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Genetic Dissection of Stem Water-Soluble Carbohydrates and Agronomic Traits in Wheat under Different Water Regimes

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Abstract

Drought is a major environmental stress threatening wheat (*Triticum aestivum* L.) productivity worldwide. Although drought impedes wheat performance at all growth stages, it is more critical during the flowering and grain-filling phases and results in substantial yield losses. In this context, stem water-soluble carbohydrates (SWSC) were dissected at flowering and grain filling stages under drought stress (DS) and well-watered (WW) conditions using a population consisted of 116 wheat accessions in this research. The main goal was to dissect the genetic basis of water-soluble carbohydrates and the agronomic traits using association mapping approach and identify linked molecular markers. The results showed significant and positive correlations for stem water-soluble carbohydrates at grain filling (SWSCG) with accumulating efficiency of stem water-soluble carbohydrates (AESWSC) and grain filling efficiency at the late stage (GFEL). The accumulating and grain filling efficiency at grain filling stage could play an important role for SWSC especially under DS condition. Four favorable alleles for plant height (PH) and grain yield (GY) were identified in two water environments. *Xbarc78-4A*$_{163}$ and *Xbarc78-4A*$_{155}$ were variant alleles for PH which were identified in both water regimes. Whereas *Xwmc25-2D*$_{151}$ and *Xgwm165-4B*$_{191}$ positively linked with GY in WW. Although *Xwmc420-4A*$_{121}$ and *Xwmc112-2D*$_{215}$ were alleles for stem water-soluble carbohydrates at flowering (SWSCF) and SWSCG in DS but the frequency were < 5% so they were considered as rare alleles. These SSR markers which explained significant level of phenotypic variability for chosen traits could be used for selection of genotypes in wheat breeding programs through marker-assisted selection.

Keywords: association mapping, drought, stem water-soluble carbohydrates, SSR markers, wheat

1. Introduction

Wheat is one of the three major cereals. Global annual production is 727.87 million metric tons in 2014-2015 (USDA, 2016). To ensure food for the rapidly growing world population, wheat production needs to double by 2050 (Alexandratos & Bruinsma, 2012). Further increases in wheat production depend on higher yields rather than an increase in cropping area (Araus et al., 2003). Declining water resources challenge this notion as water availability impacts heavily on crop yields (Kang et al., 2008). Among all the abiotic stress factors that limit crop productivity, drought is the most devastating one and the most difficult to breeders' efforts. Breeding efforts in the past, to improve drought tolerance has been hindered by its quantitative genetic basis and the poor understanding of the physiological basis of yield under water-limited conditions (Tuberosa & Salvi, 2006). Genomic approaches enable the identification and selection of chromosome regions harboring genes/QTLs (Quantitative trait loci) controlling agronomic traits and yield in crops (Collins et al., 2008; Cooper et al., 2009; Tuberosa & Salvi, 2006). Among such approaches, association mapping is increasingly being adopted as a method complementary to traditional bi-parental linkage mapping to identify genotype-phenotype associations *i.e.* molecular marker-trait associations (Sorrells & Yu, 2009; Waugh et al., 2009).

Water-soluble carbohydrates (WSC) in wheat stems are mainly composed of fructan, sucrose, glucose, and fructose, in with fructan is the major component at the late stage of WSC accumulation phase (Ruuska et al., 2006). The WSC can be accumulated in the stem and leaf sheath of cool-season cereals *e.g.* wheat, barley

(*Hordeum vulgare*), and oats (*Avena sativa*) during the period from stem elongation to early phase of grain filling and serve as temporary carbohydrate reserves, commonly called the stem carbohydrate reserves (Blum, 1998; Gebbing, 2003). In general, WSC accumulate until 10~20 days after anthesis, and the reserved WSC can reach more than 40% of total stem dry weight in wheat (Rebetzke et al., 2008).

The contribution of WSC to final yield and kernel size is 10~20% of total grain weight under normal condition (Gebbing & Schnyder, 1999). Drought stress during grain filling, often involving not only water stress but also heat, inhibits current assimilation and damages photosynthetic organs, especially leaves. When photosynthetic activity is suppressed, the reserved WSC play a more important role in partial compensation of the reduced carbon supply. In addition, drought induced reserved WSC mobilization with higher efficiency, potentially contributing up to 70% of grain dry matter (Goggin & Setter, 2004; Rebetzke et al., 2008). Therefore WSC is a major contributor to wheat grain yield and grain size in all environments but especially where photosynthesis is compromised as occurs where water is limiting (McIntyre et al., 2012). Grain yield depends on carbon from two resources: flag leaf photosynthesis and remobilization of water-soluble carbohydrates, mainly fructans, from the wheat stems (Yang & Zhang, 2006). In term, high water-soluble carbohydrate content in the stem has been suggested as a selection criterion for use inbreeding. WSC QTL have been reported in rice (Nagata et al., 2002; Takai et al., 2005), wheat (Rebetzke et al., 2008; Yang et al., 2007), maize (Thévenot et al., 2005), barley (Teulat et al., 2001) and perennial rye grass (Turner et al., 2006). With the rapid increases in number of molecular markers, association analysis has become an important tool for dissection of complex traits (Bradbury et al., 2007).

The main objective of this research is to find out the favorable alleles responsible for expression of water-soluble carbohydrates and important agronomical traits by association mapping to help breeders to improve their breeding programs through marker-assisted selection.

2. Methods

2.1 Plant Materials and Field Trials

The plant material was a population consisted of 116 winter wheat genotypes, which were collected from the different regions of China, including landraces, advanced lines and modern cultivars released from 1940s to 2000s. The wheat accessions were planted in the experiment station of the Institute of Crop Science, Chinese Academy of Agricultural Sciences at Changping (116°13′E; 40°13′N), Beijing in September 25th 2013 and harvested in the mid of June 2014.

The experimental unit was a plot consisted of four rows of 2 m length with row spaced 30 cm apart. Forty seeds were sown in each row. The field plots were subjected to two water treatments: full irrigation (well-watered, WW) and rain fed (drought stress, DS) recorded 163 mm of rainfall during 2013~2014 growing season. The WW plots were watered with 750 m^3/ha (75 mm) at the pre-overwintering, booting and flowering stages. The amount of rainfall was insufficient during each corresponding period. A randomized complete block design with three replicates was used for both WW and DS treatments. Plants were scored for the following agronomic, physiological and developmental traits: days to heading (DTH), days to flowering (DTF), flag leaf area (FLA), chlorophyll content at flowering (CCF), chlorophyll content at grain filling (CCG), plant height (PH), peduncle length (PL), spike length (SL), length of second internode from the top (LSIT), length of internodes below second internode from the top (LIBSIT), number of spikes per plant (SP), number of spikelets per spike (NSPS), sterile spikelet at the top (SST), sterile spikelet at the middle (SSM), sterile spikelet at the base (SSB), number of grains per spike (NGS), grain yield per plant (GY), grain yield per plot (GYP) and thousand-grain weight (TGW).

2.2 Phenotyping of Stem WSC and TGW

The five main stems were cut from the soil surface at two morphological stages, viz. flowering and mid-grain filling (14 days after flowering). Samples were taken from the mid-part of second row of each plot. Leaf blades were removed and the stems with leaf sheaths were cut into two parts *i.e.* the stem and the spike. The fresh samples were killed at 105 °C for 30 minutes and then keep at 80 °C to dehydrate until a constant dry weight. Stem samples of each accession were chipped into 2~5 mm length. The stem WSC was determined by the near-infrared reflectance spectroscopy (NIRS) regression models (Wang et al., 2011). Briefly, at the first step, partial least square regression models for predicting WSC in the target parts of wheat were developed using selected wavelength regions, spectroscopic pretreatments and the latent variables included in each model. The total amounts of WSC (mg/g dry weight) in each sample were also measured by chemical assay (anthrone colorimetric assay), and used for the cross validation. The NIRS regression models were highly accurate in determination of the true values of WSC measured by chemical assay in the wheat organs tested, according to

high coefficients of determination of the true values of WSC measured by chemical assay in the wheat organs tested, according to high coefficients of determination ($R^2 > 0.992$) and low root mean square errors of prediction (RMSEP < 0.228).

We obtained stem WSC at the flowering (SWSCF) and mid-grain filling (SWSCG) stages. Accumulating efficiency of SWSC (AESWSC) was estimated by [(SWSCG–SWSCF)/SWSCG] × 100%. Spikes corresponding to main stem samples were collected at the mid-grain filling for each accession to obtain thousand-grain weight (TGWG). In addition, the grain filling efficiencies at the early period (before 14 days after flowering) (GFEE) and the grain filling efficiencies at the late period (from 14 DAF to grain maturity) (GFEL) were assessed by (TGWG/TGWM) × 100% and [(TGWM–TGWG)/TGWM] × 100%, respectively. TGWM: thousand-grain weight at maturity stage.

2.3 Genotyping by SSR Markers

Seedling leaves were used as the experimental material. Leaf samples were collected from one hundred and sixteen genotypes. Experiment set *in vitro* and twenty seeds placed in each petri plate. First leaves were collected for DNA extraction. DNA extracted by DNA Quick Plant System Kit (Tiangen Bio. Co. LTD, Beijing, China). Selection of 92 SSR markers based on evenly distributed on different chromosomes and/or linked with WSC or investigated agronomic traits. These SSR markers were found on chromosome 1A, 1D, 2A, 3B, 4A, 4B, 5A, 6B, 7A, 7B and 7D, respectively. SSR analysis was conducted using 92 primer pairs directed to the amplification of di- and tri nucleotide microsatellite loci, originally developed in bread wheat within five different research programs, WMS Wheat Microsatellite (Röder et al., 1998), WMC Wheat Microsatellite Consortium (Gupta et al., 2002), BARC USDA-ARS Beltsville Agricultural Research Center (Song et al., 2005), CFA and CFD (Guyomarc'h et al., 2002; Sourdille et al., 2001). Among 92 primers, sixty primer pairs for wheat microsatellite loci were synthesized by Sangon Biotech (Shanghai) Company. For each primer pair, the sequence of original forward primer was redesigned by adding a universal M13-tail (5'CACGACGTTGTAAAACGAC-3') to their 5' ends. Thirty-two primers were used as fluorescent markers. The universal M13 primers were labelled with different fluorescence dyes *i.e.* blue and green.

The PCR was conducted in a total volume of 15 µl, containing 20 ng genomic DNA, 10 X supplied PCR buffer including 1.2 mM of $MgCl_2$, 2 mM of dNTPs, 5 unit/µl of *Taq* DNA polymerase, 2 µM of primer. Amplification reactions were conducted using a Senso Quest lab cycler. Gradient PCR was used to determine optimal annealing temperature for each primer pair. The M13 primers were labelled with different fluorescent dyes i.e. blue and green, allowing done PCR again with the following reaction mixture containing 162 µl M13 dye (AB Applied Biosystem, USA), PCR buffer 9 µl, *Taq* DNA polymerase 7.2 µl. Added 2.1 µl of above mixture in 96 well plate that already amplified at above mentioned conditions. This mixture was further amplified under the following conditions 94 °C for 5 min; 16 cycles of 94 °C for 45 s, 56 °C for 45 s and 72 °C for 45 s; followed by a 10 min extension at 72 °C. The PCR products were analyzed by electrophoresis in 2% agarose gel. The PCR were carried out separately for each microsatellite and the mixture of PCR products of three different markers with different dyes was made for simultaneous detection of the amplified alleles. Sequencing of 92 simple-sequence-repeat (SSR) loci has done by ABI3730 DNA analyzer. The PCR products were analyzed by Gene Mapper Software. The polymorphism information content (PIC) values (Botstein et al., 1980) were calculated using Power Marker software v3.25 (Liu & Muse, 2005). Population structure was estimated by STRUCTURE v2.3.2 using data from 92 SSR markers. Twenty subpopulations (k = 1 to 20) were set with a burn-in period of 50,000 iterations and a run of 500,000 replications of Markov Chain Monte Carlo after burn in. The Δk method was applied according to LnP(D) in STRUCTURE, and the output and result were estimated (Pritchard et al., 2000).

2.4 Marker-Trait Association and Statistical Analysis

Association between markers and traits was calculated using a general linear model (GLM) method in TASSEL v2.1 (Yu et al., 2006). The population structure matrix (Q) obtained from the STRUCTURE software and relative kinship matrix (k matrix) derived from the unlinked marker data estimated by TASSEL v2.1 were combined to covariate in the association tests to reduce false positive rate. The significant marker-trait associations were declared by $P \leq 0.01$ and the magnitude of the allele effects were evaluated by R^2-marker. Analysis of variance (ANOVA) was conducted using SPSS 16.0. Pearson's correlation coefficient among the traits under two water regimes were calculated by SPSS 16.0. Broad sense heritability (h^2_B) was computed by QTL IciMapping (http://www.isbreeding.net/).

3. Results

3.1 Drought Stress Induces SWSC

On the basis of the ANOVA, the SWSC of 116 genotypes at flowering and grain filling stages were highly affected by the DS. The mean square value of SWSCG under drought stress condition was significantly higher than that under well-watered condition, but the traits such as AESWSC, GFEE, GFEL, and TGWG showed non-significant differences (Table 1).

Table 1. Mean squares for various traits under two water regimes

Traits	MS	CV(%)
SP	14.26^{***}/**12.72^{***}**	0.26/**0.23**
SL	2.94^{***}/**3.92^{***}**	0.13/**0.14**
PL	56.37^{***}/**89.08^{***}**	0.22/**0.20**
LSIT	21.73^{***}/**42.23^{***}**	0.19/**0.18**
LIBSIT	251.32^{***}/**487.62^{***}**	0.31/**0.32**
NSPS	4.97^{***}/**4.85^{***}**	0.08/**0.08**
SST	0.65^{***}/**0.28^{***}**	1.21/**1.19**
SSM	0.007^{*}/**ND**	3.97/**ND**
SSB	1.41^{***}/**0.65^{***}**	0.34/**0.56**
NGS	80.21^{***}/**36.12^{***}**	0.17/**0.13**
GYP	100726.78^{***}/**199650.55^{***}**	0.38/**0.40**
TGW	84.34^{***}/**65.97^{***}**	0.18/**0.15**
PH	658.11^{***}/**1176.94^{***}**	0.21/**0.20**
DTH	12.49^{***}/**15.98^{***}**	0.01/**0.02**
DTF	9.61^{***}/**15.26^{***}**	0.01/**0.01**
FLA	5.73^{***}/**12.43^{**}**	0.21/**0.22**
GY	978.30^{***}/**1867.46^{***}**	0.26/**0.27**
CCF	38.42^{***}/**32.94^{***}**	0.08/**0.07**
CCG	85.52^{***}/**92.572^{NS}**	0.15/**0.23**
SWSCF	2351.22^{***}/**2563.55^{***}**	0.17/**0.23**
SWSCG	3972.17^{***}/**3643.75^{***}**	0.17/**0.22**
AESWSC	1124.08^{NS}/**1126.19^{NS}**	0.55/**0.63**
GFEE	1141.58^{NS}/**1115.19^{NS}**	0.27/**0.33**
GFEL	1117.45^{NS}/**1135.62^{NS}**	0.24/**0.27**
TGWG	1108.06^{NS}/**1155.11^{NS}**	0.29/**0.31**

Note. MS: mean squares; ** and *** indicate $P = 0.01$ $P = 0.001$, respectively; CV: coefficient of variation; NS: non-significant; ND: not determined; un bold value shows under DS while bold value shows under WW conditions; DS: drought stress; WW: well-watered; SP: number of spikes per plant; SL: spike length; PL: peduncle length; LSIT: length of second internode from the top; LIBSIT: length of internodes below second internode from the top; NSPS: number of spikelet per spike; SST: sterile spikelet at the top; SSM: sterile spikelet at the middle; SSB: sterile spikelet at the base; NGS: number of grains per spike; GYP: grain yield per plot; TGW: thousand-grain weight; PH: plant height; DTH: days to heading; DTF: days to flowering; FLA: flag leaf area; GY: grain yield per plant; CCF: chlorophyll content at flowering; CCG: chlorophyll content at grain filling; SWSCF: stem water-soluble carbohydrates at flowering stage; SWSCG: stem water-soluble carbohydrates at grain filling stage; AESWSC: accumulation efficiency of stem water-soluble carbohydrates; GFEE: grain filling efficiency at the early stage; GFEL: grain filling efficiency at the late stage; TGWG: thousand-grain weight at grain filling stage.

SWSC at flowering stage ranged from 140.75 to 298.5 mg/g whereas SWSC at grain filling stage ranged from 140.63 to 346.37 mg/g under drought stress (Figure 1). It is reported that stem water-soluble carbohydrates are a major carbon source for grain filling under drought stress (Zhang et al., 2016). In addition, stem water-soluble carbohydrates buffer wheat grain yield against drought stress for photosynthesis during the grain filling stage (Li

et al., 2015). In the present study, moderate to high broad sense heritability for SWSC at both stages under WW and DS conditions were estimated ranged from 0.76~0.78 to 0.71~0.80 (Appendix 1).

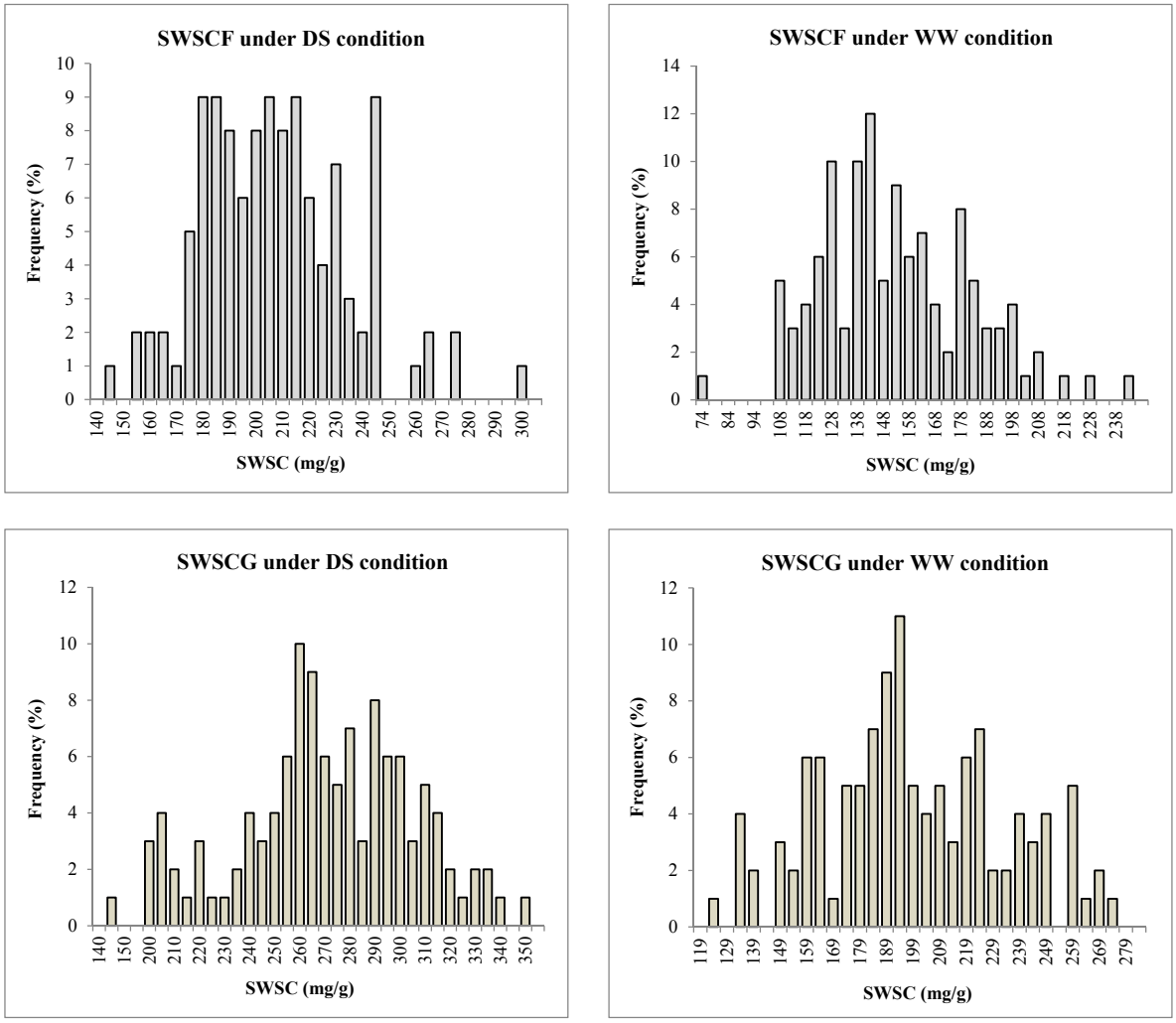

Figure 1. Frequency distribution of SWSC at flowering and grain filling stages under DS and WW condition

Note. DS: drought stress; WW: well-watered; SWSC: stem water-soluble carbohydrates; SWSCF: stem water-soluble carbohydrates at flowering stage; SWSCG: stem water-soluble carbohydrates at grain filling stage.

3.2 SWSC Correlated with Other Traits

Correlation coefficients among all traits under the two water regimes are given in Table 2. The traits associated with SWSC at two different growth stages such as SWSCF and SWSCG were highly significant correlation with each other under WW and DS conditions. SWSCF showed a highly significantly negative correlation with AESWSC under both water conditions. While, SWSCF had a significantly positive relationship with thousand-grain weight at grain filling (TGWG) and maturity (TGWM) under DS condition. In WW condition, SWSCF were also significantly positive correlation with TGWG and TGWM but had smaller values of correlation. However, SWSCG exhibited a highly positive correlation with AESWSC, TGWM and grain filling efficiency at late stage (GFEL) in both environments.

Table 2. Pearson's correlation coefficients for traits under two water regimes

Traits	SWSCF	SWSCG	AESWSC	TGWG	TGWM	GFEE	GFEL
SWSCF		0.402**	-0.472**	0.294**	0.300**	0.085	0.084
SWSCG	0.486**		0.540**	0.153**	0.460**	-0.155**	0.337**
AESWSC	-0.462**	0.511**		-0.147**	0.130*	-0.227**	0.229**
TGWG	0.130*	0.099	-0.042		0.341**	0.758**	-0.385**
TGWM	0.293**	0.576**	0.298**	0.159**		-0.328**	0.727**
GFEE	-0.026	-0.182**	-0.182**	0.870**	-0.325**		-0.887**
GFEL	0.145**	0.386**	0.268**	-0.610**	0.677**	-0.916**	

Note. Values in the upper right portion are for DS; those at the lower left are for WW. *: indicates $P = 0.05$, and **: $P = 0.01$.

There was a positive and significant correlation between SWSCG and TGWG under DS while non-significant correlation was noted for the same trait under WW condition. Whereas SWSCG had a significant and negative association with grain filling efficiency at early stage (GFEE) under two water conditions. AESWSC was significantly correlated with GFEE and GFEL under both conditions. TGWG had a highly significant correlation with GFEE (r^2 = 0.758** and 0.870**) in both environments DS and WW. Whereas TGWG and GFEL was negative correlation, the effects were opposite for TGWM with GFEE and GFEL under two water conditions (Table 2).

3.3 SSR Marker Polymorphism and Population Structure

Ninety two SSR markers were scored across 116 wheat accessions to proceed to the population structure assessment and association analysis. A total of 1600 alleles were amplified among the 116 wheat accessions, and the number of alleles per locus ranged from 3~49 with an average of 17.39. Frequencies of the 92 SSR loci ranged from 0.08 (*Xwmc757*) to 0.95 (*Xgwm515*). Besides, the average PIC value was 0.71 with a range of 0.09~0.96. The analysis of population structure was inferred with the STRUCTURE 2.3.2 software (Figure 2A). According to the method of Evanno et al. (2005), Δ*k* was plotted against the number of subpopulations *k* (Figure 2B). The maximum value of Δ*k* occurred at *k* = 5, such that *k* = 5 (five subpopulations) was defined to provide the optimal structure (Figure 3).

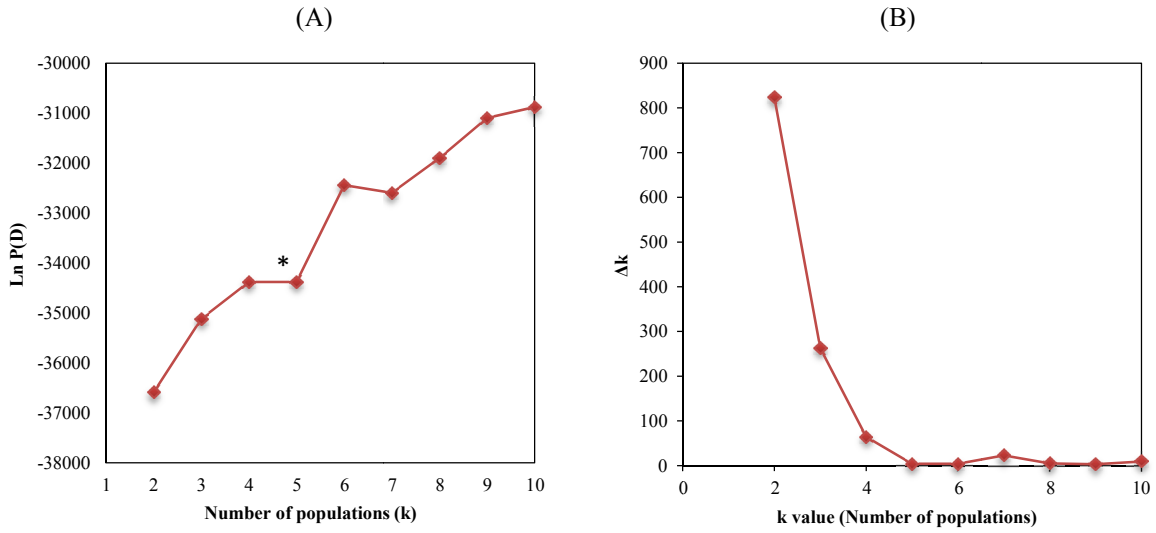

Figure 2. Population structure analysis of 116 wheat accessions based on 92 SSR markers

Note. (A) Estimated Ln P(D) by STRUCTURE; (B) Estimated Δ*k* of STRUCTURE analysis.

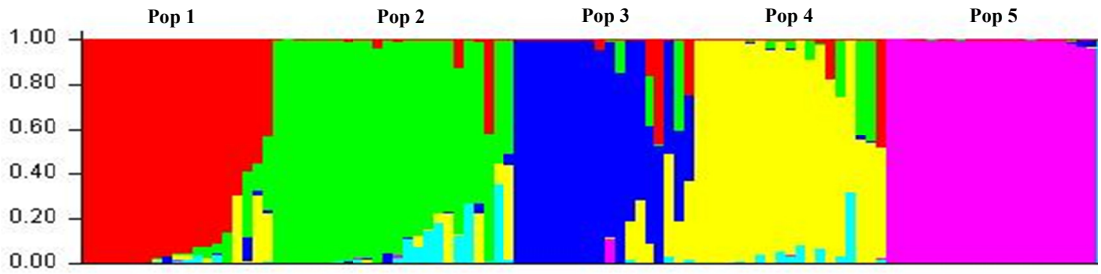

Figure 3. Population structure analysis of 116 wheat accessions based on 92 SSR markers. Five subpopulations inferred by structure analysis. Each of the 116 genotypes is represented by a different colors indicate different subpopulation

3.4 Marker-Trait Associations

Marker-trait association was tested through the general linear model. Based on the critical *P*-value less than 0.01 with 92 SSR markers for traits, identified 31 marker-trait associations (MTAs) involving 17 SSR markers distributed on the nine chromosomes for 13 traits and the R^2 ranges from 0.15 to 0.44. Among the MTAs, 12 SSR markers were specific for single traits, and the rest consisted of associations of up to five traits (Table 3). LIBSIT under DS was involved in the highest number of (5), distributing on the different chromosomes (2D, 3B, 4A and 5A respectively), and MTAs (4) were found for LIBSIT under WW condition on the chromosomes (2A, 2D, 3B and 4A respectively), followed by TGWG (4) in DS. The fewest MTAs were associated with LSIT (1) in both conditions. Each trait was associated with at least one (CCG, DTH, GY, GYP, LSIT, PL, SP, SWSCF and SWSCG) and the maximum five (LIBSIT) chromosomes.

The highest number of associated markers were found on chromosome 2D, 4A and 5A (3 each), followed by 3B and 4B (2 each), with the least on 1A, 1D, 2A and 7D (1 each). Under drought stress condition, LIBSIT was significantly associated with 5 markers with R^2 ranged from 0.18 to 0.27. The 5 MTAs were spread over chromosomes 2D (18 cM), 3B (61 cM), 4A (7cM, 71 cM), 5A (52 cM). Under well-watered condition, LIBSIT was significantly associated with 4 markers with R^2 ranged from 0.21 to 0.26. The 4 MTAs were found on chromosomes 2A, 2D, 3B and 4A. TGWG was significantly associated with 4 markers with R^2 ranged from 0.27 to 0.42 under DS. The 4 MTAs were observed on chromosomes 1D, 3B, 5A and 7D. PH was significantly associated with 3 markers with R^2 ranged from 0.18 to 0.23 under DS. The 3 MTAs were identified on chromosomes 2D (18 cM), 4A (7 cM, 71 cM). However under WW condition PH was significantly associated with only one marker with R^2 0.21. This MTA was on 4A (71 cM). TGW was significantly associated with 2 markers with R^2 ranged from 0.26 to 0.30 under DS, and from 0.28 to 0.31 under WW condition. The 2 MTAs for TGW in DS were located on 4B (31 cM), 5A (61 cM), and under WW on 4A (18 cM) and 4B (31 cM) chromosomes. Only one marker was identified associated with the other individual trait.

Xwmc25-2D was associated with LIBSIT under both conditions, whereas it was also associated with GY under WW and PH under DS. *Xbarc78-4A* was significantly associated with PH and LIBSIT under both conditions. *Xwmc420-4A* was significantly associated with LIBSIT, PH, PL, and SWSCF under only DS while LSIT under both conditions. *Xgwm149-4A* was significantly associated with TGW under both conditions whereas it was also associated with SP under DS. *Xgwm285-3B* was significantly associated with LIBSIT in both DS and WW conditions.

Table 3. Favorable alleles associated with phenotypic traits

Water regimes	Traits	Chr.	Locus	Chr. Position (cM)	Favorable allele (bp)	Frequency (%)	Mean ± SE	*P*-value	Effect
DS	DTH	1A	*Xbarc148*	57	163	0.86	205±0	0.0001	Negative
					Others	99.14	209±0.1		
	TGWG	1D	*Xwmc432*	23	197	0.86	20.5±0	0.0007	Positive
					others	99.14	9.9±0.1		
	LIBSIT	2D	*Xwmc25*	18	181	0.86	52.0±0	0.0006	Positive
					others	99.14	28±0.8		
	PH	2D	*Xwmc25*	18	181	0.86	109±0	0.0003	Positive
					others	99.14	75±1.3		
	SWSCG	2D	*Xwmc112*	28	215	0.86	346.3±0	0.0009	Positive
					others	99.14	267.7±3.3		
	LIBSIT	3B	*Xgwm285*	61	245	0.86	45.7±0	0.0005	Positive
					others	99.14	30.0±0.8		
	TGWG	3B	*Xbarc164*	70	214	0.86	20.5±0	0.0003	Positive
					others	99.14	9.9±0.1		
	LIBSIT	4A	*Xbarc78*	71	163	5.17	45.9±1.2	0.0000	Positive
					others	94.83	29.8±0.8		
	PH	4A	*Xbarc78*	71	163	5.17	100±2.9	0.0002	Positive
					others	94.83	75±1.3		
	LIBSIT	4A	*Xwmc420*	7	123	0.86	52.0±0	0.0000	Positive
					others	99.14	30.8±0.8		
	LSIT	4A	*Xwmc420*	7	121	1.72	19.5±0	0.0001	Positive
					others	98.28	16.3±0.2		
	PH	4A	*Xwmc420*	7	123	0.86	109±0	0.0000	Positive
					others	99.14	76±1.3		
	PL	4A	*Xwmc420*	7	121	1.72	27.4±0	0.0005	Positive
					others	98.27	21.4±0.4		
	SWSCF	4A	*Xwmc420*	7	121	1.72	191.9±6.8	0.0009	Negative
					others	98.28	202.1±2.6		
	TGW	4B	*Xgwm149*	31	139	0.86	37.9±0	0.0002	Positive
					others	99.14	32.1±0.4		
	SP	4B	*Xgwm149*	31	137	0.86	16±0	0.0000	Positive
					others	99.14	10±0.2		
	LIBSIT	5A	*Xgwm293*	52	204	0.86	44±0	0.0004	Positive
					others	99.14	30.1±0.8		
	TGWG	5A	*Xgwm415*	56	136	0.86	6.6±0	0.0000	Negative
					others	99.14	9.9±0.2		
	TGW	5A	*Xgwm304*	61	216	0.86	19.0±0	0.0007	Negative
					others	99.14	32.3±0.48		
	TGWG	7D	*Xgwm295*	77	229	0.86	16.7±0	0.0005	Positive
					others	99.14	9.9±0.18		
WW	LIBSIT	2A	*Xwmc296*	49	150	0.86	61.8±0	0.0009	Positive
					others	99.14	40.9±1.1		
	CCG	2D	*Xgwm382*	100	114	1.72	45.1±0	0.0010	Positive
					others	98.28	39.8±0.6		
	LIBSIT	2D	*Xwmc25*	18	181	0.86	64.3±0	0.0006	Positive
					others	99.14	40.73±1.1		
	GY	2D	*Xwmc25*	18	151	29.31	143.39±3.6	0.0001	Positive
					others	70.69	133.1±2.8		
	LIBSIT	3B	*Xgwm285*	61	251	1.72	59.5±0	0.0008	Positive
					others	98.28	40.4±1.1		
	LIBSIT	4A	*Xbarc78*	71	140	0.86	53.8±0	0.0000	Positive

				others	99.14	41.7±1.1		
PH	4A	*Xbarc78*	71	155	15.51	84±3.5	0.0000	Negative
				others	84.49	105±1.9		
LSIT	4A	*Xwmc420*	7	121	1.72	28.4±0	0.0009	Positive
				others	98.28	23.0±0.3		
TGW	4A	*Xgwm397*	18	190	0.86	21.7±0	0.0001	Negative
				others	99.14	32.2±0.43		
GYP	4B	*Xgwm165*	28	191	78.44	768.6±26.3	0.0010	Positive
				others	21.56	652±54.34		
TGW	4B	*Xgwm149*	31	139	0.86	35.34±0	0.0003	Positive
				others	99.14	32.6±0.4		

Note. DS: drought stress; WW: well-watered; Chr. chromosome; DTH: days to heading; TGWG: thousand-grain weight at grain filling stage; LIBSIT: length of internodes below second internode from the top; PH: plant height; SWSCG: stem water-soluble carbohydrates at grain filling stage; PL: peduncle length; SWSCF: stem water-soluble carbohydrates at flowering stage; TGW: thousand-grain weight; SP: number of spikes per plant; CCG: chlorophyll content at grain filling; GY: grain yield per plant; LSIT: length of second internode from the top; GYP: grain yield per plot.

On chromosome 2D, four traits such as GY (DS), LIBSIT (DS and WW), PH (DS) and SWSCG (DS) were tagged by SSR markers *Xwmc25* (18 cM) and *Xwmc112* (28 cM). The LIBSIT (DS and WW) and TGWG (DS) were associated by *Xgwm285* (61 cM) and *Xbarc164* (70 cM) on chromosome 3B. LIBSIT (DS), LSIT (DS and WW), PH (DS), PL (DS), SWSCF (DS), TGW (WW) were tagged by the *Xwmc420* (7 cM) and *Xgwm397* (18 cM) on chromosome 4A. *Xbarc78* (71 cM) was associated with PH and LIBSIT also on chromosome 4A in both water conditions. On chromosome 4B, SP (DS) and TGW (DS and WW) and GYP (WW) were identified by markers *Xgwm149* (31 cM) and *Xgwm165* (28 cM). The LIBSIT, TGWG and TGW were separately tagged by three markers *Xgwm293* (52 cM), *Xgwm415* (56 cM) and *Xgwm304* (61 cM) on chromosome 5A under DS condition.

3.5 Favorable Alleles for Different Traits

Four of the 17 loci had significantly favorable allelic effect on multiple traits were identified in DS and WW conditions (Table 3). Most of the traits had < 5% frequency of the SSR marker allele in both conditions. It was considered as a rare allele. Under DS condition, 163 bp allele of *Xbarc78* on chromosome 4A (*Xbarc78-4A*$_{163}$) showed positive effects on LIBSIT and PH, respectively. This allele had a frequency of 5.17% on both traits. Under WW condition, two marker alleles *Xwmc25-2D*$_{151}$ and *Xgwm165-4B*$_{191}$ separately increased GY and GYP, but *Xbarc78-4A*$_{155}$ showed a negative effect on PH. These favorable alleles associated with important traits that could be contribute to increase wheat production in different water environments.

4. Discussion

4.1 Variation of Stem Water-Soluble Carbohydrates under Different Water Condition

The stem WSCs become more important for grain yield in cereal crops under abiotic stress (Blum, 1998; Kiniry, 1993; van Herwaarden et al., 1998). A good capacity for stem reserve and remobilization of WSC has been proposed as a drought adaptive trait in a conceptual model for drought tolerance (Reynolds, 1999). In this study, variation in SWSC among 116 genotypes at two developmental stages has been observed under two water regimes. The phenotypic means for this trait were more affected by drought stress. The means under drought stress were significantly higher than those under well-watered. The present observations were consistent with the view of Zhang et al. (2014) that reported fructan synthesis is induced by drought stress, and that drought tolerant plants can manufacture more fructans. Fructans are the major component of WSC, insert between the head groups of phospholipids, acting as compatible solutes in cells to protect cell membranes and proteins from osmotic damage (Rathinasabapathi, 2000; Vereyken et al., 2001).

In our research, SWSCF and SWSCG under drought stress were overall higher than those under well-watered condition. The tolerant cultivars activate their protection mechanisms faster and more efficiently than the sensitive ones to cope with stress conditions (Goggin & Setter, 2004; Gupta et al., 2011). WSC mobilizes from the stem during the later phase of grain filling and thus can become an important source of assimilate for grain yield in wheat under terminal drought stress conditions (Blum, 1998). Stem WSC accumulation is influenced by

environmental factors (Blum, 1998; Ruuska et al., 2008; Ruuska et al., 2006). However, considerable genotypic variation in stem WSC concentration has been observed in wheat (Ruuska et al., 2006; Xue et al., 2008).

4.2 Heritability of Stem Water-Soluble Carbohydrates

In the present study, moderate to high broad sense heritability for SWSC at two stages (flowering and grain filling) under WW and DS conditions were estimated ranged from 0.76~0.78 to 0.71~0.80. High heritability indicates potential for phenotypic selection of WSC among families in breeding programs that target adaptation to terminal droughts (Rebetzke et al., 2007). The ability to store and remobilize large amounts of WSC to grain has been suggested as a selection criterion for wheat breeding due to its high heritability and positive linear ship with grain yield (Dreccer et al., 2009; Gupta et al., 2011).

4.3 Correlation of Stem Water-Soluble Carbohydrates and Grain Yield Traits

Mobilization of WSC during grain filling can potentially contribute about 20% of the final grain weight under non-stress conditions, and up to 70% or more of grain dry matter under drought stress in wheat (Goggin & Setter, 2004). It has also been reported that stem WSC concentration at anthesis or shortly after anthesis (*i.e.* at the stem WSC accumulation phase) is a good indicator of positive association between WSC level and grain weight or yield in wheat (Foulkes et al., 2007). Our research exhibited that SWSCF showed significantly negative correlation with AESWSC while positive association with TGWG and TGWM under both normal and stress conditions. In contrast, SWSCG had significant correlation with all traits such as AESWSC, TGWG, TGWM, GFEE and GFEL under both conditions with the exception of TGWG under WW condition that reflected non-significant association between SWSCG and TGWG. It is suggested that SWSCG could play a key function in the subsequent release of carbohydrates from stem to grain. Current results are in conformity by Yang et al. (2007). WSC are recognized as an important source of grain dry matter for grain filling, especially when current photosynthesis is inhibited by drought stress. Water deficit during grain filling stimulates senescence of the whole plant and enhances mobilization of reserved WSC to the grains (Araus et al., 2002; Guoth et al., 2009).

Negative correlation was observed between SWSCG and GFEE, whereas there was a positive association of SWSCG with GFEL under both environments. It is suggested that SWSCG could play an important role in grain filling of wheat. AESWSC exhibited positive correlation with TGWM and GFEL in both drought stress and well-watered conditions. It indicated that accumulation of water-soluble carbohydrates increased the grain filling efficiency at the later stages and that contributed to heavier the grains in term of thousand-grain weight. Xue et al. (2008) also reported a positive and significant relationship with WSC and grain weight in wheat lines.

In this study, thousand-grain weight and grain filling efficiency under drought stress was slightly lower than those under well-watered condition during the early grain filling period. Li et al. (2015) reported higher thousand-grain weight with increased grain filling efficiency under drought stress as compared to well-watered condition during the early grain filling period. Water deficit at grain filling induces carbon mobilization from tillers to the main stem ear (Blum et al., 1994). Rebetzke et al. (2007) reported that wheat progeny with high WSC produced higher grain weight and larger diameter, significantly reducing grain shriveling. WSC accumulation and remobilization are influenced by many factors, making the relationship between WSC and TGW more complex.

4.4 Genetic Polymorphism

A total of 1600 alleles were identified from 92 SSR loci when scored on 116 genotypes. High levels of polymorphism were observed for the markers, with a range of 3~49 and average of 17.39 alleles per marker locus, which indicated that the diversity of wheat accessions in this study was relatively high. The microsatellite markers presented high level of PIC in comparison with other markers in wheat (Gupta et al., 2008). It is suggested that genomic SSR markers is powerful for the evaluation of genetic polymorphism, similar results were obtained in 103 wheat accessions (Liu et al., 2010).

4.5 Marker Allele-Trait Associations

4.5.1 Chromosome 4A

We identified 17 SSR marker loci which significantly associated with agronomic and physiological traits. Among them, chromosome 4A was most important, six traits were associated with three loci, including *Xwmc420-4A* (7 cM), *Xgwm397-4A* (18 cM) and *Xbarc78-4A* (71 cM). Four classes of marker pairs were defined on the basis of the map positions determined by Marone et al. (2012): class 1 (tight linkage; distance, < 10 cM); class 2 (moderate linkage; distance, 10~20 cM); class 3 (loosely linked; distance, 20~50 cM); and class 4 (independent pairs; distance, > 50 cM). It is suggested that *Xwmc420* and *Xgwm397* moderately linked however *Xbarc78* behaved as an independent locus.

The present research results indicated that *Xwmc420-4A* was associated with LSIT under both drought stress and well-watered conditions, and also associated with LIBSIT, PH, PL, and SWSCF in drought stress. *Xwmc89*, a marker locus at the same position as *Xwmc420*, was reported that significantly associated with all grain related QTLs and explained the high proportion of phenotypic variation (Kirigwi et al., 2007). A SSR marker *Xwmc48* was identified associating with QTL for grain yield (Kirigwi et al., 2007). In addition, Liu et al. (2010) identified a QTL for PH nearby marker *Xwmc420*. In current study, Crossa et al. (2007) found a DArT marker *wPt8271*, which close to *Xbarc70* and *Xbarc78*, was associated with GY, whereas Liu et al. (2010) reported for grains per spike and thousand-kernel weight.

4.5.2 Chromosome 5A

The next important chromosome in this research was 5A. It was tagged by three SSR markers namely *Xgwm293* (52 cM), *Xgwm415* (56 cM) and *Xgwm304* (61 cM). The distance among these markers were less than 10 cM. Yang et al. (2007) detected QTL with flanking marker of *Xwmc524~Xgwm595* for TGWG, it was on the same chromosome as our studies but with different position and also was associated with same trait as in this study. Furthermore, (Maccaferri et al., 2011) found *Xbarc197* located on chromosome 5A (53 cM) adjacent with *Xgwm293* (52 cM) and also nearby *Xgwm415* and *Xgwm304* (56 and 61 cM apart), was associated with grain yield using association analysis. In our study, *Xgwm293*, *Xgwm415* and *Xgwm304* were associated with LIBSIT and TGWG and TGW under DS, while TGW is one of the most important factors affecting grain yield.

4.5.3 Chromosome 2D

Chromosome 2D is important for yield and yield components and also for stem water-soluble carbohydrates. Three SSR markers *i.e. Xwmc25* (18 cM), *Xwmc112* (28 cM) and *Xgwm382* (100 cM) were associated with GY (WW), PH (DS), LIBSIT (DS and WW), SWSCG (DS) and CCG (WW). The distance between two markers were 10 cM so it could be considered as moderately linked according to the classification of Marone et al. (2012) however *Xgwm382* was away from these markers. It acted as an independent marker. A marker *Xcfd17* associated with WSC under WW with 64 cM was apart from our finding but on the same chromosome 2D (Zhang et al., 2014).

In addition to this, Li et al. (2015) detected a marker *Xgwm261-2D* was on 23 cM and it was nearby a marker *Xwmc112-2D* (28 cM) was associated with SWSCG under terminal DS. The distance of these two markers was close and also was associated with the same trait. This finding was strengthened by our result. According to Rebetzke et al. (2007), the QTL on chromosome 2D was also mapped for WSC. It was also supported by Yang et al. (2007), but they reported QTL for WSC away from those markers that we found, and also it was reported at flowering stage. Dodig et al. (2012) identified SSR marker *Xgwm484* (41 cM) for chlorophyll content at grain filling but far distance from that marker we reported. In current study, *Xgwm382* was located at 100 cM and associated with chlorophyll content at grain filling under well-watered condition.

4.5.4 Chromosome 4B

Chromosome 4B were tagged by two SSR markers *i.e. Xgwm149* (31 cM) and *Xgwm165* (28 cM) were associated with SP (DS), TGW (DS and WW) and GYP (WW). These two markers are tightly linked with each other. In the present study, *Xgwm149* was associated with SP (DS) and TGW in both environments whereas *Xgwm165* was associated with GYP under WW condition only while SP, TGW and GYP are the most important traits that affecting grain yield. A QTL was reported for TGW under post-anthesis drought stress on chromosome 4BL (Nezhad et al., 2012).

4.5.5 Chromosome 3B

We detected that *Xgwm285* (61 cM) and *Xbarc164* (70 cM) tightly linked with each other. They were associated with LIBSIT (DS and WW) and TGWG (DS) on chromosome 3BL. Whereas *Xgwm389* was associated with tiller number on 3BS (Dodig et al., 2010).

4.5.6 Chromosome 1A

In our study, identified a marker *Xbarc148* (57 cM) was associated with DTH (DS) on chromosome 1A. A QTL for GY was mapped on chromosome 1A (Quarrie et al., 2005). A marker *Xgwm99* (126 cM) was reported associating with GY on chromosome 1A (Dodig et al., 2012).

4.5.7 Chromosome 1D

We identified that *Xwmc432-1D* (23 cM) was associated with TGWG under DS. An SSR locus *Xgwm337* (48 cM) was associated with GW on the same chromosome but far from *Xwmc432* (Groos et al., 2003). A QTL for lodging resistance was reported on chromosome 1D in a wheat DH population (Verma et al., 2005).

4.5.8 Chromosome 2A

Marker *Xwmc296* (49 cM) on chromosome 2A was associated with LIBSIT in WW condition. It was detected QTL for PL on the same chromosome with different SSR marker *Xgwm294* (76 cM) (Brbaklić et al., 2015).

4.5.9 Chromosome 7D

In an interval map of 77 cM on chromosome arm 7DS, SSR marker *Xgwm295* was identified for TGWG in DS. Previously reported QTL linked with TGW under terminal drought stress that close to *Xgwm295* marker (Nezhad et al., 2012).

4.6 Favorable Alleles for Different Traits

Xbarc78-4A was associated with LIBSIT and PH under DS, however it was only associated with PH under WW condition. It was considered as favorable alleles for these two traits. For well-watered condition, two favorable alleles were also identified on chromosome 2D and 4B that associated with GY and GYP. These traits are important for the contribution to improve the production of wheat.

5. Conclusions

It is inferred that SWSC were accumulated more at grain filling stage in DS and it is considered as increase fructans for self-protection. High heritability estimated for SWSCG under drought stress. The ability to accumulate WSC and high heritability in grain filling stage suggested as a selection criterion for wheat breeding. Total of seventeen significant marker-trait associations for 13 traits were detected. Chromosomes 2D, 4A and 5A are the most important with respect to traits and loci distance. *Xwmc25*, *Xwmc112* and *Xgwm382* were associated with GY (WW), PH (DS), SWSCG (DS) and CCG (WW). The next is 4A found two markers *i.e. Xwmc420* and *Xgwm397* are moderately linked with each other. *Xwmc420* was associated with PH, PL and SWSCF for DS, whereas *Xgwm397* was associated with TGW for WW condition. Another chromosome is 5A, three SSR markers namely *Xgwm293*, *Xgwm415* and *Xgwm304* were associated with TGW under DS.

Four favorable alleles were identified in two water environments, including $Xbarc78\text{-}4A_{163}$ increasing plant height in drought stress, but $Xbarc78\text{-}4A_{155}$ decreased plant height under well-watered condition. $Xwmc25\text{-}2D_{151}$ and $Xgwm165\text{-}4B_{191}$ were considered as favorable alleles for increasing grain yield under well-watered condition. All these markers are firstly reported with the traits in our study and expected to be helpful for marker assisted selection in wheat improvement.

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Appendix

Appendix 1. Descriptive statistics and broad sense heritability (h^2_B) for agronomical and physiological traits under both DS and WW conditions

Traits	Water regimes	Mean ± SD	Range	CV (%)	h^2_B
DTH	DS	209±3.09	200~219	0.01	0.36
	WW	213±3.42	204~224	0.02	0.39
DTF	DS	217±2.63	209~224	0.01	0.42
	WW	222±3.13	213~231	0.01	0.53
CCF	DS	52.20±4.18	36.80~64.34	0.08	0.81
	WW	55.20±3.94	43.84~66.64	0.07	0.78
CCG	DS	44.36±6.83	2.80~60.24	0.15	0.59
	WW	40.28±9.34	6.44~58.38	0.23	0.72
FLA	DS	9.79±2.06	5.87~16.82	0.21	0.37
	WW	14.0±3.14	7.81~28.77	0.22	0.30
PH	DS	76±15.66	36~118	0.21	0.80
	WW	100±20.26	50~142	0.20	0.93
SP	DS	10±2.63	4~21	0.26	0.91
	WW	12±2.80	6~24	0.23	0.88
SL	DS	8.10±1.03	5.40~10.60	0.13	0.97
	WW	8.68±1.23	5.00~12.30	0.14	0.97
PL	DS	21.32±4.73	11.40~34.60	0.22	0.96
	WW	29.07±5.74	14.80~46.80	0.20	0.97
LSIT	DS	16.19±3.00	9.20~26.20	0.19	0.96
	WW	22.9±4.23	13.40~49.20	0.18	0.96
LIBSIT	DS	30.37±9.53	8.8~57.00	0.31	0.97
	WW	41.20±13.19	12.8~68.00	0.32	0.97
SST	DS	0.51±0.61	0~3	1.21	0.63
	WW	0.38±0.45	0~3	1.19	0.44

SSM	DS	0.02±0.07	0~0.6	3.97	0.21
	WW	0±0	0~0	ND	ND
SSB	DS	2.27±0.76	0.20~4.80	0.34	0.88
	WW	1.23±0.69	0~3.80	0.56	0.41
NGS	DS	34±5.85	19~59	0.17	0.85
	WW	35±4.44	23~49	0.13	0.54
GY	DS	106.37±27.66	38.79~209.61	0.26	0.31
	WW	136.69±36.63	54.17~272.16	0.27	0.38
GYP	DS	577.36±219.59	67.83~1312.45	0.38	0.78
	WW	753.52±302.41	40.18~1603.01	0.40	0.81
TGWG	DS	9.99±2.85	5.96~20.57	0.29	0.57
	WW	9.04±2.76	4.57~15.61	0.31	0.46
TGW	DS	32.10±5.73	18.03~42.63	0.18	0.81
	WW	32.56±5.00	20.40~45.70	0.15	0.86
SWSCF	DS	204.67±34.90	140.75~298.50	0.17	0.71
	WW	150.31±35.01	73.65~242.65	0.23	0.78
SWSCG	DS	268.06±44.54	140.63~346.37	0.17	0.80
	WW	196.04±42.50	119.42~271.09	0.22	0.76
AESWSC	DS	22.05±12.09	-58.30~44.24	0.55	0.47
	WW	21.21±13.35	-13.35~50.47	0.63	0.49
GFEE	DS	31.61±8.53	9.00~91.16	0.27	0.44
	WW	28.23±9.20	9.94~86.27	0.33	0.52
GFEL	DS	0.48±11.73	-52.26~28.62	0.24	0.69
	WW	4.33±11.82	-51.62~27.36	0.27	0.73