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PROSPECTS FOR MARKER-ASSISTED SELECTION OF IMPROVED DROUGHT RESPONSES IN WHEAT

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Summary. Drought stress and high temperatures are an increasing problem in Southeast Europe. For example, in parts of Serbia this season the wheat crop was too short to combine because of an extremely dry winter. Therefore, it is important to identify sources of improved drought and heat resistance to incorporate into the local wheat breeding programmes. Molecular markers allow the haplotype of each genotype to be determined. By testing for association of allelic variation in a particular marker with variation in the phenotype, it is possible to "tag" traits for improved stress resistance so that they can be incorporated more efficiently into new varieties. This is being done with 96 accessions from the extensive collection of wheat genetic resources at the Institute of Field and Vegetable Crops, Novi Sad. These wheats have already been phenotyped for a large number of traits, including several associated with drought resistance. The allele at 47 microsatellite (SSR) loci was determined and allelic variation tested for association with phenotypic variation. Highly significant allele associations with flowering date and stem height were identified with SSR loci close to photoperiod (*Ppd*) and vernalisation (*Vrn*) genes and the major dwarfing gene (*Rht-D1*). A highly signicant allele association with flowering date was also found on chromosome 6D with SSR psp3200. This appears to be a new region found to regulate flowering time. In a subset of the genotypes, one marker, psp3071, on chromosome 6AL

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showed significant association with yield under drought in Southeast Serbia. The yield component most associated with this effect was thousand grain weight. We are using this information on allele associations to make selected crosses to initiate programmes of breeding for improved drought resistance.

***Key words*:** Marker-assisted selection, drought responses, wheat, fingerprinting, microsatellite (SSR)

***Abbreviations*:** CATR – Centre for Agricultural and Technological Research, DNA – deoxyribonucleic acid, MAS – marker-assisted selection, QTL – quantitative trait locus, SSR – simple sequence repeat (microsatellite), TGW – thousand grain weight.

Introduction

Drought is generally accepted to be the most widespread abiotic stress experienced by crop plants, and is becoming an increasingly severe problem in many regions of the world. Thus, Yugoslavia is experiencing a gradual decline in rainfall and an increase in aridity (Popoviç *et al*., 1997; Savic and Salvai, 1997). Also, seasonal rainfall for winter crops (October-July) at three sites in Serbia (Novi Sad, Kragujevac and Zajeãar) has experienced an average decrease from 511 mm to 453 mm during the period 1981/2 to 1995/6. The more drought-prone Timok region of Southeast Serbia has experienced a gradual decline in annual rainfall during the last 30 years of about 190 mm (Jevtiç and Milijiç, 1997), accompanied by an increase in the number of tropical days (when maximum temperatures exceed 30 ºC) from 10 to over 40 during the same period. In consequence, wheat yields in the Timok region can be reduced to little over 2 $t.ha^{-1}$ because of the combined effects of insufficient water, high temperatures and warm winds (Dragoviç *et al*., 1997). This season (2001/2002) in the Timok region, for example, the ground was so dry at sowing time that many wheat crops didn't emerge until February and the height of the crop at harvest in the north-east of the Timok region was too low to combine.

With this background of a gradual decline in rainfall and increased aridity in much of Yugoslavia it is important for breeders to consider crop drought resistance to ensure that their new varieties have the best opportunity for achieving their genetic potentials. Although breeders are continuing to improve the yield potential of wheat in Yugoslavia, progress in increasing wheat yields for the more droughted regions of Yugoslavia is more difficult to achieve.

The wheat collection of the Institute of Field and Vegetable Crops in Novi Sad, Yugoslavia comprises about 2 500 genotypes from more than 50 countries. The most divergent part of the collection is the Core collection which consists of 710 wheat genotypes from 38 countries. These genotypes have been evaluated during the 1993–

2000 period for 54 agronomical, morphological, physiological and other traits in field and controlled conditions. For two years, a sub-set of 195 of these genotypes was trialled at the Agricultural Research Institute "Serbia" Centre for Agricultural and Technological Research (CATR) in Zajeãar, Southeast Serbia to study responses to drought stress and identify good parents for a breeding programme to improve drought resistance in wheat.

With the development of molecular marker technologies during the last decade it is now possible to fingerprint genotypes with markers to characterise each one in terms of the marker allele at a number of locations around the genome (determining the haplotype), eg. Becker and Heun, 1995; Paglia and Morgante, 1998). There are also examples (Ivandiç *et al.*, 2002; Liviero *et al.*, 2002) where specific alleles at some loci have been found to be associated with ecotypes better adapted to droughted environments.

The objectives of the present work were to characterise a contrasting selection of wheat genotypes from the Novi Sad Core collection with a number of simple-sequence repeat (SSR) microsatellites and to look for associations between specific alleles and variation in the expression of traits important for drought resistance. Such associations would be essential prerequisites for marker-assisted selection (MAS) of traits in a breeding programme for improved drought resistance. Here we present preliminary evidence for such associations as a test of the concept.

Materials and Methods

Field trials

A two-year trial with 195 cultivars and land races of bread wheat was carried out at the CATR, Zajeãar, using three watering regimes: fully irrigated, rain-fed and under a rain-out plot shelter with single rows of each genotype in three replications. Standard agronomic practices were used to provide adequate nutrition and keep the plots free of diseases. The rain-out shelter was erected over the plot during the winter months and was provided with a 1 m deep ditch around the edge to prevent rain coming off the shelter from seeping into the plot. Plants were scored for yield and its components, flowering date and stem height (year2).

A second set of 96 genotypes (with 33 common to the first set) was selected from the Core collection at Novi Sad for screening with SSRs. These genotypes were chosen on the basis of contrasting expression for one or other of 12 traits, plus eight control varieties that had previously been tested with SSRs (Table 1). These genotypes (except for six identified by "*" in Table 1) were also trialled at the CATR, Zajeãar for one year using fully irrigated, rain-fed and plot-sheltered treatments, with three rows per genotype in two replications. Although many agronomic traits were recorded, only data for flowering date and stem height are reported here.

Table 1. Names of the 96 genotypes scored with SSRs, their countries of origin and the traits for which they were selected.

Genotype	Origin	Trait (high/low)	Genotype	Origin	Trait (high/low)
Acciaio	ITA	Grain fill time (low)	NS 22/92*	YUG	Leaf angle (high)
Ai-bian	JPN	Grain wt/ear (low)	NS 33/90	YUG	Tillers/plant (low)
Al KanTzao	CHN	Flowering (early)	NS 46/90	YUG	Tillers/plant (low)
Ana	CRO	Embryo size (low)	NS 55-25	YUG	Low temp. (low)
Avalon	GBR	Control variety	NS 559	YUG	Grain fill time (low)
Bankuty 1205	HUN	Seed size (high)	NS 602	YUG	Embryo size (high)
BCD 1302/83	MDA	Low temp. (low)	NS 63-24	YUG	Flowering (early)
Benni multifloret	USA	Grains/ear (high)	NS 66/92	YUG	Grain wt/ear (high)
Bezostaya 1	RUS	Control variety	NS 74/95	YUG	Grain wt/ear (high)
Brigand	GBR	Control variety	NS 79/90	YUG	Biomass yd. (high)
Cajeme 71	MEX	Low temp. (low)	Peking 11	CHN	Biomass yd. (high)
Capelle Desprez	FRA	Control variety	Phoenix	USA	Grains/ear (low)
Centurk	USA	Leaf angle (low)	PKB Krupna*	YUG	Grain fill rate (high)
Ching-Chang 6	CHN	Tillers/plant (low)	Pobeda	YUG	Seed size (low)
Cook	AUS	Grain fill rate (high)	Purd./Loras	USA	Peduncle (long)
Don.polupatuljasta	RUS	Embryo size (high)	Purdue 39120	USA	Grains/ear (low)
Durin	FRA	Control variety	Purdue 5392	USA	Tillers/plant (high)
F 4 4687	ROM	Embryo size (low)	Red Coat	USA	Low temp. (low)
Florida	USA	Leaf angle (high)	Renesansa	YUG	Seed size (low)
Gala	ARG	Grain fill time (high)	Rusalka	BGR	Grain fill rate (high)
HAYS 2	USA	Embryo size (low)	Siete Cerros*	MEX	Low temp. (high)
Helios	USA	Low temp. (high)	Saitama 27	JPN	Flowering (early)
Highbury	GBR	Control variety	Sava	YUG	Control variety
Hira	IND	Biomass yd. (low)	Semillia Eligulata	USA	Grains/ear (low)
Holly E	USA	Tillers/plant (high)	Slavija	YUG	Leaf angle (high)
Hope	USA	Control variety	Sofija	YUG	Embryo size (high)
Inia 66	MEX	Low temp. (high)	Sonalika	IND	Seed size (high)
INTRO 615	USA	Flowering (late)	Suwwon 92	IND	Grain fill time (low)
Ivanka*	YUG	Leaf angle (high)	Szegedi 768	HUN	Low temp. (low)

Genotype	Origin	Trait (high/low)	Genotype	Origin	Trait (high/low)
Kite	AUS	Grain fill rate (high)	Tibet Dwarf	TIB	Grain fill rate (low)
L 1/91	YUG	Embryo size (low)	Timson	AUS	Low temp. (high)
L 1A/91	YUG	Embryo size (low)	TJB 990-15	GBR	Biomass yd. (high)
L - 1	HUN	Biomass yd. (low)	Tom Thumb	TIB	Grain wt/ear (low)
Lambriego Inia	CHL	Grains/ear (high)	T. compactum	LV†	Grain fill time (low)
Lr 10	USA	Flowering (late)	T. sphaerococc	USA	Grain fill rate (low)
Lr 12	USA	Grain fill rate (low)	Triple Dirk B	AUS	Low temp. (low)
Magnif 41	ARG	Tillers/plant (high)	Triple Dirk B*	AUS	Low temp. (low)
Mexico 17 bb	MEX	Tillers/plant (low)	Triple Dirk S	AUS	Low temp. (high)
Mexico 3	MEX	Grain fill rate (high)	UC 65680	USA	Grain fill time (high)
Mexico 120	AUS	Flowering (early)	UPI 301	IND	Seed size (high)
Minister Dwarf	AUS	Grain wt/ear (low)	Vel	USA	Grains/ear (low)
Mina	YUG	Seed size (low)	Vireo “S”	MEX	Tillers/plant (low)
Mironovska 808	UKR	Low temp. (low)	WWMCB 2	USA	Embryo size (high)
Nizija	YUG	Embryo size (low)	ZG 1011	CRO	Grains/ear (high)
Norin 10/Brev.14	USA	Grain wt/ear (low)	ZG 987/3	CRO	Grains/ear (high)
Norin 10*	JPN	Seed size (high)	ZG K 238/82	CRO	Grain wt/ear (high)
Nov. Crvena	YUG	Embryo size (low)	ZG K 3/82	CRO	Grains/ear (high)
Nova Banatka	YUG	Tillers/plant (low)	ZG K T 159/82	CRO	Tillers/plant (low)

* Not included in the field trial; † - Local variety.

Microsatellites

A set of 37 microsatellite primer pairs were used (Table 2) covering all three wheat genomes and all 42 chromosomes. These were selected at random from those optimised for use with an ABI 377 Sequencer at the John Innes Centre, Norwich, UK. Genomic DNA was extracted from seedlings grown at the John Innes Centre using a Retsch MM300 Mixer Mill and a Dneasy 96 Plant Kit (Qiagen), using the protocol provided in the Dneasy 96 Plant Kit Handbook 08/99. DNA was quantified according to the SYBR Green method of Hopwood *et al.* (1997). The standard protocol of sample preparation for an ABI 377 Sequencer was applied, and the gels were run using a standard ABI 377 Sequencer procedure. Three or four SSR primer pairs were multiplexed for each run on the machine, with SSRs labelled with different fluors. The output from the sequencer was analysed with Genescan™ software to measure the molecular size of each SSR allele.

Significance of associations between marker alleles and traits at a particular locus was tested by sorting trait data according to the allele at the locus and using one-way ANOVA of trait data corresponding to each allele, provided it was present in at least five genotypes. Only one marker (psp5153) gave only one allele class with at least five genotypes represented, so this SSR was not analysed.

Results and Discussion

The results of PCR amplification of microsatellite loci in 96 genotypes using 37 wheat microsatellite primer pairs are summarised in Table 2. In total, 47 loci and 380 alleles were detected, with an average of 8.0 alleles per locus. Every SSR gave at least three alleles, though not necessarily well-distributed amongst genotypes, and four primer pairs (gwm46, gwm369, gwm484 and gwm539) gave over 15 alleles per locus, indicating that wide genetic variability had been accumulated amongst these genotypes.

To test the effectiveness of the concept of association between marker alleles and trait allele means, flowering date and stem height for the 96 genotypes were examined. As these two traits are known to be regulated, at least in part, by major genes showing Mendelian inheritance (*Ppd* and *Vrn* genes for flowering time and *Rht* genes for height), it was expected that SSR loci close to these genes would show association between variation in the trait and specific alleles.

The results of tests for trait-allele associations for 46 of the loci (psp3151 was not sufficiently polymorphic) are shown in Figure 1. As predicted, significant associations between flowering date and alleles were present for gwm257 and gwm484 (both $P < 0.001$) on the short arm of chromosomes 2B and 2D, respectively, where the photoperiod sensitivity genes *Ppd-B1* and *Ppd-D1*, respectively, are located, as well as with gwm186 ($P < 0.01$) on chromosome 5A, close to the vernalisation gene *Vrn-A1*. A weaker allele association with flowering time (significant at $P < 0.05$) was also found with SSR psp3153 on the short arm of chromosome 2A, close to where *Ppd-A1* is known to be located.

Alleles of psp3103 on the short arm of chromosome 4D were highly significantly associated ($P < 0.001$) with variation in stem height. This SSR locus is very close to the major dwarfing gene *Rht-D1*, and genotypes carrying allele 10 (molecular size 164.6) were on average 11 cm shorter than the height for any other allele class, and 27 cm shorter than the height of the tallest allele class: 47.4, 58.9, 68.5, 73.9 and 74.1 cm for alleles of molecular size 164.6, 182.2, 178.3, 168.4 and 180.2 respectively. At least four genotypes carrying allele 10 (Ai-bian, Norin 10/Brevor 14, Suwon-92 and Tibet Dwarf) are known to contain dwarfing alleles of *Rht-D1* (B. Kobiljski, unpublished data, and J. Flintham, John Innes Centre, personal communication).

However, no significant allele associations with stem height were identified with SSRs known to map close the *Rht-B1* on chromosome 4BS (psp3078 and one locus

Table 2. Primer pairs, chromosome locations, number of loci and number of alleles/locus for each SSR.

Primer pair	Chromosome location	Number of loci	Number of alleles/locus
Gwm3	3DL	1	6
Gwm11	1BS	1	9
Gwm46	7BS+?	3	5+17+3
Gwm99	1AL	1	9
Gwm130	7AS+?	2	5+6
Gwm155	3AL	1	9
Gwm160	4AL+?	2	8+13
Gwm161	3DS	1	7
Gwm165	4AS, 4BL, 4DL	3	5+5+11
Gwm186	5AL	1	11
Gwm190	5DS	1	8
Gwm192	4AL, 4BL,4DL	3	5+5+11
Gwm257	2BS	1	5
Gwm292	5DL	1	6
Gwm295	7DS	1	10
Gwm325	6DS	1	10
Gwm337	1DS	1	13
Gwm357	1AL	1	5
Gwm369	3AS	1	18
Gwm389	3BS	1	13
Gwm458	1DL	1	4
Gwm484	2DS	1	21
Gwm539	2DL	1	17
Gwm540	5BS	1	9
Gwm626	6BL	1	4
Psp3009	6BS	1	4
Psp3030	7BL	1	5
Psp3050	7AS	1	8
Psp3071	6AL	1	9
Psp3078	3BL, 4BS	1	6
Psp3088	2AL	1	6
Psp3103	4DS+?	2	5+11
Psp3123	7DL	1	3
Psp3153	2AS+?	2	4+7
Psp3200	6DS	1	8
Wmc56	3BL	1	6
Wmc73	5BL	1	6
Total		**47**	**380**

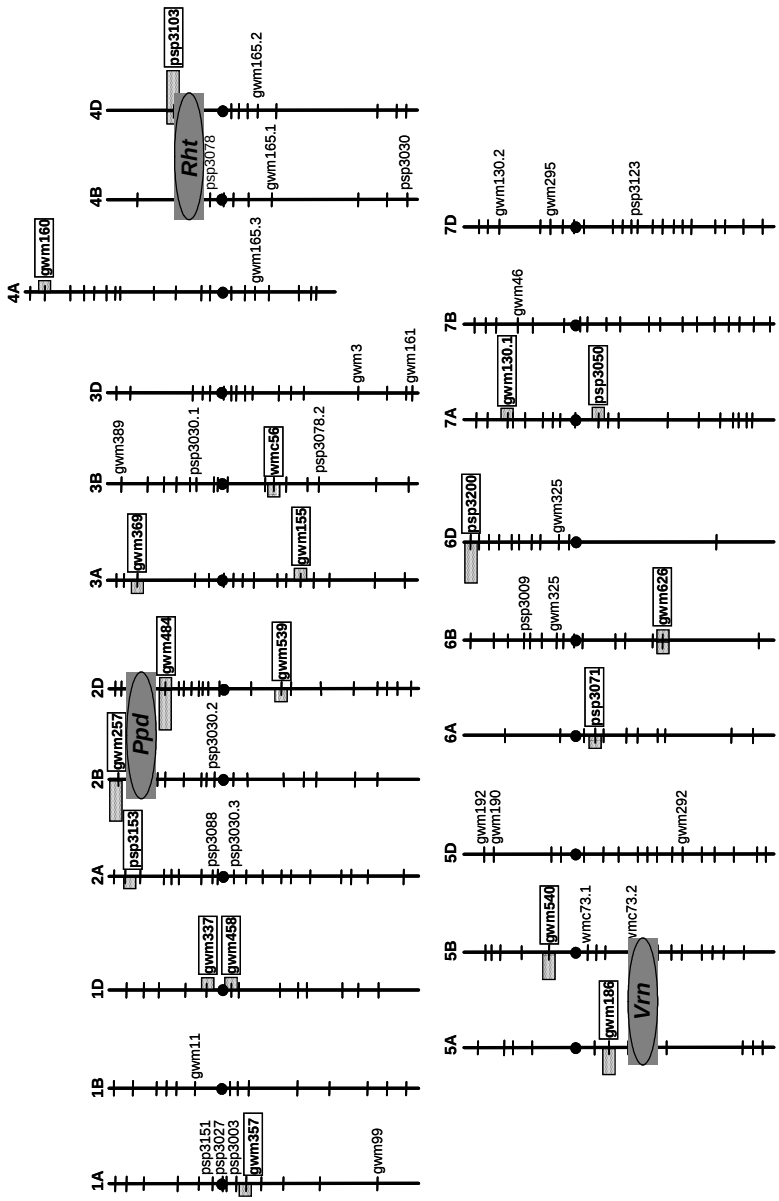

Figure 1. SSRs used for wheat genotype fingerprinting and their chromosomal locations. Bars to the left of chromosomes show significant associations with heading date, and bars to the right of chromosomes show significant associations with plant height. The length of bar shows the relative level of significance. All associations significant at $P<0.05$ are shown. Short tick marks on each chromosome indicate the location of other SSRs mapped onto wheat. The approximate positions of centromeres are indicated by filled circles. Ovals indicate the regions of the genome carrying genes for flowering time (*Ppd*, *Vrn*) and height (*Rht*).

of gwm165), despite the presence of several genotypes amongst the 96 known to carry *Rht-B1*. This result was not affected by removing from the analysis the four genotypes known to contain extreme dwarfing alleles on chromosome 4DS. Perhaps, because the allele of psp3078 (the closest marker to *Rht-B1*) known to be present in genotypes carrying *Rht-B1* alleles (eg. Siete Cerros and Highbury) was the most abundant allele (allele 2 of molecular size 158.2, present in 44 of the 96 genotypes), the effect on height was diluted by a large number of other genotypes carrying allele 2, but not containing an *Rht-B1* allele.

In addition to these regions of the wheat genome which are already known to contain major genes for flowering time and height, two other loci having highly significant ($P < 0.01$) allele associations with flowering time were identified: gwm540 and psp3200 on chromosomes 5BS and 6DS respectively. Although the vernalisation sensitivity gene *Vrn-B1* is on chromosome 5BL, an SSR with two loci on the long arm of chromosome 5B (wmc73), and presumably closer to *Vrn-B1* than gwm540, showed no significant allele-flowering date association. Thus, it seems unlikely that the association is because of close linkage with *Vrn-B1*. There may be another flowering time gene on chromsome 5BS, though this needs further confirmation.

No major flowering time effect has yet been reported on the short arm of chromosome 6D of wheat, though an effect on the long arm of barley 6H has been found (Laurie *et al*., 1995; Snape *et al.*, 2001; Stracke and Börner, 1998). Although the association of flowering date with psp3200 could be a sampling artefact, ANOVA gave a probability for the association occurring by chance of less than 1 in 28,000,000, with at least 10 genotypes present in each allele class and a difference in mean relative flowering dates for allele 3 (molecular size 176.0) and allele 5 (molecular size 170.2) of 12.0 days (5.6 and 17.6, respectively). This apparently new source of flowering time gene(s) on chromosome 6DS could provide new opportunities for breeders to adapt their varieties better to the local conditions, and under drought conditions early flowering is generally advantageous

Having established that association genetics between SSR allele and both flowering time and stem height gave both predicted and novel results, the allele analysis was extended to genotypes trialled over two years in the field at CATR, Zajeãar. Thirty-three of the 195 genotypes had been scored with SSRs. Yields of these 33 genotypes under droughted (plot shelter) conditions at Zajeãar were well-distributed amongst yields for all genotypes (Figure 2). Over both years, yields for all 195 genotypes (calculated as $t.ha^{-1}$) in irrigated plots varied from 2.5 to 11.9 $t.ha^{-1}$ (mean yields 7.3 and 6.6 $t.ha^{-1}$ in 1998 and 1999, respectively) and under the shelter they varied from 1.9 to 9.0 $t.ha^{-1}$ (mean yields 5.4 and 5.2 $t.ha^{-1}$ in 1998 and 1999, respectively) (Figure 2). Yields in the rainfed treatments were intermediate between those of the irrigated and sheltered plots (6.4 and 5.6 $t.ha^{-1}$ in 1998 and 1999, respectively). Mean yields for the two years under the plot shelter for genotypes scored with SSRs varied from 1.9 to 8.5 $t.ha^{-1}$. These mean yields under the plot shelter were analysed for association with SSR alleles.

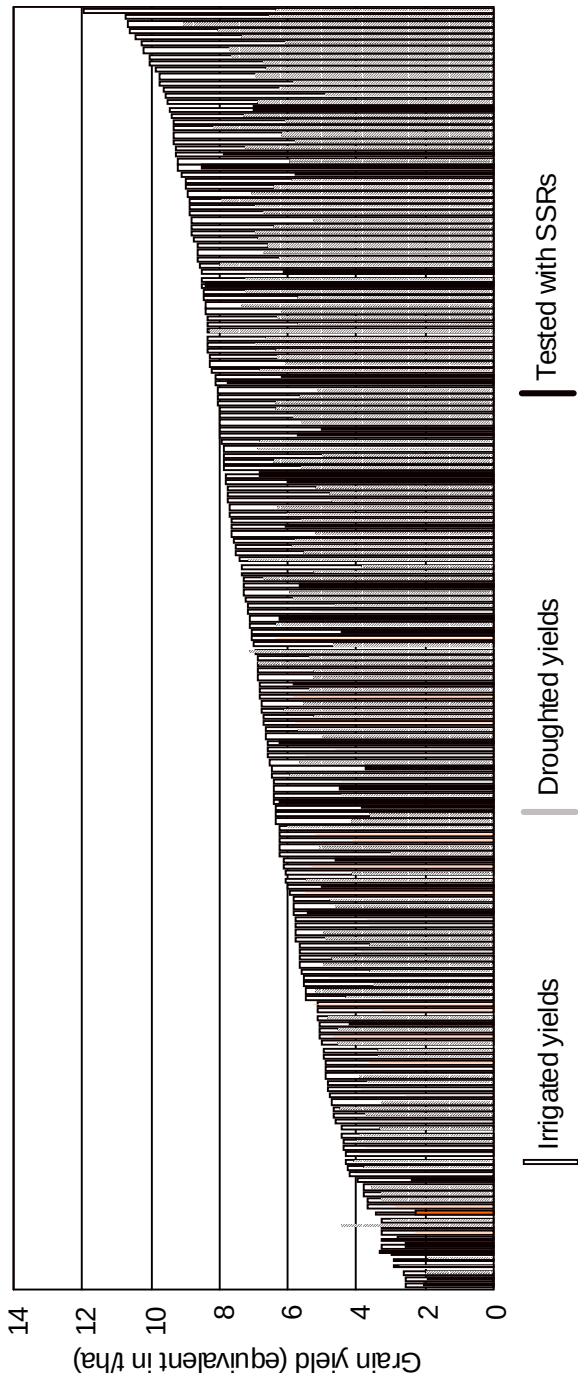

Figure 2. Yields of 195 wheat genotypes (meaned over two years) from trials under irrigated (open bars) and droughted (shaded bars) conditions in Zajeãar, and ranked according to yields under irrigated conditions. Of 195 genotypes, 33 were scored with SSR primers (solid bars).

Examining allele associations with the 33 genotypes was not possible at every one of the 47 loci because the distribution of alleles amongst genotypes did not always provide at least five genotypes in at least two allele classes. Nevertheless the majority of loci could be examined for allele associations with yield and the yield components ears m^{-3}, grains ear^{-1} and thousand grain weight (TGW).

Flowering date and stem height were also examined for comparison with the full set of 96 genotypes. However, the only SSR locus showing a significant allele association with either trait was psp3200 on chromosome 6DS ($P < 0.01$), and this was despite the fact that allele 5 (the latest flowering allele) was missing from the 33 genotypes. Lack of significance for the other flowering time and stem height loci was probably because alleles at each locus associated with the highest and lowest trait means using 96 genotypes were represented by fewer than five genotypes in the reduced dataset.

The only significant allele-trait association found for yield under drought (plot shelter) conditions with the 33 genotypes was with psp3071, situated on chromosome 6A long arm. Four alleles represented by six to eight genotypes gave mean yields varying from 3.9 to 6.4 $t.ha^{-1}$; differences between means significant at $P < 0.05$ (Table 3). The same level of significance for these allele-yield associations with psp3071 was obtained in a subsequent year's field trial using 28 of these genotypes in larger plots (data not presented). The association between alleles of psp3071 and mean yield was, however, not significant for genotypes in the irrigated trials ($P = 0.07$).

Examining psp3071 allele associations with yield components showed the association with yield to be due largely to variation in TGW ($P < 0.001$), with a smaller, but significant effect due to grains ear^{-1} ($P < 0.05$). The variation in TGW was independent of the flowering date (correlation not significant), showing that it was likely to be due to variation in assimilation rate by the ear rather than variation in the duration of grain filling.

A QTL for yield ($P < 0.01$) has recently been reported around the centromere of chromosome 6A of durum wheat by Blanco *et al.* (2002). One of their RFLP loci located within the region of this QTL (Xrsq805) maps only 3.2 cM from psp3071 in

Table 3. a) Allele means for yield and its components ears m^{-2}, grains ear^{-1} and TGW for psp3071 and b) ANOVA for each trait.

a)

Allele (molecular size)	Genotypes per allele	Trait means			
		Yield (t/ha)	Ears/square, m	Grains/ear	TGW
1 (169.5)	6	3.9	479.5	24.1	34.0
3 (165.5)	7	5.8	552.4	25.0	42.6
5 (161.6)	6	4.3	410.0	29.5	34.4
8 (155.8)	8	6.4	486.4	30.4	43.3

b)

Trait	Source of Variation	SS	df	MS	*F*-ratio	*P*-value
Yield	Between alleles	27.985	3	9.328	4.3760	0.0141
	Within alleles	49.029	23	2.132		
	Total	77.015	26			
Ears per square metre	Between alleles	65772.9	3	21924.3	1.5777	0.222
	Within alleles	319625	23	13896.7		
	Total	385398	26			
Grains per ear	Between alleles	205.639	3	68.5464	4.6207	0.0114
	Within alleles	341.2	23	14.8348		
	Total	546.839	26			
TGW	Between alleles	514.319	3	171.44	8.8102	0.00045
	Within alleles	447.561	23	19.4592		
	Total	961.88	26			

a doubled haploid population from the hexaploid wheat cross Chinese Spring x SQ1 (Quarrie *et al.*, unpublished data). A highly significant QTL for TGW has been found on chromosome 6B long arm in several field trials of this doubled haploid population (Quarrie, unpublished data), and its location is homoeologous to psp3071 on chromosome 6AL, though no significant effect on TGW in this population has been found on chromosome 6AL.

With regard to prospects for MAS of improved drought responses in wheat, our SSR results have shown that associations between marker alleles and traits can be identified and that the technique can identify new and useful sources of variation in traits. Crosses have already been made between wheat genotypes carrying different alleles at the psp3071 locus and contrasting in grain size to test whether subsequent selection on the basis of variation in grain size can be monitored using specific alleles of psp3071.

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