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Full Length Research Paper

Identification of informative simple sequence repeat (SSR) markers for drought tolerance in maize

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Maize is moderately sensitive to drought. Drought affects virtually all aspects of maize growth in varying degrees at all stages, from germination to maturity. Tolerance to drought is genetically and physiologically complicated and inherited quantitatively. Application of molecular-marker aided selection technique for improvement of drought tolerance would accelerate breeding progress by increasing selection efficiency. One of the most important aims in plant breeding is to determine the chromosomal regions related to drought tolerance. Therefore, 38 maize hybrids were planted in two separate experiments with well-watered (WW) and water-stressed (WS) conditions at grain filling period using a randomized complete block design (RCBD) with three replications. Drought tolerance indices such as stress susceptibility index (SSI), mean productivity (MP), tolerance (TOL) and stress tolerance index (STI) were used to evaluate the susceptibility and tolerance of the hybrids. Also, to assess the genetic relationships among the 38 maize hybrids used in the evaluation and breeding for drought tolerance, and to determine informative markers for drought tolerance, 12 microsatellite primers were used. Genomic DNA was extracted with the CTAB method and PCR was performed based on the common method for microsatellite markers. PCR products were separated using 6% polyacrylamide denaturing gel. Stepwise multiple regression analysis was used to determine the chromosomal regions related to drought tolerance. A total of 40 simple sequence repeat (SSR) alleles (bands) with a mean of 3.33 alleles per locus were identified. Polymorphism information content (PIC) of the 12 SSR loci ranged from 0.23 (Phi080) to 0.79 (UMC2359), with a mean PIC of 0.53. The analysis also led to identification of informative SSR markers, namely UMC1862 (bin 1.11), UMC1719 (bin 4.10-4.11), UMC1447 (bin 5.03), UMC2359 (bin 9.07) and UMC1432 (bin 10.02), which significantly contributed to the differentiation of the drought tolerant and susceptible genotypes analyzed in the study. These SSR markers could be further validated and potentially deployed in molecular marker-assisted breeding for drought tolerance in maize.

Key words: Maize, drought tolerance, informative markers, microsatellite, simple sequence repeat (SSR).

INTRODUCTION

Maize (*Zea mays* L.) is an important crop to which a large extent of cultivable land has been allocated. As regard the under cultivation area, the amount of production and yield per area, maize ranks 3rd in the world after wheat and rice. Using genetic resources and increasing the tolerance of genotypes against biotic and abiotic environmental stresses will increase the production.

Improving the drought tolerance had been studied by many researchers. Previous achievements were not so notable due to the complexity of drought tolerance improvement, insufficient genetic variation for drought tolerance, complex interaction of drought with environmental factors and lack of effective selection techniques (Shiri et al., 2010a, b). Currently, with advances in germplasm improvement, evaluation techniques for genetic heredity and with the use of molecular markers, the improvement of drought tolerance and other a-biotic stresses has been facilitated.

Drought tolerance like other environmental stresses in higher plants is a complex genetic and physiologic trait. Most plant processes which are critical in drought tolerance have little inheritance and show a continual variation and are also under the influence of environmental conditions.

Previous genetic studies revealed that both additive and dominance gene effects in inheritance are included in almost all traits related to drought (Shiri et al., 2010a, b). Identifying the complete-linked molecular markers with target gene and mapping its chromosome locus is an important goal in plant breeding for gene cloning and marker-aided selection. Associations between markers and traits were first reported in maize by Stuber and Moll (1972) using isozymes.

Recent developments in plant molecular genetics have provided plant breeders with powerful tools to identify and select Mendelian components underlying both simple and complex agronomic traits (Dekkers and Hospital, 2002). Application of the markers leads to facilitation of breeding programs and will play a major role in yield increase. The advent of abundant DNA-based molecular markers allowed the construction of genetic maps (Helentjaris et al., 1986).

In maize, most research efforts have been directed toward the development of microsatellite marker systems for genetic mapping and germplasm analysis (Taramino and Tingey, 1996; Phelps et al., 1996). Due to their variability, abundance, and wide microsatellites in the genomes distribution, microsatellites can be used for making a full genetic map for the maize. Microsatellite markers are among those DNA markers that show genetic differences between genotypes in the DNA molecule and due to their high polymorphism, they are a proper tool for evaluating genetic diversity in different plants including maize (Barbosa-Neto et al., 1996). Microsatellites are known as repeating sequences of 2 to 6 bp, which make segments of 20 to 100 bp (Agrawal et al., 1999; Goldstein and Schlotterer, 1998).

Sequences surrounding microsatellite loci, within a species or within species of a genus are barely preserved in relative ones and they can be used for the primer design in order to amplify microsatellites (Barriere et al., 2001; Bernardo, 2001). The frequency of microsatellites in the genome, high level of allele variation in the microsatellite locus and the ease of their application makes them distinctive from genetic markers. The other feature is their co-dominance and the detection of heterozygote from homozygote (Ovesna et al., 2002).

Senior et al. (1998) reported that microsatellite markers in maize show a high level of polymorphism, and they can be used for investigation of genetic variation in this plant. Matsuoka et al. (2002), used microsatellite markers for an evolution study in maize. By sequencing the alleles, it was found that there exists a complex pattern of mutation in the microsatellite regions. Genetic diversity in inbred was less than 0.01 which shows their self fertilization nature. Moreover, it was suggested that the genetic diversity in maize has been reduced in comparison with its wild relatives due to its domestication. Chin et al. (1996) found that among the sequences of 2 and 3 nucleotides of microsatellites, the 3 nucleotides ones had the highest frequency in the maize genome. Also, in another study performed by Kantety et al. (1995) on maize, the most frequent polymorphism was found in the repetition of 2 and 3 nucleotides.

The study on mapping or tagging presents information about the number of genes controlling the trait and the place of these genes in linkage map. Deby et al. (2009) in an effort to identify SSR markers of drought tolerance in 24 tropical maize lines with different responses to drought stress, came to the conclusion that dupssr12, umc1042, bnlg1866, umc1056, dup13, umc1069, umc1962, bnlg-1028 and c1344 markers were among those drought related markers in the susceptible and drought tolerant genotypes under investigation.

In maize, about 148 quantitative trait loci (QTLs) for grain yield have been detected. However, fewer QTLs were identified under water-stressed conditions (about 20 QTLs) (Maize Genetics and Genomics Database, 2010). Considering the complexity of the physiological pathways of both yield and drought tolerance, it is necessary to identify and verify the chromosomal loci of grain yield coding and drought tolerance in different levels of water stress (Xiao et al., 2005).

This experiment was conducted to identify informative SSR markers related to grain yield of maize hybrids in well watered and water stressed conditions, to measures the drought tolerance and to introduce related markers to provide genetic maps for future programs.

MATERIALS AND METHODS

This study included two parts: the evaluation of grain yield in well watered (WW) and water stressed (WS) conditions in grain filling stage in the farm, and also the genetic evaluation of the hybrids under investigation using microsatellite markers (SSR) *in vitro*.

Field experiment

The seeds of 20 maize inbred lines were obtained from Seed and Plant Improvement Institute of Iran. In line x tester fashion, 18 female inbred and two male testers (K3653/2 and K3615/1) were crossed through controlled pollination to produce 36 hybrid progenies in 2007. The parents were KLM77008/1-3-3-1-2-2-1 (L1), KLM77012/4-1-1-4-1-2-1 (L2), KLM77021/4-1-2-1-2-1-2 (L3), KLM77029/8-1-1-1-2-1-5 (L4), KLM77029/8-1-1-1-2-2-2 (L5), KLM76004/3-5-1-2-2-1-1-1 (L6), KLM76012/1-3-1-1-1-2-1-1 (L7), K74/2-2-1-3-1-1-1-1 (L8), K74/2-2-1-4-4-1-1-1 (L9), K74/2-2-1-19-1-1-1-1 (L10), K74/2-2-1-21-2-1-1-1 (L11), K74/2-2-1-21-3-1-1-1 (L12), K74/1 (L13), K3545/7 (L14), K3544/4 (L15), K3640/6 (L16), KLM75010/4-4-1-2-1-1-1 (L17), KLM76010/1-13-1-2-1-1 (L18), K3653/2 (T1) and K3615/1 (T2).

36 generated late maturity maize hybrids along with two checks (S.C.704 and S.C.700) were planted in two experiments with WW and WS at grain filling period in Pars Abad-e-Moghan (39° 41' N 47° 32' E, with 40 to 50 m above from sea level), Ardebil, Iran in 2008, using an RBCD design with three replications. The plot was made up of four rows of 5 m in length with the distance between rows and hills of 75 and 18 cm, respectively. The pedigree, yield of hybrids in WW condition, yield of hybrids in WS condition and grain filling duration of the studied hybrids are given in Table 1. In WW condition, the irrigation was performed nine times based on the crop water requirements during growth period, but in WS condition, the irrigation was done six times from the planting time till the end of

Table 1. Pedigree, yield of hybrids in well watered condition (Y_P), yield of hybrids in water stressed condition (Y_S), grain filling duration in WW condition (GFD_{ww}) and grain filling duration under water stressed condition at grain filling stage condition (GFD_{ws}) of the 38 maize hybrids used in this study.

Code	Hybrid	Y_P	Y_S	GFD_{ww}	GFD_{ws}
1	KLM77008/1-3-3-1-2-2-1× K3653/2	10.26	6.00	52	41
2	KLM77012/4-1-1-4-1-2-1 × K3653/2	8.17	5.77	50	37
3	KLM77021/4-1-2-1-2-1-2 × K3653/2	7.13	4.75	49	37
4	KLM77029/8-1-1-1-2-1-5 × K3653/2	8.86	6.21	50	41
5	KLM77029/8-1-1-1-2-2-2 × K3653/2	9.23	5.79	52	40
6	KLM76004/3-5-1-2-2-1-1-1 × K3653/2	7.67	5.37	52	47
7	KLM76012/1-3-1-1-1-2-1-1 × K3653/2	8.16	6.01	51	36
8	K74/2-2-1-3-1-1-1-1 × K3653/2	11.19	6.11	46	39
9	K74/2-2-1-4-4-1-1-1 × K3653/2	8.65	5.03	49	38
10	K74/2-2-1-19-1-1-1-1 × K3653/2	9.32	5.07	52	35
11	K74/2-2-1-21-2-1-1-1 × K3653/2	8.87	5.69	54	45
12	K74/2-2-1-21-3-1-1-1 × K3653/2	8.65	6.00	51	39
13	K74/1 × K3653/2	7.84	5.76	48	41
14	K3545/7 × K3653/2	8.22	5.95	48	39
15	K3544/4 × K3653/2	8.08	6.04	48	37
16	K3640/6 × K3653/2	6.98	5.98	45	39
17	KLM75010/4-4-1-2-1-1-1 × K3653/2	9.12	6.62	50	39
18	KLM76010/1-13-1-2-1-1 × K3653/2	8.70	6.19	52	40
19	KLM77008/1-3-3-1-2-2-1× K3615/1	7.35	5.99	46	42
20	KLM77012/4-1-1-4-1-2-1 × K3615/1	6.80	5.96	48	39
21	KLM77021/4-1-2-1-2-1-2 × K3615/1	6.60	5.21	46	37
22	KLM77029/8-1-1-1-2-1-5 × K3615/1	6.09	5.19	46	40
23	KLM77029/8-1-1-1-2-2-2 × K3615/1	7.88	4.70	47	38
24	KLM76004/3-5-1-2-2-1-1-1 × K3615/1	8.44	5.18	49	41
25	KLM76012/1-3-1-1-1-2-1-1 × K3615/1	7.96	4.96	45	41
26	K74/2-2-1-3-1-1-1-1 × K3615/1	7.92	4.78	45	36
27	K74/2-2-1-4-4-1-1-1 × K3615/1	7.41	6.11	47	37
28	K74/2-2-1-19-1-1-1-1 × K3615/1	7.71	5.81	49	39
29	K74/2-2-1-21-2-1-1-1× K3615/1	9.11	5.31	47	39
30	K74/2-2-1-21-3-1-1-1 × K3615/1	8.71	5.52	48	40
31	K74/1 × K3615/1	7.60	5.47	46	43
32	K3545/7 × K3615/1	6.99	4.91	46	38
33	K3544/4 × K3615/1	8.48	6.29	45	38
34	K3640/6 × K3615/1	7.41	6.32	45	38
35	KLM75010/4-4-1-2-1-1-1 × K3615/1	9.24	5.83	46	38
36	KLM76010/1-13-1-2-1-1 × K3615/1	8.83	5.53	49	41
37	SC700 (drought susceptible check)	6.79	6.37	47	38
38	SC704 (drought tolerant check)	9.74	7.27	45	37
Mean		8.21	5.71	48	39

the flowering period and then, in order to apply water stress, irrigation was withheld completely from the end of flowering till crop maturity (grain filling stage). The duration of water stress varied from 30 to 47 days depending on the grain filling duration of the different hybrids. The environmental severity degree is estimated with SI (stress intensity) and maximal rate of SI is one. In this study, SI was 0.30, so stress intensity was moderate. Grain yield was determined under both well-watered and water-stressed conditions at grain filling period experiments and was used as Yp and Ys, respectively. Yp and Ys were used for the calculated drought

stress indices as described by Shiri et al. (2010a, b).

DNA extraction and SSR assay

Total genomic DNA was extracted from two to three young fresh leaves at 4 to 5 leaves stage using the cetyl trimethylammonium bromide (CTAB) method according to Saghai-Maroof et al. (1984), with minor modifications. The quantity and quality of DNA were evaluated by a UV-spectrophotometer. 12 SSR primers were chosen based on repeat unit and bin location to provide uniform coverage of the entire maize genome from the MaizeGDB database (Maize Genetics and Genomics Database, 2010). Amplification reaction products were separated on a 6% denaturing polyacrylamide gel. The amplified fragments were detected by the silver staining method as described by Bassam et al. (1991). For subsequent statistical analysis, in order to obtain a binary matrix, polymorphic bands amplified by SSR markers were scored as present (1) or absent (0). The generated data matrices were subjected to statistical analysis using SPSS (18) and POPGEN analytical software.

RESULTS

Field evaluation

The analysis of variance according to line × tester method revealed significant difference among the lines, testers and line × tester interaction for grain yield in both conditions and for all indices (data was not shown). This indicated that both additive and non-additive (dominance) gene effects were important in the genetic expression of all the indices and grain yield in both WW and WS conditions. The GCA/SCA ratio was less than unity for all of the indices and grain yield in both conditions; this means that these characters were governed predominantly by non-additive component. Also, the narrow sense heritability estimates were generally lower than the broad sense heritability, indicating the presence of non-additive gene action. These components can be exploited by hetreotic breeding programme. Grain yield recorded high genetic variance value under WW condition when compared to those under WS condition. Also, narrow and broad sense heritability estimates in WW condition were higher than those of the WS condition (Shiri et al., 2010a, b). In this study, the grain yield varied from 6.09 t ha^{-1} (in hybrid KLM77029/8-1-1-1-2-1-5 × K3615/1) to 11.19 t ha^{-1} (in hybrid K74/2-2-1-3-1-1-1-1 x K3653/2) in WW condition and from 4.70 t ha^{-1} (in hybrid KLM77029/8-1-1-1-2-2-2 × K3615/1) to 7.27 t ha^{-1} (in hybrid SC704) in WS condition. Mean grain yield under WW condition was 8.21 t ha^{-1}, while in WS condition was 5.65 t ha^{-1}, indicating a reduction of 30% in comparison to the normal irrigation condition (Table 1). Overly, based on yield in WW and WS conditions and bipolt analysis, hybrids KLM77029/8-1-1-1-2-1-5 × K3653/2, K74/2-2-1-3-1-1-1-1 × K3653/2, KLM75010/4-4-1-2-1-1-1 × K3653/2 and SC704, especially hybrid K74/2-2-1-3-1-1-1-1 x K3653/2, were the best hybrids in this study (Shiri et al., 2010a, b).

SSR marker analysis

In this study, microsatellite markers were used for the investigation of the genetic diversity of 38 maize hybrids. To this end, 12 primer pairs of microsatellite were used, which had relatively high polymorphism in available literatures. Primer selection was conducted in a way that each 10 chromosomes of maize cover at least one representative primer. Using the primer pairs, genomic DNA was amplified and polymorphism was found among the genotypes (Table 2 and Figure 1).

Among the 38 studied hybrids, 40 bands were amplified using 12 primer pairs of microsatellites. The number of alleles were between 2 to 6 and the most frequent allele was related to Phi031 ,UMC1877 and UMC2359 primers, with 6, 5, 5 alleles, respectively (Table 2).

Polymorphism information content (PIC) for each SSR marker was determined as described by Senior et al. (1998). PIC is synonymous with gen-diversity and shows the discriminatory power of a marker by the number of alleles of marker and the relative frequency of these alleles in the studied population (Senior et al., 1998). This parameter measures the diversity of alleles in each gene locus which is $1-\sum f_i^2$. In this formula, f_i is the frequency of i-th allele in a locus. In this study, PIC for microsatellite loci had a fluctuation of 0.23 to 0.79, having an average of 0.53. Among the 4 types of the used repeaters, the highest average PIC was for 4 nucleotides with the value of 0.72. Shanan index of the 12 SSR loci ranged from 0.39 (Phi080) to 1.58 (UMC2359), with a mean Shanan index of 0.94 (Table 2).

Multi-regression based on stepwise method was used in order to identify the relationship between the grain yields in WW and WS conditions, drought tolerance indices with molecular data and identification of markers and chromosomal loci that can be potentially related with drought tolerance indices. The results of this stepwise regression are shown in the Table 3.

Given these results, we can state that the UMC2359 primer was correlated with the grain yield in the well-watered condition. This primer was located on chromosome 9 and in the bin area 9.07, and justified almost 13.5% of the phenotype variance of grain yield in this condition. In the water-stressed condition, UMC2359, UMC1432, UMC1862 and UMC1719 primers were associated with grain yield. These four primers were located in the bin area of 9.07, 10.02, 1.11, and 4.10(4.11), respectively, and justified almost 67% of phenotype variance of grain yield in WS condition (Table 3).

TOL and stress susceptibility index (SSI) indices are mostly associated with plant survival mechanism and less genotype sensitivity than yield potential. The primer associated with both indices was NC133. This primer could justify 14.9 and 19% of the phenotype variance of SSI and TOL indices, respectively. The primer NC133 was located on chromosome 2 and bin area 2.05 (Table 3).

Table 2. Allele numbers, effective allele numbers, bin location, polymorphic index content (PIC), Shanan index and motif for SSR markers.

Primer	Motif	Bin location	Allele number	Effective allele number	PIC	Shanan index
UMC1862	(GA)8	1.11	5	3.5	0.72	1.39
NC133	GTGTC	2.05	4	1.93	0.48	0.91
UMC1501	(AAG)5	3.05	2	1.89	0.47	0.66
UMC1719	(GCG)5	4.10-11	4	2.8	0.64	1.13
UMC1447	(CTT)4	5.03	2	1.54	0.35	0.53
Phi031	GTAC	6.04	6	4.48	0.78	1.62
BNLG1617	AG(16)	6.05	2	1.41	0.29	0.47
UMC1333	(CAG)4	7.03	3	2	0.49	0.87
UMC1545	(AAGA)4	7.00	3	2.97	0.66	1.09
Phi080	AGGAG	8.08	2	1.29	0.23	0.39
UMC2359	(AAAAG)4	9.07	5	4.7	0.79	1.58
UMC1432	(AG)6	10.02	2	1.85	0.46	0.65
Mean	-	-	3.33	2.53	0.53	0.94

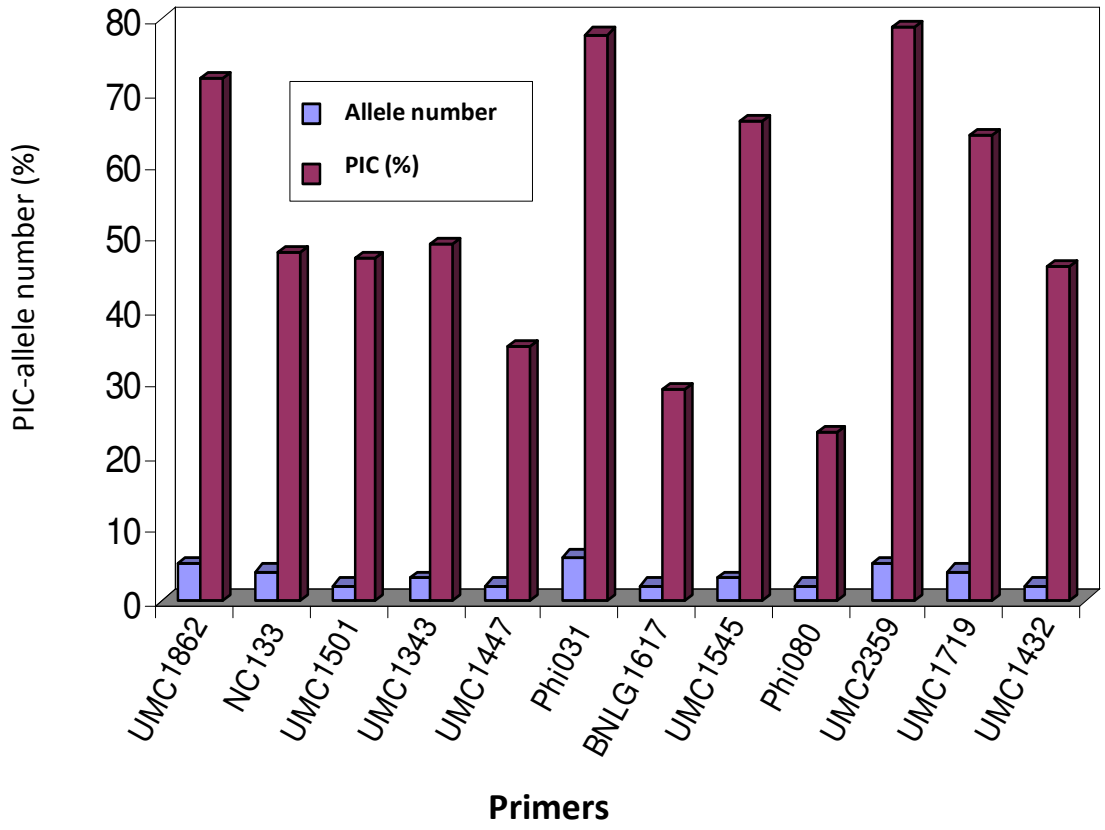

Figure 1. The percentage of polymorphism and the number of alleles related to each allele.

MP and STI indices were associated with yield potential in contrast to TOL and SSI indices. The primers of UMC1447 and UMC2359 were found to be related with MP index; both of them covered 29% of the MP index changes. Primers of UMC1447 and UMC2359 with justification of 37% of the phenotype variance were also located in the coding chromosome region of STI index. Primers of UMC1447 and UMC2359 were in the bin area of 5.03 and 9.07, respectively. Generally, it seemed that according to the results of this study, coding genes of grain yield were located on chromosomes 9, 10, 1, 4 and 5. UMC2359, UMC1432, UMC1862, UMC1719, UMC1447

Table 3. Regression coefficient (b), R2, adjusted R2 and P value of marker entered in stepwise regression in order to detect informative markers with grain yield and drought tolerance index.

Trait	Primer	B	R^2	Adjusted R^2	P value
YP	UMC2359	0.368	0.135	0.111	0.023
YS	UMC2359	0.546	0.299	0.258	0.002
	UMC1432	0.418	0.382	0.327	0.001
	UMC1862	0.756	0.585	0.52	0
	UMC1719	0.822	0.675	0.612	0
TOL	NC133	0.435	0.19	0.167	0.006
SSI	NC133	0.386	0.149	0.125	0.017
MP	UMC1447	0.389	0.151	0.128	0.016
	UMC2359	0.543	0.295	0.254	0.002
STI	UMC1447	0.54	0.292	0.251	0.002
	UMC2359	0.61	0.372	0.317	0.001

YP, Yield of a hybrid in well watered condition; YS, yield of a hybrid in water stressed condition; TOL, tolerance; SSI, stress susceptibility index; MP, mean productivity, STI: stress tolerance index.

markers were located more than other markers in the coding area associated with drought tolerance (Table 3).

DISCUSSION

Drought stress leads to reduction of genetic variance components and heritability, hence, in this case, the selection to drought tolerance with classic methods was not be effective enough and it was better to use molecular methods as complementary, since they are not influenced by environmental conditions.

The results of this study will be helpful in giving basic information about indirect selection of traits through associated markers. However, to ensure the existence of linkage between marker and different traits, it is required to provide segregation populations such as DH (doubled haploid), F2 (second filial) and RIL (recombinant inbred line), so that linkage maps will be made according to these populations and then the gene loci of the trait controllers in the chromosome will be determined (Naghavi et al., 2009).

Certainly, the marker information of this study will be effective in making the linkage maps to select primers properly. Some of these markers are used in breeding projects, but lack of enough time and proper linkage between agronomical traits and molecular markers is among the most important limitations in identifying associated markers with agronomical traits (Gupta et al., 2005). In addition, by using informative markers associated with drought tolerance for markers with identified locus, drought tolerance can be transferred by crossing with producing chromosome replacement lines.

In this study, the SSR marker associated with grain yield in the well-watered condition was UMC2359 which was located in bin area 9.07 while in water stressed condition, primers of UMC2359, UMC1432, UMC1862 and UMC1719 in bin area of 9.07, 10.02, 1.11, and 4.10(4.11), respectively, were the related markers to grain yield. From previous reports on QTL mapping for grain yield, it was found that the number, chromosomal loci or effects of QTL were different under the different ecological conditions.

Xiao et al. (2005) identified two putative QTLs for grain yield under the well-watered regime. These 2 loci were located on chromosomes 1 and 9, respectively, and accounted for 21% of the phenotypic variance. Only 1 QTL associated with grain yield was detected on chromosome 9 under the water-stressed regime and explained 13.8% of the phenotypic variance. Ribaut et al. (1997) examined QTLs for yield in tropical maize under 3 irrigation regimes, and 5 and 4 QTLs were detected under intermediate and severe stresses, respectively while in the study of Agrama and Mounir (1996), the chromosomal areas effective in drought tolerance were located on chromosomes 1, 3, 5, 6 and 8 and they identified five putative QTLs for yield.

Despite the relative similarity between the results of this study and that of Xiao et al. (2005) investigation and other studies, some controversial results were due to grain yield in maize, which is a quantitative trait and is controlled by numerous genes, so it can be considered that many areas of maize chromosomes are engaged in forming and identifying grain yield. In addition, literatures and previous projects verify this conclusion (Xiao et al., 2005; Barriere et al., 2001; Ribaut et al., 1997).

Also, in this and previous studies, it was noted that markers associated with grain yield in the well-watered and water-stressed conditions were different from each other. This suggests that the regulation and expression of genes was different under the 2 water regimes. Therefore, selection for yield improvement under WW condition only, would not be very effective for yield improvement under WS condition.

It is recommended that the primers introduced in Table 3 should be applied for future studies, so that sections of genomes with close relation with drought tolerance will become separated by particular molecular techniques and be used in molecular studies including QTL analysis. This approach provided appropriate tools for breeding drought tolerance in maize.

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