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Associations between anther-culture response and molecular markers on chromosomes 2H, 3H and 4H of barley (Hordeum vulgare L.)

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Abstract Segregation distortion is a common phenomenon among anther-culture-derived plants and it has been suggested that the distorted areas may contain genes affecting survival in anther-culture. Segregation of 111 markers was checked in an androgenetic barley (*Hordeum vulgare* L.) progeny and a linkage map was constructed. Thirty one progeny lines were tested for their anther-culture ability and associations between molecular markers and anther-culture traits were established. Two regions on chromosomes 2H and 4H were associated with anther response, three on 2H (two areas) and 3H with plant regeneration rate, and one on 4H with spontaneous diploidization. The chromosomal regions controlling anther-culture response and the regions where distorted segregation was found were not always the same.

Key words Regeneration · Mapping · Distorted segregation

Introduction

Anther-culture involves the induction of embryoid formation from immature pollen and the regeneration of embryoids to plantlets, and both of these characters have been shown to be under independent genetic control (Henry and De Buyser 1985; Agache et al. 1989). Albinism, common in cereal anther-culture, is also genetically controlled (Tuvesson 1989). Genetic differences may also occure in the rate of spontaneous diploidization, which is important for the practical use of barley anther-culture. In wheat (*Triticum aestivum* L.) genes controlling anther-culture traits have been located on chromosome arms using translocation and substitution lines (Ghaemi et al. 1995; Sibikeeva and Sibikeev 1996). Recently, molecular markers have been employed to map genes controlling somatic tissue-culture traits in rice (*Oryza sativa*) (Taguchi-Shiobara et al. 1997), maize (*Zea mays* L.*)* (Armstrong et al. 1992), wheat (Ben Amer et al. 1997) and barley (Komatsuda et al. 1995; Mano et al. 1996). Anther-culture response in maize, namely the ability to induce embryoids from microspores, has also been mapped (Cowen et al.1992; Wan et al. 1992; Beaumont et al. 1995).

Segregation distortion is a common phenomenon in anther culture-derived barley (Thompson et al. 1991; Graner et al. 1991; Heun et al. 1991; Zivy et al. 1992). Usually the distortion observed has been in favor of the allele of the parent performing better in anther-culture (Foisset and Delourme 1996). Anther-culture is supposed to invoke selection on the random sample of gametes contained by the anthers, and it has been suggested that the distorted genomic areas may contain genes affecting survival in anther-culture (Thompson et al. 1991; Zivy et al.1992; Barua et al. 1993) though direct evidence for this hypothesis has not been provided.

In the present study, a molecular map consisting of 85 markers was produced, based on the anther culture-derived progeny of 200 barley lines. The segregation of 111 markers was tested in the doubled-haploid progeny and, with a subset of markers, was compared with segregation in F_2 progeny from the same cross. In addition, a selection of 31 doubled-haploid progeny lines were tested for their anther-culture ability (anther response, plant regeneration, green plant ratio and spontaneous diploidization) to locate genes controlling anther-culture response in barley.

Materials and methods

Marker analysis

A cross was made between two doubled-haploidized six-row spring barleys, Rolfi and Botnia, and 200 doubled-haploid lines were produced from the F_1 by anther-culture according to Man-

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ninen (1997). Sixty two F_2 progeny from the same cross were also used. DNA of the doubled-haploid lines was extracted from foliage of 10–14 day old seedlings with a modified CTAB-method (Poulsen et al. 1993). A quicker small-scale CTAB-method according to Tinker et al. (1993) was used to isolate DNA from the F_2 progeny. Amplification reactions for RAPDs were performed as in Manninen and Nissilä (1997). Amplification products were separated on 1.4% agarose gels with electrophoresis (50 V, 16 h). Clones from A. Graner, Gatersleben, Germany, M. Sorrells, Cornell University, Ithaca, USA, and A. Kleinhofs, Washington State University, Pulman, USA, were used as probes in RFLP-analysis. Southern blotting and hybridization were both performed according to the manufacturer's instructions for the Hybond N+ -membranes employed (Amersham LIFE SCIENCES, Version 2.0). The microsatellites HVM3, HVM36, HVM60 were amplified according to Liu et al. (1996) and analyzed with an ALF DNA sequencer using the Fragment Manager computer program (Pharmacia Biotech, Sweden).

Segregation of 94 RAPD markers, 14 RFLP markers and three microsatellite markers in the doubled-haploid progeny was tested against an expected 1:1 ratio by χ^2 analysis. A segregation test in the F_2 progeny was based on 26 evenly spaced RAPD markers and an expected 3:1 ratio was used. The amplification of the RAPD bands was first tested on the heterozygous F_1 generation. JoinMap 2.0 (Stam and Van Ooijen 1995) was employed for map construction. Recombination fractions were converted to centiMorgans with Haldane's mapping function.

Anther-culture

A set of 31 doubled-haploid lines, together with the parental varieties, was tested for their anther-culture response. Lines were selected according to their marker profile in the three major distorted chromosomal regions; lines represented all combinations between these areas. Recombination within each distorted chromosomal region was restricted among the selected lines. Anthers were cultured according to the best method established by Manninen (1997). Anthers from several spikes were distributed among 6-cm Petri dishes (50 anthers per dish) and a total of 500 anthers per genotype were used. The number of responsive anthers was recorded after 4-weeks incubation in darkness at +25° C. The Petri dishes were transferred to light and the number of green and albino plantlets induced during the next 4-weeks incubation were recorded. For lines producing over five viable green plants the ploidy level of regenerants was checked using flow cytometry as described by Rokka et al. (1995); 500–7000 nuclei were analysed in each sample.

Statistical analysis

For all anther-culture traits, except the proportion of diploid green plants, data were recorded using each Petri dish as a separate replication. The data were non-normally distributed with distributions strongly skewed towards zero, and genotypes had unequal variances for all the traits analysed. Therefore, all anther-culture experiments were analysed using non-parametric tests, which make no assumptions about the underlying distribution of the data. Mann-Whitney and Kruskall-Wallis tests are non-parametric alternatives to the *t*-test and a one-way analysis of variance for independent samples. Both of these non-parametric tests use ranks instead of the observations when calculating the test statistics (Steel and Torrie 1980). A Kruskall-Wallis test was employed to detect the effect of the doubled-haploid line for the numbers of responding anthers, the plants per responding anthers, the proportion of green plants and proportion of diploid green regenerants. A set of 34 evenly distributed marker loci were tested for their allelic effect on anther-culture response using Mann-Whitney U statistics. All statistical analyses were run using SPSS version 6.1.

Results and discussion

The parents of the doubled-haploid progeny differed in 28 of 180 (15.6%) clone-enzyme combinations; these RFLP data were partly extracted by M. Auranen (unpublished). Fourteen RFLP loci were mapped based on these differences and 104 of 1275 (8%) amplified RAPD bands, as well as 3 of 17 (18%) amplified microsatellites, differed between Rolfi and Botnia.

Markers were first grouped using a 5.0 LOD threshold and afterwards groups and unlinked anchor markers were joined to form chromosomes based on previous information on anchor-marker locations. Eighty five markers were mapped to linkage groups keeping the jump threshold at 3.0 in JoinMap (Fig. 1). The linkage map constructed in the Rolfi × Botnia cross covered 654 cM compared with the total length of 1060 cM of the barley consensus map (Qi et al. 1996). Markers tended to cluster to certain areas of the genome, leaving others uncovered. On chromosome 3H six markers mapped exactly to the same position. No markers additional to CDO393 could be mapped to chromosome 1H, and large gaps existed on other chromosomes. Other areas were well covered with closely linked markers. Adding new markers appeared inefficient in filling the gaps: new RAPD markers tended to map to the same areas as the previous ones and polymorphic RFLPs and microsatellites were not easily found outside these regions. This may be at least partly due to lack of polymorphism in the cross used; Rolfi and Botnia are both six-rowed spring barley varieties from the Finnish barley germplasm, which is known to be narrow-based (Manninen and Nissilä 1997). The coancestry coefficient of Rolfi and Botnia is 0.135 and the amount of polymorphism in molecular markers was very low.

Of the 111 markers analysed in the doubled-haploid progeny 48 (44.1%) showed distorted segregation (χ^2-test, $P \leq 0.05$) (Fig. 1). When a Bonferroni corrected significance threshold ($P \leq 0.00045$) was used, 25 loci (22.5%) still showed distorted segregation. The majority of these loci were located on chromosome 3H in a 40-cM segment surrounding the BCD828 RFLP marker; Rolfi alleles were over-represented. The same region on chromosome 3H is distorted on the Igri x Franka map, which is based on androgenetic doubled-haploid lines (Graner et al. 1991). Igri alleles are favoured and Igri is known to respond better than Franka in anther-culture. Distorted segregation on chromosome 3H has also been reported in other androgenetic mapping progenies (Barua et al.1993; Becker et al. 1995). On chromosome 7H there was a 25-cM segment of distorted segregation in the telomeric region of the short arm. In this region 67% of the alleles were inherited from Botnia. This chromosomal region is distorted too in the Proctor × Nudinka map (Heun et al. 1991), also based on androgenetic doubled-hapoids. The ksuD22 and MWG865 RFLP markers on chromosome 2HL segregated favouring the Rolfi alleles (73% and 66%). This chromosomal region overlaps with that hav-

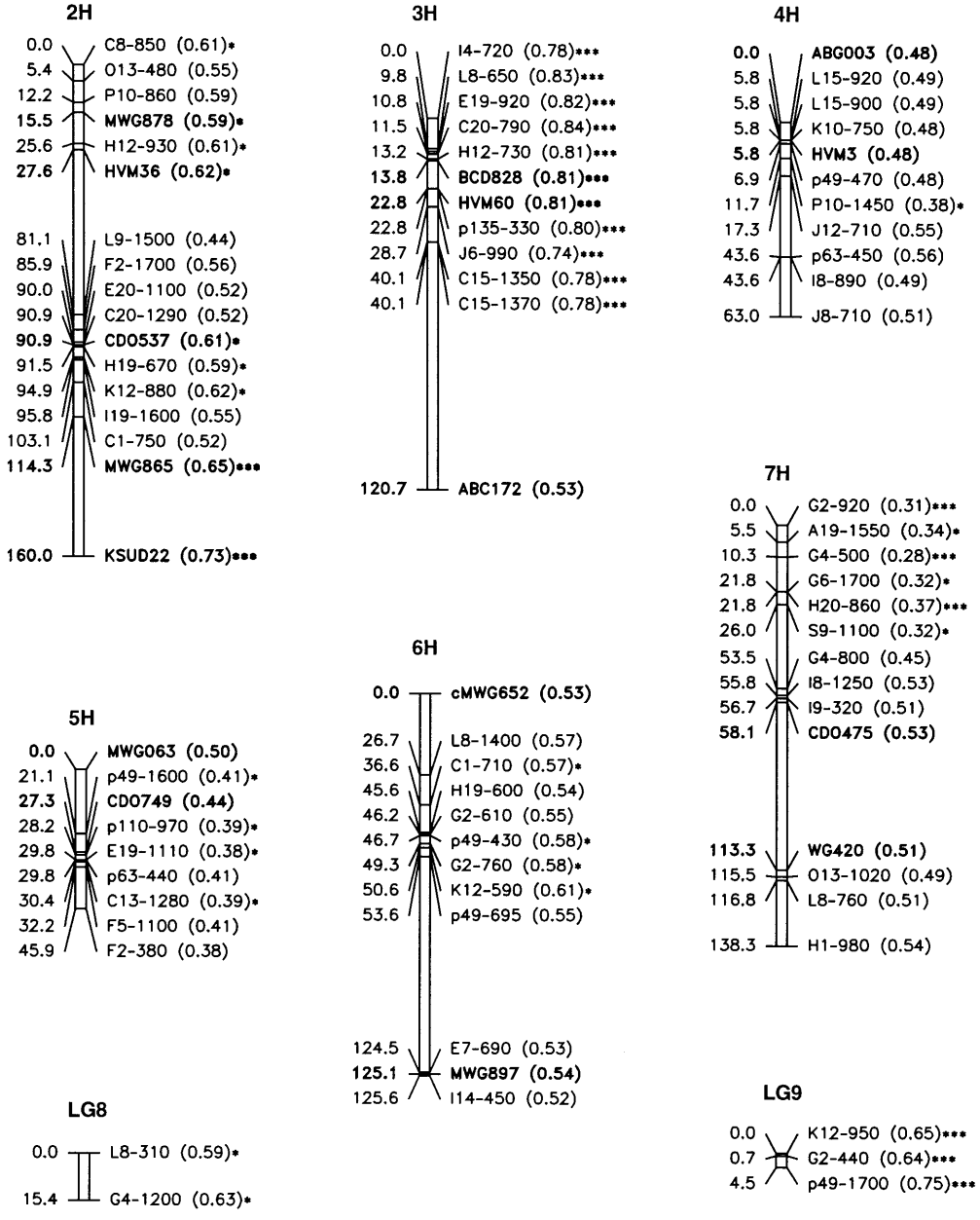

Fig. 1 Linkage map of Rolfi × Botnia. Second-round maps calculated with a jump threshold of 3.0 and a triplet threshold of 6.0 in Joinmap are presented. Anchor markers (RFLP, microsatellites) are shown in *bold*. The frequency of the Rolfi allele is shown in *parenthesis* and significant deviation from Mendelian segregation is marked by *asterisks* ($P < 0.0024$ ***, $0.0024 < P < 0.05$ *). Distances between markers are in centiMorgans on the left side of the chromosomes

ing an excess of Franka variants in the Igri × Franka map (Graner et al. 1991). In three closely linked RAPD markers not assigned to any chromosome an excess of Rolfi alleles (67%) was observed. When the less-significantly distorted ($0.00045 < P < 0.05$) markers were considered, segregation favouring the Botnia marker alleles (60%) was observed on chromosome 5HS around CDO749, as was segregation favouring Rolfi alleles on chromosome 2H centromeric region around CDO537 (60%) and on the short arm surrounding MWG878 (60%). In both Igri × Franka and Steptoe × Morex androgenetic progenies' skewed segregation has been observed for regions around CDO749 and in Igri × Franka for regions around CDO537 (Graner et al.1991; Devaux et al. 1995).

For a comparison of segregation a subset of 25 RAPD markers was tested in the F_2 from the same cross. Three of the markers (12%) did not segregate according to expectation (3:1) when analysed with a χ^2-test ($P \leq 0.05$) and, when the Bonferroni-corrected significance level ($P \leq 0.0024$) was used, only one marker, L8–760, was significantly distorted (Botnia alleles in excess). L8–760 is located on chromosome 7HL near WG420; this region was not distorted in the doubled-haploid progeny. G6–1700, which was the other one of the less-severely skewed markers in the F_2 progeny, is located on 7HL in the region which was also distorted in the doubled-hap-

Table 1 Anther-culture response of Rolfi and Botnia. Means for RA% (percentage of responsive anthers), PPRA (plants per responsive anther), GP% (green plant percentage) and DIPL% (percentage of diploid green plants) are given. *P*-values for Mann-Whitney U test statistiscs are shown below the means

Barley variety	Cultured anthers	RA%	PPRA	GP%	DIPL%
Rolfi	500	7.8	2.8	16.7	100
Botnia	500	16.6	1.3	15.4	42
P-value		0.023	0.031	0.875	

loid progeny, but the direction of distortion differed between the two progenies.

Markers with distorted segregation have been found in many types of barley mapping-progenies: anther culture-derived (Graner et al. 1991; Heun 1991; Barua et al. 1993; Becker et al. 1995), doubled-haploids produced by the bulbosum-technique (Devaux et al. 1995; Kjær et al. 1995), F_2/F_3 (Graner et al. 1991), and recombinant inbred lines (Teulat et al. 1998, Qi et al. 1998). The percentage of distorted markers varies from 7 to 44%, but seems to be greatest in androgenetic progenies. The Proctor × Nudinka map is an exception, with only 10% skewed RFLP markers (Heun et al. 1991) and 8% of skewed AFLP markers (Becker et al. 1995), although this map is based on anther culture-derived lines.

Rolfi and Botnia were both recalcitrant in anther-culture, regeneration rates being 3.6 and 3.2 green plants per 100 anthers, respectively. Parental varieties differed significantly for anther response and regeneration rate per responsive anthers (Table 1.). In the doubled-haploid progeny the effect of the genotype was highly significant for the number of responsive anthers, the number of plants per responsive anther and the proportion of green plants ($P < 0.001$, Kruskal-Wallis test). When the effect of each marker allele on these parameters was tested with a selection of 36 evenly distributed markers, mean ranks for each line were used and the percentages of diploid green plants were included in the analysis. When the Bonferroni-corrected significance threshold ($P \leq 0.00035$) for a repeated Mann-Whitney U-test was employed, only one marker-trait association was found, namely between ABG003 and anther response on chromosome 4H. When a more relaxed significance threshold ($P < 0.05$) was used, ten markers were associated with anther culturability, as shown in Table 2.. Markers on chromosome 3H were associated with plant production per responsive anther, those lines with Rolfi alleles regenerating more plants, although many of these plants were albinos. With all these markers an excess of Rolfi alleles was seen in the doubled-haploid progeny. Three markers on chromosome 4H were associated with the numbers of responsive anthers, Botnia alleles giving higher rates. The percentage of diploids was associated with ABG003 on 4H, Rolfi alleles being favourable; Rolfi was the better parent for this character (Table 1). On chromosome 2H two chromosomal regions were associated with the plant production per responsive anther: one on the short arm (C8–850), where Botnia alleles were favoured, and another in the centromeric region (CDO537, C1–750), where Rolfi alleles gave higher a response. Putative markers for anther response were found in the centromeric region of chromosome 2H (CDO537, K12–880), Botnia alleles being associated with the favourable effect. All the distorted markers on chromosome 2H had an excess of Rolfi alleles. The green plant to albino plant ratio was not associated with any marker, probably due to a lack of variation in the parental varieties for this character (Table 1). No association of markers with any of the anther-culture traits was observed on chromosomes 5H, 6H and 7H, even though markers with distorted segregation were identified on all of these chromosomes. In contrast, associations with anther response were established on chromosome 4H, where Mendelian segregation was observed. On chromosome 4H the selection pressures caused by anther-culture may have been in opposite directions since Botnia alleles were favourable for anther response and Rolfi alleles for spontaneous diploidization. Due to the small number of doubled-hapoid lines studied, it is unlikely that all loci affecting anther culturability and causing segregation distortion could be detected. This is especially the case for the percentage of diploid green plants since only 12 doubled-haploid lines and 5–20 individuals per line were studied.

Genes controlling somatic tissue culture traits have earlier been mapped in barley. Komatsuda et al. (1993, 1995) located a locus (*Shd1*) affecting shoot differentiation rate from immature embryos on chromosome 2H. Mano et al. (1996) found two QTLs controlling callus growth and four QTLs for shoot regeneration from immature embryo cultures. Interestingly, two of the shoot regeneration loci, *Qsr1* on 2H and *Qsr2* on 3H, are located in the same positions where associations between markers and regeneration of plantlets was found in the Rolfi × Botnia cross. This may indicate that at least some of the same genes are involved in plant regeneration from somatic and androgenetic tissues.

In summary, two chromosomal regions associated with anther response were found on chromosomes 2H and 4H, the Botnia alleles being associated with the favourable effect in both cases. Three regions were associated with plant regeneration on chromosomes 2H (two regions) and 3H, Rolfi alleles being favourable in two and Botnia alleles in one of the cases. Rolfi was the parent with higher rates of regeneration. One marker associated with the diploidization rate was found on chromosome 4H. No association could be found between the significantly skewed markers on 7H and any of the anther-culture traits. A chromosomal region contributing to anther-culture traits was found on 4H, which segregated in a Mendelian fashion in the doubled-hapoid progeny. This indicates that, in our doubled-hapoid progeny, the observed segregation distortion could be explained by genes affecting anther-culture for chromosome 3H; but for the remainder of the distorted genomic regions there is no adequate explanation.

Table 2 Putative markers[a] for genes affecting anther-culture response (RA% = percentage of responsive anthers, PPRA = plants per responsive anther, DIPL% = percentage of diploid green plants)

Chromosome	Marker	Frequency of Rolfi allele	*P*-value for χ^2-test	Anther-culture trait	Rolfi allele		Botnia allele		*P*-value for M-W U test
					Mean	Mean rank	Mean	Mean rank	
2H	C8–850	0.614	0.0013	PPRA	2.9	12.3	3.5	19.7	0.022
	CDO537	0.610	0.0027	RA%	2.0	11.4	10.2	19.1	0.015
				PPRA	3.8	18.9	2.2	11.9	0.028
	K12–880	0.623	0.0005	RA%	4.6	12.8	6.7	19.4	0.045
	C1–750	0.515	0.6629	PPRA	4.3	19.4	2.5	11.9	0.018
3H	I4–720	0.782	0.0000	PPRA	3.7	16.1	2.1	10.0	0.047
	L8–650	0.827	0.0000	PPRA	4.0	18.6	2.0	10.9	0.019
	BCD828	0.815	0.0000	PPRA	3.5	18.6	2.1	10.9	0.019
4H	ABG003	0.480	0.5967	RA%	2.5	9.3	10.1	19.7	0.000
				DIPL%	96.3	10.0	69.1	4.5	0.012
	P49–470	0.480	0.5657	RA%	1.8	11.0	9.4	20.7	0.002
	J12—710	0.547	0.2524	RA%	2.0	10.8	5.4	18.8	0.008

[a] Other markers used as classifying variables in the Mann-Whitney test were ABC172, C1–710, C15–1350, CDO475, CDO749, cMWG652, F2–1700, G2–440, G2–920, H1–980, H12–930, H20–860, HVM36, HVM60, I8–890, KSUD22, L8–310, L8–1400, MWG063, MWG865, MWG878, MWG897, P49–430, P49–695, P49–1600, and WG420

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