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QUANTITATIVE TRAIT LOCI (QTLs) ANALYSIS OF DROUGHT TOLERANCE AT GERMINATION STAGE IN A WHEAT POPULATION DERIVED FROM SYNTHETIC HEXAPLOID AND OPATA

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ABSTRACT

Drought is a major limiting factor affecting productivity. Wheat is a major crop and staple food in Pakistan. Genetic linkage map construction based on linked DNA markers spanning whole wheat genome and subsequently QTL mapping for drought tolerance is a way forward to enhance breeder's ability for effective selection. An F_8 population derived from the cross of OPATA x SH-349 has been used. An experiment was conducted at germination stage under controlled conditions. The drought was induced by15% PEG nutrient solution in acid washed sand medium. Microsatellite DNA markers data were used for linkage maps construction. Morphological data under drought and non-stressed conditions along with linkage maps data were used for QTL mapping. The results of QTL analysis using single marker analysis showed 14 SSR markers linked to QTLs for five traits in both drought and control condition. Using simple interval mapping and composite interval mapping 12 QTLs for different traits of interest were found. Implications of found QTLs and linked markers are discussed.

Key words: Drought, SSR, PCR, QTL mapping, wheat.

INTRODUCTION

Wheat is staple food in Pakistan and has central position among grain crops. It is sown on 9.042 thousand hectare and contributes 3.1% to GDP. It is major food grain crop in Pakistan. Its production must be enhanced to meet the food security needs of the growing population of Pakistan.

Simultaneous improvements for stress tolerance and yield have posed a problem in wheat improvement through conventional breeding methods. Fortunately, progress in molecular genetics has provided plant breeders with a rapid and powerful alternative approach in selection (Lin *et al*., 2005). Molecular markers are important tool for creating genetic linkage maps and have provided a significant increase in the amount of genetic knowledge in many cultivated plant species. Linkage maps have increased our ability to map QTLs (Collard *et al*., 2005).

The drought tolerance in wheat is a quantitative (complex) trait and it is controlled by many genes. These genes have an additive effect towards overall drought tolerance. Quantitative trait loci control different morpho-physiological characters responsible for drought tolerance mechanisms. Like water absorption ability is enhanced with the help of deep and strong root system, water loss is decreased by favorable shoot morpho-physiological traits, and leaf rolling to reduce radiation absorption (Xiao *et al.*,1996). Moreover, these characters segregate independently from each other (Mitra, 2001). The advent in molecular breeding has facilitated to tag these QTLs with molecular markers enhancing breeder's ability of gene pyramiding for increased drought tolerance (Tuberosa and Salvi, 2006). Once a marker-trait association has been established, marker assisted selection reduces or eliminates dependence on environment for the selection, which is a problem in conventional breeding programs dealing with drought tolerant cultivar production (Moose and Mumm, 2008).

Additionally, the expressions of complex traits like drought vary with growth stage of plant. Identification of stress tolerance at both the germination and seedling stages is particularly important (Mano and Takeda, 1997). Germination is a crucial stage for plant establishment (Song *et al.,* 2008). Low germination may lead to poor stand establishment, resulting in lower grain yields. The seedling stage is generally the most sensitive phase of plant development, and almost all work on stress tolerance in different crop species has included plant assessment at this stage (Song *et al.,* 2008; Tlig *et al.,* 2008).

QTL analysis could be done from F_2 population but double haploid (DH) and RILs are mostly preferred (Prioul *et al.,* 1997). The present project was designed to determine phenotypic responses of recombinant inbred lines (RILs) of hexaploid wheat population at

germination under non stressed and drought conditions, genotype RILs with SSR markers, and analyze QTLs for drought tolerance at germination stage.

MATERIALS AND METHODS

Studies were conducted on a population of recombinant inbreed lines (RILs) developed from a cross between wheat cultivar Opata and SH-349. The Opata is relatively drought susceptible and SH-349 is relatively drought tolerant parent. The population was kindly contributed by Wheat Wide Crosses Program, NARC Islamabad. Phenotyping was done at germination stage using 92 RILs under controlled conditions. Experiment was conducted in completely randomized design with three repeats. Properly cleaned plastic pots (5 x 4 inches) with holes at their bottom were filled with washed and autoclaved sand. Pots were placed in trays in which half strength nutrient solution (Epstein, 1972) was added with or without added 15% PEG (polyethylene glycol) for drought and non-stressed conditions, respectively. These trays were placed in growth room with14/10 light and dark duration at 25°C. Nutrient solution was replenished regularly. From third day onward the number of germinated seeds were counted on daily basis till no more seed was germinating. Those seeds were considered as germinated which had plumule length more than 50 percent of the seed length. After 28 days of plantation when no more seed was germinating, seedlings were weighed and lengths were measured. Dry weight was also recorded. The phenotypic data for root length, shoot length, dry biomass, germination percentage and germination rate (Maguire, 1962) were analyzed using software program Minitab V-13 and analysis of variance was done using two factor factorial analysis in completely randomized design (CRD).

DNA isolation was done by CTAB method (Doyle and Doyle, 1987). DNA quantification was done on 1% agarose gel by visual comparison of λ DNA standards of 20, 50, 100, 200 and 300 ng/µl samples. The samples were diluted to 20 ng/µl. PCR was done by making reaction mixture containing 1X PCR Buffer 2 µl, 5 mM $MgCl_2$ 2.4 µl, 0.2 mM each of dATP, dGTP, dCTP and dTTP 0.4 µl, 5 pmole each of reverse and forward primers, 1 unit of *Taq* DNA polymerase and 20 ng of DNA sample with total volume of 20 µl of reaction mixture.

Parents of the population (Opata and SH-349) were subjected to microsatellite marker analysis using more than two hundred markers. Only those markers were genotyped in the progeny which had shown polymorphism in the parents. The recombinant inbreed lines were genotyped with 39 markers.

The phenotypic means of the genotypic groups (A and B) declared on the basis of marker genotypes were compared statistically using simple regression analysis. The coefficient of determination value pertains to the association of marker with the QTL being detected. On the basis of p-values obtained the significance of a QTL being detected was declared. The Single point analysis in computer program QTL cartographer V 2.5 was used for this analysis.

Markers data and morphological data at germination stage were imported to QTL cartographer V 2.5.10 for analysis using import data option. Five hundred permutation tests were conducted between trait data and marker data to find the LOD (Log Likelihoods of Odds) threshold value for simple interval mapping and composite interval mapping. Simple interval mapping and composite interval mapping were then performed at threshold value of 2.7 (threshold obtained after 500 permutations) for all linkage groups and all traits simultaneously.

RESULTS AND DISCUSSION

Phenotypic data: The phenotypic data was collected for shoot length (SL), root length, (RL), dry biomass (DB), germination rater (GR) and germination percentage (GP). All the parameters recorded under control (C) and drought conditions (D) were statistically highly significant for all genotypes under two treatments (Table 1). Data for some phenotypic traits showed skewness either positive or negative (Table 2). The data was normalized by taking $\log_{10}$ or Square root of the data before QTL analysis.

Table 1. Mean squares and level of significance of all the traits studied in RILs under drought and control conditions.

Source of Variation	Shoot length	Root length	Dry Biomass	Germination Rate	Germination Percentage
Genotype (G)	222.2 **	48.9**	0.0325**	12.96**	2804.2**
Treatment (T)	27040.5**	2857.4**	2.3565**	1356.456**	115023.5**
G x T	116.8**	27.6^{ns}	0.0316**	7.427**	1356.8**

^{ns} non significant ** Significant at 0.01, * significant at 0.05 level

Table 2. Minimum, maximum and means ± standard deviation of all the traits studied.

Trait	Minimum	Maximum	Mean value ± Standard deviation	Skewness in data*
SLC (cm)	3.5	40	29.40 ±6.372	-545.18
RLC (cm)	2	21	11.98±2.846	-30.39
DBC (g)	0.05	0.525	0.2451± 0.090	0
GRC	0	10.1422	5.530±2.224	-3
GPC	0	100	82.16±23.79	-19520.66
SLD (cm)	5	32	18.87±5.690	100.56
RLD (cm)	4	16	10.07±2.888	19.67
DBD (g)	0.011	0.242	0.0975±0.0575	0.010
GRD	0	5.69219	1.962±1.359	1.34
GPD	0	100	49.3±28.65	6028.55

* Data with high positive or negative skewness were normalized before QTL analysis.

N.B. SLC- Shoot length under control, RLC-Root length under control, DBC-dry biomass under control, GRC-germination rater under control and GPC- germination percentage under control condition. SLD- Shoot length under drought, RLD-Root length under drought, DBD-dry biomass under drought, GRD-germination rater under drought and GPD- germination percentage under drought condition.

Molecular data: In total 39 markers were genotyped in RILs population which were polymorphic in parents. According to band sizes two genotypic classes were obtained which were A (like parent 1) and B (like parent 2).

QTL analysis

Single marker analysis: QTL analysis was performed by single marker analysis using software package QTL Cartographer version 2.5.10. The software was provided with data for all morphological traits for both drought and control conditions. The data of RILs genotyped by 39 SSR markers were also provided to software program. This software analyze the data and uses statistical tool simple linear regression model $y = b_0 + b_1 x + e$. The aim of analysis was to test either marker is linked to any trait of interest or not. This was done by determining if b_1 is significantly different from zero. The F statistic compares the null hypothesis H_0: $b_1 = 0$ to an alternative hypothesis H_1: $b_1 \neq 0$.

The * and ** showed the significance level 5% and 1%, respectfully. L_0 and L_1 were likelihoods which assumed that null hypothesis H_0 is nested within H_1. Two nested hypothesis were compared by test statistic likelihood ratio test (LRT). The "Likelihood Ratio Test (LRT) Statistic" was $-2\ln (L_0/L_1)$. The coefficient of regression (R^2) tells how much phenotypic variation is explained by the marker and on the basis of these values the QTLs are declared either major or minor. The details of markers linked to different traits studied are summarized in Table 3.

Three markers were linked to shoot length trait under control condition (SLC) which were WMC-153, WMC24 and WMC-367 explaining 9%, 10% and 4% phenotypic variation. WMC-24 was also linked to shoot length under drought (SLD) condition explaining 11% phenotypic variation. One marker WMC-454B was also found to be linked to SLD condition explaining 7% phenotypic variation. Four markers WMC-153, WMC-367, WMC24 and CFA-2123 were found to be linked to root length under control (RLC) condition. Only one marker WMC-24 was found to be linked to root length under drought (RLD) condition. WMC-24 was linked to root length and shoot length under control and drought condition so it seems to be linked to a gene which controls root length and shoot length in both conditions. Two markers WMC- 476 and WMC- 24 were linked to dry biomass in control (DBC) condition. The markers WMC-357, WMC-484, WMC-177, WMC-160, WMC-407 and WMC-556 had a linkage with dry biomass in drought (DBD) condition. All these markers were significant at 5%. No major QTL was identified. Germination rate was highly affected by drought condition. Four markers WMC-160, WMC-24, WMC-407, and WMC-479 were linked to germination rate under control (GRC) condition. Only one marker was linked with germination rate in drought (GRD) which was WMC-160. The QTL linked to WMC-160 explains phenotypic variation 7 percent and 4 percent in drought and control conditions respectively. Germination percentage was linked to WMC-153, WMC-24, and WMC-479 in control (GPC) condition among these QTL linked to WMC-153 explains the phenotypic variation up to 12%. In drought condition germination percentage (GPD) was linked to two markers WMC-457 and WMC-160. These both markers are minor QTLs and explain phenotypic variation up to 7 percent each.

The data was collected at germination stage for the performance of the RILs in drought and control conditions. Drought affects plant traits differently at different plant stages (Hall *et al.*, 1991). Identifying QTLs for stress tolerance at different growth stages was necessary. The marker explaining most variation in the

trait of germination percentage under control condition is WMC-153. It explains the phenotypic variation by a higher R^2-value at 1% level of significance. Marker WMC-153 proves to be linked to traits of root length and shoot length under control conditions and explaining a phenotypic variation with R^2-value of 0.09 and 0.096 respectively. This allele is likely to be contributed by the parent SH-349 because this parent is drought tolerant. Kumar *et al.* (2010) used this marker WMC-153 for screening for leaf rust genes (Lr24 + Lr28).

Another marker WMC-24 was linked to shoot length, root length, germination rate, dry biomass, and germination percentage under control condition. This marker was significantly linked to root length and shoot length in both conditions. This allele could be contributed by SH-349 because this parent shows vigorous growth at germination stage. The R^2-values obtained for root length in control and drought conditions were 0.058 and 0.108 respectively, which shows that this QTL explains the phenotypic variation more under drought condition than in control conditions. For shoot length this pattern persists and R^2-values obtained was 0.096 and 0.11 for control and drought conditions, respectively.

Table 3. QTLs detected using single marker analysis. Likelihood Ratio Test (LRT) and regression coefficient (R^2).

Trait	Linked marker	LRT-Value	Significance level	R^2
SLC	WMC-153	6.118	*	0.090
	WMC-367	5.568	*	0.038
	WMC-24	7.34	**	0.096
RLC	WMC-153	10.060	**	0.130
	WMC367	5.972	*	0.028
	CFA-2123	4.795	*	0.057
	WMC-24	4.406	*	0.058
DBC	WMC-476	4.126	*	0.056
	WMC-24	5.787	*	0.077
GRC	WMC-160	4.608	*	0.042
	WMC-24	4.323	*	0.05
	WMC-407	4.267	*	0.057
	WMC-479	4.941	*	0.067
GPC	WMC-153	8.592	**	0.122
	WMC-24	4.097	*	0.052
	WMC-479	3.997	*	0.054
SLD	WMC-454 B	4.998	*	0.078
	WMC-24	7.079	**	0.110
RLD	WMC-24	7.380	**	0.108
DBD	WMC-357	5.255	*	0.092
	WMC-484	5.890	*	0.090
	WMC-177	4.637	*	0.039
	WMC-160	6.167	*	0.099
	WMC-407	5.564	*	0.090
	WMC-556	4.181	*	0.068
GRD	WMC-160	4.767	*	0.068
GPD	WMC-457	4.854	*	0.071
	WMC-160	5.048	*	0.073

At 0.05 and ** at 0.01 level of significance.
SLC= Shoot length under control, RLC=Root length under control, DBC= Dry biomass under control, GRC= Germination rate under control and GPC= Germination percentage under control. The other trait terms ending in D stands for under drought.

Marker WMC-407 was linked to germination rate and dry biomass in control and drought conditions respectively. In a study conducted by Mason *et al.* (2010) this marker was linked to kernel weight and grain filling duration, at chromosome 2A with a R^2-value of 0.109, and 0.150, respectively. In the study WMC-160 was found to be linked with germination rate under control condition with R^2-value of 0.042 and was linked to dry biomass, germination rate and germination percentage in drought condition with R^2-values of 0.099, 0.068 and 0.073. This marker was reported by Mason *et al.* (2010) on chromosome 5B and it is said to be linked with leaf length, days of flowering with a R^2-value of 0.13 and 0.21, respectively.

A marker WMC-479 was found to be linked to germination rate under control in the study with a R^2-

value of 0.067 at 5% level of significance. This marker was reported by (Båga *et al.*, 2007) on 2B chromosomes and with a fragment size of 179 bp and according to them this marker was previously reported on 7A chromosome. According to (Båga *et al.*, 2007) CFA2123 was also linked to 7A chromosome. In current study CFA2123 was linked to RLC with R^2-value of 0.057. WMC-457 was reported on 4D chromosome.

Another marker of interest was WMC-357 which was linked to QTL for dry biomass in drought condition and this marker had a R^2-value of 0.092 at 5% level of significance. This means that this marker explains 9% phenotypic variation in RILs. This was reported on chromosome 5D by Mason *et al.* (2010). WMC-367 was linked to root length under control and has a R^2-value of 0.028 which indicates it could be a minor QTL. This marker WMC-367 was reported to be mapped on 1B by Genc *et al.* (2010). Another marker WMC-476 was linked to dry Biomass under control with R^2-value of 0.056 and was reported to be linked to 7B chromosome by Mason *et al.* (2010).This study identified at least one marker for 10 traits measured. Maximum numbers of linked markers which were six were identified to trait dry biomass in drought condition. Only one marker was found to be linked with germination rate in drought condition which is WMC-160.

Simple interval mapping and Composite interval mapping: More QTLs were found in composite interval mapping (CIM) than simple interval mapping (SIM) showing more detection power of composite interval mapping (Table 4). QTL (Qrlc1I) has been found for root length under control in simple interval mapping at LOD 2.0 (Table 4). R^2 value was 0.12 for this QTL which showed its 12% contribution towards total phenotypic variation. It was linked to marker WMC-153. QTL found for germination percentage under control (Qgpc1I) was also linked with marker WMC-153 and it had R^2 value as the QTL for root length. This QTL was found at LOD of 1.8. QTL for root length (Qrlc1C) found in composite interval mapping (CIM) is linked with marker WMC-153. It had LOD score of 2.5 (Table 4). QTL for shoot length (Qslc1C) under controlled conditions has also been found in CIM. This was linked with marker WMC-367 with R^2 value of 0.09 and LOD value of 1.9. Two QTLs have been found for dry biomass (Qdbc1Ci and ii) under controlled conditions by CIM at LOD of 3.0 and 1.9, respectively. One of these QTL was linked with marker WMC-177 and the other was linked with WMC-476. R^2 value for first QTL was 0.10 and for second QTL is 0.01. Under controlled conditions QTL has been mapped by CIM for germination rate (Qgrc2C). This QTL was linked with marker WMC-319 with LOD score was 2.2.

Composite interval mapping (CIM) found two QTLs for germination percentage (Qgpd1Ci and ii) at LOD 1.9 and 2.9 under drought conditions. One QTL was linked to marker WMC-153 and the other QTL was linked to marker WMC-476. R^2 value for one of these QTL was 0.12. For dry biomass one QTL (Qdbd1C) was found by CIM under drought conditions. This QTL linked with marker WMC-171 with LOD value of 3.0. Under drought conditions one QTL has been found for germination rate (Qgrd2C) at LOD 3.1 and the other has been found for germination percentage (Qgpd2C) at LOD 3.0 by composite interval mapping. Both these QTLs were linked with marker WMC-160.

Table 4. QTLs found in simple interval mapping (I) and Composite Interval Mapping (C) under controlled and drought conditions.

S.No.	QTLs	Trait	Linked marker	LOD score	R^2
1	Qrlc1I	RLC	WMC-153	2.1	0.12
2	Qgpc1I	GPC	WMC-153	1.8	0.12
3	Qrlc1C	RLC	WMC-153	2.5	0.12
4	Qslc1C	SLC	WMC-367	1.9	0.09
5	Qdbc1Ci	DBC	WMC-177	3.0	0.10
6	Qdbc1Cii	DBC	WMC-476	1.8	0.01
7	Qgrc2C	GRC	WMC-319	2.2	0.01
8	Qgpd1Ci	GPD	WMC- 153	1.9	0.12
9	Qgpd1Cii	GPD	WMC-476	2.9	0.01
10	Qdbd1C	DBD	WMC-171	3.0	0.09
11	Qgrd2C	GRD	WMC-160	3.1	0.10
12	Qgpd2C	GPD	WMC-160	3.0	0.06

SLC= Shoot length under control, RLC=Root length under control, DBC= Dry biomass under control, GRC= Germination rate under control and GPC= Germination percentage under control. The other trait terms ending in D stands for under drought.

For the development of mapping population synthetic Hexaploid has been used which has been produced by wide crosses between durum wheat and D genome source *Aegilops tauschii* (Mujeeb-Kazi *et al.*, 1996). The genome D is reported for abiotic stress tolerance in wheat crop (Gorham *et al.*, 1997). We have used SSR markers mostly reported on D genome. Thirty nine polymorphic score able markers have been found in this project, most of them belong to different chromosomes of D genome. The reason for less number of polymorphic markers is that D genome show less polymorphism than other two genomes of wheat (Gao *et al.*, 2004).

Ten QTLs for different traits have been found under controlled and drought conditions as a result of composite interval mapping which clearly depicted more power and reliable results of composite interval mapping as compared to simple interval mapping. One QTL has been found for root length at LOD 2.5 under controlled conditions by composite interval mapping linked to marker WMC-153 similar to simple interval mapping (Table 4) and single marker analysis (Table 3). Similarly, QTL for shoot length under control condition was linked to marker WMC-367 which was also shown by single marker analysis (Table 3).

For dry biomass, by CIM, two QTLs have been found under controlled conditions at LOD of 3.0 and 1.8. One QTL has been found to be linked with WMC-177 and the other was linked to WMC-476. WMC-476 was also detected linked to dry biomass under control by single marker analysis. WMC-177 has been mapped on 2A chromosome (Jing *et al.*, 2007) and was linked to protein content (Zlatska *et al*., 2008), seedling biomass (Genc *et al.*, 2010), spikelet per spike and 1000 kernel weight (Yao *et al.*, 2009).

One QTL has been mapped for germination rate under controlled conditions by CIM. It was linked with marker WMC-319. Flanking markers linked to this marker were WMC-159 and WMC-160. WMC-160 is linked to germination rate under control in single marker analysis (Table 3). Under drought conditions CIM mapped two QTLs for germination percentage. One of these QTLs was linked to WMC-153 and the other was linked to WMC-476. Single marker analysis also showed linkage between WMC-153 and germination percentage under control. One QTL linked to dry biomass has been found by CIM under drought condition which has LOD value of 3.0. This QTL was linked with marker WMC-171. In previous work marker WMC-171 was found to be mapped on 2B chromosome and it showed R^2 value of 0.81 for fusarium head blight resistance (Löffler *et al.*, 2009).

Two QTLs for two different traits have been found under drought conditions. One was linked to germination rate and the other is linked with germination percentage. Both of these QTLs were linked with WMC-160. LOD value for germination rate was 3.1 while for germination percentage was 3.0. R^2 values for both of these QTLs was 0.01 which indicates minor contribution of these QTLs towards total phenotypic variation in population. Mason *et al*. (2010) found that marker WMC-160 was linked with leaf length and days of flowering and had a R^2 value of 0.13 for leaf length and 0.21 for days of flowering.

Marker WMC- 153, WMC-476 and WMC-160 are linked with more QTLs associated with different traits under controlled and drought than any other marker. WMC-153 was linked to root length and germination percentage under controlled conditions in simple interval mapping results. It was also linked with QTL mapped for root length under controlled conditions and to germination percentage under drought conditions by composite interval mapping. WMC-476 was linked with QTL mapped by CIM for dry biomass under controlled conditions and to germination percentage under drought conditions.WMC-160 was linked with germination rate as well as germination percentage under drought conditions found by composite interval mapping.

It can be concluded that composite interval mapping is more accurate and powerful than simple interval mapping because we were able to find QTLs for only two traits when simple interval mapping was performed i.e. root length and germination percentage. On the other hand, we have found QTLs for five different traits i.e. root length, shoot length, dry biomass, germination percentage and germination rate under controlled and drought conditions when we used CIM.

Here we report markers related to drought tolerance in a population (OPATA x SH349) which can be helpful in increasing the selection efficiency of drought tolerant plants in future research strategies after these marker have been validated across diverse environments and genetic background, hence time and resources can be saved. However, more DNA markers are needed to be mapped in this population for increased genomic coverage and detailed QTL mapping at other growth stages for drought tolerance.

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