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Full Length Research Paper

Quantitative trait loci (QTL) analysis of flag leaf senescence in wheat (*Triticum aestivum* L.) with microsatellite DNA markers under water-stressed condition

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The objective of this study was to detect quantitative trait loci (QTL) associated with drought tolerance in wheat genotypes by simple sequence repeat (SSR) markers and to provide valuable information for marker assisted selection. SSR markers linked to flag leaf senescence (FLS) was identified in two DNA pools, which were established using F_2 mapping population, resulting from a cross between a drought sensitive genotype 'Variant-11' and drought tolerant genotype 'Veery'. Parents were screened initially with 34 SSR primer pairs. The linkage map was constructed with the six linked markers into one linkage group covering 82.7 cm. QTL detection with analysis of variance showed that all of the six markers were significantly associated with drought tolerance in this population. Single marker regression (SMR) analysis revealed that R-square percentage ranged from 39.3% (*Xgwm339*) and 12.3% (*Xgwm577*). Simple interval mapping (SIM) located a QTL for leaf flag senescence, between markers interval *Xgwm566* and *Xgwm339*, while composite interval mapping (CIM) indicated a QTL location between the interval marker *Xgwm296* and *Xgwm566*. The SSR markers can be used for the detection of QTLs quantitative trait loci linked with flag leaf senescence as indicator for drought tolerance.

Key words: Flag leaf senescence, *Triticum aestivum* L., SSR markers, simple interval mapping (SIM), composite interval mapping (CIM), quantitative trait loci (QTL).

INTRODUCTION

Wheat (*Triticum aestivum* L.) production is adversely affected by drought in 50% of the area under production in the developing, and 70% in the developed countries (Trethowan and Pfeiffer, 2000). As water resources are likely to decline in the coming decades (Zhao et al., 2008), the areas devoted to wheat production will be increasingly threatened by water availability. Hence, improving wheat adaptation to drought will acquire a greater socio-economic importance across the globe than it currently has.

Leaf senescence is the sequence of biochemical and physiological events, comprising the final stage of leaf development from the mature, fully extended state, until

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Abbreviations: CIM, Composite interval mapping; **FLS,** flag leaf senescence; **QTLs,** quantitative trait loci; **SIM,** simple interval mapping; **SMR,** single marker regression; **SSR,** simple sequence repeats.

death. It is induced, either by internal hormonal factors related to ageing or, prematurely by external environmental factors, such as high temperature and drought (Chandler, 2001). In bread wheat, flag leaf senescence (FLS) relates to the period of reallocating resources from the source to the sink, during grain filling. Since flag leaf photosynthesis in wheat contributes about 30 to 50% of the assimilates for grain filling (Sylvester-Bradley et al., 1990), the onset and rate of senescence are important factors for determining yield potential (Evans, 1993). Though, mapping quantitative trait loci for FLS as a yield determinant in winter wheat, under optimal and drought-stressed environments, have been identified, using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers, which revealed the genetic control of this trait and the QTLs identified on chromosome 2D, associated with better performance under drought (Verma et al., 2004).

The usual method to locate and compare loci regulating quantitative traits loci (QTLs) requires a segregating population of plants with each one genotyped with molecular markers (Quarrie et al., 1999). However, plants from such segregating populations can also be grouped according to phenotypic expression of a trait and tested for differences in allele frequency between the population bulks: bulk segregant analysis (BSA) (Quarrie et al., 1999; Brauer et al., 2006). A molecular marker showing polymorphism between the parents of the population, and which is closely linked to a major QTL regulating a particular trait, will mainly co-segregate with that QTL, that is, segregate according to the phenotype, if the QTL has a large effect (Michelmore et al., 1991; Quarrie et al., 1999; Mackay and Caligari, 2000; Brauer et al., 2006).

In several cereal species, genetic linkage maps have allowed the identification of regions controlling some traits related to the response to drought. Different segregating populations from maize, rice, sorghum, barley, durum (tetraploid) wheat and sugar cane (amongst others) have been studied for many different criteria or quantitative characters, such as phenology, plant architecture, metabolic pathways, water-use efficiency or carbon isotope discrimination (Grausgruber et al., 2005; Rooney, 2004; Hash et al., 2003; Kiani et al., 2007). Molecular markers improve the efficiency of breeding, by allowing manipulation of the genome through marker-assisted selection. In order to identify molecular markers for flag leaf senescence, it is first necessary to construct a genetic map as a tool for discovering the genetic factors as quantitative trait loci (QTL); though QTLs influencing senescence have been identified in sorghum (Tuinstra et al., 1997; Crasta et al., 1999; Xu et al., 2000; Kebede et al., 2001) maize (Beavis et al., 1994), winter wheat (Verma et al., 2004), and spring wheat (Milad et al., 2011).

The objectives of this study was to detect QTL linked with flag leaf senescence as indicator, for drought tolerance in wheat genotypes, by the SSR markers, and to provide valuable information for marker assisted selection.

MATERIALS AND METHODS

Plant materials and genomic DNA extraction

The wheat genotypes used in the study were sensitive genotype 'Variant-11' and tolerant genotype 'Veery'. 'Variant-11' was derived from 'Gemmiza-1' cultivar, using somaclonal variation tool (Barakat et al., 2005). The wheat cultivar 'Veery' is highly tolerant to drought (Rajaram et al., 1996).

The two wheat genotypes that had contrasting response to drought stress were crossed to generate a F_1 seeds during winter season 2006 at the Experimental Farm Station, Faculty of Agriculture, Alexandria University, Alexandria, Egypt. The F_1 seeds population derived from the cross ('Veery' X 'Variant-11') were obtained. The F_2 seeds were obtained by selfing, during winter season 2007.

Genomic DNA was extracted from fresh leaves of individual F_2 plants and their parents, using the Saghai-Maroof et al. (1984) method.

Growing condition and drought tolerance evaluation

In 2008 winter growing season 100 plants from F_2 population and their parents were planted in polyethylene bag under greenhouse condition. The polyethylene bag with dimensions (13 cm diameter, 15 cm height) was used to grow single wheat plants in a greenhouse experiment. They were filled with sandy soil (3.5 kg) and were given the total amount of daily irrigation, until reaching booting stage. Drought tests were carried using 50% of the amount of daily irrigation. Daily irrigation water requirements were calculated by CROPWAT software (Smith, 1991) from agro-meteorological data of the studied area and crop coefficient (Kc) of wheat as follows:

$$ETo = \frac{0.408\Delta(Rn - G) + \gamma \frac{37}{Thr + 273} u2\,(e^{\circ}\,(\mathrm{Thr}) - \mathrm{e}a)}{\Delta + \gamma(1 + 0.34\mathrm{u}2)}$$

$$ETc = \mathrm{Eto} \times \mathrm{Kc}$$

Where, ETc is the evapo-transpiration for crop; Kc is the crop coefficient; ETo is the reference evapo-transpiration (mm houre^{-1}); Rn is the net radiation at the grass surface (MJ m^{-2}h^{-1}); G is the soil heat flux density (MJ m^{-2}h^{-1}); Thr is the mean hourly air temperature (°C); Δ is the saturation slope vapor pressure curve at Thr (Kpa °C^{-1}); γ is the psychrometric constant (Kpa °C^{-1}); e^{o} (Thr) is the saturation vapor pressure at air temperature Thr; ea is the average hourly actual vapor pressure and and u2 is the average hourly actual wind speed (ms^{-1}).

Calculated ETc, (crop evapotranspiration), which is equal 100% of daily water consumption use for the wheat, was used to calculate irrigation requirements with the following equation:

Daily irrigation requirements (IR) = ETc + 15% (leaching requirements)

The data of daily IR was adjusted to the volume of polyethylene bags used, and the following (Table 1) show the volume of daily IR

Table 1. Daily IR cm^3 from 1st March to 30th April.

Day	Daily IR cm^3	50% of the amount of daily irrigation cm^3
1st to 10th March	31.9	15.95
11th to 20th March	28.2	14.1
21st to 31st March	38.9	19.45
1st to10th April	38.1	19.05
11th to 20th April	30.3	15.15
21st to 30th April	20.9	10.45

in cm^3 till the stage of flag leaf appearance, and then drought tests were carried out for 21 days. After 21 days from the stress condition, the flag leaf of the main tiller of each plant was obtained during morning hours when leaves were fully turgid. The percentage of flag leaf area remaining green GFLA (%) was measured by using the leaf area meter (Portable Living Leaf Area Meter, Model: YMJ, Zhejiang Top Instrument Company Limited). These assessments were carried out by the same operator in the population to avoid any bias between operators influencing results.

Bulk segergant analysis and construction of the genetic linkage map

For SSR analysis, PCR was performed using publicly available Xgwm (Roeder et al., 1998), SSR analysis with bulk segregant analysis including PCR reaction, gel electrophoresis was performed following the protocol described by Michelmore et al. (1991) to find markers linked to flag leaf senescence gene as indicator for drought tolerance. The bovine serum albumin (BSA) approach was used to compare two pooled DNA samples of individuals (Michelmore et al., 1991). In order to perform BSA for identification of markers closely linked to the flag leaf senescence gene, we selected 10 resistant and 10 extreme susceptible F_2 individuals to construct resistant bulk (RB) and susceptible bulk (SB), respectively. The young leaves were selected to extract genomic DNA, using the CTAB method (Murray and Thompson, 1980; Rogers and Bendich, 1988).

The SSR markers were verified to fit Mendel segregation ratios (3:1) by the Chi-square test. The genetic map was constructed with Map manager QTX Version 0.22 (Meer et al., 2002), linkage groups were created by the command “make linkage group”. The Kosambi mapping function (Kosambi, 1994) was applied to transform recombination frequencies into centiMorgans (cM) as map distances. The genetic map was drawn by the QGene program (Nelson, 1997).

QTL mapping methods

All the QTLs analysis methods were performed with the software package QGene (Nelson, 1997). QTL analysis was performed by one way ANOVA for each marker to be identified as putatively associated with flag leaf senescence (this was done to confirm association between the marker and flag leaf senescence loci). R-square explained by individual locus was determined by SMR. SIM and CIM to evaluate markers intervals, putatively associated with trait phenotypes. With CIM markers outside, the intervals are considered as cofactors. By removing the effects of these cofactors, the location and effect of a QTL within the interval can be better estimated. The default parameters were used and allowed the QGene to select the cofactor. To determine the critical LOD thresholds for SIM and CIM mapping, a permutations test with 1000 permutations was performed with significance level of 0.05.

RESULTS

Segregation analysis and linkage map construction

Out of the thirty four SSR primers pairs used in this study, only 7 primer pairs generated polymorphism between the two parents and their bulk. Each of these polymorphic markers were used to genotype all the 100 F_2 population individuals. All clearly distinguishable polymorphism bands, ranging from 90 to 170 bp, were treated as dominant markers that P_1 was absent (0) and P_2 was present (1) and scored (Figures 1 and 2). The goodness-of-fit of observed F_2 data to theoretically expected segregation ratios was tested, using Chi-square tests. The expected segregation ratios for dominant markers are presented in Table 2. Chi-square test revealed that 6 markers accorded with the expected ratio of 3:1, and only one pair marker *Xgwm182* (left primer TGA TGT AGT GAG CCC ATA GGC and right primer TTG CAC ACA GCC AAA TAA GG) showed significant distortion from the expected ratio (Table 2). The linkage map of the F_2 population was constructed by Map manager QTX Version 0.22 (Meer et al., 2002), spans a total of genetic distance of 82.7 cM (Kosambi cM), with 6 markers which were distributed on one linkage group (Figure 3).

QTL analysis

The association of the six polymorphic SSR DNA markers pairs, with flag leaf senescence as an indicator for drought tolerance was analyzed on the F_2 mapping population by ANOVA (Table 3). All of the six markers were significantly associated with drought tolerance in this population. Marker *Xgwm339* (left primer AAT TTT CTT CCT CAC TTA TT AAA and right primer CGA ACA ACC ACT CAA TC) and *Xgwm293* (left primer TAC TGG TTC ACA TTG GTG CG and right primer TCG CCA TCA CTC GTT CAA G) were showed the highest F-value (31.348) and (28.081), respectively and also had the highest percentage of phenotypic variances explained by each QTL (calculated as R-square) 39.3 and 36.7%, respectively (Table 3). SIM located a QTL for leaf flag senescence gene between marker interval *Xgwm566* (left primer TCT GTC TAC CCA TGG GAT TTG and right

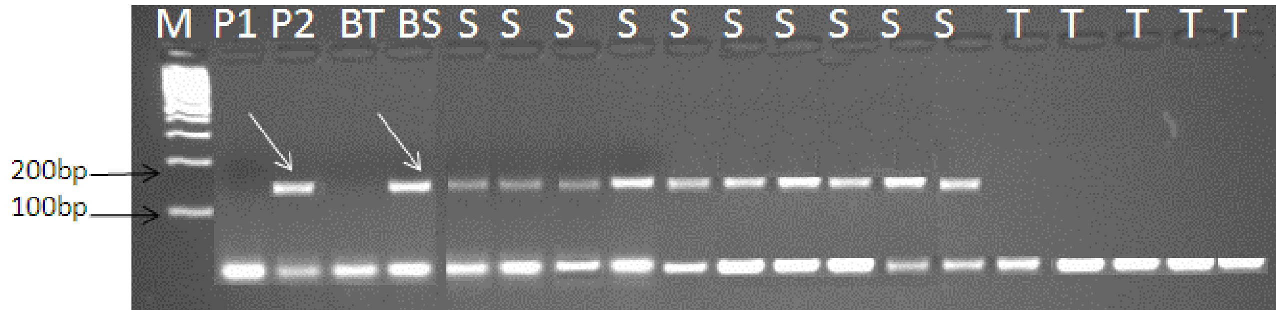

Figure 1. Polymorphism detected by SSR marker *Xgwm566* for leaf flag senescence in F_2 population. M, DNA molecular weight marker (the unit is bp); followed by P_I and P_2 parents Veery and Variant-11, respectively. BT is the bulk tolerant; BS is the bulk susceptible; S_{1-10}, F2 susceptible plants; S $_{1-5}$, F2 resistant plants. The polymorphism of P1 and P2 at about 130 bp.The white arrow indicates the polymorphism band.

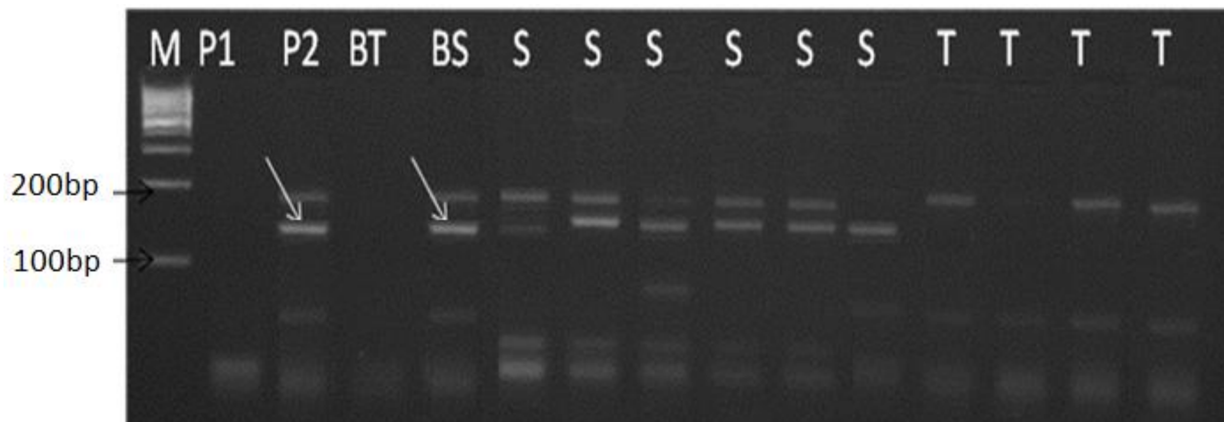

Figure 2. Polymorphism detected by SSR marker *Xgwm577* for leaf flag senescence in F_2 population. M, DNA molecular weight marker (the unit is bp);followed by P_I and P_2 parents Veery and Variant-11, respectively. BT is the bulk tolerant; BS is the bulk susceptible; S_{1-6}, F2 susceptible plants; T $_{1-5}$, F2 resistant plants. The polymorphism of P1 and P2 at about 120 bp. The white arrow indicates the polymorphism band.

Table 2. F_2 segregation pattern of flag leaf senescence in the cross of variant-11 and veery.

SSR marker	Observed number (O)	Expected number (E)	χ^2 [(O-E)^2 / E]
Xgwm566	74:26	75:25	0.053
Xgwm339	68:32	75:25	2.613
Xgwm577	83:17	75:25	3.413
Xgwm293	70:30	75:25	1.333
Xgwm296	76:24	75:25	0.053
Xgwm30	68:32	75:25	2.613
GWM182	42:48	75:25	28.213

Critical value of Chi-square at level of significance 0.05 = 3.841.

primer CTG GCT TCG AGG TAA GCA AC) and *Xgwm339* (left primer AAT TTT CTT CCT CAC TTA TT AAA and right primer CGA ACA ACC ACT CAA TC), this interval within the two markers had the highest LOD score 17.36 and the estimated distance between them was 2 cM (Table 4).

After the use of two markers Xgwm293 and Xgwm339 as cofactors, a CIM indicated that a QTL located between the interval of marker *Xgwm296* (left primer AAT TCA ACC TAC CAA TCT CTG and right primer GCC TAA TAA ACT GAA AAC GAG) and *Xgwm566* (left primer TCT GTC TAC CCA TGG GAT TTG and right primer CTG

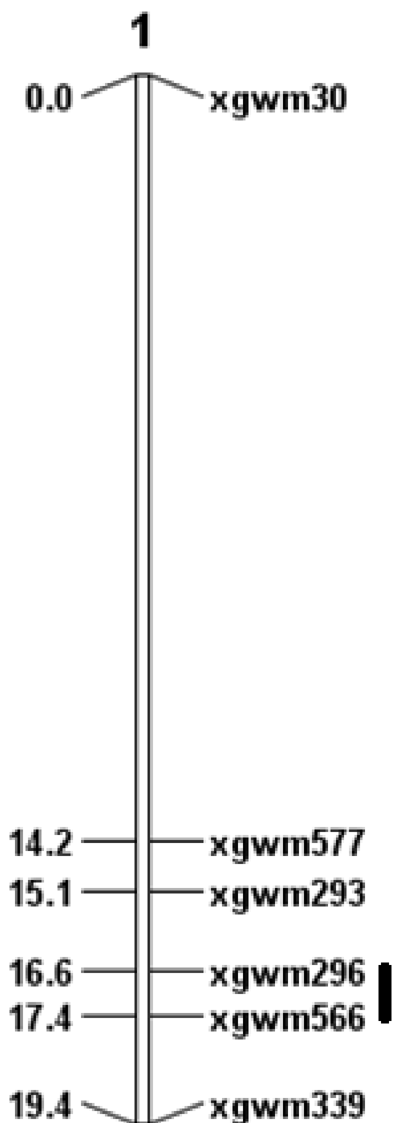

Figure 3. Linkage map of the wheat (*Triticun aestivum* L) population F_2 (variant11 × veery) constructed with six polymorphic SSR markers and 100 lines. The putative flag leaf senescence quantitative trait locus region is shown as shaded bar. Genetic distances are given on the left side of the linkage group in centiMorgans (cM) and markers are given on the right side of the linkage group.

GCT TCG AGG TAA GCA AC), the interval between the two makers showed the highest LOD score 11.49, the estimated distance between them was 0.8 cM (Table 4). A positive value for the additive QTL effect in both interval analysis methods (SIM and CIM) indicated the presence of the tolerant allele from the tolerant parent Veery, which increases the value of the phenotype (Table 4).

DISCUSSION

The timing of FLS is an important determinant of yield under stress and optimal environments. The drought induced premature leaf senescence, as has been commonly observed in other studies (Nooden et al., 1997; Buchanan-Wollaston, 1997). Cereal genotypes have been shown to exhibit differences in flag leaf senescence under drought, which affect yields, in sorghum (Rosenow and Clark, 1981), maize (Baenziger et al., 1999) and durum wheat (Hafsi et al., 2000). QTL analysis based on a genetic map, derived from 48 doubled haploid lines, using (SSR) markers, revealed the genetic control of this trait (Verma et al., 2004). In this investigation, we aimed to use SSR DNA markers for mapping quantitative trait loci for flag leaf senescence gene as indicator for drought tolerance in wheat under drought stress, which are described in the wheat F_2 population (Veery x Variant-11).

The three widely-used methods for detecting QTLs are single-marker analysis, simple interval mapping and composite interval mapping (Liu, 1998; Tanksley, 1993). Single-marker analysis (also 'single-point analysis) is the simplest method for detecting QTLs associated with single markers. The statistical methods used for single-marker analysis include t-tests, analysis of variance (ANOVA) and linear regression. Linear regression is most commonly used because the coefficient of determination (R^2) from the marker explains the phenotypic variation arising from the QTL linked to the marker. This method does not require a complete linkage map and can be performed with basic statistical software programs. In this study, the analysis of variance revealed significant associated between the polymorphic SSR DNA markers with flag leaf senescence in this population. Marker *Xgwm339* and *Xgwm293* were showed the highest F-value (31.348) and (28.081), respectively and therefore these two markers are mostly likely linked to flag leaf senescence trait (Table 3). The results of the percentage of phenotypic variances explained by each QTL (calculated as R-square) showed similar results with the F-test. The R-square percentage revealed that the marker *Xgwm339* had the highest percentage (39.3%) followed by the marker *Xgwm293* (36.7%) (Table 3).

However, the major disadvantage with the previous two methods is that the further a QTL is from a marker, the less likely it will be detected. This is because recombination may occur between the marker and the QTL. This causes the magnitude of the effect of a QTL to be underestimated (Tanksley, 1993). The use of a large number of segregating DNA markers covering the entire genome (usually at intervals less than 15 cM) may minimize both problems (Tanksley, 1993). SIM method makes use of linkage maps and analyses intervals between adjacent pairs of linked markers along chromosomes simultaneously, instead of analyzing single markers (Lander and Botstein, 1989). The use of linked

Table 3. Single marker analysis (SMA) associated with flag leaf senescence QTLs using QGene (Nelson, 1997).

Locus name	Position(cM)	SMR [-log p(F)]*	SMR (F)	SMR (%R^2)
Xgwm30	0	5.681	15.016	23.6
Xgwm577	14.2	2.758	6.785	12.3
Xgwm293	15.1	9.621	28.081	36.7
Xgwm296	16.6	4.56	11.723	19.5
Xgwm566	17.4	8.328	23.521	32.7
Xgwm339	19.4	10.501	31.348	39.3

*-log p (F) for 0.05, 0.01 and 0.001 are 1.30, 2.00 and 3.00, respectively.

Table 4. QTLs and its additive effect detected by SIM and CIM for flag leaf of F_2 mapping population (variant11 × veery) under water stress condition.

QTL analysis method	The distance between the interval	Marker interval	Additive effect	LOD
SIM	2 cM	*Xgwm 566 - Xgwm339*	+51.35	7.36
CIM	0.8 cM	*Xgwm296 - Xgwm566*	+33.92	1.49

markers for analysis compensates for recombination between the markers and the QTL, and is considered statistically more powerful, compared to single-point analysis (Lander and Botstein, 1989; Liu, 1998). Many a times, the allele size of the marker as reported in this study by SIM indicated that the most likely position for the QTL is within the interval between *Xgwm566* and *Xgwm339* (Table 4). CIM has become popular for mapping QTLs. This method combines interval mapping with linear regression and includes additional genetic markers in the statistical model, in addition to an adjacent pair of linked markers for interval mapping (Jansen, 1993; Jansen and Stam, 1994; Zeng, 1993, 1994). The main advantage of CIM is that it is more precise and effective at mapping QTLs, compared to single-point analysis and interval mapping, especially when linked QTLs are involved. In this study (CIM) indicated that the most likely position for the QTL is within the interval between the two markers *Xgwm296* and *Xgwm566*. Within this interval, flag leaf senescence could be located which effect drought resistance, to discover that gene; this will require further map based cloning experiments.

Although there have been few studies on the inheritance of flag leaf senescence in wheat under optimal conditions, additive gene effects have been demonstrated in the genetic control of flag leaf area duration (Simon, 1999). Genetically determined late onset of leaf senescence in sorghum (*Sorghum bicolour* L.) (Borrell et al., 2000a, b), maize (*Zea mays* L.) (Baenziger et al., 1999), and durum wheat (*T. durum* L.) (Benbella and Paulsen, 1998; Hafsi et al., 2000) has increased yield under water-stressed environments. In this study a positive value for the additive QTL effect in both interval analysis methods indicated the presence of the tolerant allele from a tolerant parent Veery, which increases the value of the phenotype (Table 4). Tight linkage between molecular markers and gene for flag leaf senescence can be of great benefit to drought tolerance breeding programs by allowing the investigator to follow the DNA markers (PCR-based markers) through early generation, rather than waiting for phenotypic expression of the tolerance genes. Molecular markers that are closely linked with target alleles present a useful tool in plant breeding, since they can help to detect the tolerant genes of interest without the need of carrying out field evaluation. Also, it allows for screening big number of breeding materials at early growth stages and in short time.

The present study indicated that SSR markers, combined with bulked segregant analysis, could be used to identify molecular markers linked to the flag leaf senescence gene as indicator for drought tolerance in wheat. Once these markers are identified, they can be used in wheat breeding programs as a selection tool in early generations.

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