

## Identification of QTLs for four physiological traits in an advanced backcross population of wheat under drought stress

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### Abstract

Advanced backcross quantitative trait locus analysis was applied to identify QTLs for chlorophyll content, flag leaf senescence, cell membrane stability and the abscisic acid content traits under water-stressed conditions in BC<sub>2</sub>F<sub>2</sub> wheat families. The parents and 75 BC<sub>2</sub>F<sub>2</sub> families were evaluated phenotypically for drought tolerance using two irrigation treatments [0.25 and 0.75 m<sup>3</sup> (H<sub>2</sub>O) m<sup>-2</sup> (soil)]. The polymorphism among parental genotypes and 75 BC<sub>2</sub>F<sub>2</sub> families were tested using 40, 98 and 400 different TRAP, SRAP and SSR primer combinations, respectively. Mapping analysis produced 14 QTLs, in which a single QTL explained 9-39% of the phenotypic variation. These QTLs distributed on eight chromosomes. Four QTLs were significantly associated with chlorophyll content and distributed on four chromosomes. Three QTLs significantly influenced flag leaf senescence and mapped on chromosomes 1B, 2B and 3B. Two QTLs for cell membrane stability were identified and mapped on each chromosome of 2B and 6B. Five QTLs for abscisic acid content were identified on chromosomes 2B, 3B, 5B, 6B and 3D. All of the QTLs for the four physiological traits had a positive additive effect except for flag leaf senescence which had negative additive effects. The results also showed that the regression analysis for the relationship between the TRAP, SRAP and SSR markers and the phenotypes of BC<sub>2</sub>F<sub>2</sub> families for the four physiological traits was highly significant. Therefore, the TRAP, SRAP and SSR markers linked to the QTL for the drought tolerance can be further used in breeding for drought tolerance in wheat.

**Keywords:** abscisic acid content, physiological traits, QTL, SRAP, SSR, TRAP, *Triticum aestivum*.

**Abbreviations:** AB-QTL\_Advanced Backcross Quantitative Trait Locus; BSA\_Bulk Segregant Analysis; LOD\_Likelihood Ratio; QTL\_Quantitative Trait Locus; SSR\_Simple Sequence Repeat; SRAP\_Sequence-Related Amplified Polymorphism; TRAP\_Target Region Amplification Polymorphism.

### Introduction

Drought is a major abiotic stress that affects wheat (*Triticum aestivum* L.) production in many regions of the world. Maintaining a high yield in drought conditions has, therefore, become a priority, particularly when considering global environmental changes and the increase in world population (Takeda and Matsuoka, 2008). However, the physiological basis of yield maintenance under drought conditions remains poorly understood (Tuberosa and Salvi, 2006). Drought tolerance is a quantitative trait with complex phenotype and genetic control (McWilliam, 1989). Therefore, understanding the genetic and physiological bases of drought tolerance in crop plants are a prerequisite for developing superior genotypes through conventional breeding. Chlorophyll is one of the major chloroplast components for photosynthesis. The relative chlorophyll content has a positive relationship with photosynthetic rate. Flag leaf senescence is associated with

improved yield and transpiration efficiency under water-limited conditions in wheat (Verma et al., 2004). The cell membrane stability (CMS) is one of the main cellular targets common to different stresses (Levitt, 1980). Abscisic acid (ABA) has been demonstrated to play an important role in plant response to water stress. An increase in ABA content was generally associated with decreased stomatal conductance and grain yield but increased leaf temperature (Tuberosa et al., 1998).

DNA molecular markers based on the molecular quantitative genetics provide an opportunity to study quantitative traits such as drought tolerance through quantitative trait loci (QTLs) analysis. Molecular markers proved to be an important way to increase selection efficiency and there are good prospects for marker-assisted selection in improving drought responses in wheat (Quarrie et al., 2003). Recently, identification of new TRAP and SRAP markers linked to chlorophyll content, leaf

senescence and cell membrane stability in water- stressed wheat have been reported (Elshafei et al., 2013, Saleh et al., 2014). These markers have been linked to yield components as well (Moustafa et al., 2014). Therefore, application of QTL analysis to study the physiological traits will improve our understanding of genetic factors that influence these complex traits.

Advanced backcross QTL analysis (AB- QTL analysis) was proposed as a molecular-breeding method that integrates QTL analysis with germplasm development in crosses between adapted and wild germplasm (Tanksley and Nelson, 1996). Using the AB- QTL method, many QTLs have been identified and transferred from wild germplasm to elite breeding lines in cereal crops (Septiningsih et al., 2003; Ho et al., 2002; Huang et al., 2004). AB-QTL analyses have been successfully executed to locate favorable exotic QTL alleles that could improve agronomic traits in wheat (Huang et al., 2004; Liu et al., 2006; Kunert et al., 2007). Here, our report is the first to identify TRAP, SRAP, and SSR markers for the physiological traits under water- stressed condition in BC<sub>2</sub>F<sub>2</sub> wheat families. The objectives of this investigation were to identify TRAP, SRAP, and SSR markers linked to chlorophyll content, leaf senescence, cell membrane stability and the ABA concentration traits under water- stressed conditions in BC<sub>2</sub>F<sub>2</sub> wheat families using bulked segregant analysis.

## Results and Discussion

### *Phenotypic analysis of physiological traits*

Analysis of variance revealed significant differences in trait means among wheat genotypes and water treatments for the four physiological traits; chlorophyll content, flag leaf senescence, cell membrane stability and abscisic acid content (Suppl. Table 1). The parental lines exhibited contrasting phenotype for the four physiological traits. The difference between the mean of the four physiological traits of the two parents under well-watered and drought- stress were significant (Table 1). In general, the parent Veery had higher mean values for chlorophyll content, cell membrane stability and abscisic acid content than the parent Yecora Rojo under both water treatments. However, the parent Yecora Rojo had higher mean values for flag leaf senescence than the parent Veery under both water treatments (Table 1).

The mean of the BC<sub>2</sub>F<sub>2</sub> families for chlorophyll content and abscisic acid content were midway between Veery and Yecora Rojo under both water treatment (Table 1). However, the mean of the BC<sub>2</sub>F<sub>2</sub> Families for the cell membrane stability was near to the mean of the lowest parent under both water treatments (Table 1). It was noticed that the mean of the BC<sub>2</sub>F<sub>2</sub> Families for flag leaf senescence was over the highest parent under both water treatments (Table 1).

In this study, the physiological traits associated with drought tolerance appeared to be quantitatively inherited as shown by the nearly continuous distribution of 75 BC<sub>2</sub>F<sub>2</sub> families derived from Veery × Yecora Rojo (Fig. 1). The continuous distribution of the physiological traits indicated that those traits should be polygenic in nature. Transgressive segregations were also observed in the BC<sub>2</sub>F<sub>2</sub> families for all the investigated traits (Fig. 1). The distribution of the all physiological traits in the present investigation was continuous in BC<sub>2</sub>F<sub>2</sub> Families, showing their quantitative nature (Fig. 1). Meanwhile, a

transgressive segregation was found between the BC<sub>2</sub>F<sub>2</sub> families, indicating that favorable alleles governing target traits had been widely separated in the BC<sub>2</sub>F<sub>2</sub> families. Therefore, the distributive character of phenotypic data was suitable for QTL analysis.

In the present study, Veery and Yecora Rojo differed considerably in their chlorophyll content when grown under both drought stress and well- watered conditions, indicating that chlorophyll content can be an efficient selection parameter for drought tolerance of photosynthesis. Several investigators reported that water deficit can destroy the chlorophyll and prevent its creation (Beltrano and Ronco, 2008; Nikolaeva et al., 2010). Leaf senescence is a highly regulated physiological process that leads to leaf death. Drought-induced leaf senescence contributes to nutrient remobilization during stress; thus, allowing the rest of the plant to benefit from the nutrients accumulated during the life span of the leaf (Munné-Bosch and Alegre, 2004). In the present investigation, the parent Yecora Rojo had larger values than Veery for the reduction speed of flag- leaf (RFC) as indicator for flag leaf senescence. Cereal genotypes have been shown to exhibit differences in flag leaf senescence under drought, which affect yields (Hafsi et al., 2000). The CMS has been extensively used as selection criterion for different abiotic stresses including drought and high temperature in wheat (Rahman et al., 2006). The obtained results in the present study indicated that Yecora Rojo had lower CMS than Veery at the same level of stress. Several investigators reported that differences in CMS might result from differences in leaf structure (Kocheva et al., 2014), cell-wall composition (Buanafina, 2009), the degree of membrane lipid saturation (Kumar et al., 2012) and epicuticular wax coating (Tripathy et al., 2000). ABA induces stomatal closure and reduces water loss via transpiration, which leads to an increased tolerance to water stress (Yang et al., 2010). Our results in the present study showed that the two parents Veery and Yecora Rojo differed considerably in their ABA content, when grown under both drought stress and well- watered conditions, indicating that ABA content can be an efficient selection parameter for drought tolerance. The results in the present study did not differ from what has been reported before that water stress causes ABA accumulation in stressed plants (Ünyayar et al., 2004).

### *QTL analysis*

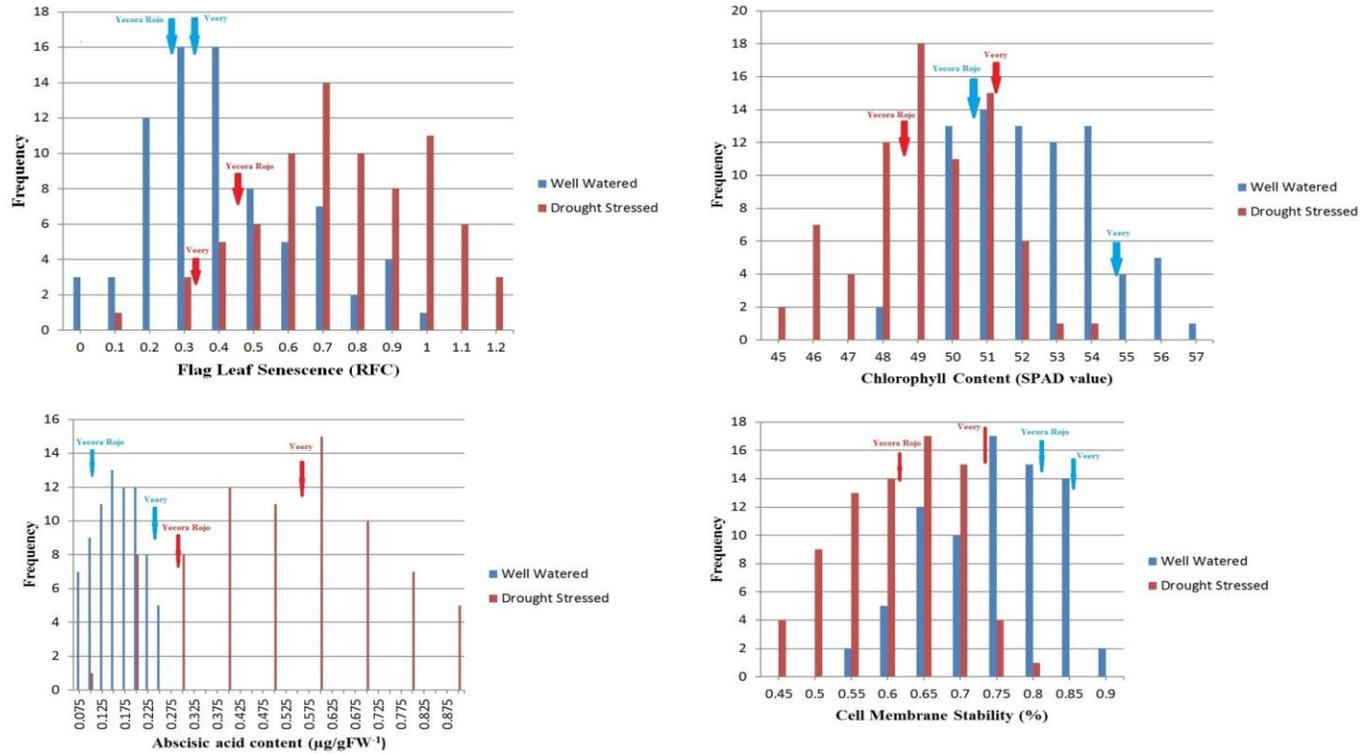
Out of 120 different TRAP primers, 200 different SRAP primers and 400 SSR primers used in this study, only 40, 60 and 100 primer pairs, respectively, generated polymorphisms between the two parents; Veery and Yecora Rojo. Each of these markers was used to screen DNA bulks of ten tolerant and ten sensitive BC<sub>2</sub>F<sub>2</sub> families, according to the physiological traits. The primers that showed reproducible polymorphism were used to test the 75 BC<sub>2</sub>F<sub>2</sub> families of the Veery × Yecora Rojo population. Mapping analysis produced 14 QTLs, including a single QTL that could explain 9- 39% of phenotypic variation (Table 2, Fig. 2). These QTLs distributed on eight chromosomes (Table 2).

Four QTLs for chlorophyll content (CH) were detected on chromosomes 4A, 3B, 6B and 7D. The largest portion of the total phenotypic variation (R<sup>2</sup>= 39%) was explained by marker

**Table 1.** Means± standard deviation, ranges of the 75 BC<sub>2</sub>F<sub>2</sub> families derived from Veery X Yecora Rojo population and parents for chlorophyll content (SPAD), flag leaf senescence (RFC), cell membrane stability (%) and abscisic acid content (µg/ g FW) under well- water and drought stress condition.

Traits	Treatment	Parent Variety		Significant	BC <sub>2</sub> F <sub>2</sub> Families		
		Veery	Yecora Rojo		Min	Max	Mean
Chlorophyll content	Well- watered	54.9± 0.01	50.7± 0.25	**	45.2	58.1	52.2± 2.45**
	Drought- stressed	51.2± 0.50	48.6± 0.21	**	44.1	56.4	49.8 ± 2.07**
Flag leaf Senescence	Well- watered	0.30± 0.01	0.34± 0.02	**	0.01	1.27	0.56± 0.28**
	Drought- stressed	0.33± 0.02	0.47± 0.03	**	0.10	1.30	0.82± 0.25**
Cell membrane stability	Well- watered	85.00± 0.03	81.00± 0.04	**	46.00	99.00	74.00 ± 0.11**
	Drought- stressed	74.00± 0.02	61.00± 0.04	**	43.00	82.00	64.00 ± 0.09**
Abscisic acid content	Well- watered	0.231 ± 0.002	0.111 ± 0.001	**	0.078	0.275	0.173 ± 0.051**
	Drought- stressed	0.577 ± 0.004	0.280 ± 0.001	**	0.096	0.966	0.577 ± 0.211**

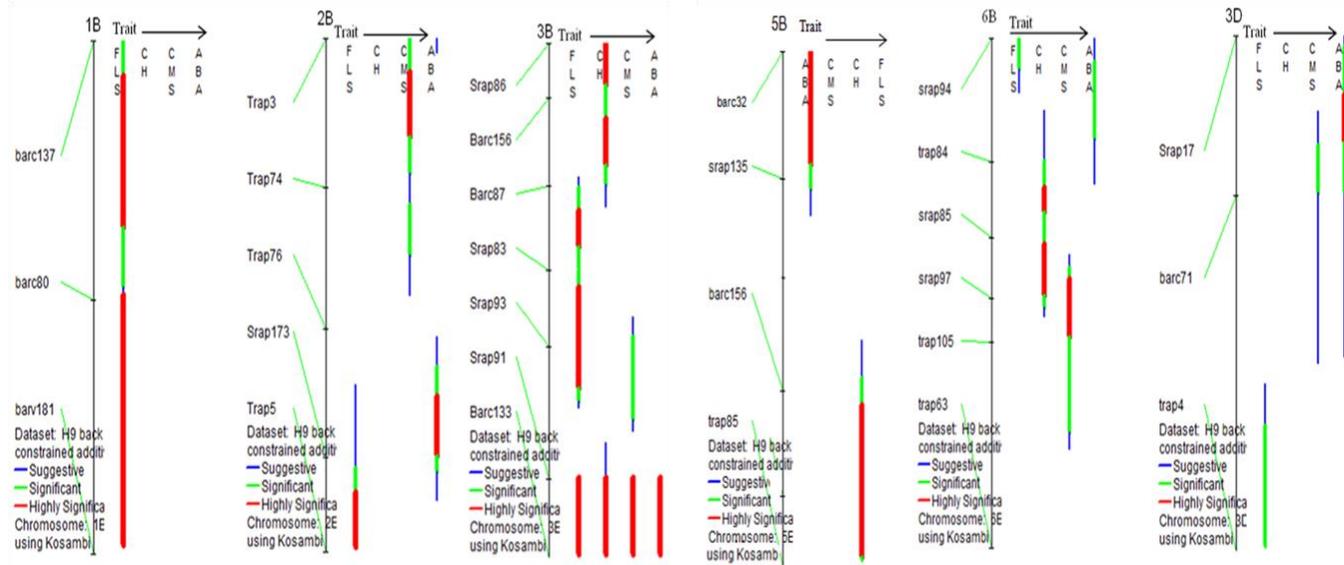
\*\* Significant at 0.01 probability level



**Fig 1.** Frequency distributions of the for chlorophyll content (SPAD), flag leaf senescence (RFC), cell membrane stability (%) and abscisic acid content (µg/ g FW) under well- water and drought stress condition.

**Table 2.** Genetic characteristics of QTL related to chlorophyll content (CH), flag leaf senescence (FLS), cell membrane stability (CMS) and abscisic acid content (ABA) traits in the 75 BC<sub>2</sub>F<sub>2</sub> families.

QTLs	Chromosome location	Marker interval	Nearest marker	Position (CM)	LOD	R <sup>2</sup>	P value	Additive effect
QCH- ds	4A	Barc52- Sraps113	Barc52	28.5	3.5	16	0.00020	2.92
	3B	Barc156- Sraps86	Barc156	24.4	5.1	19	0.00003	1.7
	6B	Srap85- Sraps97	Srap85	16.6	8.3	23	0.00001	1.84
	7D	Barc76- Barc126	Barc76	13.7	9.8	39	0.00000	2.38
QFLS- ds	1B	Barc80- Barc137	Barc80	24.4	5.1	15	0.00021	-0.39
	2B	Trap5- Sraps173	Trap5	19.1	6.3	27	0.00000	-0.28
	3B	Srap93- Sraps83	Srap93	19.5	6.3	31	0.00000	-0.28
QCMS-ds	2B	Trap74- Trap3	Trap74	26.5	4.2	16	0.00015	0.07
	6B	Srap97- Trap105	Srap97	17.6	7.4	33	0.00000	0.1
QABA- ds	2B	Srap173- Trap76	Srap173	22.5	5.5	26	0.00000	0.22
	3B	Srap91- Barc133	Srap91	27.0	4.3	9	0.00432	0.14
	5B	Barc32- Sraps135	Barc32	21.8	5.9	26	0.00000	0.22
	6B	Trap84- Sraps94	Trap84	22.8	4.6	21	0.00002	0.21
	3D	Srap17- Barc71	Srap17	27.9	4.1	27	0.00000	0.22



**Fig 2.** Integrated linkage map for four physiological traits; chlorophyll content (CH), flag leaf senescence (FLS), cell membrane stability (CMS) and the abscisic acid content (ABA) traits based on BC<sub>2</sub>F<sub>2</sub> Families of Veery x Yecora Rojo Population.

interval Barc76- Barc137 with an additive effect of up to a 2.38 SPAD increase from the Veery alleles (Table 2).

Three QTLs significantly influenced flag leaf senescence and mapped on chromosomes 1B, 2B and 3B (Table 2). The negative additive effects for flag leaf senescence indicate that the sensitive parent 'Yecora Rojo' alleles are in the direction of increasing the trait. The phenotypic variation explained by individual QTLs ranged from 15 to 31% (Table 2).

Two QTLs for cell membrane stability were identified and mapped on each chromosome of 2B and 6B (Table 2). The phenotypic variation for cell membrane stability explained by these QTLs was 16% and 33% with a LOD value of 4.2 and 7.4, respectively. For the QTLs, the Veery alleles increased the values of cell membrane stability (Table 2).

Five QTLs for abscisic acid content (ABA) were identified on chromosomes 2B, 3B, 5B, 6B and 3D. The phenotypic variation for ABA content explained by these QTLs ranged from 9% to 27% with a corresponding LOD of 4.3– 4.1 (Table 2). All of the QTLs for abscisic acid content trait had a positive additive effect indicating contribution of alleles increasing the ABA content trait by the tolerant parent Veery. In addition, the positive additive effects indicated the relative importance of additive gene effects in controlling the abscisic acid content trait for drought tolerance in BC<sub>2</sub>F<sub>2</sub> families.

The results also showed that the regression analysis for the relationship between the TRAP, SRAP and SSR markers and the phenotypes of BC<sub>2</sub>F<sub>2</sub> families for the four physiological traits was highly significant. This indicates that the TRAP, SRAP and SSR markers were associated with the physiological traits as an indicator for drought tolerance genes. The genetic distance between the TRAP, SRAP and SSR markers and drought tolerance genes were determined and ranged from 13.7 to 28.5 cM (Table 2). Therefore, these TRAP, SRAP and SSR markers were linked to the QTL for the four physiological traits as an indicator for drought tolerance genes. QTL identification based on linkage studies identified chromosomal regions, not individual genes, which may affect a trait. Linkage analysis in plants typically localizes QTLs within 10 to 20 cM intervals because of the limited number of recombination events that occur during the construction of mapping populations and the cost for propagating and evaluating a large number of lines (Holland, 2007). 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). Bernardo, (2008) reported that the QTL mapping literature has shown that if a breeders can develop a mapping population of 100-150 progenies derived from an F<sub>2</sub> or backcross population between two inbreds, they obtain reasonably good phenotypic data for the traits of interest, and genotype the population with markers spaced about 10 to 15 cM apart. Then, an analysis of the phenotypic and marker data with an appropriate statistical method will almost always lead to the identification of at least a few markers associated with each trait of interest. Recently, Zhang et al. (2013) reported that a QTL with negative additive effect for root number trait was detected under osmotic stress at a location 11 cM away from the QTL locus on chromosome 7B.

Results in the present investigation indicated that all of the QTLs using TRAP, SRAP and SSR markers for leaf chlorophyll content, cell membrane stability and abscisic acid content had a positive additive effect indicating contribution of

alleles increasing the chlorophyll content, cell membrane stability and abscisic acid content by the tolerant parent Veery. In addition, the positive additive effects indicates the relative importance of additive gene effects in controlling leaf chlorophyll content, cell membrane stability and abscisic acid content as an indicator for drought tolerance in BC<sub>2</sub>F<sub>2</sub> families. The negative additive effects for flag leaf senescence indicate that the sensitive parent 'Yecora Rojo' alleles are in the direction of increasing the trait. In the present study, the TRAP markers were assigned to chromosomes 2B, 5B, 6B and 3D in agreement with previous report (Li et al., 2007). Also, SRAP markers were assigned to chromosomes 2B, 3B, 5B, 6B and 3D in agreement with previous report (Li et al., 2007). In addition, the SSR markers were assigned to chromosomes 4A, 1B, 3B, 5B, 3D and 7D in agreement with previous report (Gupta et al., 2002; Röder et al. 1998). Golabadi et al., (2011) reported that homoeologous groups of chromosomes 2, 3, 5 and 7 of wheat contain a number of genes that are important for tolerance to abiotic stress. Previously, Yang et al., (2007) reported that four additive QTLs controlling chlorophyll content under conditions of both rainfed and well-watered mapped on chromosomes 1A, 5A, and 7A at grain filling stage. The QTL for flag leaf senescence was discovered on the chromosomes 2B and 2D and the QTLs on chromosome 2D associated with better performance under drought stress (Verma et al., 2004). Žur et al. (2012) found significant ( $p < 0.01$ ) association with markers localized on chromosome 4A and 5A for unstressed plants and on chromosomes 3A, 3B and 5B for low temperature treated plants.

Identification of associated molecular markers at a major locus contributing to water-stress tolerance would be useful for the indirect selection of wheat plants for water-stress tolerance (Visser, 1994). However, identifying molecular markers associated with important genes or traits in most instances requires screening of a relatively large number of individuals in the population (Lawson et al., 1994). Bulk segregant analysis (BSA) was originally developed to overcome this difficulty (Michelmore et al., 1991), because comparing bulk samples is easier than evaluating many individuals in different populations (Moustafa et al., 2014). In the present study, we were able to identify several types of molecular markers associated with the four physiological traits in wheat under water-stress as indicator for drought tolerance gene in wheat. These markers might be used for marker-assisted selection. The present results support the idea that BSA can provide fast detection of molecular markers linked to genes of interest.

## Materials and Methods

### Plant materials

A set of 75 BC<sub>2</sub>F<sub>2</sub> families derived from a cross between Veery cultivar as the donor (drought tolerant cultivar introduced from CIMMYT) and Yecora Rojo as the recurrent parent (drought sensitive cultivar developed in USA and recommended for environment of Saudi Arabia since 1981) was used in this study. Yecora Rojo is a high yield, 2- gene dwarf cultivar but is very sensitive to environmental factors, such as drought stress, especially during the grain filling period (Barakat et al., 2010).

### **Field evaluation**

Phenotypic evaluations were made on 75 BC<sub>2</sub>F<sub>2</sub> families and their parents (Veery and Yecora Rojo) for tolerance to drought under field condition. Two water regimes were established after germination on the basis of free- surface water evaporation pan monitored at the weather station next to the field at the Agricultural Research Station of King Saud University (Dierab, near Riyadh, 24° 42N, 44° 46E, 400Alt.). The control treatment was irrigated frequently when accumulative evaporation reached 50 mm to 100% of field capacity while the drought treatment was irrigated with 33% of field capacity when accumulative evaporation reached 150 mm. The total accumulated irrigation water were 750 mm (7500 m<sup>3</sup>/ ha) for control treatment and 250 mm (2500 m<sup>3</sup>/ ha) for drought stress. The irrigation regimes were applied two weeks after sowing. Seeds were sown in plots consisting of 3 rows 3 m long, and 0.2 m apart with seeding rate of 14 g m<sup>-2</sup> for each wheat genotype. Fertilizers were applied at the rate of 120 kg N and 80 kg P<sub>2</sub>O<sub>5</sub> ha<sup>-1</sup>. Four physiological traits; leaf chlorophyll content, flag leaf senescence, cell membrane stability and ABA concentration were determined.

### **Leaf chlorophyll content (CH)**

Leaf chlorophyll was determined at the heading stage using a chlorophyll meter (SPAD-502, Konica sensing, IL, USA). Six flag leaves for each BC<sub>2</sub>F<sub>2</sub> family and parents per replicate in well-watered and drought-stress conditions were measured according to Peng et al. (1993).

### **Flag leaf senescence (FLS)**

Leaf chlorophyll content representing the degree of leaf senescence in wheat was measured using chlorophyll meter (SPAD-502, Konica sensing, IL, USA). Six flag-leaves for each BC<sub>2</sub>F<sub>2</sub> family and parents were selected to evaluate the flag leaf chlorophyll content at heading (FCH). 35 days after heading, the same flag leaves were used to determine the chlorophyll content at maturity (FCM). The indicator for flag leaf senescence (FLS) was calculated according to Dwyer et al. (1991):  $FLS = (FCH - FCM) / 35$ . All average values for each line were used for QTL analysis.

### **Cell membrane stability (CMS)**

Medium part of flag leaves (three plants/replicate) was collected from field plots. Samples collected (2 cm segments) were washed three times in deionized water to remove electrolytes adhered on the surface according to the protocol of Blum and Ebercon, (1981). The samples were then kept in a capped vial (20 ml) containing 10 ml of deionized water and incubated in the dark for 24 h at room temperature. The conductance was measured with a conductivity meter (HQ14d, Portable Meter, HACH Company, USA). After the first measurement the vials were autoclaved for 15 min to kill the leaf tissue and release the electrolytes. After cooling, the second conductivity reading was taken. These two measurements were carried out individually for all the samples from both the control and stress treatments. CMS was calculated as the reciprocal of cell-membrane injury following Blum and Ebercon (1981):  $CMS\% = [(1-(T_1/T_2)) / (1-(C_1/C_2))] \times 100$ , where, T and C refer to the stress and control samples,

respectively; the subscripts 1 and 2 refer to the initial and final conductance readings, respectively.

### **Quantifying ABA**

The accumulation of ABA under well-water and drought stress treatment in leaves tissues in wheat was studied by quantifying ABA using HPLC (Muthurajan et al., 2011). Five leaf tissues from five different plants for each genotype (the first leaf below the flag leaf) were collected from well- water and drought stress treatment plants, separately, after the anthesis stage in the milk stage (Zadoks 75). Tissues were freeze-dried for 24 h and stored at -80 °C. The leaves tissues were then ground using liquid N, dissolved in 80% methanol, and incubated at 4 °C for 12 h. The debris was pelleted by centrifugation at 3,000 rpm for 5 min and the methanol in the supernatant was evaporated using a rotary flash evaporator. To this extract, an equal volume of phosphate buffer (pH 8.0) was added and the pH was adjusted between 8 and 9 using 0.1 N potassium hydroxide. The mixture was then extracted twice with ethyl acetate by adding an equal volume of ethyl acetate and centrifugation at 3,000 rpm for 5 min. The ethyl acetate fraction containing chlorophyll was discarded and pH of the pooled extract was adjusted between 2 and 3 using 0.2 N HCl and evaporated in a rotary evaporator. Then, the residue was dissolved in 4 ml of methanol and used for HPLC [Agilent technologies (USA), column SB- C1B (1.8µm, 4.8 × 150mm)] quantification of ABA. The peak areas were measured, and the ABA concentration was quantified using the standard curve obtained using chemical- grade ABA (Sigma).

### **DNA extraction and PCR amplification**

Frozen young leaves (500 mg) were ground to a powder in a mortar and a pestle with liquid nitrogen. The DNA extraction was done using the CTAB method (Saghai-Marooof et al., 1984). Five leaves from five different plants were sampled for DNA extraction for each line at BC<sub>2</sub>F<sub>2</sub> family.

Forty different TRAP primer combinations (Hu and Vick, 2003), 98 different SRAP primer combinations (Li and Quiros, 2001) and 400 SSR primers (Röder et al., 1998, Gupta et al., 2002) were used in this study. The PCR reaction mixture consisted of 20 to 50 ng genomic DNA, 1× PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.1 mM of each dNTP, 0.5 µM primer, and 1 U *Taq* polymerase in a volume of 0.025 cm<sup>3</sup>. After incubation at 94 °C for 5 min, 5 cycles were performed with 94°C for 1 min, 35°C for 1 min, and 72°C for 1 min 40 s. Further, the similar 35 cycles were performed with exception for the annealing temperature at 50°C and a final extension at 72°C for 7 min, for SRAP and TRAP program of PCR cycle. The program of PCR cycle for SSR analysis included an initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min; annealing at 50, 55 or 60°C (depending on the individual microsatellite primer) for 1 min; and extension at 72°C for 2 min followed by a 17-min final extension at 72°C. Amplification products were electrophoretically resolved on 2-3 % (m/v) agarose gels containing 0.1 µg cm<sup>-3</sup> ethidium bromide and photographed on a UV trans- illuminator.

### **Bulked segregant analysis**

Bulked- segregant analysis (BSA) was used in conjunction with TRAP, SRAP and SSR analysis (Michelmore et al., 1991) to

find markers linked to genes of the physiological traits under drought stress. Tolerant and sensitive bulks were prepared from BC<sub>2</sub>F<sub>2</sub> families individuals by pooling aliquots containing equivalent amounts of total DNA, approximately 50 ng/ml from each of ten sensitive and ten tolerant BC<sub>2</sub>F<sub>2</sub> families selected according to phenotypic assessments. Then, TRAP, SRAP and SSR primers were screened on the parents and the two bulk DNA samples, from which some primer combinations revealed bands that were polymorphic, not only among parental genotypes but also between the pair of the bulk DNA. The name and sequence of all polymorphic successful markers are listed in supplementary Tables (Suppl. Table 2, Suppl. Table 3 and Suppl. Table 4). Based on the evaluations of DNA bulks, individual BC<sub>2</sub>F<sub>2</sub> families were analyzed with co-segregating primers to confirm TRAP, SRAP and SSR markers linkage to the physiological traits as an indicator for drought tolerance genes.

#### Data and linkage analysis

Analysis of variance was performed using the SAS 9.1 program. The ANOVA was estimated for all traits according to Steel and Torrie, (1980). Map manager QTX v. 0.22 (<http://manager.roswellpark.org/mm/QTX.html>) was used to analyze the linkage relationship of the detected TRAP, SRAP, and SSR markers. Linkage was detected when a log of the likelihood ratio (LOD) threshold of 3.0 and maximum distance was 50 cM. Kosambi's mapping function was used. QTL were identified using composite interval mapping provided by MAP MANAGER QTX. Genetic loci with the most significant effect for each QTL were assembled into multiple regression models using PROC REG of SAS v. 9.1 software packages (SAS Institute, Cary, NC, USA) to determine the total amount of the phenotypic variation explained (Nelson, 1997).

#### Conclusion

Our results from the physiological traits showed approximately normal distributions. This showed clear continuous and transgressive segregation. Frequency distribution of the traits showed transgressive segregation in both directions. Our results also indicated that TRAP and SRAP and SSR markers, combined with bulked segregant analysis could be used to identify molecular markers linked to four physiological traits; leaf chlorophyll content, flag leaf senescence, cell membrane stability and abscisic acid content, as indicators for drought tolerance genes in wheat. The marker-assisted selection with TRAP, SRAP and SSR markers might be useful for developing improved cultivars.

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