

QTL MAPPING OF WHEAT DOUBLED HAPLOIDS FOR CHLOROPHYLL CONTENT AND CHLOROPHYLL FLUORESCENCE KINETICS UNDER DROUGHT STRESS IMPOSED AT ANTHESIS STAGE

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Abstract

Drought stress is one of the major environmental constraints to crop plants including wheat worldwide. Synthetic hexaploid can act as a vehicle for improving crop tolerance against biotic and abiotic stresses. Doubled haploid population consisting of one hundred and forty individuals derived from cross of Opata and SH223 was used in the present study to identify genomic regions associated with various quantitative attributes of physiological nature. Doubled haploid mapping population was phenotyped for chlorophyll content and chlorophyll fluorescence kinetics under control and drought stress imposed at anthesis stage. Genotyping of population was accomplished by utilizing two hundred and sixty one polymorphic Gaterslaben wheat microsatellites and Beltsville agriculture research center simple sequence repeats. Linkage map of doubled haploid population comprising of 19 linkage groups and covering map length of two thousands six hundred and twenty six (2626) cM was constructed using map maker software. Major and minor QTLs associated with quantitative traits were identified using QGene software. Major QTL for chlorophyll content (*QTc.wwc-1B-S11*) of doubled haploid mapping population under anthesis drought stress was mapped on chromosome 1B and explained 10.09 percent of phenotypic variation at LOD score of 5.5. Seven major and minor QTLs for PCFK of doubled haploids were identified on chromosome 1B, 7A and 7D under control and drought stress at anthesis stage. The identified QTLs are of prime importance for high resolution mapping in synthetic hexaploid wheat. Genomic synteny of doubled haploids was observed with rice chromosome 2, 4, 7 and maize chromosome 7 owing to occurrence of orthologous QTLs for chlorophyll content and chlorophyll fluorescence respectively.

Key words: QTL mapping, Wheat, Chlorophyll, Stress, Haploids.

Introduction

Drought stress is amongst the major impediments to crop production in many regions, especially the arid and semiarid regions and is intricate to predict and mitigate (Ahmad *et al.*, 2004). Water is becoming limiting factor for sustained production of wheat and other crops. Therefore, breeding for enhanced drought tolerance in wheat is an essential entity for achieving improved crop productivity and greater food security for the hundreds of millions of rural poor (Ortiz *et al.*, 2008; Noorka *et al.*, 2013). Genetic diversity of yield related attributes can be harnessed through utilization of synthetic hexaploid wheat. Plant responses to biotic and abiotic stresses can be additive, synergistic or antagonistic and are affirmed by quantifying different quantitative and qualitative traits (Tuberosa & Salvi, 2006). Wheat yield is positively associated with number of grains (Razzaq *et al.*, 2013; Nawaz *et al.*, 2013).

Breeding of superior drought tolerant wheat varieties can be harnessed through molecular markers, genomics and post genomic strategies (Nguyen *et al.*, 2004). Chlorophyll a and b are important photosynthetic pigments for absorption and exploitation of light energy to perform photosynthetic act (Thomas *et al.*, 2005). The maintenance of chlorophyll content in crops under different environmental constraints might be an effective strategy to increase biomass and grain yield (Wang *et al.*, 2008a; 2008b). Therefore, understanding the genetic mechanism of chlorophyll content and chlorophyll fluorescence parameters would be very effective for yield

improvement in wheat. Chlorophyll fluorescence parameters are used to detect subtle changes in the activity of photosynthetic apparatus of different genotypes of crop plants (O'Neill *et al.*, 2006).

Wheat is allohexaploid and has enormously large genome of 16x10,9 bp/1C and more than eighty percentage of genome is constituted of repetitive sequences (Gill *et al.*, 1996). Molecular markers have been extensively utilized to map quantitative trait loci associated with different agronomic and phenological attributes of wheat. However, very little information is available for genetic dissection and chromosomal localization of genes regulating physiological characteristics, especially component of photosynthesis. QTLs for some important physiological traits involved in dehydration tolerance, including water use efficiency (Hao *et al.*, 2003) osmotic adjustment (Morgan & Tan, 1996) chemical desiccation tolerance (Börner *et al.*, 2003) and Chlorophyll content (Cao *et al.*, 2004) were mapped on chromosomes 7A and 7D. Cao *et al.*, (2004) detected seven QTLs for chlorophyll content on chromosomes 2B, 4A, 5B, 6A, 7A, and 7D under Nitrogen sufficient environment, while nine QTLs were identified for chlorophyll content on chromosomes 2D, 3A, 4B, 5B, and 6A when wheat seedlings are grown under Nitrogen deficient environment. Yang *et al.*, (2007) mapped four additive QTLs controlling chlorophyll content under water deficient and well watered on chromosomes 1A, 5A, and 7A in wheat. Few QTL studies have been reported so far on CF parameters in wheat (Yang *et al.*, 2007; Liang *et al.*, 2010; Zhang *et al.*, 2010; Czyczyło- Myszka *et al.*, 2011; Kumar *et al.*, 2012; Li *et al.*, 2012). The present study was

aimed to identify the quantitative trait loci associated with chlorophyll content and chlorophyll fluorescence parameters of doubled haploid population developed by the cross of Opata x SH223.

Materials and Methods

Plant material: The germplasm studied was comprised of one hundred and fifty individuals of doubled haploids developed by cross of Opata and SH223 by Dr. Abdul Mujeeb kazi in National Agriculture Research Center (NARC) Islamabad. F₁ individuals were derived by cross of SH223 and Opata. Healthy tillers of F₁ individuals were selected from the field at booting stage and central floret of each spikelet was removed. Pollens were removed with the help of fine forceps and emasculated plants were placed in water. Emasculated spikelet's were pollinated with freshly collected maize pollen after two days of emasculations. Twenty four hours following pollination plants were sprayed with 2,4-Dichloro acetic acid solution and embryo rescue was accomplished after sixteen days. Haploid embryos were allowed to grow on MS medium yielding haploid seedlings. Five to seven cm long haploid seedlings were then transferred to pots containing soil for acclimation. Seedlings at tillering stage were transferred from soil to beaker containing 1,000 ml colchicine solution for eight hours in order to induce diploidy and shuffled back to soil which finally yielded doubled haploids.

Experimental design: Seeds were sown in jiffies for each line at the rate of three to four seeds per jiffy. Twelve days old seedlings of doubled haploids were transplanted into field and greenhouse. There were two treatments i.e. control and drought stress imposed at anthesis stage. Control condition retained regular mode of irrigation whereas stress at anthesis stage was given by withholding water from 68 to 88 days after sowing and different physiological and biochemical parameters of drought mapping population were recorded at maturity. Field and greenhouse experiments were performed in NARC, Islamabad.

Chlorophyll content was estimated by the method as described by Arnon (1949). Acetone (80%) was used for extraction of chlorophyll content and impurities were eliminated by centrifugation. Chlorophyll a and b were calculated by measuring absorbance at 663 and 645 nm.

At anthesis stage, flag leaves of doubled haploid mapping population were used for measurement of chlorophyll fluorescence with the help of chlorophyll fluorometer. Chlorophyll fluorescence of doubled haploids was measured using FIM 1500 apparatus (Analytical Development Company, UK). The transients were induced by red light of 3000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, provided by an array of six light emitting diodes (peak 650 nm), which focused on the exposed area of the sample (4 mm in diameter). All samples of doubled haploids were dark adapted for 30 minutes prior to the fluorescence measurement. Chlorophyll fluorescence traits Fo, minimum fluorescence yield of PS II; Fm, maximum fluorescence yield of PS II; Fv, variable fluorescence yield, calculated as Fm-Fo; Fv /Fm, maximum quantum

yield of PS II, calculated as (Fm-Fo)/Fm were recorded under control and drought stress imposed at anthesis stage.

DNA extraction: Fifteen days old seedlings of the doubled haploid population were used for the extraction of genomic DNA using the CTAB method described by Doyle and Doyle (1987) with modifications. Samples were freeze-dried for the disintegration of cell wall which were then ground in a mixer twice for 30 second. Samples were incubated in CTAB extraction buffer for 45 minutes. CTAB extraction buffer comprising of 2.0 percentage weight / volume 100 mM Tris-HCL having pH 8.0, CTAB, 1% Na₂S₂O₃, 1.4 M NaCl, 0.2% *b*-mercaptoethanol and 20 mM EDTA was used for DNA extraction.. Protein traces were removed by the addition of Chloroform: isoamyl alcohol in a ratio of 24:1. This step was repeated twice for the complete elimination of protein. Acetate mix comprising of 10 M ammoniumacetate and 3 M sodiumacetate and isopropanol were used for the precipitation of DNA. DNA was dissolved in Tris acetate buffer comprising of 1 mM EDTA and 10 mM Tris-HCl. RNA was removed by the addition of RNAase. DNA was stored at -20°C for 12 hours and then incubated at 4 °C. Samples were diluted before use for microsatellite analysis. Genomic DNA was run on 1% agarose gel at 100v for 90 minutes in 1 X TAE buffer. A molecular ladder (1Kb) was run as standard for comparison. Staining was accomplished by using ethidium bromide and visualized under ultra violet light in a transilluminator. DNA was quantified on agarose gels with a known DNA molecular weight standard. According to the band width, DNA was diluted for further steps of PCR experiment.

Microsatellite analysis: Polymerase chain reaction of wheat microsatellite consortium, Gatersleben wheat microsatellite (gwm) and Beltsville agriculture research center (barc) and microsatellite markers were performed by methods as described by Roder *et al.*, (1998b) and Somers *et al.*, (2004). Amplification of DNA was accomplished in a 96 well thermocycler each comprising of DNA, deoxynucleotide, forward and reverse primers, MgCl₂, PCR buffer and Taq DNA Polymerase. Double helix was converted into single strands by denaturation at 94°C for 3 minutes. Primers were attached at annealing step carried out round about 50 to 60°C. New nucleotides were added to annealed primers at extension step which was accomplished at 72°C. Automated laser fluorescence ALF express sequencer was used for detection of simple sequence repeats. A mixture of 1.5 ul of PCR product and 2ul internal standard was denatured at 96°C for two minute. Denatured product was cooled on ice. ReproGel was used for preparation of polyacrylamide gel. The gel was run for two hours depending on the expected fragment size in gel doc containing 1X Tris-borate EDTA buffer. The gels were re-used four to five times depending on the quality of the gel that was determined by a parameter called laser value indication on the machine. During electrophoresis, the fluorescently labelled fragments in each lane migrate downwards through the gel. The fixed laser beam passes through the glass spacer located at thermoplate of the glass cassette. The beam is

directed in to the gel perpendicular to the direction of band migration. The laser beam excites the fluorescently labelled PCR product and the light emitted is detected by photodetectors located behind the gel. Since, there were 40 photodetectors, one for each lane: for each run forty samples were loaded including one well for external standard. Fragment Analyzer Version 1.02 was used for size calculation of Microsatellite fragment. Secondly, three or four peaks as external standard, which was loaded on the well number one and were detected on first line of output were defined as external size standard and then the internal size standard peaks on the rest of 39 wells were adjusted to external size standards. An external standard comprising of 73, 122, 196, 231 base pair was loaded in first line. Primers were also visualized by running sample on 1.5% of agarose gel.

Genetic linkage map construction: Computer program Join map version 3 was used for the construction of linkage map (Lander and Botstein, 1989) by evaluating genotypic data of one hundred and forty lines of doubled haploid mapping population. Markers were grouped by applying two point analyses with grouping command with minimum LOD value of 3.0 and recombination value of $0=0.40$. Wheat SSR map from Roder *et al.*, (1998a) was used as reference map for assigning markers to the chromosomes. Non significant linkage was declared if gap between markers is greater than 50 cM. The approximate position of centromere on the linkage map was sketched by their location on map published by Sourdille *et al.*, 2004.

QTL analysis: QTL localization and characterization for different physiological and biochemical attributes of doubled haploid mapping population was followed by using Interval mapping approach (Lander & Botstein, 1989) based on multiple regression which relates phenotypic data with marker interval (Haley & Knott, 1992). Mapping analysis was performed at lower LOD in order to avoid the repetition of QTLs. Statistically important attributes related to QTL mapping were reported.

Results and Discussions

Genotyping of doubled haploids: Fig. 1 depicts the results of polymorphism and monomorphism among SH223 and opata for primer category of Beltsville Agriculture Research Center, Gaterslaben Wheat Microsatellites and Gaterslaben D genome Microsatellites on agarose gel electrophoresis. Gaterslaben wheat microsatellite 912 with an internal standard of 73 and 231 base pair is an example of multiallelic monomorphic loci for Opata and SH223 (Fig. 2) where as Gaterslaben wheat microsatellites 297 indicates polymorphism of seven base pair among parents (Fig. 3). Figure 4 is an example of null allele segregation for wheat microsatellites 1263 where as Fig. 5 describes allele segregation in doubled haploid population for primer wheat microsatellite 938.

Five hundred and sixty simple sequence repeat markers were applied for parental screening through agarose and poly acryl amide gel electrophoresis. A new SSR based linkage map of hexaploid wheat was constructed based on double

haploid individuals derived from Opata and SH223. The parents were analyzed with five hundred and sixty (560) simple sequence repeats pairs. Out of 560 tested SSRs, eighteen (18) SSRs did not show amplification in parents. Remaining five hundred and forty two (542) showed amplification in parents and were identified as monomorphic or polymorphic. Two hundred and sixty one (261) polymorphic simple sequence repeats were applied on doubled haploid mapping population for generating linkage map. Chromosomes 1B and 4A had the highest number of polymorphic loci where as chromosome 4D were having lowest number of loci. Two hundred and sixty one of the polymorphic loci were mapped on 19 linkage groups with a total map length of 2626 cM, which covered 65 percentage of genome according to an estimation by Sourdille *et al.*, (2003), who suggested that the entire map length of common wheat was about 4,000 cM. The average of chromosome length and number of loci per chromosome was 130 cM and 10 respectively. Quarrie *et al.*, (2005) developed a genetic map comprising of five hundred and sixty seven (567) markers including SSR, RFLP and AFLP. These molecular markers were assigned to 21 linkage groups and yielded map length of 3521.7 cM with an average chromosomal length of 168 cM. The map length of doubled haploids derived from Opata and SH223 for genome A, B and D was 900, 915 and 811cM respectively. Distribution of markers across the genome was not uniform. There were gaps of more than 30 cM on chromosomes 2D, 3B, 3D, 5D, 6A and 7B. Gaps on chromosomes and partial coverage of some chromosomal arms like 1AL, 4BS, 6BL has also been reported by Paillard *et al.*, (2003). Gaps have been reported in intraspecific (Torada *et al.*, 2006) or interspecific (Messmer *et al.*, 1999) crosses used for generating linkage maps in wheat species. Roder *et al.*, (1998b) and Gupta *et al.*, (2002) reported gaps by mapping GWM and WMC primer pairs on ITMI population and mapped less polymorphism on D genome. Only two loci were mapped on chromosome 4D of doubled haploids. Torada *et al.*, (2006) mapped less number of loci on chromosome 4D. Partial coverage of chromosome 4D has been observed by Gross *et al.*, 2003. Hai *et al.*, 2008 developed genetic linkage map of doubled haploid lines for 19 chromosomes of hexaploid wheat except chromosome 4D and 6B due to low level of polymorphism. Doubled haploid production through maize culture exhibits low level of marker segregation distortion as compared to pollen culture technique (Quarrie *et al.*, 2005).

Analysis of the phenotypic values: The phenotypic values and statistical data for chlorophyll content and chlorophyll fluorescence kinetics under drought stress is shown in Table 1. Between the two parental varieties, C, Fo, Fm, Fv and Tm of SH223 was higher than those of Opata under drought stress where as population showed transgressive segregation. The simple correlation coefficients among the traits are presented in Table 2. C is significantly positive correlated with Fo, Fm and Fv. Fo is significantly positive correlated with Fm, and significantly negative correlated with Fv/Fm. Fm is significantly positive correlated with Fv and Fv/Fm. Fv is significantly positive correlated with and Fv/Fm.

Polymorphism among Opata and SH223

Monomorphism among Opata and SH223

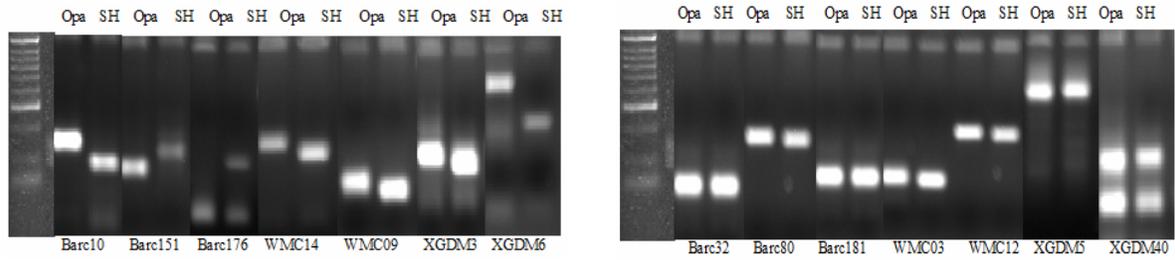


Fig. 1. Polymorphism and monomorphism of simple sequence repeats for SH223 and Opata on agarose gel electrophoresis.

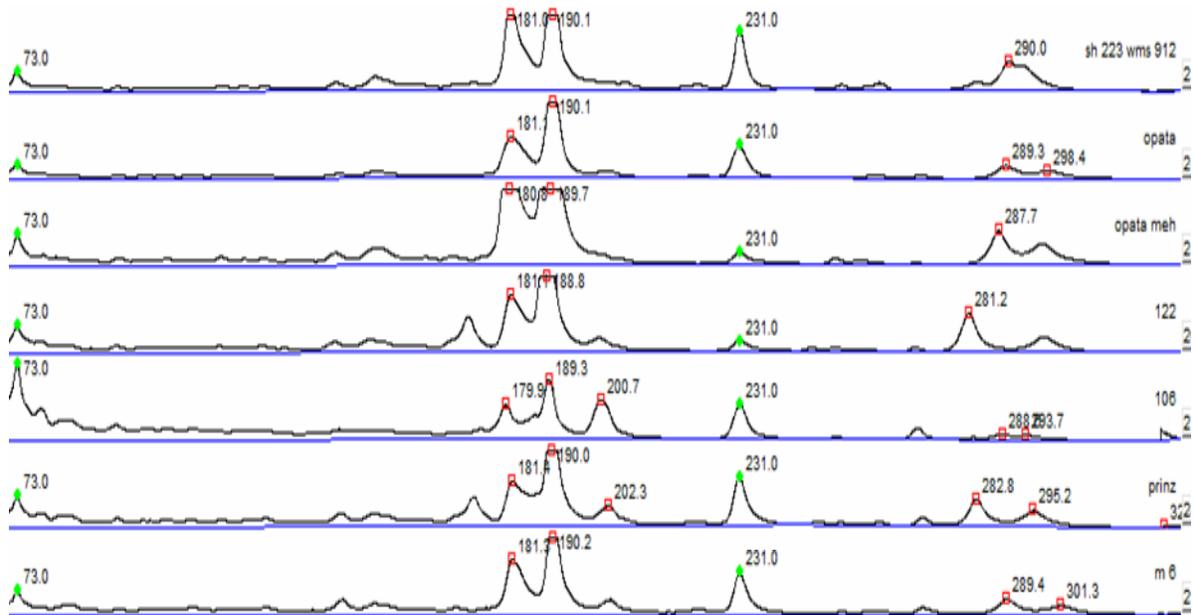


Fig. 2. Fragment analysis of wheat microsatellites 912.

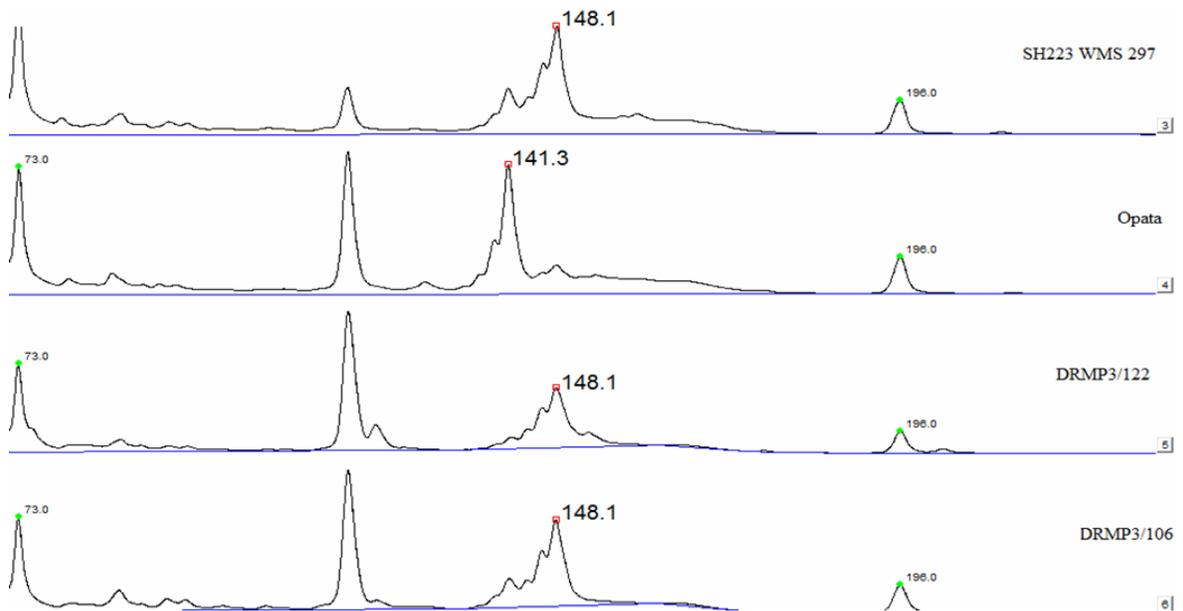


Fig. 3. Polymorphism among parents for wheat microsatellites 297.

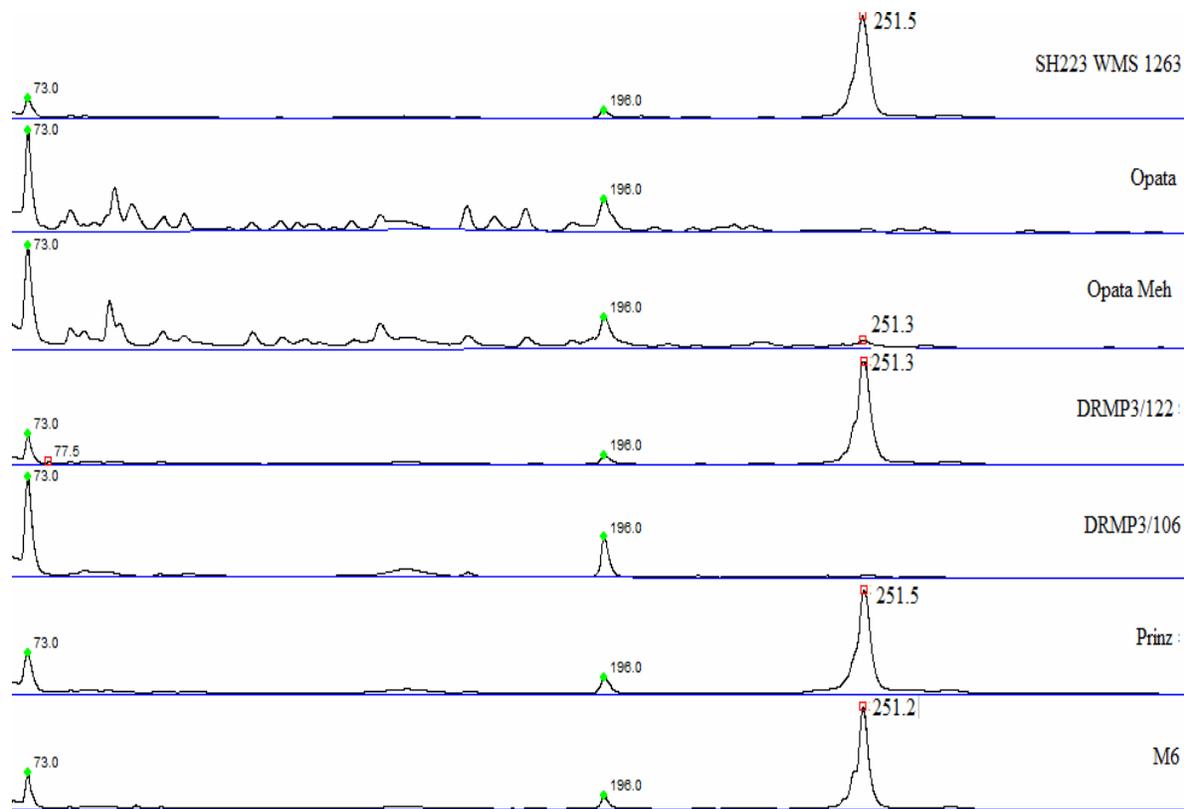


Fig. 4. Null allele segregation for primer wheat microsatellite 1263 in parents.

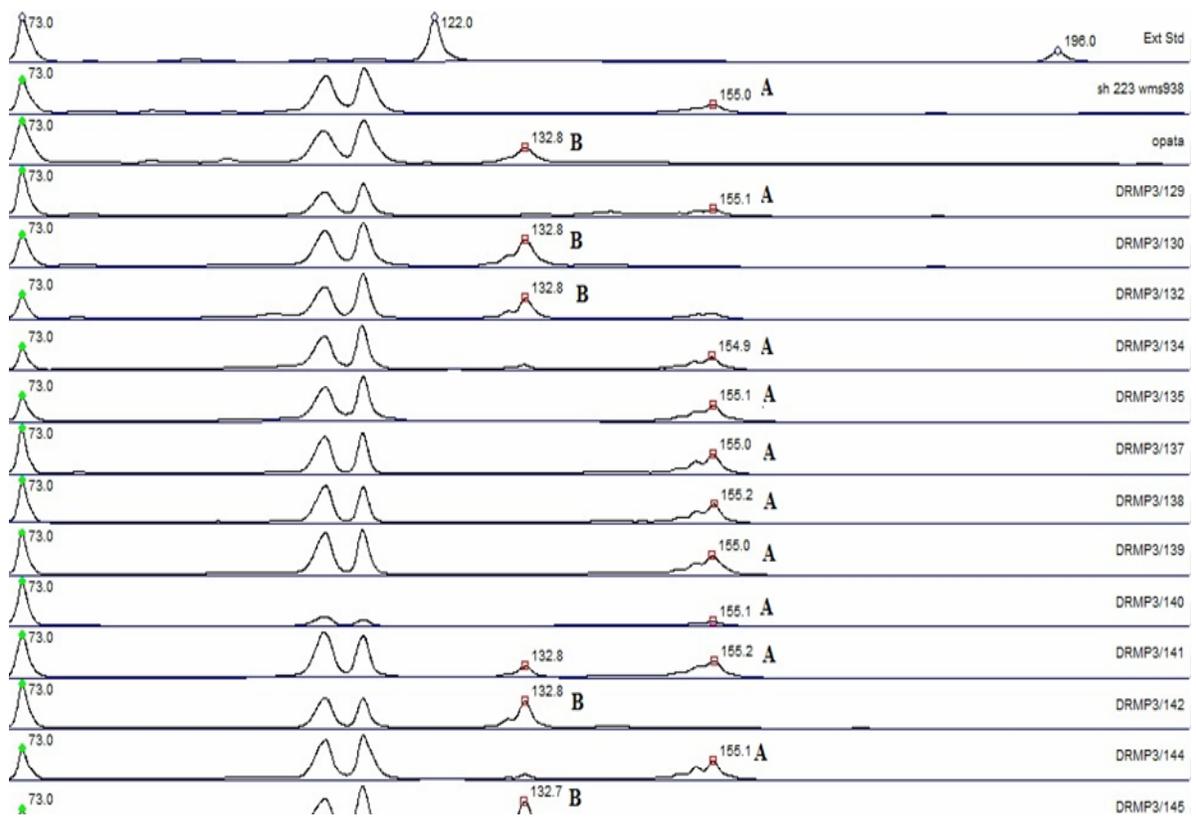


Fig. 5. Allele segregation in doubled haploid population for primer wheat microsatellite 938.

Table 1. Descriptive statistics of doubled haploids for Total chlorophyll content (mg/g) and chlorophyll fluorescence attributes under drought stress.

	SH223	Oyata	Mean	Std Devi	Mini.	Maxi.	CV	Prob
F0	740	689	765	28.58	529	878	6.75	0.001
Fm	3856	3517	3526	147.24	2970	3696	5.892	0.0001
Fv	3221	2978	2825	125	1915	3211	7.618	0.001
Fv/Fm	0.66	0.7	0.68	0.009	0.54	0.65	9.083	0.001
Total Chlorophyll	1.49	1.65	0.73	0.25	0.51	1.75	8.58	0.001

Table 2. Co relation matrix of Doubled haploids for Total chlorophyll content (mg/g) and chlorophyll fluorescence attributes under drought stress.

	C	F0	Fm	Fv	Tm	Fv/Fm
F0	0.24					
Fm	0.41	0.2				
Fv	0.39	0.04	0.73			
Tm	0.05	0.11	0.25	0.2		
Fv/Fm	0.06	-0.42	0.35	0.42	0.1	
Total chlorophyll content (mg/g)	0.12	1.26	0.29	0.29	0.1	0.18

QTL Analysis: Two minor and one major QTLs were mapped for total chlorophyll content of doubled haploid mapping population derived from SH223 and Oyata on chromosomes 1B, 3B and 4A under control condition as represented in Fig 6. The explained variance for these QTLs ranged from 5.34 percentage to 14.35 percentage. The first minor QTL with additive effect of 0.34 from SH223 for chlorophyll content was mapped on chromosome 1B under control experiment and explained only 5.34 percentage of phenotypic variation (Table 3). The second minor QTL *QTc.wwc-3B-C11* for this attribute was localized on chromosome 3B and explained 8.90 percentage of R^2 . The major QTL for total chlorophyll content under control experiment of 2009 was identified on chromosome 4A and explained 15.35 percentage of phenotypic variation. Seven QTLs for chlorophyll content were detected on chromosomes 2B, 4A, 5B, 6A, 7A, and 7D under nitrogen (N) sufficient environment, where as nine QTLs were localized for this attribute on chromosomes 2D, 3A, 4B, 5B, and 6A under N deficient environment (Cao *et al.*, 2004). Three minor QTLs for this attribute were mapped on chromosome 2B, 7A and 7B of double haploid mapping population under drought stress at anthesis stage with R^2 in the range of 5.57 to 10.09. Both parents contributed positive allele for this parameter. Eight additive QTLs for chlorophyll a content were detected on chromosomes 1B, 4A, 5D, 6B, and 7A in wheat by Zhang *et al.*, 2009.

The first major QTL for chlorophyll content (*QTc.wwc-1B-S11*) of doubled haploid mapping population under anthesis drought stress was mapped on chromosome 1B and explained 10.09 percentage of phenotypic variation at LOD score of 5.5. The main allele for this QTL was contributed by Oyata. Two more QTLs (*QTc.wwc-5B-S9* and *QTc.wwc-7B-S9*) for this trait were mapped on chromosomes 5B and 7B under drought stress at anthesis stage and explained 10.09 and 12.30 percentage of phenotypic variation. The positive allele for QTL located on chromosome 5B was contributed by SH223 where as allele for QTL located on chromosome 7B was attributed

by Oyata. Four additive QTLs controlling chlorophyll content were identified on chromosomes 1, 5 and 7 of genome A under control and well deficient condition in wheat by Yang *et al.*, 2007.

Drought is one of the major impediments to crop plants having detrimental impact on photosynthesis in due of ravages of reactive oxygen species on electron transport chain of the photosystem II. Efficiency of PSII can be vindicated by parameters of chlorophyll fluorescence kinetics (PCFKs) (Zhang *et al.*, 2005). Chlorophyll fluorescence of double haploid population are adversely affected under drought stress except *Fo* having significant increment under water deficient environment. Low PCFKs values were observed under drought stress than control condition except *Fo* (Demming *et al.*, 1987; Hong *et al.*, 1999; Zhao *et al.*, 2005; Yang *et al.*, 2007). Correlation coefficients among PCFKs of doubled haploid mapping population are significantly decreased under water deficient environment while making comparison with control condition. Traits associated with photosynthetic rate exhibit polygenic inheritance and are controlled by quantitative trait loci (Hervé *et al.*, 2001; Fracheboud *et al.*, 2002; Cao *et al.*, 2004; Juenger *et al.*, 2005) and QTL mapping identifies chromosomal regions associated with these quantitative traits (Alonso-Blanco & Koornneef, 2000).

Seven major and minor QTLs for PCFK of doubled haploids were identified on chromosome 1B, 7A and 7D under control and drought stress at anthesis stage as shown in Table 4. One QTL controlling *Fo* under control condition is located on 1B at LOD value of 6.15. The favorable allele for this QTL was originated from Oyata and explained 10.67 percentage of phenotypic variation. QTL (*QFm.wwc-7A-C11*) explained 8.56 percentage of variation at LOD scores of 3.5. The positive allele for this QTL was originated from Oyata. The QTL for *Fv* (*QFv.wwc-7D-C9*) was positioned at 72 cM with in marker interval of Xwmc42-Xwmc14 and explained 7.89 percentage of R^2 at LOD value of 4.5 under control condition. The main source of this QTL was from parent B i.e. Oyata.

Two QTLs for F_0 attribute were detected on chromosomes 1B and 7D for drought stress at anthesis stage. The first QTL (*QF₀.wwc-1B-S11*) explained 7.89 percentage of phenotypic variation for F_0 where as second QTL located on 7D chromosome explained 6.13 percentage of variation. Only one QTL (*QF_v/F_m.wwc-7D-S9*) for Fv/Fm for doubled haploid mapping population was detected on chromosome 7D under anthesis drought stress. This QTL was originated from Opata and explained 9.13 percentage of the phenotypic variation at LOD value of 2.5. One QTL for Fv/Fo (*QF_v/F_o.wwc-7D-S11*) explained 10.23 percentage of phenotypic variation under drought stress and was mapped on chromosome 7D. The favorable allele for this QTL was advocated from Opata. The genomic region on chromosome 1B flanking by markers Xbarc60 and Xwms31 is considered an important region in due of harboring QTLs related to F_0 under control and drought stress experiment of 2009 and 2011 respectively. Chromosomes 1B, 7A and 7D are considered important chromosomes in respective of harboring QTLs for PCFKs, water water use efficiency (Hao *et al.*, 2003) and chlorophyll content (Cao *et al.*, 2004). Yang *et al.*, 2007 identified 18 additive QTLs for chlorophyll fluorescence attributes on eight chromosomes of hexaploid wheat with their variance in the range of 7.27 percentage to 72.72. QTLs detected under control and drought stress not only provide information for the polygenic inheritance and their interaction with environment but also helps to identify molecular markers closely linked with quantitative traits.

The present study was aimed to dissect the complex quantitative inheritance of physiological attributes of wheat under control and drought stress imposed at anthesis stage. Genotyping was accomplished using simple sequence repeat markers. Major and minor QTLs associated with chlorophyll content and PCFK of doubled haploids were identified on chromosome 1B, 7A and 7D. These identified QTLs are of prime importance for high resolution mapping in synthetic hexaploid wheat. These identified genomic regions needs to be further saturated with molecular markers for precise localization of QTLs governing the inheritance of these polygenic traits. Further, genetic and transcriptome characterization of this mapping population could be helpful for the identification of genomic regions closely associated with avoidance strategy of wheat under water deficient environment. Introgression of these resistant loci into adapted genetic backgrounds through marker assistant selection is a promising tool for plant evolution, gene cloning and transgenic crop improvement.

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