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Identification of new TRAP markers linked to chlorophyll content, leaf senescence, and cell membrane stability in water-stressed wheat

M.S. SALEH[1], A.A. AL-DOSS[2], A.A. ELSHAFEI[2], K.A. MOUSTAFA[2], F.H. AL-QURAINY[1], and M.N. BARAKAT[2,3]*

Botany and Microbiology Department, College of Pure Science[1] and Plant Production Department, College of Food and Agriculture Sciences[2], King Saud University, Riyadh 11451, Riyadh, Saudi Arabia
Crop Science Department, Faculty of Agriculture, University of Alexandria, Alexandria, Egypt[3]

Abstract

In order to identify target region amplification polymorphism (TRAP) markers linked to three physiological traits in wheat (*Triticum aestivum* L.), the segregating F_4 population from the cross between drought-sensitive (Yecora Rojo) and drought-tolerant (Pavon 76) genotypes was made. The parents and 150 F_4 families were evaluated phenotypically for drought tolerance using two irrigation treatments [2.5 and 7.5 $m^3(H_2O)$ m^{-2}(soil)]. Using 40 different TRAP primer combinations tested for polymorphism in parental and F_4 family genotypes, the results revealed that quantitative trait locus (QTL) for chlorophyll content was associated with TRAP 5, TRAP 14, and TRAP 20 and explained 18, 16, and 23 % phenotypic variation, respectively. The genetic distance between chlorophyll content QTL and TRAP 5, TRAP 14, and TRAP 20 were 12.3, 19.8, and 13.6 cM, respectively. QTL for flag leaf senescence was associated with TRAP 2, TRAP 3, TRAP 15, and TRAP 16 and explained 33, 27, 28, and 23 % phenotypic variations, respectively. The genetic distance between flag leaf senescence QTL and TRAP 2, TRAP 3, TRAP 15, and TRAP 16 were 9.4, 14.7, 18.1, and 17.3 cM, respectively. QTL for cell membrane stability was associated with TRAP 8, TRAP 9, and TRAP 37 and explained 27, 30, and 24 % phenotypic variation, respectively. The markers TRAP 8, TRAP 9, and TRAP 37 had genetic distances of 17.0, 10.0, and 9.0 cM, respectively. Therefore, these TRAP markers can be used in breeding for drought tolerance in wheat.

Additional keywords: drought tolerance, genetic distance, QTL, target region amplification polymorphism, *Triticum aestivum*.

Introduction

Drought is a major abiotic stress that affects wheat (*Triticum aestivum* L.) production in many regions of the world (Takeda and Matsuoka 2008). However, the physiological basis of yield maintenance under drought conditions remains poorly understood (Tuberosa and Salvi 2007). 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 is a prerequisite for developing superior genotypes through conventional breeding. In addition to selection for field performance, selection for physiological traits related to drought tolerance is essential since water-limited environments are notably variable from year to year. Among the physiological traits correlated with performance under drought and recognized as useful measures of drought tolerance in cereals crops are chlorophyll content (Shen *et al.* 2001, Guo *et al*. 2008), flag leaf senescence (Verma *et al.* 2004, Barakat *et al.* 2013), and cell membrane stability (Blum and Ebercon 1981).

Chlorophyll content (ChlC) has a positive relationship with photosynthetic rate (Guo and Li 1996) and several investigators suggested that maintaining higher ChlC for a longer period of time is one of the strategies for increasing crop production, particularly under water-limited conditions (Benbella and Paulsen 1998, Verma *et al.* 2004). Flag leaf senescence (FLS) is associated with improved yield and transpiration efficiency under water-limited conditions in wheat (Verma *et al.* 2004).

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Abbreviations: ChlC - chlorophyll content; CMS - cell membrane stability; FLS - flag leaf senescence; LOD - likelihood ratio; QTL - quantitative trait locus; TRAP - target region amplification polymorphism.
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* Corresponding author; e-mail: mnrbarakat@yahoo.com

The cell membrane stability (CMS) is one of the main cellular targets common to different stresses (Levitt 1980). The extent of its damage is inversely used as a measure of tolerance to various stress factors in plants, such as heat, drought (Blum and Ebercon 1981, Reynolds *et al.* 1994, Fokar *et al.* 1998), and salinity (Leopold and Willing 1983). Therefore, application of quantitative trait loci (QTLs) analysis to study the physiological traits will improve our understanding genetic factors that influence these complex traits.

The molecular markers provide tools to study quantitative traits such as drought tolerance through 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 (Quarrie *et al.* 2003). In recent years, some QTLs for physiological traits under drought stress have been detected in various crop plants (Ribaut *et al.* 1997, Courtois *et al.* 2000, Baum *et al.* 2003, Verma *et al.* 2004, Milad *et al.* 2011). Marker assisted selection may reduce problems associated with genotype × environment interactions, improve the selection efficiency, and facilitate combining different tolerance traits into a single efficient genotype.

Target region amplification polymorphism (TRAP) was developed and is used in genetic mapping (Hu and Vick 2003, Liu *et al.* 2005). Previously, TRAP has also been successfully used to estimate the genetic diversity in genetic stocks of wheat (Xu *et al.* 2003, Al-Doss *et al.* 2011). Our report is the first to identify TRAP markers for the physiological traits under water-stress. We report here our results on identifying new TRAP markers linked to genes controlling ChlC, FLS and CMS.

Materials and methods

A set of 150 recombinant wheat (*Triticum aestivum* L.) inbred lines (RILs, at F4) 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 (Barakat *et al.* 2010).

The 150 recombinant inbred lines and the parents were tested for tolerance to drought under field conditions. 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 [2.5 and 7.5 $m^3(H_2O)$ m^{-2}(soil)] were applied two weeks after sowing.

Flag leaf ChlC was determined at the heading stage (ChlCH) using a chlorophyll meter (*SPAD-502*, *Konica Sensing*, IL, USA). Six flag leaves for each RILs and parents per replicate in well-watered and drought-stress conditions, respectively, were measured at heading stage according to Peng *et al.* (1993). Mean values for each line were used for QTL analysis. The changes in leaf ChlC represent the degree of leaf senescence. Therefore, ChlC of the same six flag leaves for each RILs and parents were measured at 35 d after heading to determine the ChlC at maturity (ChlCM). The indicator for flag leaf senescence (FLS) was calculated according to Dwyer *et al.* (1991): FLS = (ChlCH - ChlCM)/35. All average values for each line were used for QTL analysis.

Medium parts of flag leaves (2 cm) from three plants per replicate were collected from field plots and 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 cm^3) containing 10 cm^3 of deionized water and incubated in the dark at room temperature for 24 h. The conductance was measured with a portable conductivity meter (*HQ14d*, *HACH Company*, Loveland, 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. The control samples gave a measure of leakage solely due to the cutting and incubation of leaf discs. The conductance of the stress sample was a measure of electrolyte leakage due to water stress and was assumed to be proportional to the degree of injury to the membranes. Cell membrane stability (CMS) [%] = $[(1 - (S_1/S_2))/(1 - (C_1/C_2)] \times 100$, where S and C refer to the stress and control samples and subscripts 1 and 2 refer to the initial and final conductance readings, respectively.

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-Maroof *et al.* 1984). Forty different TRAP primer combinations (Hu and Vick 2003) were used in this study. The PCR reaction mixture consisted of 20 to 50 ng genomic DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.5 μM primer, and 1 U *Taq* polymerase in a volume of 0.025 cm^3. 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. Amplification products were electrophoretically resolved on 1.5 % (m/v) agarose gels containing 0.1 μg cm^{-3} ethidium bromide and photographed on a UV trans-illuminator.

Bulked-segregant analysis (BSA) was used in conjunction with TRAP analysis (Michelmore *et al.* 1991) to find markers linked to genes of selected physiological traits under drought stress. Tolerant and sensitive bulks were prepared from RILs (F4 generation)

individuals by pooling aliquots containing equivalent amounts of total DNA, approximately 50 ng cm^{-3} from each of ten sensitive and ten tolerant RIL plants selected according to on phenotypic assessments. Then, TRAP 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. Based on the evaluations of DNA bulks, individual RIL plants were analyzed with co-segregating primers to confirm TRAP marker linkage to the physiological traits as an indicator for drought tolerance genes.

Map Manager QTX v. 0.22 software (Meer *et al.* 2002) was used to perform composite interval mapping (CIM) (Zeng 1994) 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 v. 9.1* software packages (*SAS Institute*, Cary, NC, USA) to determine the total amount of the phenotypic variation explained (Nelson 1997).

Results

The analysis of variance indicated that there were statistically highly significant differences ($P = 0.01$) for leaf ChlC, FLS, and CMS among water treatments and wheat genotypes. Also the interactions between water treatments and genotypes for all physiological traits were highly significant.

The parents, Pavon76 and Yecora Rojo, significantly differed for all physiological traits under both control and water stress conditions. The differences among RILs were highly significant for all the traits under both growth conditions (Table 1).

Out of 40 different TRAP markers used in this study, only 17 primer pairs were polymorphic 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. Three TRAP markers (TRAP5, TRAP14, and TRAP20) were identified for ChlC (Table 2). The amplification profiles of the TRAP primer pairs were characterized in the F_4 families and their parents. The TRAP5, TRAP14, and TRAP20 generated one polymorphic fragment at 270, 190, and 490 bp, respectively, which was present only in the tolerant bulk and Pavon76 and missing in the sensitive bulk and Yecora Rojo (Fig. 1).

Four TRAP markers (TRAP2, TRAP3, TRAP15, and TRAP16) were identified for flag leaf senescence in F_4 families and their parents (Table 2). The TRAP2, TRAP3, TRAP15, and TRAP16 generated one polymorphic fragment at 570, 250, 300, and 280 bp, respectively, which was present only in the tolerant bulk and Pavon76 and was missing in the sensitive bulk and Yecora Rojo (Fig. 1).

Three TRAP markers (TRAP8, TRAP9, and TRAP37) amplified polymorphic bands for cell membrane stability (Table 2). The TRAP8, TRAP9, and TRAP37 generated one polymorphic fragment at 290, 290, and 260 bp, respectively, which was present only in the tolerant bulk and Pavon76 and was missing in the sensitive bulk and Yecora Rojo (Fig. 1).

To check potential for co-segregation of DNA fragments and tolerant phenotypes, multiple regression analysis was carried out in order to confirm an association between the TRAP markers and the selected physiological traits in all 150 F_4 families. The relationships between TRAP5, TRAP14, and TRAP20 and leaf ChlC were highly significant and explained 18, 16, and 23 % of the variation, respectively. Also, the TRAP2, TRAP3, TRAP15, and TRAP16 markers were significantly ($P < 0.01$) associated with FLS and explained 23, 27, 28, and 23 % of the variation, respectively. In addition, the TRAP8, TRAP9, and TRAP37 markers were significantly ($P < 0.01$) associated with the CMS and explained 27, 30, and 24 % of the variation, respectively (Table 2). This indicates that the TRAP markers were associated with the physiological traits considered as indicators for drought tolerance.

Table 1. Chlorophyll content [rel. units], flag leaf senescence, and membrane stability [%] under well-watered and drought stress conditions in the parents and 150 RILs (F4 families). Minimum, maximum, and mean ± SE, $n = 3$. Differences between two parents or among RILs significant at $P = 0.05$ (*) or $P = 0.01$ (**).

Traits	Treatment	Parents		RILs (F4 lines)		
		Pavon76	Yecor Rojo	min	max	mean
Chlorophyll content	well-watered	52.5 ± 1.96	48.4 ± 0.30*	43.6	57.8	51.4 ± 2.32**
	drought-stressed	53.1 ± 0.15	49.7 ± 0.81**	44.4	56.2	50.7 ± 0.19**
Flag leaf senescence	well-watered	0.12 ± 0.02	0.24 ± 0.03**	0.03	0.80	0.29 ± 0.16**
	drought-stressed	0.15 ± 0.07	0.56 ± 0.14*	0.02	0.98	0.37 ± 0.18**
Cell membrane stability	well-watered	0.75 ± 0.03	0.64 ± 0.03**	0.45	0.99	0.81 ± 0.11**
	drought-stressed	0.73 ± 0.02	0.51 ± 0.06**	0.25	0.96	0.66 ± 0.13**

Table 2. Genetic characteristics of QTL related to chlorophyll content (ChlC), flag leaf senescence (FLS), and cell membrane stability (CMS) traits as indicators of drought tolerance in the 150 F_4 families of Pavon76 × Yecora Rojo hybrids.

Trait	Marker	Primers sequence	QTL [cM]	LOD	R^2 [%]	P value	Add. effect
ChlC	TRAP5-5B	F: TGAGTCCAAACCGGAAT R: CAGGCAAGACGCAAGGTG	12.3	14.8	18	0.0001	1.36
	TRAP14-5A	F: GAGTCCAAACCGGAGC R: CCCTCCACCAATCACAAT	19.8	7.9	16	0.0429	1.80
	TRAP20-6B	F: TGAGTCCAAACCGGTAA R: GAGGAAGACGACGAGGAGT	13.6	14.1	23	0.0001	1.89
FLS	TRAP2-2A	F: TGAGTCCAAACCGGAAT R: CGGACAGTGGCGGAGTTA	9.4	15.3	33	0.0001	0.19
	TRAP3-1D	F: TGAGTCCAAACCGGAAT R: GGCGAACTCCGACATCTT	14.7	11.7	27	0.0001	-0.16
	TRAP15-2D	F: TGAGTCCAAACCGGAGC R: TCCTACAAACATTGCCTACT	18.1	8.8	28	0.0077	-0.18
	TRAP16-3B	F: TGAGTCCAAACCGGAGC R: TTCTTCCTCCCGCTCATCCT	17.3	9.8	23	0.0168	-0.23
CMS	TRAP8-4A	F: TGAGTCCAAACCGGAAT R: TTCTTCCTCCCGCTCATCCT	17.0	11.2	27	0.0005	0.10
	TRAP9-5A	F: TGAGTCCAAACCGGAGC R: TCACCCGCACCTTCTTCC	10.0	17.2	30	0.0001	0.15
	TRAP37-3B	F: AGTAACCCACCGCCTCCTTC R: CAGGCAAGACGCAAGGTG	18.4	9.0	24	0.0137	0.11

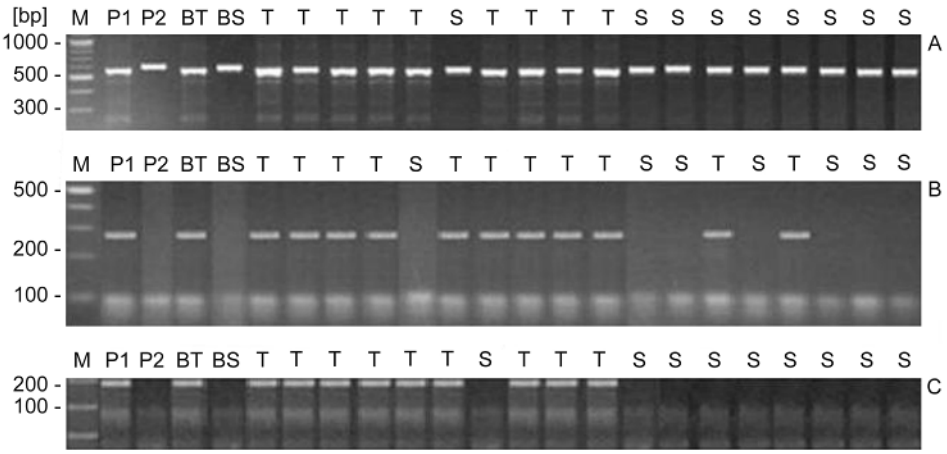

Fig. 1. Selective genotyping of F_4 families of Pavon76 × Yecora Rojo wheat hybrids with the TRAP5 (*A*), TRAP3 (*B*), and TRAP9 (*C*) markers for drought tolerance: M - molecular mass marker, P1 - Pavon76, P2 - Yecora Rojo, BT - tolerant bulk; BS - sensitive bulk, T - F_4 tolerant lines, S - F_4 sensitive lines.

The genetic distance between the ten TRAP markers and drought tolerance genes were determined (Table 2). Therefore, these TRAP markers were linked to QTLs for the selected physiological traits. All of the QTLs for leaf ChlC and CMS had a positive additive effect indicating contribution of alleles increasing the leaf ChlC and CMS by the tolerant parent Pavon76 (Table 2). Positive additive effect of the QTL on chromosomes 4A, 5A, 3B, 5B, and 6B indicated contribution of QTL alleles in these loci from the tolerant parent. In addition, the positive additive effects indicated the relative importance of additive gene effects in controlling the leaf ChlC and CMS for drought tolerance in F_4 families. The negative additive effects for FLS indicated that the sensitive parent Yecora Rojo alleles were in the direction of increasing the trait. Our results also show that the allelic contribution to the physiological traits QTLs came from both parents.

Discussion

The development of wheat cultivars with high yield potential under drought stress conditions is a major aim for wheat breeding programs. Breeding for complex traits needs to take into account various factors, such as

understanding the genetic, physiological, and molecular basis of the traits including interactions among the different component traits with the environments (Tester and Langridge 2010). DNA markers associated with the genomic regions are promising environmentally insensitive tool for selecting genotypes with increased drought tolerance. Molecular markers linked to the drought tolerance trait represent a more reliable tool for selecting drought tolerance genotypes at early stages.

Leaf ChlC is considered as an important characteristics of the plant health or integrity of the photosynthetic apparatus (Krause and Weiss 1991, Clark *et al.* 2000) and provides a rapid and accurate method of detecting plant tolerance to drought stress (Percival and Sheriffs 2002). In the present study, the two parents Pavon76 and Yecora Rojo differed considerably in their ChlC when grown under both drought stress and well-watered conditions (Table 1). Also the Yecora Rojo had higher speed of ChlC reduction during senescence than Pavon76. The differences in flag leaf senescence under drought affect yield in sorghum (Rosenow and Clark 1981), maize (Baenziger *et al.* 1999), and durum wheat (Hafsi *et al.* 2000). In addition, Yecora Rojo had lower CMS than Pavon76 at the same level of stress. Several investigators reported that differences in CMS may result from differences in leaf structure (MacRae *et al.* 1986), cell-wall composition (Jarvis *et al.* 1988), the degree of membrane lipid saturation (Tal and Shannon 1983) and epicuticular wax (Sutter and Langhans 1982).

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 a relatively large number of individuals in the population (Lawson *et al.* 1994). Bulked segregant analysis (BSA) was originally developed to overcome such difficulty since comparing bulk samples is easier than evaluating many individuals in different populations (Altinkut *et al.* 2003, Barakat *et al.* 2011, 2013, Milad *et al.* 2011). BSA was first reported by Michelmore *et al.* (1991) to identify RAPD markers tightly linked to genes for resistance to lettuce downy mildew.

In this study, mapping and identifying QTL for leaf ChlC, FLS, and CMS traits as indicators for drought tolerance in wheat were described in the population of wheat hybrids (Pavon76 × Yecora Rojo) using TRAP markers. Using BSA, we were able to identify several molecular markers associated with the three selected physiological traits in wheat under water-stress. We identified three (TRAP5, TRAP14, and TRAP20), four (TRAP2, TRAP3, TRAP15, and TRAP16), and three (TRAP8, TRAP9, and TRAP37) markers linked to leaf ChlC, FLS, and CMS, respectively. QTLs were associated with above mentioned markers and explained from 16 to 33 % of the phenotypic variation for these physiological traits. Therefore, these markers should be useful for marker-assisted selection since they can help to detect the tolerant genes of interest without the need of carrying out field evaluation. They also allow screening large breeding material at early growth stages and in a short time.

In the present study, the markers TRAP5, TRAP14, TRAP20, TRAP2, TRAP3, TRAP15, TRAP16, TRAP8, TRAP9, and TRAP37 were assigned to chromosomes 5B, 5A, 6B, 2A, 1D, 2D, 3B, 4A, 5A, and 3B, respectively. Homoeologous groups of chromosomes 2, 3, 5, and 7 of wheat contain a number of genes that are important for tolerance to abiotic stresses (Dubcovsky *et al.* 1995, Golabadi *et al.* 2011). Previously, Cao *et al.* (2004) detected seven QTLs for ChlC on chromosomes 2B, 4A, 5B, 6A, 7A, and 7D under nitrogen sufficient environment, whereas nine QTLs are identified on chromosomes 2D, 3A, 4B, 5B, and 6A when wheat seedlings are grown under N deficiency. Yang *et al*. (2007) reported that four additive QTLs on chromosomes 1A, 5A, and 7A control ChlC under rain-fed and well-watered conditions at grain filling stage. The QTLs for FLS were discovered on the chromosomes 2B and 2D and the QTLs identified on chromosome 2D are associated with better performance under drought stress (Verma *et al.* 2004). Recently, Barakat *et al.* (2013) reported that QTLs for FLS are associated with one RAPD marker, four ISSR markers, and one SSR marker and are located on the 2D chromosome.

The present study identifies for the first time the TRAP markers that were linked to leaf ChlC, FLS, and CMS were indicators for drought tolerance genes in wheat and might be further considered in wheat breeding programs.

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