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IDENTIFICATION OF GENOMIC REGIONS CONFERRING DROUGHT TOLERANCE IN BREAD WHEAT USING ISSR MARKERS

RANA HAROON MAQSOOD[1], MUHAMMAD WAQAS AMJID[2], MUHAMMAD ASIF SALEEM[3], GHULAM SHABBIR[2*] AND IHSAN KHALIQ[1]

[1]Department of Plant Breeding & Genetics, University of Agriculture, Faisalabad
[2]Department of Plant Breeding & Genetics, PMAS-Arid Agriculture University Rawalpindi
[3]Department of Plant Breeding and Genetics, Bahaudin Zakaria University, Multan
[*]Corresponding author's email: drgshabbir@gmail.com; Cell number: 0321-5327916

Abstract

Drought stress is one of ever escalating and disastrous situation for plantadaptations under changing climate. Quantitative Trait Loci (QTL) analysis was done to identify chromosomal locations containing QTLs for photosynthetic rate, relative water content and cell membrane stability under drought stress conditions. An F_2 population was developed from an intraspecific cross between a drought tolerant genotype (Chakawal-50) and a drought susceptible genotype (9436) of *Triticum aestivum*. A total of 30 ISSR markers were used to screen both parents. Only 4 ISSR markers were found polymorphic which were used to score 180 F_2 plants. A total of 73 bands produced were found polymorphic from these 4 markers using capillary electrophoresis. One QTL was found linked to Photosynthetic rate on chromosome 3A, one to relative water contents on chromosome 4D and one to cell membrane thermo-stability on chromosome 2B, respectively. As these traits were also positively correlated with thousand grain weight, so indirectly these QTLs might improve plant yield under limited water conditions. Therefore, these QTLs may be used through marker assisted selection while breeding wheat under limited water conditions.

Key words: ISSR markers, Drought, Photosynthetic rate, Relative water contents, Cell membrane thermo-stability, Capillary electrophoresis.

Introduction

Wheat is an important crop in Pakistan, whichis grown in irrigated and rain-fedareas, often exposed to abiotic stresses including drought, heat, salinity etc (Anon., 2016-17).Water availability is decreasing (Vorosmarty *et al.*, 2000) so there is a need to develop cultivars with higher productivity under limited water availability. The knowledge about genetic modifications during onset of stressesin certain physiological traits related to yield is very important for the development of high yielding cultivars. Genomics tools are useful for plant breeders to evaluate and find the chromosomal location of certain loci responsible for high yield under drought condition.

Plant response to drought is a complex phenomenon (Asif *et al.*, 2005). Drought affects plant osmotic potential and ionic equilibrium resulting in complete or partial blockage of photosynthesis. Consequently,plant remains unable to produce glucose for its survival resulting in plant cell death (Reddy *et al.*, 2004).Plants developed stress management systems at three levels. At first level plant uses escape mechanism, at second level plant uses avoidance and at final level plant develop tolerance and resistance to stress (Farooq *et al.*, 2009). Plants also cope with water stress at cellular level and molecular level by some proteins and some transcription factors. Plants react to all kind of stresses by some physiological, biochemical and molecular changes through changed and enhanced gene expression (Wahid *et al.*, 2007). At cellular level plants make some changes in cell cycle, cell division, adjust membrane system and modify its cell wall. Plants also change its metabolism by osmotic adjustment to maintain turgor pressure and scavenging ROS (Bartels & Sunkar, 2005). Some amino acids are involved in osmoregulation of cell during scarcity of water in cells mainly proline and glycine betaine (Brosche *et al.*, 2005).

Plants also regulate stress inducible genes which protect plant by detoxifying enzymes, producing transporter proteins, osmoprotectants, transcription factors, protein kinases and phosphatases (Bibi *et al.*, 2010). These genes are LEA, HSPs, MAP kinase, CBF/DREB families, aquaporin and transporters (Choi *et al.*, 2000). In plant stress responses, different hormones like ethylene, ABA and jasmonic acid are also produced (Peleg and Blumwald, 2011). Marker assisted selection is a tool in genomics era to select genotypes of interest by using short sequences of DNA (markers) present near or tightly linked to the gene of interest. These markers are used to find the QTLs (quantitative trait loci) related to quantitative traits(Collard & Mackill, 2008).

Many QTLs has already been reported in wheat. Five markers were found linked to chlorophyll contents, flag leaf senescence and cell membrane stability using SARP marker (Elshafei *et al.*, 2013). One QTL for photosynthetic rate and two for cell membrane thermo-stability were found on chromosome 2A (Malik & Malik, 2015). Cell membrane stability was linked with QTLs on chromosome 7A (Ciuca & Petcu, 2009). Eleven SSR markers were linked to relative water contents, awn length, 1000-grain weight, coleoptile length, extrusion length and shoot length in wheat (Ahmad *et al.*, 2014).ISSR marker UBC-811_{850bp} was associated with spike length and ISSR marker UBC-881_{450bp} was associated with grain yield and biological yield. 1000-kernal weight was associated with UBC-811_{775pb} and UBC-840_{1530pb}. UBC-840_{535bp} was associated with number of spikes per square meter (Motawea *et al.*, 2015). Five QTLs were detected for spike length and one QTL was associated with number of grains per spike using ISSR markers in wheat (Khaled & Hamam, 2015). QTLs related to Photosynthetic rate and water stress tolerance has also been reported but these are not many QTLs to cover whole phenotypic variation in these characters because these are much complex traits (Elshafei *et al.*, 2013; Malik

& Malik, 2015). Being a developing country, in Pakistan not much work has been done on QTL mapping for water stress related traits. Present study was conducted to find QTLs linked to ISSR markers (Khaled *et al.*, 2015) related to Photosynthetic rate, relative water content and cell membrane thermo-stability using an F_2 population of a cross of Bread Wheat as well as to identify the genetic mechanism controlling these traits.

Materials and Methods

Plant material and field layout: F_2 population was developed from an intraspecific cross between water stress tolerant genotype Chakwal-50 (P_1) and susceptible genotype 9436 (P_2) of *Triticum aestivum*. The parents, F_1 and F_2 populations were grown in the field under drought stress conditions using Randomized Complete Block Design (RCBD) with three replications at the department of Plant Breeding and Genetics, University of Agriculture, Faisalabad (Latitude = 31°25′45″N, Longitude = 73°4′44″E, Altitude = 184.4 m). Field was flood irrigated four times but half of normal irrigation (1.5 Acre inches per irrigation) water was applied to maintain stress. Same experiment was repeated and used as control. Control experiment was irrigated four times with normal irrigation (3 Acre inches per irrigation). Three rows of one meter length each was sown for both parents and F_1 while for the F_2, twenty lines were sown for each replication. Two seeds per hole were planted with dibbler at plant to plant and row to row distance of 15 and 30 cm, respectively. Thinning was done to retain one plant per hole after germination.

Phenotyping: Ten plants each for both parents and F_1 and 180 plants for F_2 were tagged. At the anthesis stage, data were recorded for relative water contents, excised leaf water loss, cell membrane stability and photosynthetic rate. Relative water contents of leaf samples were measured by adopting the procedure and formula used by Barrs & Weatherley (1962), excised leaf water loss as by Clarke & McCaig (1982) and Cell membrane thermo-stability by the method proposed by Saadalla *et al.* (1990). The fully matured leaves below flag leaf were selected to record the data. Flag leaf was used for recording photosynthetic rate using Portable Photosynthetic System CIRAS-3. 1000-grain weight was measured using a weighing balance after harvesting.

Genotyping: Mature leaves were excised early in the morning and were transported to the laboratory in ice and stored at -20°C. The standard CTAB method was used for DNA extraction (Rogowsky *et al.*, 1991). PCR amplification was done using ISSR primers (Khaled *et al.*, 2015) by the method prescribed by Godwin *et al.* (1997). The DNA of both parents and F_1 were initially amplified using 30 ISSR primers to find polymorphism between the parents after running on 2% agarose gel electrophoresis. The primers showing maximum polymorphic bands were selected. A total of 4 unambiguous polymorphic primers were selected and used to screen F_2 population for QTL mapping. These 4 ISSR primers were then labeled with different (6-FAM, VIC, NED, PET) florochroms (Table 4). 6-FAM was obtained from Integrated DNA Technologies (IDT) and other florochroms were obtained from ABI (Applied Biosystems).PCR products were resolved using an Applied Biosystems/Hitachi 3130x1 genetic Analyser and Genescan 1200 Liz Size Standard with 60 seconds standard injection time. DNA fingerprints obtained from capillary electrophoresis were evaluated using the GeneMapper software v3.7 as well as by manual scoring of patterns and peaks (Campbell *et al.*, 2011). Each fluorescent peak from capillary run was treated as individual band and the size scored using the GeneMapper software v3.7.

Linkage and QTL analysis: A linkage map was developed by using Joinmap3.0 software (Van Ooijen & Voorrips, 2001). The Kosambi mapping function (Kosambi, 1943) was used to convert recombination frequency to genetic map distance (centiMorgan, cM). QTL Cartographer2.5 software program was used to identifyQTLs by using test statistics composite interval mapping (CIM) calibrated at 1000 permutations test to calculate the critical F-value at a 5% significance level. Walk speed of 0.5cM was used to determine threshold LOD value to declare a QTL. An LOD value of 3 was used as threshold. Input files for each program containing the linkage map, the phenotypic and molecular marker data were prepared per the instructions given in the manuals (Van Ooijen & Voorrips, 2001; Basten *et al.*, 2004). The proportion of observed phenotypic variance attributable to a QTL was estimated by the coefficient of determination (R^2) from corresponding model (Basten *et al.*, 2004).

Results

Phenotyping: Diverse parents were selected after screening of 50 wheat genotypes collected from different research institutes of Pakistan. Analysis showed that both parents were significantly diverse from each other. There were clear differences between tolerant and susceptible line for all traits. F_2 population developed from crossing of these two genotypes was suggested to be highly segregating and good population to dissect morphological traits genetically.

Data collected from all tagged plants at anthesis stage were tested using F test. Analysis of variance showed that significant differences were present between all generations including parental lines for all traits. F_2 segregating data (Table 1) for traits cell membrane thermo-stability, excised leaf water loss, relative water content, photosynthetic rate and thousand grain weight was normally distributed under water stress conditions (Fig. 1).

Correlation studies: Correlation studies (Table 2) revealed that cell membrane stability was negatively correlated with excised leaf water loss and positively correlated with all other traits under study. Excised leaf water loss was negatively correlated with all traits used in this study. Relative leaf water content was positively correlated with photosynthetic rate and thousand grain weight. Photosynthetic rate was also positively correlated with thousand grain weight.

Table 1. Means values and population effects for relative water contents (RWC), excised leaf water loss (ELWL), cell membrane thermo-stability (CMT), photosynthetic rate (PR) and 1000-grain weight (TGW) under drought conditions.

Traits	Generations				Population effect
	P_1	P_2	F_1	F_2	
RWC	62.87	49.29	63.90	55.18	**
ELWL	1.73	4.73	2.93	3.14	**
CMT	84.41	78.98	81.20	82.12	**
PR	29.81	24.48	28.46	29.82	**
TGW	41.38	36.04	41.73	39.60	**

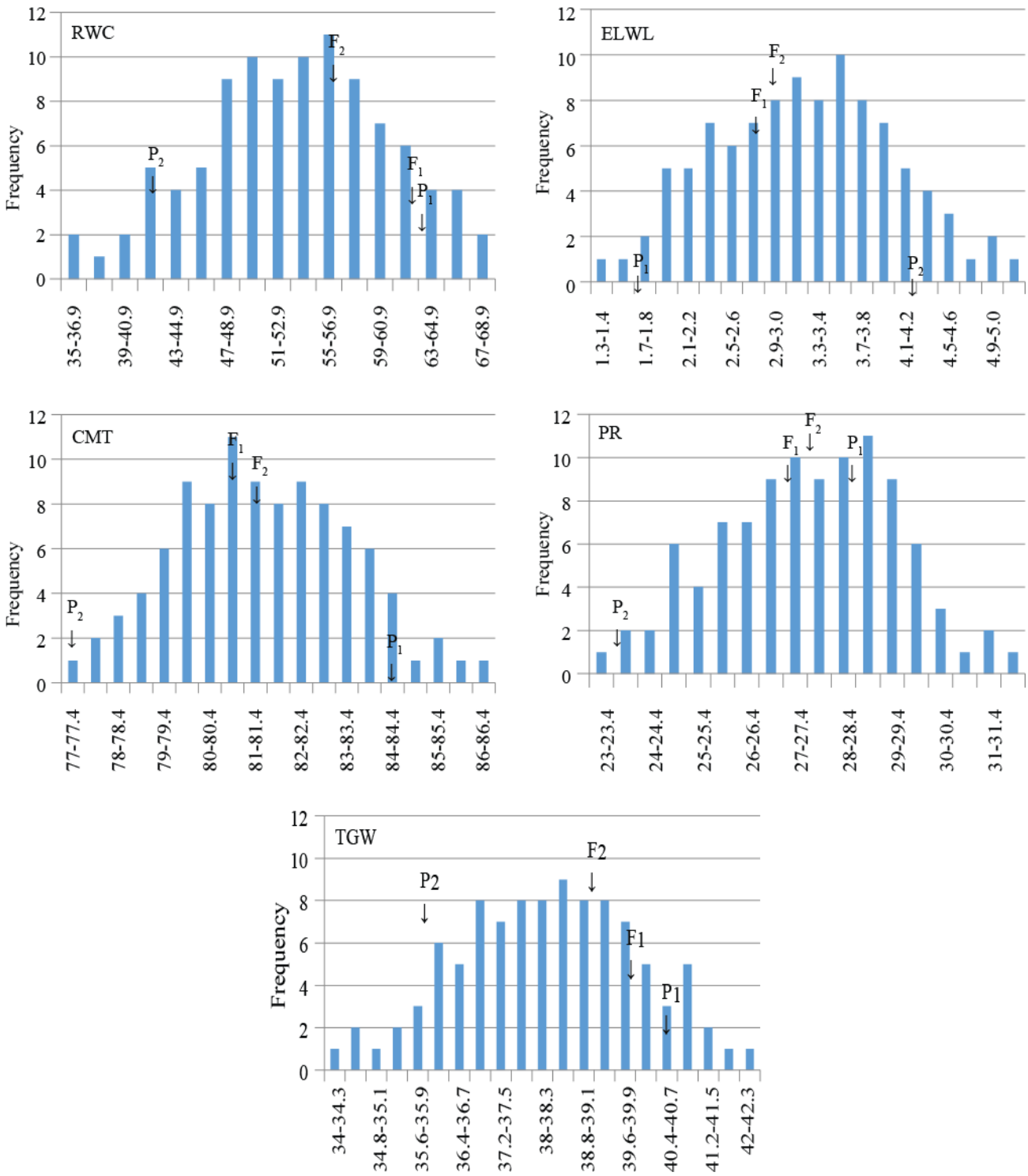

Fig. 1.Frequency distribution of the F_2 population for relative water contents (RWC), excised leaf water loss (ELWL), cell membrane thermo-stability (CMT), photosynthetic rate (PR) and 1000-grain weight (TGW) under drought conditions.

Table 2. Phenotypic correlation for relative water contents (RWC), excised leaf water loss (ELWL), cell membrane thermo-stability (CMT), photosynthetic rate (PR) and 1000-grain weight (TGW) under drought conditions.

Traits	CMT	ELWL	RLWC	PR
ELWL	-0.2227*			
RWC	0.2709**	-0.4287**		
PR	0.1499	-0.1355	0.3778**	
TGW	0.5773**	-0.3542**	0.7324**	0.4284**

Table 4. Sequences of PCR primers used in this study.

Primer	Sequence
UBC-808	5´-6-FAM-AGAGAGAGAGAGAGAGC-3´
UBC-826	5´-VIC-ACACACACACACACACC-3´
UBC-833	5´-NED-GAGAGAGAGAGAGAGAT-3´
AD2	5´-PET-AGCAGCAGCAGCAGCAGCG-3´

Table 5. ISSR data table of primers utilised, total number of scorable and polymorphic bands, and percentage polymorphism detected within wheat F_2 population.

Primer	Total no. of scorable bands	Total no. of polymorphic bands	% Polymorphism
UBC-808	196	26	13
UBC-826	158	15	9
UBC-833	143	20	14
AD2	126	12	10

Genotyping: Four ISSR primers were used which produced 73 polymorphic bands out of a total of 623 bands for both parents with an average of 18 polymorphic bands out of 152 total bands per marker using capillary electrophoresis (Table 5). Only 10% polymorphism was detected between the parents in this cross of bread wheat. It was shown by chi-square goodness of fit that 61 bands were segregated with a segregating ratio of 3:1, all the remaining which bands were independently segregating and were not mapped.

Linkage map construction: All the 61 bands were mapped on 12 linkage groups (LG). The numbers of bands in each linkage group ranged from 3-10 with an average distance of 32cM per linkage group. A total of 390cM distance was covered by all the linkage groups produced in this experiment. The average distance between contiguous bands was 6.5cM. The linkage groups LG1 was assigned to chromosome 3A, LG2 was assigned to chromosome 7A, LG3 was assigned to 6A, LG4 was assigned to 4D, LG5 was assigned to 7D, LG6 was assigned to 7B, LG7 was assigned to 3B, LG8 was assigned to 1D, LG9 was assigned to 2B, LG10 was assigned to 5A, LG11 was assigned to 4B, LG12 was assigned to 6D.

QTL mapping: A total of three QTLs were significantly associated with three morphological and physiological traits in this QTL analysis (Table 3). One QTL linked to UBC-808 named QPr3AC was identified for photosynthetic rate on chromosome 3A at LOD 4.9 explaining 30.2% of phenotypic variation. One QTL linked to AD2 named QRwc4DC was identified for relative water contents on chromosome 4D with a LOD score of 12.3 explaining 3.2% of phenotypic variation. One QTL linked to UBC-833 named QCmt2BC was identified for cell membrane thermo-stability for chromosome 2B with a LOD score of 18.6 explaining 7.5% of phenotypic variation (Fig. 2). Traits like photosynthetic rate, relative water contents and cell membrane stability showed positive additive effects with the value of 3.2, 2.7 and 1.35 respectively. All these additive effects were positive showing that all the genes which contributed for these QTLs were from resistant parents (Chakwal-50).

Discussion

This experiment was conducted on Pakistani acclimatized germplasm using molecular markers to find some locus on chromosomes which were linked with some morphological and physiological traits. In this study 30 ISSR markers were used to screen both diverse parents. However, out of 30 only 4 were selected which showed highly polymorphic and reproducible bands on 2% agarose gel. During survey of F_2 population, 4 selected markers produced only 73 polymorphic bands (10%) out of 623 scoreable bands using capillary electrophoresis. This was quite low frequency of genetic variation in intraspecific crosses which was also suggested in previously (Amjid *et al.*, 2015). Reasons behind the low polymorphism is perhaps directional selection by breeders for few specific traits. This could be due to unintentional and intentional phenotypic selection for similar type of genotypes in the past. Germplasm used nowadays has little variation due to directional selection.

In this study 3 QTLs were identified as linked with relative water contents, cell membrane thermo-stability and photosynthetic rate. QTL for photosynthetic rate was identified on chromosome 3A, showed that this chromosome might be important for biomass production. QTL showed 30.2% of total phenotypic variation present in F_2 population for photosynthetic rate was controlled by this QTL. Some QTLs for photosynthetic rate on chromosome 2A, 7A, 6A and 7D using SSR markers were already identified in *Triticum aestivum* (Ilyas *et al.*, 2014; Malik & Malik, 2015). QTL identified in present study strongly suggested that apart from these previously explored genomic regions, chromosome 3A would also be important contributor for photosynthetic rate as suggested in previous studies using RFLP marker (Zhang *et al.*, 2010).

Table 3. Biometrical parameters of individual QTLs under drought conditions.

Trait	QTL	Distance (cM)	Nearest marker	Chromosome name	LOD	Additive effects	R^2 Value
Photosynthetic rate	qtlPR	8.6	$UBC\text{-}808_{606}$	3A	4.9	3.2	30.2
Relative water contents	qtlRWC	12.9	$Ad2_{108}$	4D	12.3	2.7	3.21
Cell membrane thermostability	qtlCMT	12.4	$UBC\text{-}833_{516}$	2B	18.6	1.35	7.45

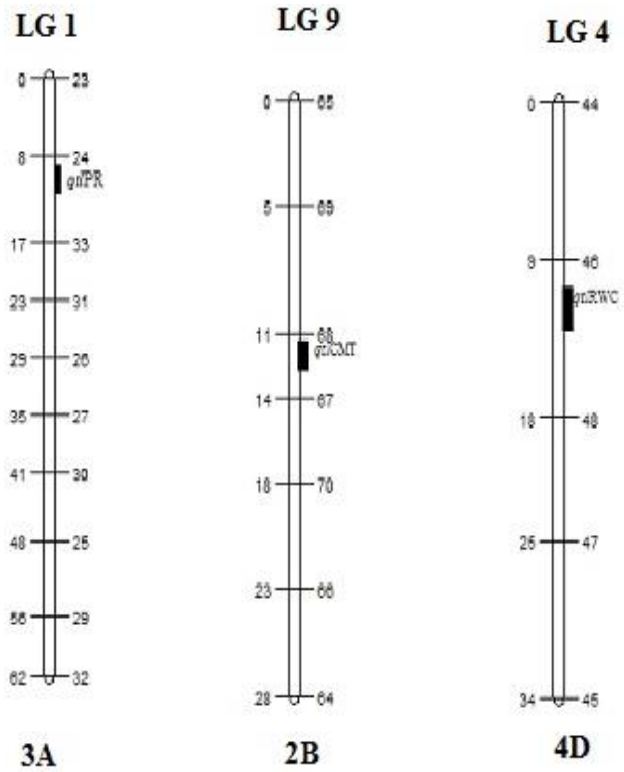

Fig. 2. Linkage groups showing QTLs for relative water contents, cell membrane stability and photosynthetic rate under drought conditions.

QTL for relative water contents was found on chromosome 4D, strongly suggested that this chromosome could be important for drought tolerance as some genes for leaf water contents were identified on this chromosome. This QTL exhibit only 3.21% of phenotypic variation present in F_2 population for this trait. Present study strongly suggested that chromosome 4D should be given due importance in upcoming breeding programs and further be analyzed for QTLs identification under water stress conditions. D genome originated from *Aegilops tauschii* which has been much important for drought tolerance and other abiotic and biotic stress tolerance (Sohail *et al.*, 2011). QTLs for relative water contents have already been identified using SSR markers on chromosome 2A, 5A and 5D of wheat in previous researches (Ahmad *et al.*, 2014; Malik & Malik, 2015).

Cell membrane thermo-stability was linked with QTL on 2B. This QTL exhibit 7.45% of total phenotypic variation present in F_2 population for this trait. This chromosome was also important regarding to drought tolerance. Previous studies showed that QTLs for this trait were located on chromosome 2A, 5A, 7A, 1B, 2B, 3B and 1D under water stress conditions (Ciuca & Petcu, 2009; Elshafei *et al.*, 2013). It could be suggested that genes reside on these QTLs related to these traits were mostly contributed by dominant parent (Chakawal-50) as revealed by positive additive effects. So, this genotype should also be given due importance in further breeding programs. Previous studies also verified results presented in this study (Ahmad *et al.*, 2014; Amjid *et al.*, 2015; Malik & Malik, 2015).

QTLs identified for same traits or using same molecular markers in different crops could be beneficial to check the reliability of QTLs in different genetic backgrounds. This could also be used to find evolutionary history, homologs and homeologs between these crops. These QTLs in different species of same genus could enable us about evolutionary pathway and ancestry of these modern species. Photosynthetic rate, relative water contents and cell membrane has already found linked to certain QTLs in different crops such as barley (Teulat *et al.*, 2001), sunflower (Herve *et al.*, 2001), *Arabidopsis* (Juenger *et al.*, 2005), cotton (Amjid *et al.*, 2015; Saleem *et al.*, 2015) and rice (Gomez *et al.*, 2006). 3 QTLs for relative leaf water content in soybean was found by Virginia *et al.* (2012). (Teulat *et al.*, 2001) found 9 QTLs in barley related to relative water contents. (Abdi *et al.*, 2013) identified 2 QTLs under drought stress conditions and 6 QTLs under normal conditions in sunflower for relative leaf water content. Tripathy *et al.* (2000) identified 9 QTLs in rice for Cell membrane thermo-stability on different chromosomes. Eleven QTLs were identified for relative water contents in rice by Courtois *et al.* (2000). Teng *et al.* (2004) revealed 2 QTLs for transpiration rate in rice. 2 QTLs were identified for stomatal conductance in cotton by (Ulloa *et al.*, 2000). Amjid *et al.* (2015) reported two QTLs for relative leaf water contents and a QTL for cell membrane stability in cotton Saleem *et al.* (2015) also unveiled a QTL for relative water contents in cotton. QTLs identified in this study could be tested in different genetic backgrounds and used to find homologs and homeologs in different crops.

Correlation studies showed that photosynthetic rate, relative water contents and cell membrane thermo-stability were positively correlated with thousand grain weight indicating that increase in these traits might result in increase of wheat yield. Increase in photosynthetic activity would also increase yield of the crop. Cell membrane stability would allow plant to withstand and produce high yield under water stress conditions. As shown by these correlation studies, QTLs for these traits might influence wheat yield indirectly. QTLs should be assessed based on their effect in increasing yield under water stress environment. QTLs found in present study were linked to each of three genomes i.e., A, B and D in *Triticum aestivum*. Genome B and D might be more important under water stress conditions as these host the genes for water contents and cell membrane stability.

These QTLs related to physiological traits could help in wheat breeding which could withstand any level of water stress and harsh environment. For the validation of these QTLs further experimentations should also be done under different climatic conditions as well as in different genetic backgrounds with a shared parent. Present experiment was done using Pakistani acclimatized germplasm; future studies should also be done using germplasm from different countries. In future, these QTLs might be validated as well as these DNA bands could be sequenced to develop gene specific markers. These QTLs could also be introgressed into genetic background of different high yielding wheat genotypes under water stress conditions to boost wheat yield under water stress conditions.

Conclusion

Positive correlation between relative water contents, cell membrane stability, photosynthetic rate and thousand grain weight revealed that QTLs found in present study could increase yield under harsh environments. These QTLs could be beneficial for plant breeders to develop higher yielding and drought tolerant wheat varieties. Same QTLs related to certain traits in different species and different crops showed the way for evolutionary, homologs and hoshameologs studies. It could also help in confirming pedigree of different polyploid species of same genus.

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