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Protein markers for anther culturability in barley

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Abstract Two-dimensional electrophoresis of proteins from a recombinant population of anther culture-derived doubled haploid lines identified 4 loci or linkage groups showing a deviation from an expected 1:1 segregation. It was hypothesized that these markers are linked to genes involved in the process of haploid plant production and that the deviation was due to a selection for alleles conferring higher anther culture response. To check this hypothesis, the anther culturability of 50 of the doubled haploid lines and their two inbred parents was assessed. It was found that 2 of the loci which had a distortion of segregation showed a significant effect on anther culture response, the most efficient allele being the most frequent in both loci. In addition, 2 more markers associated with anther culturability were found. One of the first mentioned 2 loci and one of the latter 2 were found to be linked to genes involved in both embryoid production and subsequent green plant regeneration. The remaining two were linked to genes involved only in green plant regeneration. Of the 4 favorable alleles 3 were inherited from one parent.

Key words *Hordeum vulgare* · Anther culture · Linkage · Two-dimensional electrophoresis markers

Introduction

Anther culture is a technique used for doubled haploid (DH) production in barley. Both environmental and genetic factors may influence DH production, and a great deal of effort has been devoted to increasing the efficiency of producing them (reviewed by Pickering and Devaux 1992). Many investigators have studied the inheritance of this character in barley. Foroughi-Wehr et al. (1982) showed that the technique involves two main mechanisms separately inherited, the induction of embryoids and their subsequent regeneration into plantlets. From a complete diallel cross experiment among seven barley cultivars, Powell (1988) detected significant additive and dominance effects for percentage of responding anthers and for green or albino plants produced per 100 anthers cultured. Investigating 22 reciprocal and 1 single hybrid, Larsen et al. (1991) found that genetic variation for embryo and green plant regeneration could be explained by general combining ability. The value of a parental barley line appears to be its suitability for predicting the level of anther culture response expressed in a cross. Most of the barley yellow mosaic virus-resistant DH lines produced by Foroughi-Wehr and Friedt (1984) were derived from F_1 hybrids having an anther culture-responsive parent.

The segregation of anther culturability characters among plants obtained from single crosses has been studied in different species. Dunwell et al. (1987) found that the percentage of responding anthers was significantly different between F_2 plants in barley. The quantitative inheritance of different components of anther culturability, including percentage of responding anthers, embryoid production, and plant regeneration, has been reported in wheat (Lazar et al. 1984, Deaton et al. 1987), triticale (Charmet and Bernard 1984), maize (Petolino and Thompson 1987, Petolino et al. 1988, Afele and Kannenberg 1990), and rice (Quimio and Zapata 1990). In addition, transgressive segregation for green plant regeneration has been reported by Hou (1992) in a F_2 population of barley, and Agache et al. (1988) identified transgressive DH lines combining high embryo production and regeneration ability through anther culture in wheat.

Thus, it is possible to transfer anther culturability by selective crosses. However, the assessment of anther culturability is very labor intensive: at least 2 months of tissue culture are required to obtain valid results, and the procedure is environmentally sensitive. An alternative method would be to identify markers linked to genes involved in

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the anther culture response. In maize, Cowen et al. (1992) identified four RFLP loci tagging embryoid production.

Distortions of segregation for genes encoding polypeptides were found in a barley DH population derived from a cross between the two inbred lines 'Kaskade' and 'DH8293' (Zivy et al. 1992). It was hypothesized that these markers are genetically linked to factors involved in the process of haploid production that have been submitted to selection during this process. They could therefore be used as markers of anther culturability. The aim of the study presented here was to test this hypothesis by culturing the anthers of DH lines that differed in the presence or absence of the selected alleles.

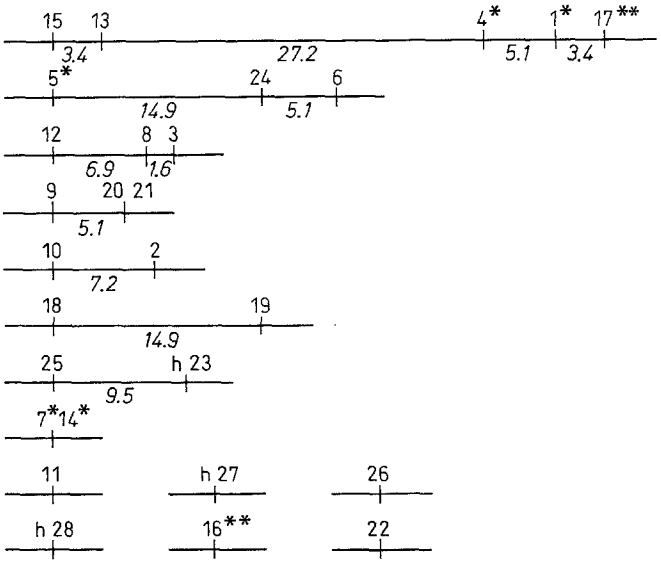
Materials and methods

Fifty DH lines derived from the F_1 hybrid between 'Kaskade' and 'DH8293' were selected from a population of 62 DH lines investigated by two-dimensional electrophoresis (Zivy et al. 1992). In this previous study, 28 protein markers were arranged in a genetic map (Fig. 1). Taking into account approximately 40 cM at both ends of the 14 independant loci and linkage groups, they estimated the total length of the marked genome to be between 730 and 1270 cM. According to the length of published high-resolution genetic maps (Graner et al. 1991; Heun et al. 1991; Kleinhofs et al. 1993), the length of the barley genome is between 1250 and 1450 cM. Although our map did not mark the genome with high resolution, it enabled the identification of four chromosome regions showing a significantly distorted segregation: 17-1–4, 5, 7–14, and 16 (see Fig. 1). The 50 DH lines selected for the present study were those that did not show recombination within the linkage group 17-1–4. They were divided into 13 groups on the basis of their genotype at 17-1–4, 5, 7–14, and 16. The number of DH lines within a group ranged from 1 to 7.

After vernalization, 2–4 plants of 'Kaskade', 'DH8293' and of each F_1-derived DH line were raised in a greenhouse. Plant growth conditions and anther culture were the same as for the production of the F_1-derived DH lines (Zivy et al. 1992). Spikes were sampled from each DH line within a group with a total of 14–36 spikes per group being used. Numbers of anthers, embryoids, and green plants were recorded.

Statistical analyses were conducted using the generalized linear model (GLM) procedure in the statistical analysis system (SAS 1988). Analyses of variance were performed on the 52 genotypes (DH lines and two parents), except for loci 2, 13, and 16, which had one missing value, and h23, which had two missing values.

Fig. 1 Linkage map. (*numbers in italics*, distances in cM, * locus showing a significant deviation to 1:1 segregation ($P< 0.05$), ** locus showing a highly significant deviation to 1:1 segregation ($P< 0.01$) (from Zivy et al. 1992))



Results

Anther culturability of 'Kaskade', 'DH8293', and their F_1-derived DH progeny

Anther culturability was defined as the number of green plants per 100 anthers (%GPL/ANTH). Two intermediate steps were also computed: number of embryoids per 100 anthers (%EMB/ANTH) and number of green plants per 100 embryoids (%GPL/EMB).

A large amount of variation was found among the 52 genotypes for the three variables. The distribution was relatively normal for %EMB/ANTH, but not for %GPL/EMB and %GPL/ANTH, which were skewed. Few genotypes produced large quantities of green plants, and many produced only small numbers. Of the 52 genotypes 13 did not produce any green plants at all. 'Kaskade' and 'DH8293' produced relatively small numbers of embryoids, but 'DH8293' produced more green plants than 'Kaskade'.

There was no correlation between %EMB/ANTH and %GPL/EMB, but both characters were positively correlated to %GPL/ANTH with r=0.547 and 0.826, respectively, thereby being significant at the 1% level. The skewness of %GPL/EMB and %GPL/ANTH led us to use the transformed variables Log(%GPL/EMB+1) and Log(%GPL/ ANTH+1) for variance and regression analyses (Steel and Torrie 1980).

Marker associations with anther culturability

The means for %GPL/ANTH for the different lines, grouped according to their genotype at the four loci that showed a deviation to the 1:1 segregation, are indicated in Table 1. One-way analyses of variance were performed separately for each locus. Loci 17 and 5 showed highly significant effects (P=0.0004 and P=0.0033, respectively, Table 2). In both cases, the selected allele from 'DH8293' resulted in a higher yield of green plants, as illustrated in Fig. 2A and B. Loci 7 and 16 did not show any significant effect.

Lines having the 4 selected alleles were the most efficient (see Table 1). Of the 5 top performing lines, 4, including the best one, have these alleles. The number of genotypes studied was too small to study the interaction between the 4 loci. However, in the one-way analysis of variance among the 13 groups defined according to genotype at the 4 loci, the contrast between genotypes having

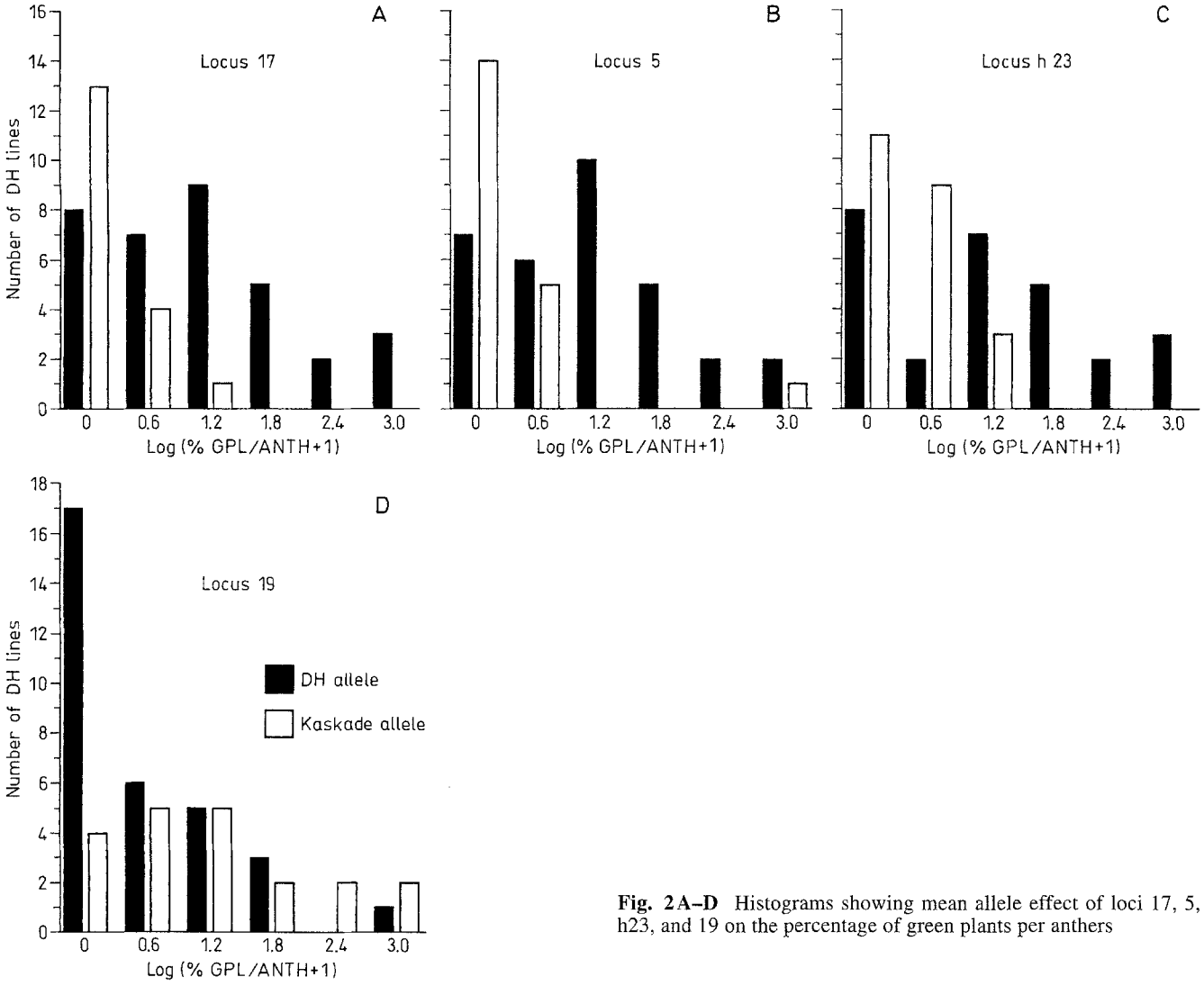


Fig. 2A–D Histograms showing mean allele effect of loci 17, 5, h23, and 19 on the percentage of green plants per anthers

Table 1 Means for anther culturability characters according to the genotype at loci showing distorted segregation. One DH line had data missing at locus 16 and was excluded from this table

Group	Locus[a] 17	7	5	16	Number of selected alleles	Number of DH lines	% EMB/ ANTH	% GPL/ EMB	% GPL/ ANTH
1[b]	0	0	0	1	1	3	158.5	0.155	0.137
2	0	0	1	0	1	1	48.0	0.234	0.110
3	0	1	0	0	1	4	143.0	0.049	0.095
4	1	1	0	0	2	1	112.9	0.353	0.400
5	1	0	1	0	2	5	366.1	0.935	3.288
6	1	0	0	1	2	2	117.3	0.911	0.765
7	0	0	1	1	2	3	257.6	0.241	0.597
8	0	1	0	1	2	3	114.6	0.151	0.113
9[c]	1	1	1	0	3	6	150.7	1.547	2.260
10	1	1	0	1	3	7	332.0	0.606	4.477
11	0	1	1	1	3	4	147.0	0.510	0.968
12	1	0	1	1	3	5	260.1	0.935	2.028
13	1	1	1	1	4	7	296.8	3.859	11.251

[a] 1, Presence of selected allele; 0, absence of selected allele: 17, 7, and 5 in 'DH8293', and 16 in 'Kaskade'
[b] 'Kaskade' is in group 1

Table 2 Effect of the most significant marker of different linkage groups on the percentage of green plants per anther. Analyses of variance were done on the log-transformed variable, but means shown here are on the untransformed variable (*P* Probability of Fisher's test, *R-square* ratio of the sum of squares explained by the locus to the total sum of squares)

Locus	*P*	R-square	Mean for DH8293 allele	Mean for Kaskade allele
17	0.0004	0.221	4.48	0.38
5	0.0033	0.160	3.90	1.72
h23	0.0009	0.207	5.32	0.66
19	0.0102	0.125	1.55	5.47

the selected allele at the 4 loci and those having the selected allele only at loci 17 and 5 was not significant (P=0.09): selected alleles at loci 7 and 16 did not significantly modify %GPL/ANTH in the presence of selected alleles at loci 17 and 5.

Of the other loci revealing polymorphism between 'Kaskade' and 'DH8293' (Zivy et al. 1992) but not different from a 1:1 ratio in the F_1-derived DH progeny, 6 (15,

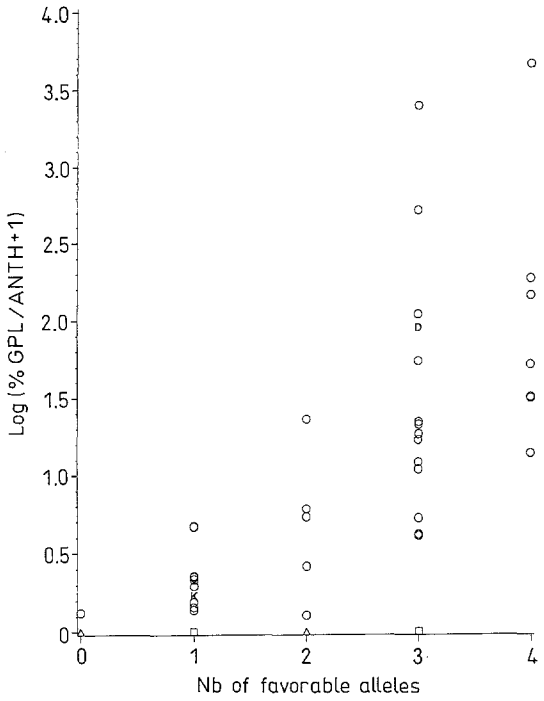


Fig. 3 Percentage of green plants per anther as a function of the number of favorable alleles at the 4 loci showing a significant effect (*K* 'Kaskade', *D* 'DH8293', ○ 1 DH line, △ 2 DH lines, □ 4 DH lines)

Table 3 Mean effects of alleles of different linkage groups on the percentages of embryos per anther and of green plants per embryoid. For the percentage of green plants per embryoid, analyses of variance were done on the log-transformed variable, but means shown here are on the untransformed variable.

Locus	*P*	Mean for DH8293 allele	Mean for Kaskade allele
%EMB/ANTH			
17	0.0232	263.3	155.6
5	0.4339	240.3	203.2
h23	0.4967	242.5	209.8
19	0.0354	188.3	286.3
%GPL/EMB			
17	0.0017	1.53	0.23
5	0.0061	1.52	0.38
h23	0.0010	1.80	0.32
19	0.0506	0.77	1.58

13, 25, h23, 19 and 18) also had a significant effect on anther culturability. Loci 15 and 13 were tightly linked and located 27.2 cM from group 17-1–4 (Fig. 1). Thus, the significant effect of loci 15 and 13 can be explained by linkage with locus 17, which showed a highly significant effect (see above). This hypothesis is strengthened by the fact that there was no significant difference between genotypes having the favorable allele at locus 17 and at loci 15 and 13, and those having the favorable allele only at locus 17.

Loci h23-25 constitute an independant linkage group on the derived map (Fig. 1), although linkage with loci 25 and h23 was apparent but not significant when using the LOD score of 3 to build the map. Thus, the significant effect found for loci 5, 25, and h23 could be due to the fact that they actually mark the same chromosomal segment. There was no significant difference between genotypes having the favorable allele at loci 5 and h23-25 and those having the favorable allele at locus 5 only. On the other hand, h23, which would be further than 25 from 5, had a higher effect on anther culturability (P=0.002 vs 0.033). Therefore, the hypothesis that the linkage group h23-25 and locus 5 tag different genes involved in %GPL/ANTH cannot be excluded. The effect of h23 is illustrated in Fig. 2C.

The 2 linked loci 19 and 18 did not show linkage with any other marker (Fig. 1). In contrast with the other markers showing a significant effect, the favorable allele originated from 'Kaskade'. They mainly affected %GPL/ANTH in the percentage of anthers producing no green plants. This was confirmed by a chi-square test of independence. The effect of locus 19, the most significant one, is illustrated in Fig. 2D.

In summary, four linkage groups containing markers for anther culturability were identified. In order of decreasing significance they are: 17, h23, 5, and 19 (Table 2). A regression of %GPL/ANTH against the number of favorable alleles (0–4) was computed, and the regression was highly significant (P=0.0001). A graph of %GPL/ANTH against the number of favorable alleles is shown in Fig. 3.

The results of analyses of variance on %EMB/ANTH and %GPL/EMB for the 4 loci that showed a significant effect on %GPL/ANTH are shown in Table 3. Loci 17 and 19 showed a significant effect on both characters, while loci 5 and h23 showed a significant effect only on %GPL/EMB. As expected from the linkage between 5 and h23, these 2 loci acted in the same direction on the same character.

Discussion

In an earlier study of DH lines derived from a cross between 'DH8293' and 'Kaskade' Zivy et al. (1992) observed a significant deviation from the 1:1 segregation for markers or linkage groups 17-1–4, 5, 7–14, and 16. Alleles from 'DH8293' were found more frequently at 17-1–4, 7–14, and 5, while the 'Kaskade' form was more often found for marker 16. Two groups (17-1–4 and 5) had a significant effect on anther culturability. Linkage group 17-1–4 had a greater effect than locus 5 and also showed the most deviant segregation. For both loci, the allele originating from 'DH8293' was the most efficient. This is in accordance with the hypothesis tested in this study. The mean value for anther culturability for DH lines having the 4 selected alleles was higher than that for DH lines having other allelic combinations, although not significantly. An epistatic interaction between them cannot definitely be ruled out. However, it was not expected that all markers showing a deviation to the 1:1 segregation would have an effect on anther culturability, since some deviations could have occurred by random chance. In addition, the monogenic de-

terminism of marker 16 was uncertain. The marker that was most likely to show an effect on anther culturability was marker 17 (Zivy et al. 1992).

In addition to 17-1–4 and 5, two other linkage groups showed a significant effect on anther culturability: 18–19 and h23-25. Weak linkage was found between h23-25 and 5, and these two groups could actually mark a single chromosome segment containing a gene involved in anther culturability. More DH lines should be studied to confirm this. Several hypotheses can explain the fact that an unselected locus shows an effect on anther culturability: (1) the locus is weakly linked to a selected chromosome region, as hypothesized above for h23-25 and 5. For 18–19, the selected region would not be located on our map. (2) Epistatic interaction: the locus affects anther culturability only in the presence of another unmarked locus subjected to selection. (3) Anther effect: since the first generation of DH lines was derived from a F_1 hybrid, anther tissues were heterozygous at all of the polymorphic loci. On the contrary, the second generation of regenerants was derived from DH lines where anther tissues were homozygous. Since tapetal cells are known to play a role in microspore development (Pelletier and Ilami 1972, Sunderland and Huang 1985), it is possible that different interactions occur in the different tissues and that some genes have an effect in one tissue but not in the other one.

Two types of genes involved in anther culturability were tagged: linkage groups 18–19 and 17–1–4 were subsequently seen to have significant effects at both steps of DH production, the percentage of embryoids produced per anther and the percentage of green plants produced per embryoid, while 5 and h23-25 showed a significant effect only on the latter character.

As with 17-1–4 and 5 the favorable alleles for h23-25 originated from 'DH8293' although they originated from 'Kaskade' for 18–19. Thus 'DH8293' and 'Kaskade' can be hypothesized to contain 3 and 1 favorable alleles, respectively. This could explain the observation that transgressive DH lines were obtained in both directions for anther culturability. The difference between the means of lines having the 4 favorable alleles (%GPL/ANTH=9.73) and those having none of them (%GPL/ANTH=0.043) was very large, although the amount of variability explained by the 4 markers was 44%. The remaining unexplained portion could be due to environmental variation, which is known to have an important role in anther culture experiments (Dunwell et al. 1987; Henry and De Buyser 1990) and possibly to the fact that the markers are only partially linked to the genes responsible for the variation. In addition, one cannot exclude the occurrence of other genes, as yet unmarked, controlling this trait. It is not known whether the genetic effects evidenced in the present study are specific to the cross or not. Other crosses should be studied and/or other types of markers should be added to the present map.

Distorted segregation for genetic markers has already been reported in anther culture-derived DH progenies of maize (Bentolila et al. 1992; Wan et al. 1992), wheat (Agache et al. 1989; Müller et al. 1989; Devaux et al. 1990), and barley (Graner et al. 1991; Heun et al. 1991; Thompson et al. 1991). The same phenomenon was observed among barley DH lines derived by the *Hordeum bulbosum* method (Kleinhofs et al. 1993). In most of these studies, the loci have been proposed as potential markers for haploid production capability. The results of the present study confirm this hypothesis.

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