PHS susceptible	C	Zen-type, 2174-type	PHS resistance
	Vp1B1	83 bp InDel	Vp1B1-83_IND	Ins	*Vp1Ba,b*	PHS susceptible	Del	*Vp1B1c*	Slightly PHS res
	Vp1B1	193 bp InDel	Vp1B1-193_IND	Del	*Vp1Bb*	PHS resistance	Ins	*Vp1ba,c*	PHS suceptibe
Drought tolerance	*TaDreb-B1*	A/C	TaDreb_SNP	A	*TaDREB-B1a*	Tolerant	C	*TaDREB-B1b*	Susceptible
	SST-4D	A/G	SST4D-1093	A	TaSST-D1a	High WSC	G	TaSST-D1b	Low WSC
	SST-A2	GTT/ATA	SST-A2	GTT	TaSST-A2a	High WSC	ATA	TaSST-A2b	Low WSC
	1feh-w3	C/T	fehw3_SNP	C	Westonia type	High expression	T	Kauz type	Low expression
	COMT-3B	T/G	COMT3B_882_SNP	T	COMT-3Ba	High lignin	G	COMT-3Bb	Low lignin
	AWN-5A	T/G	AWN_BWc8266_5AL	T	awn	Absence	G	AWN	Presence

Leaf rust	Lr9	T/A	Lr9_SNP	T	Lr9-	susceptible	A	Lr9+	resistant
	Lr14a	T/A	ubw14	T	*Lr14a-*	Susceptible	A	*Lr14a+*	Resistant
	Lr21	T/C	Lr21-1346-C/T	T	*Lr21-*	Susceptible	C	*Lr21+*	Resistant
	Lr34	TCC InDel	Lr34_TCCIND	Ins	*Lr34-*	Susceptible	Del	*Lr34+*	Resistant
	Lr37/Yr17/Sr38	A/G	VPM_SNP	A	*Lr37+*	resistant	G	*Lr37-*	susceptible
	Lr47	C/G	Lr47-1	C	Lr47-	Susceptible	G	*Lr47+*	Resistant
	Lr67	G/C	CSTM4_67G	G	*Lr67+*	Resistant	C	*Lr67-*	Susceptible
	Lr68	T/C	Lr68-2	T	*Lr68+*	Resistant	C	*Lr68-*	Susceptible
	Lr46	C/G	Lr46_JF2-2A	C	*Lr46-*	Susceptible	G	*Lr46+*	Resistant
Stem rust	*Sr36*	T/C	Sr36/Pm6_8068	T	*Sr36-*	Susceptible	C	*Sr36+*	Resistant
	Sr2	G/A	Sr2_ger9 3p	G	*Sr2-*	Susceptible	A	Sr2 (Hope)	Resistant
Powdery mildew	*Pm21*	T/A	Stpk_75_SNP	T	*Pm21+*	Resistant	A	*Pm21-*	Susceptible
Fusarium head blight	*Fhb1*	InDel	Fhb1_KSU	Del	*Fhb1+*	Resistant	Ins	Fhb1-	Susceptible
Tan spot	*Tsn1*	Null/G	Tsn1	Null	*Tsn1+*	Insensitive	G	*Tsn1-*	Sensitive
Root leision nematode	Rlnn	T/A	Rlnn1_SNP	T	*Rlnn1+*	susceptible	A	*Rlnn1-*	resistant
Virus	Sbm1	C/T	*SbmP_6061*	C	*Sbm+*	susceptible	T	*Sbm-*	resistant
Eye spot	*Pch1*	InDel	*CU8*	VPM1	*Pch1+*	Resistant	Hobbit Sib	*Pch1-*	Susceptible
Soil born diseases	*Cre8*	C/G	*Cre8_SNP*	C	*Cre8-*	susceptible	G	*Cre8+*	resistant
Rye translocation	*1B.1R*	G/A	1B1R_scm9	scm9	1B.1R	+	Housekeeping	1B1B	-
HMW-GS	*Glu-A1*	G/A	*gluA1.1_1594*	G	Ax2*, Null	Strong gluten	A	1	Weak gluten
	Glu-A1	InDel	*gluA1.1_1883*	A	2*, 1	-	G	Null	Strong gluten
	Glu-B1	C/T	Bx13_SNP	C	Bx13	-	T	Non Bx13	-
	Glu-D1	C/G	Glu-D1d_SNP	C	2+12 or other	Weak gulten	G	5+10	Strong gluten
Quality	*Wbm*	C/T	Wbm_SNP	C	*wbm-*	Low expression	T	*wmb+*	High expression
Grain color	Tamyb10-A1	T/C	Tamby10-A1	T	R-A1b	red allele	C	R-A1a	white allele
	Tamyb10-A1	InDel	Tamby10-Nor17	Del	R-A1a(CS)	red allele	Ins	R-A1a(Norin17)	white allele
	Tamyb10-B1	InDel	TamybR B1a-b	Ins	R-B1b	red allele	Del	R-B1a	white allele
	Tamyb10-D1	InDel	TamybR D1a-b	Ins	R-D1a	white allele	Del	R-D1b	red allele
Grain hardness	*Pina-D1*	A/G	Pina-D1_INS	A	*Pina-D1a*	Soft	G	*Pina-D1b*	Hard
	Pinb-D1	C/T	Pinb-D1_INS	C	*Pinb-D1a*	Soft	T	*Pinb-D1b*	Hard
	Pinb2-v2	InDel	Pinb2_IND	Ins	*Pinb-B2a*	Relatively soft	Del	*Pinb-B2b*	Relatively hard
Grain protein	*Gpc-B1*	A/T	GCP_DUP	A	Gpc-B1	Normal GPC	T	Gpc-B1	Increased GPC
	NAM-6A	T/C	NAM-6A-SNP1	T	A1c, A1d		C	A1a, A1b	
	NAM-6A	A/-	NAM-6A-SNP2	A	A1a, A1c		Del	A1b, A1d	
	TaGS1a	3bp InDel	TaGS1a_1447IND	Ins	Hap-I	Low TGW	Del	Hap-II	High TGW
	TaGS2-A1	239 bp InDel	TaGS2A1_239IND2	Ins	TaGS2-A1b	High TGW	Del	Low TGW	TaGS2-A1a
	TaGS2-B1	G/A	TaGS2B1_1936	G			A		
Waxiness	*Wx-B1*	InDel	Wx-B1_IND	Del	*Wx-B1a*	Wild-type	Ins	*Wx-B1b*	Null (mutatnt)
Polyphenol oxidase	*Ppo-A1*	C/T	PPOA1_SNP	C	*Ppo-A1a*	High PPO	T	*Ppo-A1b*	Low PPO
	Ppo-D1	G/C	PPOD1_SNP	G	*Ppo-D1b*	High PPO	C	*Ppo-D1a*	Low PPO
	PPO2-2A	T/C	PPOA2b_230	T	A2b	Low PPO	C	A2a,c	High PPO
	PPO2-2A	G/A	PPOA2c_1864	G	A2c	High PPO	A	A2a,b	Low PPO
	PPO2-2B	A/C	PPOB2c_827	A	B2c	Low PPO	C	B2a,b	High PPO
	PPO2-2B	A/C	PPOB2a_160	A	B2a		C	B2b,c	
	PPO2-2D	98 bp InDel	PPOD2_98IND	Del	D2a	High PPO	Ins	D2b	Low PPO
Phytoene synthase	*Psy-A1*	37 bp InDel	PSY-A1_IND	Ins	*Psy-A1b*	Low YPC	Del	*Psy-A1a*	High YPC
	Psy-B1	T/C	PSY_B1c_SNP	T	*Psy-B1c*	High YPC	C	*Psy-B1a or b*	Low YPC
	Psy-D1	C/T	Psy1Da-g	C	*Psy-D1g*	High YPC	T	*Psy-D1a*	Low YPC
Zeta-carotene	*Zds-A1*	G/C	ZDS-A1_SNP	G	*TaZds-A1b*	High YPC	C	*TaZds-A1a*	Low YPC
	Zds-D1	C/T	ZDS-D1_SNP	C	*TaZds-D1b*	Low YPC	T	*TaZds-D1a*	High YPC
Lipoxygenase activity	*Lox-B1*	G/C	LoxB1_SNP	G	*Lox-B1b*	Low LOX	C	*Lox-B1a*	High LOX
Lycopene	*Lyce-B1*	G/C	LYCE-B1_SNP	G	*TaLYC-B1b*	Lower YPC	C	*TaLYC-B1a*	Higher YPC
Phytoene desaturase	*PDS-B1*	C/G	PDS-B1_SNP	C	*TaPds-B1b*	Lower YPC	G	*TaPds-B1a*	Higher YPC
Peroxidase	*POD-A1*	G/A	PODA1_462_SNP	G	*TaPod-A1a*	Lower POD	A	*TaPod-A1b*	Higher POD