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Research Article

AFLP marker linked to water-stress-tolerant bulks in barley (*Hordeum vulgare* L.)

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Abstract

The amplified fragment length polymorphism (AFLP) assay is an efficient method for the identification of molecular markers, useful in the improvement of numerous crop species. Bulked Segregant Analysis (BSA) was used to identify AFLP markers associated with water-stress tolerance in barley, as this would permit rapid selection of water-stress tolerant genotypes in breeding programs. AFLP markers linked to water-stress tolerance was identified in two DNA pools (tolerant and sensitive), which were established using selected F_2 individuals resulting from a cross between water-stress-tolerant and sensitive barley parental genotypes, based on their paraquat (PQ) tolerance, leaf size, and relative water content (RWC). All these three traits were previously shown to be associated with water-stress tolerance in segregating F_2 progeny of the barley cross used in a previous study. AFLP analysis was then performed on these DNA pools, using 40 primer pairs to detect AFLP fragments that are present/absent, respectively, in the two pools and their parental lines. One separate AFLP fragment, which was present in the tolerant parent and in the tolerant bulk, but absent in the sensitive parent and in the sensitive bulk, was identified. Polymorphism of the AFLP marker was tested among tolerant and sensitive F_2 individuals. The presence of this marker that is associated with water-stress tolerance will greatly enhance selection for paraquat and water-stress tolerant genotypes in future breeding programs.

Key words: bulked segregant analysis, drought, *Hordeum vulgare*, AFLP, paraquat.

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Introduction

Water stress is one of the greatest yield-reducing factors. It causes various physiological and biochemical effects on plants (Tanaka *et al.*, 1990; Irigoyen *et al.,* 1992; Smirnoff, 1993; Bohnert and Jensen, 1996; Jamaux*,* 1997; Tabaeizadeh, 1998), and is therefore a serious problem in many parts of the world where barley, wheat, and other small-grained cereals form the staple diets (Quarrie *et al*., 1999). This is true not only in arid and semiarid regions, but also in places where total precipitation is high, but is not evenly distributed over the growing season (Ribaut *et al*., 1997). The effects of water stress upon leaf physiology could be mediated by the production and accumulation of toxic reactive oxygen intermediates. Reactive oxygen intermediates generated during water stress may represent a serious challenge to a number of cellular functions. For instance, chlorophyll destruction, serious de-organization of chloroplast fine structure, and enzyme inactivation can be due to these toxic oxygen forms (Irigoyen *et al*., 1992). These effects of water stress on the physiology of the plants is very similar to the stress caused by the peroxidizing herbicide paraquat (PQ), which leads to the production of highly toxic free radicals generated by reaction of molecular oxygen with PQ radicals formed in the chloroplast during photosynthesis (Dodge, 1971). Therefore, a close correlation is expected between the plant's tolerance to stresses imposed by water and PQ. It is also possible that drought-tolerant plants can be selected based on their response to PQ in segregating breeding populations (Altinkut *et.al*., 2001). Other morphological and physiological traits of interest with respect to water-stress tolerance mechanisms include leaf size and relative water content (RWC) (Teulat *et al*., 1997 a, b; Grainer and Tardieu, 1999; Gonzalez *et al*., 1999). In our previous study (Altinkut *et al*., 2001), we reported that leaf size, RWC and PQ tolerance

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are associated with enhanced tolerance to water-stress in barley and wheat.

Tolerance to water-stress must be incorporated into breeding material to be grown in regions of low rainfall. However, a major constraint in introducing this trait into otherwise elite genotypes is the lack of meaningful selection criteria. An alternative would be to identify molecular markers associated with water-stress tolerance, and to use these markers to indirectly select for tolerance (Courtois *et al*., 2000). Molecular marker analysis allows to identify genome segments contributing to the genetic variance of a trait, and thus to select superior genotypes at these loci, without uncertainties regarding the genotype, due to environment interaction and experimental error.

The amplified Fragment Length Polymorphism (AFLP) technique (Vos *et al*., 1995) is based on the amplification of selected restriction fragments of a total genomic digest by PCR, and separation of labeled amplified products by denaturing polyacrylamide gel electrophoresis (Becker *et al*., 1995). It has been extensively used for developing polymorphic markers linked to important agronomic traits. High reproducibility, rapid generation, and high frequency of identifiable polymorphisms make AFLP analysis an attractive technique for determining linkages by analyzing individuals from segregating populations (Goodwin *et al*., 1998; Hartl *et al*., 1999). In this study, we have used the bulked segregant analysis (BSA) technique (Michelmore *et al*., 1991) in combination with AFLPs to identify potential molecular marker(s) associated with water-stress tolerance in barley, so that these markers shall be used for selecting water-stress tolerance in segregating populations.

Material and Methods

Plant material

Eighty F_2 plants from the cross Tokak x ST 5819 and their parental lines were grown in individual pots with a soil mixture containing soil/sand/organic matter at a ratio of 1:1:1. All plants were grown in a growth chamber at 23 °C, 70% humidity, and 16 h light/8 h dark periods.

Screening the F_2 mapping population for water-stress tolerance

The methods used for selecting water-stress tolerance in barley plants were described previously (Altinkut *et al*., 2001). Briefly, to prepare bulks, leaf chlorophyll content after PQ treatment, leaf size, and RWC from the tolerant and sensitive parents of the barley cross and its F_2 population of 80 individuals were measured. Leaf size and chlorophyll content after PQ treatment were measured on the 2nd top leaf, taken from the plants during the pre-flowering stage. Leaf extracts were analyzed for determination of chlorophyll content, after treating leaves with 100 µM PQ solution, under a light intensity of 12000 lux for 24 h. RWC were measured on the 6th day of water stress.

Bulked segregant analysis with AFLPs

Freeze-dried leaf materials from F_2 individuals and parents were extracted for genomic DNA, according to the method described by Lodhi *et al*. (1994). Bulked segregant analysis with AFLP markers was used to identify markers associated with water-stress tolerance in the population Tokak x ST5819. Aliquots of DNA from seven tolerant and seven sensitive plants were combined to produce bulks.

Four AFLP reactions were performed with each primer pair on the DNA extracted from the following groups of barley plants: (1) the tolerant parent; (2) the sensitive parent; (3) the pooled DNA of the most tolerant F_2 plants; and (4) the pooled DNA of the most sensitive F_2 plants. The AFLP procedure was performed as described by Zabeau and Vos (1993) and Vos *et al*. (1995), and comprised of three steps, as follows.

Template DNA preparation: 0.5 µg DNAs from bulks and parents were restricted with 5U *Mse*I (Gibco BRL, MD, USA) (a frequent 4-base cutter) and 5 U *Pst*I (Gibco BRL) (a rare 6-base cutter) in a restriction-ligation buffer (250 mM Tris-HCI (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% w/v polyethylene glycol-8000) for 16 h at 37 °C, in a total volume of 50 µL. After checking for complete digestion, the digested DNAs were ligated to MseI adaptor (5'-GACGATGAGTCCTGAG-'3; 3'-TACTCA GGACTCAT-'5) (50 pmol) and PstI adaptor (5'-CTCGTA GACTGCGTACATGCA-'3; 3'-CATCTGACGCATGT-'5) (5 pmol), using 1 U/µL T4 DNA ligase (Gibco BRL) and 1 mM ATP (Gibco BRL), for 6 h at 37 °C. Prepared template DNAs were stored at 4 °C until required.

Pre-amplification: After adaptor ligation, pre-amplification of DNA fragments was performed using Vos *et al*.'s (1995) primer notation moo/poo non-selective primer combinations in a 25 µL reaction containing the following: 2 µL of ligated DNA, 50 ng moo primer (5'-GAC GATGAGTCCTGAGTAA-'3) and 50 ng poo primer (5'-CTCGTAGACTGCGTACATGCA-'3), 0.2 mM dNTP's, 1X PCR buffer and 0.5 U Taq polymerase (Promega, WI, USA). Samples were run in a PTC-100 MJ thermocycler (MJ Research, Watertown, MA) for 20 cycles of 94 °C (30 s), 56 °C (1 min), and 72 °C (1 min). Pre-amplification products were then diluted 5X in double-distilled H_2O, and used as templates for selective amplification.

Selective amplification: Selective amplification of the pre-amplified DNA was carried out using various selective primer combinations of moo (moo+CAC, moo+ACC, moo+CCA, moo+CAA, moo+ACG, moo+CAG, moo+CAT moo+CGA, moo+CGT, moo+CCT) and poo (poo+CCA, poo+GTT, poo+GAC, poo+TGG), with 3-base-pair extension in a 25 µL reaction containing: 5 µL diluted pre-amplification product, 50 ng selective moo primer and 50 ng selective poo primer, 0.2 mM dNTPs, 1X PCR buffer, and 0.5 U Taq polymerase (Promega). The following cycle profile ensured optimal selective amplifica-

tion: one cycle of 1 min at 94 °C, 1 min at 65 °C, and 1 min 30 s at 72 °C, followed by 11 cycles of 1 °C lower annealing temperature each cycle, and 22 cycles of 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C.

Electrophoresis

Amplification products were separated on a 6% polyacrylamide (acrylamide: bisacrylamide = 19:1) / 8 M urea sequencing gel at 1750 V for 3 h in 1X TBE buffer (0.09 M Tris-borate and 0.002 M EDTA), and detected by silver staining according to the manufacturer's instructions (Promega). Band sizes were determined by comparison with a 10 base-pair (bp) DNA ladder size standard from Gibco-BRL (MD, USA).

Results and Discussion

The main objective of this study was to determine whether different polymorphic AFLP markers could be identified in barley bulks made up based on traits associated with water-stress tolerance. Since water-stress tolerance is a quantitatively inherited trait, controlled by several genetic loci, which are difficult to measure (Forster *et al*., 2000), identification of molecular markers associated with a major locus contributing to water-stress tolerance would be useful for indirect selection for water-stress tolerance (Altinkut and Gozukirmizi, 2003). However, in most instances, identifying genetic markers associated with important genes or traits requires screening of a relatively large number of individuals in the population (Lawson *et al*., 1994). For this reason we chose to use BSA, since comparing bulk samples is more convenient than evaluating many individuals in different populations (Sweeney and Danneberger, 1994).

To constitute bulks, individual barley F_2 plants from cross between water-stress tolerant (*Hordeum vulgare* L. cv. 'Tokak' - a known water-stress tolerant cultivar) and water-stress sensitive (*H. vulgare* L. 'ST 5819' - a highly water-stress sensitive line) barley plants were used to prepare the water-stress tolerant and sensitive bulks. Each water-stress tolerant and sensitive bulk included 7 selected F_2 plants. The methods used for selecting water-stress tolerance in these barley plants were described previously (Altinkut *et al.*, 2001). Briefly, the parental barley genotypes used here differed in their leaf chlorophyll content following PQ treatment, leaf size, and RWC. These traits were scored in the barley cross F_2 population of 80 individuals (Table 1). To prepare the bulks, leaf chlorophyll content after PQ treatment was measured from the tolerant and sensitive parent of the barley cross and its individual segregating F_2 plants. Seven F_2 plants that fell in the extreme ends of the distribution for leaf chlorophyll content were then selected to prepare the tolerant and sensitive bulks. In addition, RWC and leaf size were measured in F_2 plants and their parental genotypes. As shown in Table 1, plants with higher chlorophyll content also had smaller leaves and

Table 1 - Morphological and physiological data used for making the water stress tolerant and sensitive barley and wheat bulks in both parental lines and their selected F_2 individuals. Measurements of leaf size and chlorophyll content after PQ treatment were conducted on the 2nd top leaf taken from the plants during the preflowering stage. Leaf extracts were analysed for determination of chlorophyll content after treating leaves with 100 µM PQ solution under a light intensity of 12000 lux for 24 h. Application of water stress and measurement of RWC were according to Altinkut *et al.* (2001). *Values presented for parental genotypes were averages of data taken from 20 parental plants.

	A	B	Leaf size (cm^2)	RWC (%)
Barley parental lines				
*Tokak (T)	2.0 ± 0.07	1.25 ± 0.12	20 ± 5.9	58 ± 1.3
*ST 5819 (S)	1.95 ± 0.08	0.125 ± 0.04	43 ± 7.2	45 ± 2.2
Tolerant F_2 individuals				
F_2-1	-	1.43	11	60
F_2-2	-	1.44	11	60
F_2-3	-	1.45	12.8	61.5
F_2-5	-	1.39	13.5	61.5
F_2-6	-	1.426	14	63
F_2-22	-	1.28	21.5	57
F_2-25	-	1.3	22	59.5
Sensitive F_2 individuals				
F_2-70	-	0.163	43	42
F_2-73	-	0.122	45.5	46.8
F_2-75	-	0.098	49.5	35
F_2-77	-	0.128	52.5	43
F_2-78	-	0.066	54	33
F_2-79	-	0.048	55.2	32
F_2-80	-	0.036	59	28
Wheat parental lines				
*Kirac (T)	2.95 ± 0.03	2.5 ± 0.25	39 ± 6.4	72 ± 4.4
*Sultan 95 (S)	2.65 ± 0.09	0.14 ± 0.03	65 ± 6.2	56 ± 2.9
Tolerant F_2 individuals				
F_2-1	-	3	31.2	80
F_2-2	-	3	31.2	79.6
F_2-3	-	2.9	32	80
F_2-4	-	2.9	32	78
F_2-5	-	2.88	33.6	77.3
F_2-6	-	2.85	33	76
F_2-7	-	2.8	34.5	75
F_2-8	-	2.78	35	75
Sensitive F_2 individuals				
F_2-73	-	0.9	69	45
F_2-74	-	0.6	70.9	48
F_2-75	-	0.5	70	46.5
F_2-76	-	0.55	70	48
F_2-77	-	0.6	71.2	48
F_2-78	-	0.6	72.1	48
F_2-79	-	0.5	73	47.8
F_2-80	-	0.1	75	45

A: Chlorophyll Content in untreated leaves mg/chlorophyll/fresh weight.
B: Chlorophyll Content in PQ treated leaves mg/chlorophyll/fresh weight.

Table 2 - Morphological and physiological data of parental lines* and their F_2 individuals, used to make up the water-stress tolerant and sensitive barley bulks.

	Leaf size (cm^2)	A	B	C
Tokak*	20	2	1.25	57.6
ST5819*	43	1.95	0.125	44.9
F_2-1^T	11	-	1.43	60
F_2-2^T	11	-	1.44	60
F_2-3^T	12.8	-	1.45	61.5
F_2-4^T	13.5	-	1.39	61.5
F_2-5^T	14	-	1.42	63
F_2-6^T	21.8	-	1.53	62
F_2-7^T	22	-	1.3	59.5
F_2-8	21.5	-	1.28	57
F_2-9	13	-	0.65	49.5
$F_2$10	15	-	0.17	45.3
F_2-11	15	-	0.43	50.5
F_2-12	15	-	0.5	49.5
F_2-13	15.3	-	0.54	48.6
F_2-14	16	-	0.44	48
F_2-15	16.5	-	0.55	53
F_2-16	17	-	0.41	51
F_2-17	17	-	0.47	51.3
F_2-18	18	-	0.48	55
F_2-19	19	-	0.52	52
F_2-20	19	-	0.79	49
F_2-21	19	-	0.47	52
F_2-22	19.5	-	1.12	55.5
F_2-23	20	-	0.41	51
F_2-24	21	-	0.34	47
F_2-25	22	-	0.31	49
F_2-26	22.1	-	0.42	51
F_2-27	23	-	0.38	41
F_2-28	23	-	0.4	45.5
F_2-29	23	-	1.02	53.5
F_2-30	23.2	-	0.42	45
F_2-31	24	-	1.1	57.5
F_2-32	24	-	0.9	50.5
F_2-33	24.6	-	1.21	58
F_2-34	24	-	0.32	45
F_2-35	25	-	0.92	53
F_2-36	25.8	-	0.69	48.5
F_2-37	27	-	0.84	52.5
F_2-38	27.3	-	0.2	42
F_2-39	27	-	0.4	46
F_2-40	27	-	0.94	51
F_2-41	27	-	0.65	58
F_2-42	27	-	0.35	48
F_2-43	28	-	0.24	44
F_2-44	28.7	-	1.09	53
F_2-45	29	-	0.55	42
F_2-46	29	-	0.28	43
F_2-47	29.2	-	0.23	45
F_2-48	29.8	-	0.96	52
F_2-49	30.2	-	0.98	54
F_2-50	30	-	0.2	47
F_2-51	31	-	0.57	50
F_2-52	31	-	0.46	42
F_2-53	31.6	-	1.08	59
F_2-54	31	-	0.26	45
F_2-55	31.5	-	0.86	51.2
F_2-56	32.3	-	0.91	52
F_2-57	32	-	0.56	49
F_2-58	32	-	0.73	50.8
F_2-59	32.2	-	0.21	46.8
F_2-60	32.2	-	0.27	45
F_2-61	33	-	0.23	51
F_2-62	33	-	0.92	53.9
F_2-63	34	-	0.86	50
F_2-64	35	-	0.26	48
F_2-65	35	-	0.5	47
F_2-66	35	-	0.62	56
F_2-67	39	-	0.79	50
F_2-68	41	-	0.71	49.4
F_2-69	41	-	0.61	47
F_2-70	43	-	0.16	42
F_2-71	52	-	0.92	51.5
F_2-72	45.7	-	0.27	45
F_2-73	45	-	0.9	52
F_2-74^S	45.5	-	0.12	46.8
F_2-75^S	47.5	-	0.15	48
F_2-76^S	49.5	-	0.09	35
F_2-77^S	52.5	-	0.12	43
F_2-78^S	54	-	0.06	33
F_2-79^S	55.2	-	0.04	32
F_2-80^S	59	-	0.03	28

*Values presented for parental genotypes are averages of data from 20 parental plants. A: Chlorophyll content in untreated leaves (mg/chlorophyll/fresh weight). B: Chlorophyll content in PQ treated leaves (mg/chlorophyll/fresh weight). C: RWC on 6th day of water stress.

*Values presented for parental genotypes are averages of data from 20 parental plants.

^{T}: tolerant F_2 individuals selected for bulk.

^{S}: sensitive F_2 individuals selected to constitute the bulks.

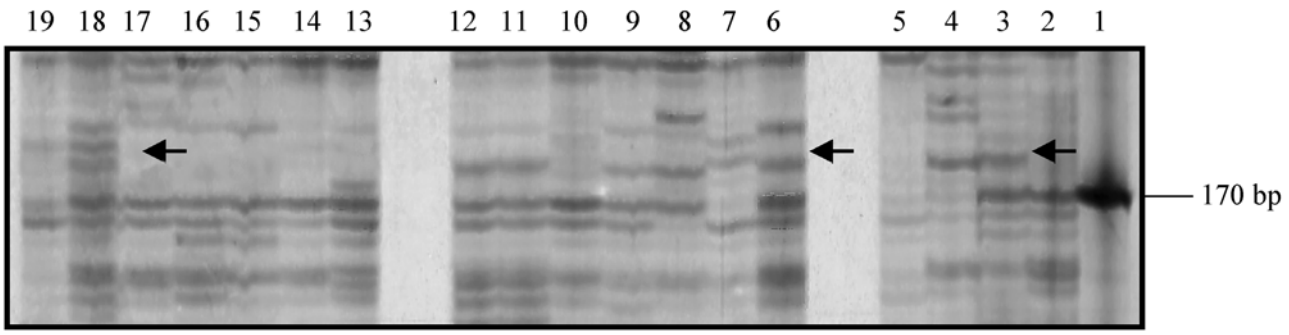

Figure 1 - Results of AFLP amplification based on the use of primers moo+CAT / poo+TGG in barley. 1-10 bp ladder, amplification from sensitive parent ST5819 (2), tolerant parent Tokak (3), tolerant bulk (4), sensitive bulk (5). 6-12: tolerant F_2 individuals, 13-19: sensitive F_2 individuals. AFLP marker is indicated with an arrow (only relevant part of the representative gel is shown).

higher RWC values. The correlation coefficient calculated for PQ tolerance and leaf size was significant (r: -0.423, $p > 0.05$). We then examined the correlation between PQ tolerance and RWC, and the correlation coefficient calculated was highly significant (r: 0.830, $p > 0.05$). Finally, the association between RWC and leaf size also showed a significant correlation (r: -0.593, $p > 0.05$).

AFLP fingerprinting was then used to identify markers potentially associated with water-stress tolerance. Combinations of ten *Mse*I primers and four *Pst*I primers, each with three selective bases, on DNA from pooled samples, were used in barley DNA. The DNA pools that we used in this study included F_2 individuals selected for their morphological and physiological traits associated with water-stress tolerance. Approximately 12000 AFLP fragments amplified from barley using a total of 40 primer combinations were analyzed by running on polyacrylamide gels. A majority of the 40 primer pair combinations produced amplification patterns, which were not different between the tolerant and sensitive bulks and their parents. However, one primer pair produced an amplification product that was present in the tolerant parent and in the tolerant bulk, but not in the sensitive one. The primer pair moo+CAT / poo+TGG amplified a DNA fragment of 172 bp, which was present in the tolerant parent and in the tolerant bulk, but not in the sensitive one. The segregation of this marker in the 14 individuals, which constituted the bulks was tested. The 172 bp fragment was present in six out of seven individuals in the tolerant bulk, and was absent in six out of seven individuals in the sensitive bulk. Only 1 sensitive plant generated the polymorphic band (Figure 1). It was significant that the single F_2 plant in the sensitive bulk which generated the 172 bp fragment, and the single F_2 plant in the tolerant bulk which did not generate the 172 bp fragment were two of the misclassified individuals, possibly due to selection error, and that, despite this, BSA was successfully used to identify the fragment. The reproducible amplification of this fragment in repeated amplifications suggests that it is associated with a major gene(s) that contributes to enhanced tolerance to water-stress-associated traits. The results also demonstrate that a BSA strategy may be useful even for the identification of markers for quantitative traits, such as water-stress tolerance. Recently, similar approaches have been used to identify markers associated with other quantitatively inherited traits, namely anther culture response in potato (Boluarte-Medina and Veilleux, 2002), economically important traits in *Pinus* (Lerceteau *et al*., 2000), and disease resistance in tomato (Peleman, 1999). However, to determine the transportability of the marker identified in this study to other genotypes, other crosses derived from different parental genotypes should be evaluated. In this regard, the present study only focussed on the barley genotype that is most commonly used in breeding programs in Turkey as a source of drought tolerance.

The marker identified in this study, once verified in other barley genotypes, would allow implementation of marker-assisted selection. We expect that this marker may be even more useful when converted into a simple-sequence PCR-based marker that can be used for large-scale water-stress tolerance screening of segregating populations.

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