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IDENTIFICATION OF NEW SSR MARKERS LINKED TO LEAF CHLOROPHYLL CONTENT, FLAG LEAF SENESCENCE AND CELL MEMBRANE STABILITY TRAITS IN WHEAT UNDER WATER STRESSED CONDITION

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Segregating F_4 families from the cross between drought sensitive (Yecora Rojo) and drought tolerant (Pavon 76) genotypes were made to identify SSR markers linked to leaf chlorophyll content, flag leaf senescence and cell membrane stability traits in wheat (*Triticum aestivum* L.) under water-stressed condition and to map quantitative trait locus (QTL) for the three physiological traits. The parents and 150 F_4 families were evaluated phenotypically for drought tolerance using two irrigation treatments (2500 and 7500 m^3/ha). Using 400 SSR primers tested for polymorphism in testing parental and F_4 families genotypes, the results revealed that QTL for leaf chlorophyll content, flag leaf senescence and cell membrane stability traits were associated with 12, 5 and 12 SSR markers, respectively and explained phenotypic variation ranged from 6 to 42%. The SSR markers for physiological traits had genetic distances ranged from 12.5 to 25.5 cM. These SSR markers can be further used in breeding programs for drought tolerance in wheat.

Keywords: Physiological traits – QTL – SSR markers – *Triticum aestivum*

INTRODUCTION

Drought is a major abiotic stress that affects wheat (*Triticum aestivum* L.) production in many regions of the world, limiting crop production in arid and semi-arid areas. Drought tolerance is a quantitative trait with complex phenotype and genetic control [16]. Therefore, understanding the genetic and physiological bases of drought tolerance in crop plants are a prerequisite for developing superior genotypes through plant breeding programs. In addition, selection for field performance is based on the selection for physiological traits related to drought tolerance. Among the physiological

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traits that are associated with wheat performance under drought stresses, chlorophyll content [14, 26]; flag leaf senescence [3, 30] and cell membrane stability [5] have been recognized as considerable indicators for drought tolerance in cereal crops. Maintaining a high yield in drought conditions has, therefore, become a priority, particularly when considering global environmental changes and the increase in world population [28]. 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 [11, 24]. However, the physiological basis of yield maintenance under drought conditions remained poorly understood [29]. Therefore, application of quantitative trait loci (QTLs) analysis to study the physiological traits will improve our understanding of genetic factors that influence these complex traits.

The molecular markers provide tools to study quantitative traits such as drought tolerance through quantitative trait loci (QTLs) analysis and are crucial in projects aiming to increase selection efficiency. Marker-assisted selection in improving drought responses in wheat was reported a few years ago [21]. In recent years, some QTLs for physiological traits under drought stress have been detected in cereal crops [4, 8, 13]. Marker assisted selection may reduce problems associated with genotype X environment interactions, improve the selection efficiency and facilitate combining different tolerance traits into a single genotype [29].

The objective of this investigation was to identify SSR markers linked to the chlorophyll content, leaf senescence, and cell membrane stability traits in wheat under water-stressed conditions and to identify QTLs for the chlorophyll content, leaf senescence, and cell membrane stability traits in F_4 families using bulked segregant analysis.

MATERIALS AND METHODS

Plant materials

A set of 150 recombinant wheat (*Triticum aestivum* L.) inbred lines (RILs, at F_4) developed from the cross between Pavon76 (drought tolerant cultivar introduced from CIMMYT) and Yecora Rojo (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 [2]. The 150 recombinant inbred lines and the parents were tested for tolerance to drought under field condition. The water regimes were established after germination on the basis of free-surface evaporation monitored at a weather station located at the Agricultural Research Station of King Saud University (Dierab, near Riyadh; 24° 42 N, 44° 46 E, 400 m above sea level). Two irrigation regimes [0.25 and 0.75 m^3 (H_2O) m^{-2} (soil)] were applied two weeks after sowing. The experiment was laid out in split-plot design with three replications. Water treatments were assigned to the main plots while the wheat genotypes distributed randomly over the sub-plots.

Measurement of physiological traits

Leaf chlorophyll content

Leaf chlorophyll content was determined at the heading stage using a chlorophyll meter (SPAD-502, Konica sensing, INC., Japan) [20], using six flag leaves for each RILs and parents in well-watered and drought-stress conditions.

Flag leaf senescence

Leaf chlorophyll content representing the degree of leaf senescence in wheat was measured using chlorophyll meter (SPAD-502, Konica sensing, INC., Japan). Six flag-leaves for each RILs 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 reduction speed of flag-leaf chlorophyll content (RFC) as indicator for flag leaf senescence was calculated as described by Dwyer et al. [10]: RFC = (FCH − FCM)/35.

Cell membrane stability

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 [5]. 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 [5]:

$$\mathrm{CMS\%} = [(1-(T_1/T_2))/(1-(C_1/C_2))]\acute{}100,$$

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.

DNA extraction

Frozen young leaves (500 mg) of 150 recombinant inbred lines (RILs, at F4) and their parents were individually ground to a powder in a mortar with liquid nitrogen. The DNA extraction was done using the CTAB method [23].

PCR amplification

400 SSR primers [15, 22] were used in this study. PCR amplication for SSR were carried out in a 20 µl reaction mixture containing 1 X buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 500 nM primer, 1U Taq polymerase, and 50–60 ng template DNA. 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. The amplification products have been electrophoresed in 2–3% agarose gels.

Bulked segregant analysis

Bulked segregant analysis (BSA) was used in conjunction with SSR analysis [18] to find markers linked to genes of physiological traits under drought stress. Tolerant and sensitive bulks were prepared from RILs (F_4 generation) individuals by pooling aliquots, containing equivalent amounts of total DNA, approximately, 50 ng/µl from each of ten sensitive and ten tolerant RILs plants selected, based on phenotypic assessments. SSR primers were tested and screened on parents and two bulk DNA samples, based on polymorphic patterns of primer combinations, not only among parental genotypes, but also between the pair of the bulk DNA. Based on the evaluations of DNA bulks, individual RILs plants were analyzed with co-segregating primers to confirm SSR markers linkage to the physiological traits as an indicator for drought tolerance genes.

Data and linkage analysis

Map Manager QTX Version 0.22 software [17] was used to perform composite interval mapping (CIM) [33] and to evaluate marker intervals putatively associated with trait phenotypes. Linkage was detected when a log of the likelihood ratio (LOD) threshold was 3.0 and maximum distance was 50 cM. The Kosambi's mapping function was used. Genetic loci with the most significant effect for each QTL were assembled into multiple regression models, using PROC REG of SAS version 9.1 software packages [25], to determine the total amount of the phenotypic variation explained [19].

RESULTS

SSR analysis

Out of 400 different SSR markers used in this study, only 120 primer pairs generated polymorphisms between the parents. Each of these markers was used to screen DNA bulks of the ten tolerant and the ten sensitive F_4 families according to the physiological traits. Twelve SSR markers were, only, amplified polymorphic bands for leaf

chlorophyll content (Table 1). Nine SSR markers were dominant markers (Xgwm617, Wmc326, Xgwm334, Barc3, Barc64, Barc122, Barc123, Barc571 and Barc170). The SSR primers Xgwm334 and Barc571 generated one polymorphic fragment at 125 and 110 bp which was present only in the sensitive bulk and Yecora Rojo (sensitive parent) and was missing in the tolerant bulk and Pavon76 (tolerant parent). However, the other dominant SSR markers generated one polymorphic fragment, which was present only in the tolerant bulk and Pavon76 (tolerant parent) and was missing in sensitive bulk and Yecora Rojo (sensitive parent). Three SSR markers were co-dominant markers (Wmc93, Barc7 and Barc176). A typical amplification pattern generated by Wmc93 was shown in Fig. 1. Among the 27 F_4 lines, nine had profiles of Pavon76, two of Yecora Rojo, and sixteen were heterozygotes (Fig. 1). The Wmc93 allele from the tolerant parent was larger than from the sensitive parent. This locus was inherited in a Mendelian co-dominant manner. There were clear co-segregations between the amplification of the larger Wmc93 allele and the F_4 lines showing the tolerant phenotypes. In the homozygous sensitive F_4 lines, only the smaller Wmc93 allele was amplified. In a proportion of tolerant F_4 lines, both the larger and the smaller alleles were amplified, these lines were presumably heterozygous. The co-dominant microsatellite marker Wmc93 was able to identify the heterozygotes, and would serve as an important tool to rapidly transfer the drought tolerance genes into other wheat cultivars. The co-dominant microsatellite marker Wmc93 was able to identify the

Table 1

Genetic characteristics of QTL related to chlorophyll content (CH) and flag leaf senescence (FLS) traits as indicator of drought tolerance in the 150 F_4 families derived from Pavon 76 × Yecora Rojo

Trait	Primers name	Locus	QTL (CM)	LOD	R^2 (%)	P value	Additive effect
CH	Xgwm617	6A	15.5	16.4	11	0.0001	0.99
	Xgwm334	6A	18.4	8.9	23	0.0001	1.65
	Wmc93	3A	24.7	10.9	33	0.0001	1.66
	Wmc326	3B	22.6	11.5	42	0.0001	1.95
	Barc17	1A	19.3	14.6	22	0.0001	1.10
	Barc170	4A	12.5	18.6	33	0.0001	1.69
	Barc122	5A	18.4	12.5	27	0.0001	1.59
	Barc3	6A	18.6	12.8	14	0.0001	1.05
	Barc64	7A	20.9	13.7	14	0.0001	1.01
	Barc571	3D	15.4	23.7	17	0.0001	0.92
	Barc123	6D	13.4	18.2	27	0.0001	1.48
	Barc76	7D	16.8	17.6	22	0.0001	1.10
FLS	Wmc93	1A	23.3	6.8	6	0.0001	–0.08
	Wmc105	6B	16.8	11.7	13	0.0001	–0.11
	Wmc167	2D	18.1	9.8	12	0.0001	–0.12
	Barc141	5A	17.4	12.7	27	0.0001	–0.41
	Barc194	1B	13.9	15.7	21	0.0001	–0.13

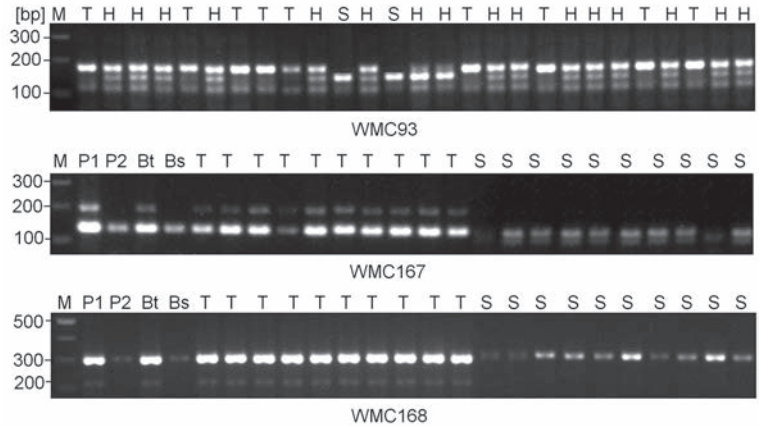

Fig. 1. Selective genotyping of F_4 families of Pavon76×Yecora Rojo wheat hybrids with Wmc93, Wmc167 and Wmc168 markers for leaf chlorophyll content, flag leaf senescence and cell membrane stability traits, respectively. M – molecular weight, P1 – Pavon76, P2 – Yecora Rojo, Bt – tolerant bulk, Bs – sensitive bulk, T – F_4 tolerant lines, S – F_4 sensitive lines, H – heterozygote

heterozygotes. The segregation ratio was 1 (37 tolerant homozygotes): 2 (72 heterozygotes): 1 (35 sensitive homozygotes) in the genotyping F_4 lines. The ratio fitted the expected Mendalian ratio, 1: 2: 1 ($\chi 2 = 0.45$).

Five SSR markers (Wmc93, Wmc105, Wmc167, Barc194 and Barc141) have been identified for flag leaf senescence (Table 1). The amplification profiles of the SSR primer pairs were characterized by the F_4 families and their parents. The SSR primers Wmc93, Wmc105, Wmc167, Barc194 and Barc141 generated one polymorphic fragment at 190, 320, 200, 170 and 270 bp, respectively, which was present only in the tolerant bulk and Pavon76 (tolerant parent) and was missing in the sensitive bulk and Yecora Rojo (sensitive parent) (Fig. 1).

Ten SSR markers were linked to cell membrane stability (Table 2). Three SSR markers were co-dominant (Barc12, Barc137 and Barc80). The other seven SSR markers were dominant markers Wmc27, Wmc168 and wmc216, which were present only in the tolerant bulk and Pavon76 (tolerant parent) and were missing in sensitive bulk and Yecora Rojo (sensitive parent) (Fig. 1).

QTL analysis

Multiple regression analysis was carried out to confirm the association between the detected SSR markers and the physiological traits as an indicator for drought tolerance genes in all 150 F_4 families. The results revealed a highly significant regression between the twelve SSR markers and leaf chlorophyll content of the phenotypes of F_4 families (Table 1). The explained variances were ranged from 11 to 42% (Table 1). Also, the Wmc93, Wmc105, Wmc167, Barc194 and Barc141 markers were signifi-

Table 2
Genetic characteristics of QTL related to cell membrane stability (CMS) trait as indicator of drought tolerance in the 150 F_4 families derived from Pavon 76 × Yecora Rojo

Primers name	Locus	QTL (CM)	LOD	R^2 (5)	P value	Additive effect
Barc12	3A	23.8	9.7	7	0.0001	0.05
Barc115	5A	16.5	12.9	14	0.0001	0.08
Barc10	5A	19.8	13.2	17	0.0001	0.08
Wmc168	7A	19.2	11.9	26	0.0001	0.09
Wmc216	1B	14.3	15.0	10	0.0001	0.06
Barc137	1B	22.5	9.5	8	0.0001	0.05
Barc80	1B	18.2	15.8	15	0.0001	0.06
Barc188	1B	20.5	9.4	10	0.0001	0.07
Barc81	1B	16.2	15.3	15	0.0001	0.07
Wmc27	2B	25.5	8.1	24	0.0001	0.10
Barc147	3B	22.9	12.4	7	0.0001	0.06
Barc164	3B	21.9	8.3	13	0.0001	0.08

cantly ($P<0.01$) associated with the flag leaf senescence and explained 6, 13, 12, 21 and 27% of the variation, respectively (Table 1). In addition, ten SSR markers (Table 2) were significantly ($P<0.01$) associated with the cell membrane stability and the explained variances were ranged from 7 to 26% (Table 2). This indicates that the SSR markers were associated with the physiological traits under investigations as an indicator for drought tolerance genes.

The linkage relationship between the twenty-eight SSR markers and the physiological traits as an indicator for drought tolerance genes were estimated, using the F_4 families, deriving from the cross, Pavon76 × Yecora Rojo. The genetic distance between the twenty eight SSR markers and drought tolerance genes were ranged from 12.5 to 25.5 cm, with range of LOD scores from 6.8 to 23.7 (Tables 1 and 2). Therefore, these SSR markers were linked to the quantitative trait loci (QTL) for the physiological traits under investigation as an indicator for drought tolerance genes.

The results in the present investigation indicated that all of the QTLs using SSR markers for leaf chlorophyll content, and cell membrane stability had a positive additive effect indicating contribution of alleles increasing the chlorophyll content, and cell membrane stability by the tolerant parent 'Pavon76' (Tables 1 and 2). Positive additive effect of the QTL, on chromosomes 1A, 3A, 4A, 5A, 6A, 7A, 1B, 2B, 3B, 6B, 2D, 3D, 6D and 7D using SSR markers, indicates contribution of QTL alleles in these loci from the tolerant parent, 'Pavon76'. In addition, the positive additive effects indicates the relative importance of additive gene effects in controlling leaf chlorophyll content cell, and membrane stability as an indicator for drought tolerance in F_4 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.

DISCUSSION

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 [31]. However, identifying molecular markers associated with important genes or traits in most instances requires screening a relatively large number of individuals in the population. Bulked segregant analysis (BSA) was originally developed to overcome such difficulty since comparing bulk samples is easier than evaluating many individuals in different populations [1, 3]. BSA was first reported by Michelmore et al. [18] to identify RAPD markers tightly linked to genes for resistance to lettuce downy mildew. In this study, mapping quantitative trait loci for leaf chlorophyll content, flag leaf senescence, and cell membrane stability traits as indicator for drought tolerance gene in wheat under drought stress are described in the population of wheat hybrids (Pavon76 × Yecora Rojo) using SSR markers. Using bulked segregant analysis (BSA), we were able to identify twenty-eight SSR markers linked to the three physiological traits (leaf chlorophyll content, flag leaf senescence, and cell membrane stability traits), as indicator for drought tolerance gene in wheat.

In the present study, the twenty-eight SSR markers were assigned to chromosomes 1A, 3A, 4A, 5A, 6A, 7A, 1B, 2B, 3B, 6B, 2D, 3D, 6D and 7D in agreement with previous report [15, 22, 27]. Homoeologous groups of chromosomes 2, 3, 5 and 7 of wheat contain a number of genes that are important for tolerance to abiotic stress [9, 12]. Previously, Cao et al. [6] detected seven QTLs for chlorophyll content on chromosomes 2B, 4A, 5B, 6A, 7A, and 7D under nitrogen (N) sufficient environment, while nine QTLs were identified for chlorophyll content on chromosomes 2D, 3A, 4B, 5B, and 6A when wheat seedlings are grown under N deficient environment. Yang et al. [32] reported that four additive QTLs controlling chlorophyll content under both rainfed and well-watered conditions were 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 identified on chromosome 2D associated with better performance under drought stress [30]. SSR markers Wmc9, Wmc596, Wmc603 and Barc108 were weakly but significantly associated with cell membrane stability after water stress [7]. Żur et al. [34] 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. Recently, Barakat et al. [3] reported that quantitative trait locus for flag leaf senescence was associated with 1 RAPD marker, 4 ISSR markers, and 1 SSR marker and were located on the 2D chromosome. Our results also show that the allelic contribution to the physiological traits QTLs came from both parents. A negative additive effect indicates that the source of the allele for flag leaf senescence was Yecora Rojo. On the other hand, the positive additive effects indicates the relative importance of additive gene effects in controlling the leaf chlorophyll content, and cell membrane stability as an indicator for drought tolerance in F_4 families.

QTLs for leaf chlorophyll content, flag leaf senescence, and cell membrane stability traits in wheat under water-stressed conditions were associated with the twenty-

eight SSR markers and explained from 6 to 42% of the phenotypic variation for three physiological traits. These markers should be useful for marker-assisted selection. 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 screening large number of breeding materials at early growth stages and in a short time.

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