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Segregation distortion and linkage studies in microspore-derived double haploid lines of *Hordeum vulgare* L.

M. Zivy [1,*], P. Devaux [2], J. Blaisonneau [1], R. Jean [3], and H. Thiellement [4]

[1] Station of Plant Genetics, CNRS-INRA-UPS, Ferme du Moulon, F-91190 Gif-sur-Yvette, France
[2] Florimond-Desprez, Laboratory of Cytology and Tissue Culture, BP 41, F-59242 Cappelle-en-Pévèle, France
[3] University of Science and Technology of Lille Flandres Artois, Laboratory of Ecological Genetics and Biology of Plant Populations, F-59655 Villeneuve d'Ascq, France
[4] Blaise Pascal University and INRA, Laboratory of Organization and Variability of the Plant Genome, F-63038 Clermont-Ferrand CEDEX 1, France

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Summary. A total of 62 doubled haploid (DH) lines was derived from a cross between tow lines of barley by anther culture. By two-dimensional electrophoresis of seedling proteins, the segregation of 28 loci in the population of DH lines was studied and a linkage map was constructed. The linkage map covered a large part of the length of the genome. A deviation to the 1:1 segregation expected in the absence of selection was observed for at least one chromosome segment. This might be linked to a gene or group of genes selected because of their involvment in the process of haploid production.

Key words: *Hordeum vulgare* – Doubled haploid lines – Segregation distortion – Linkage map – Two-dimensional electrophoresis

Introduction

One question which has arisen with the development of doubled haploid (DH) production techniques in plant breeding schemes is whether or not *in vitro* procedures, i.e., mainly male or female gametophyte culture or embryo rescue after interspecific hybridization, induce selection pressures leading to significant segregation distortions. Several experiments have been reported on these topics, e.g., in tobacco (Kumashiro and Oinuma 1985), rape (Hoffmann et al. 1982), wheat (de Buyser et al. 1985; Winzeler et al. 1987), triticale (Charmet and Branlard 1985), and maize (Lashermes et al. 1988).

In cultivated barley (*Hordeum vulgare* L.), haploid plants can be obtained through the *in vitro* culture of male gametophytes by anther culture or more recently by microspore culture, and by the technique of chromosome elimination following interspecific hybridization with *H. bulbosum* (for review, see Pickering and Devaux 1991).

Most studies using monogenetic phenotypic traits (Powell et al. 1984; Doll et al. 1989; Kjær et al. 1990) or protein encoding genes (Powell et al. 1990) as genetic markers support the hypothesis that no selection is induced by the *H. bulbosum* technique. Moreover, none of the 23 genetic markers (morphological traits, enzyme encoding genes, RFLP, and PCR loci) used by Schön et al. (1990) showed significative deviation from the 1:1 segregation in 42 DH lines derived from F_1 plants. However, Furusho et al. (1990) observed a deviation from the 1:1 ratio for resistance to powdery mildew in DH lines derived from F_1 plants, although the F_2 segregation did not deviate from 3:1. Interestingly, this deviation depended on the genotype of the parental lines.

Concerning the androgenetic method, Foroughi-Wehr and Friedt (1984) and Kao (1988) observed a deviation from the 1:1 ratio for the number of rows per spike; Powell et al. (1986) observed a deviation for three of the five genes tested, but only 14 DH lines were studied.

In this study, we have tried to answer the question of whether or not there are selection pressures during the process of DH production by anther culture. The occurrence of selection was tested for by scoring segregation distortions among protein markers separated by two-dimensional (2D) electrophoresis in a population of 62 DH lines derived from a single F_1 hybrid. As a high level of polymorphism was found for heat shock proteins (HSP) in wheat (Zivy 1987), plants were submitted to heat shock before protein extraction, to increase the number of markers.

* To whom correspondence should be addressed

Materials and methods

Plant material

The two inbred parental lines "Kaskade" and "DH8293" were crossed, and haploid and spontaneously DH plants were regenerated from the F_1 through anther culture according to Devaux (1987). The haploid plants were treated with colchicine according to Pickering (1980). At maturity, grains were collected from spontaneous DH plants and fertile colchicine-treated haploid plants. Eleven DH plants were obtained in a first series, and 51 were obtained in a subsequent series, using the same genotype and methods.

Several grains of the two parental lines and of their 62 DH progenies were sown on moist filter paper in petri dishes and allowed to germinate in the dark at a constant 20 °C. On the 7th day seedlings were submitted or not (for controls) to heat shock (3 h at 40 °C), then their aerial parts were cut off and immediately plunged into liquid nitrogen, where they were stored until extraction. Controls (no heat shock) were done only on seedlings of the parental lines, to identify HSP.

2D Electrophoresis

Proteins were extracted according to the procedure of Zivy (Damerval et al. 1986) and 2D electrophoresis was performed as described in Bahrman and Thiellement (1987). The 240 × 200 × 1 mm 2D gels bound to Gelbond were silver stained according to Heukeshoven and Dernick (1985) modified by Damerval et al. (1987) in the apparatus described by Granier and de Vienne (1986).

Gel comparisons

The two parental lines were first compared (at least four gels from different extractions), to identify spot variations reproducible enough to be scored without ambiguity with onyl one gel per genotype: only one quantitative variation was retained; all others were presence/absence variations. The segregation of these spots was then scored in the DH progeny. When technical problems prevented reliable notations on a gel, 2D electrophoresis was repeated. To take in account variations between batches of 2D gels, samples from parental lines or 1:1 mixtures of parental samples were run in all batches. Identification of HSP was done by comparison of at least four gels of each parental line with at least four gels of their respective controls.

Spot identification

Only spots found variable between the two parental lines were numbered. The identification number was suffixed with a "k" or a "d," when the spot was found specific, respectively, to Kaskade or DH8293, and not suffixed when the spot was present in both parents (quantitative variation). It was prefixed with a "h" when the spot corresponded to a HSP. When allelic relationships were found between two spots, they took the same number and different suffixes.

Linkage map

The linkage map was built with the aid of the "Mapmaker" software (Lander et al. 1987).

Results

Comparison between the two parental lines allowed the identification of 50 variable spots (Fig. 1). Five of them corresponded to heat shock proteins (h23k, h23d, h28k, h28d, h27k). All of them showed a presence/absence variation, except for spot 26, which was clearly more intense in DH8293 than in Kaskade. The segregation of these spots was then studied in the 62 DH lines. Of the 3,100 possible observations, 22 were not scored because of ambiguities or bad resolution in regions of some gels.

Allelic relationships

Identification of pairs of spots corresponding to different allelic products of the same structural gene was done according to the following criteria: (i) two allelic products must originate from different parental lines, and they cannot be both present in the same DH line; (ii) they must be relatively close to each other on 2D patterns, i.e., they have similar pI and apparent molecular masses. Accordingly, 44 spots were found to correspond to 22 pairs of allelic products (see Fig. 1). Different reasons can explain the absence of alternative allelic product for the 6 remaining spots 15d, 10k, 25k, h27k, 26, and 16k (see Discussion). The simplest hypothesis will be assumed in the following section, i.e., control by biallelic loci, one allelic form corresponding to the presence of the spot (or presence of a large spot for spot 26), and the alternative one corresponding to the absence of the spot (or presence of a small spot for spot 26). Thus, 28 loci were considered.

Linkage map

For haploid segregations, the maximum-likehood estimate of the recombination rate between two loci is equal to the observed recombination rate. This rate remains unchanged when an allelic form at one locus affects viability. The software "Mapmaker" (Lander et al. 1987) was used to build the map shown in Fig. 2. The linkage groups were defined using a lod score threshold of 3.0 (likehood for linkage between two loci 1,000 times greater than likehood for independence). The highest recombination rate taken into account was 0.283 (between loci 15 and 17). Three-point and multiple-point tests were performed in groups comprising more than two loci, to determine their order. As no significative interference was observed, the recombination rates were converted into centiMorgans using Haldane's formula: $x(r) = -1/2ln(1\text{-}2r)$ (Bailey 1961), where x is the estimated distance in Morgans and r is the recombination rate.

The 28 loci were found to fall into 14 independent loci or linkage groups. In two cases, no recombination was observed between pairs of loci: between 20 and 21, and between 7 and 14. The latter observation could be explained in a different way: there could be actually only one locus encoding polypeptides 20 and 21, and one locus encoding polypeptides 7 and 14; the difference between

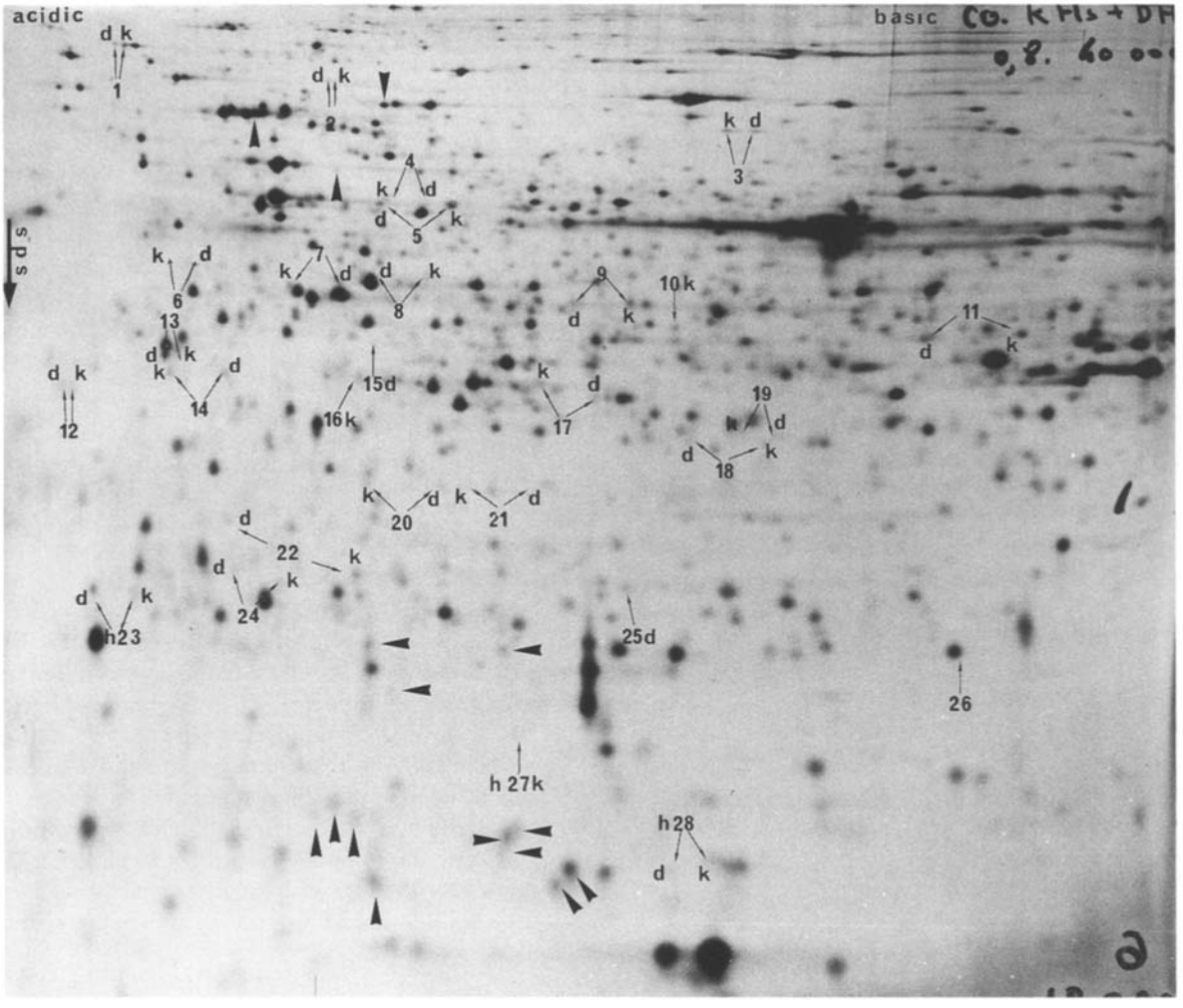

Fig. 1. 2D electrophoresis of the 1:1 mixture of proteins from the two parental lines DH8293 and Kaskade. Only variable spots are numbered. *Linked arrows* show pairs of allelic form. For identification code, see 'Material and methods'. *Large arrowheads* show non-variable HSPs. Although some faint spots are not visible on this photography, their positions is shown

the two products of the same gene could be caused by post-translational modifications. This hypothesis is unlikely for polypeptides 7 and 14, since the two polypeptides have very different physical properties, but it is possible for polypeptides 20 and 21 (see Fig. 1). The three loci encoding HSPs or controlling their synthesis were found to be unlinked. However, the HSP encoding locus h23 was found to be linked to locus 25 (9.5 cM).

Segregation distortions

Table 1 shows the frequency (f) of the DH8293 allelic form at each locus in the population of DH lines. For seven loci, a significant difference from 0.5 was found: loci 16 ($f=0.300$, $p=0.004$), 17 ($f=0.689$, $p=0.006$), 1 ($f=0.661$, $p=0.012$), 4 ($f=0.645$, $p=0.024$), 5 ($f=0.645$, $p=0.024$), 7 ($f=0.645$, $p=0.024$), and 14 ($f=0.645$, $p=0.024$). However, three of them (17, 1, and 4) were closely linked and two others (7 and 14) did not recombine at all (see Fig. 2). Thus, the seven loci actually mark the four following chromosome segments: 17-1-4, 7-14, 16, and 5. For all of them except one (locus 16), the selected form originated from DH8293.

The significance level that should be taken into account for several independent comparisons should be divided by the number of comparisons. However, the 28 comparisons were not independent, since some loci were linked. Taking into account the 14 independent linkage groups or loci, the significance level for each individual comparison is 0.4%. Two loci showed a deviation from the 1:1 segregation with a risk of about this magnitude: 16 and 17 (respectively, 0.4 and 0.6%).

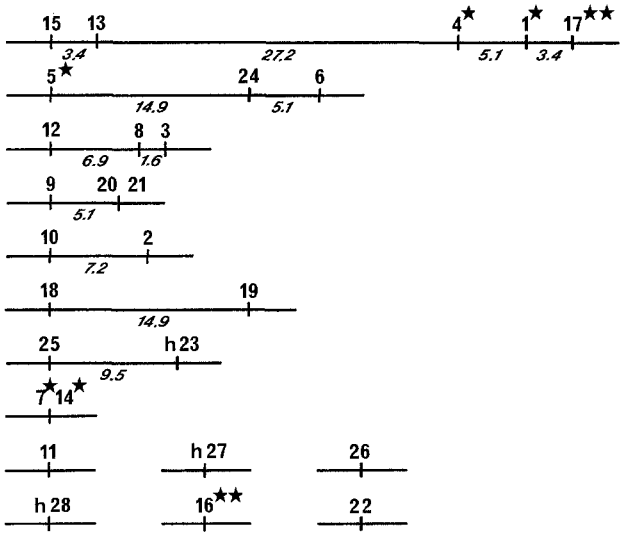

Fig. 2. Linkage map. Names of loci are the same as names of spots. Italic numbers: distances in cM. * Locus showing a significant deviation to 1:1 segregation ($P<0.05$); ** Locus showing a highly significant deviation to 1:1 segregation ($P<0.01$)

Table 1. Frequency of the DH8293 allelic form at the 28 loci in the 62 DH lines

Locus	p	nobs	freq	χ^2
1	41	62	0.661	6.45*
2	29	62	0.468	0.26
3	37	62	0.597	2.32
4	40	62	0.645	5.23*
5	40	62	0.645	5.23*
6	33	62	0.532	0.26
7	40	62	0.645	5.23*
8	38	62	0.613	3.16
9	26	62	0.419	1.61
10	27	60	0.450	0.60
11	35	62	0.565	1.03
12	36	62	0.581	1.61
13	26	61	0.426	1.33
14	40	62	0.645	5.23*
15	27	61	0.443	0.80
16	18	60	0.300	9.60**
17	42	61	0.689	8.67**
18	32	62	0.516	0.06
19	36	62	0.581	1.61
20	27	62	0.435	1.03
21	27	62	0.435	1.03
22	36	62	0.581	1.61
h23	32	58	0.552	0.62
24	32	61	0.525	0.15
25	34	62	0.548	0.58
26	36	62	0.581	1.61
h27	30	61	0.492	0.02
h28	32	61	0.525	0.15

p: number of scored presences; nobs: number of observations; χ^2: conformity to 1:1 segregation (1 *df*); Freq: frequency
* Frequency significantly different form 0.5 ($P<0.05$)
** Frequency highly significantly different from 0.5 ($P<0.01$)

Discussion

In this study, the occurrence of selection pressures was tested by looking for segregation distortions. The expected segregation for two allelic forms of one gene in the absence of selection is 1:1. Our results are in the form of a presence/absence of polypeptides (except for one quantitative variation), and our interpretation is that the presence/absence of one polypeptide corresponds to the presence/absence of an allelic form of one gene. In most cases, pairs of allelic products were observed (22 pairs), which shows that the variation was in the structural gene encoding the polypeptide. However, in six instances no alternative allelic form was found. This can still be explained by variation for the structural gene (alternative spot hidden by others or a silent gene), but it can also be explained by the variation of a regulator controlling the amount of the polypeptide. The segregation of a regulator will also be expected to be in a 1:1 ratio. However, as a polypeptide can be under the control of several regulators (Colas des Francs and Thiellement 1985), there is the probability that two or more regulators controlling the same polypeptide are different in the two parental lines; in this case, a 1:1 segregation is not expected. Thus, the use of a polypeptide spot without an alternative allelic form (and of polypeptides showing quantitative variation) is less safe for the determination of segregation distortions. For the same reasons, their localization in linkage groups is also less secure, and it cannot be determined whether the encoding gene or a regulator was localized.

Construction of a linkage map

The study of recombination frequencies allowed the construction of a linkage map showing 14 independent loci or linkage groups (6 independent loci and 8 linkage groups comprising two to five loci: see Fig. 2). The three variable loci encoding HSP were found to be independent of each other, but one of them was found linked to a locus encoding a non-HSP protein. Among 20 HSP spots observed in the parental lines, 5 were variable: as expected, the level of polymorphism in HSP was found to be higher than in non-HSP (Zivy 1987).

The localization of spots with no alternative allelic form was performed according to the simplest hypothesis, i.e., segregation of one gene encoding the protein or a regulator of its synthesis. For five of them, no difference to the 1:1 segregation was observed, indicating that this hypothesis was probably true. For the last one (spot 16), this could not be verified therefore the localization of the locus might be erroneous.

In this study, the linkage between two loci was significant if the recombination frequency was lower or equal to 0.283, which corresponds to 41.7 cM. Accordingly,

each identified linkage group or independent locus marked a chromosome segment of 41.7 cM at both ends of its position, plus the length of the linkage group itself. Thus, the total length of marked chromosomes was between 1,272 cM (if the 14 marked chromosome segments did not overlap each other) and 730 cM (maximum overlap). To our knowledge the total length of the genome of barley has not been estimated. However, those of rice and maize were estimated to be between 1,400 and 1,500 cM (McCough et al. 1988; Murray et al. 1988). Thus, a relatively large part of the genome of barley was marked with the 28 markers, and is is likely that the study of a few other crosses with the same methodology could allow the production of a complete linkage map.

Selective effects

Two chromosome segments showed a significant distortion of segregation. As one of them was represented by a spot without an alternate allelic form (spot 16k), the non-conformity to the 1:1 segregation could be interpreted in another way, as already discussed. The other one is marked by three closely linked loci (17-1-4). As there was a positive gradient of deviation to the 1:1 ratio from locus 4 to locus 17 (the frequency of the DH allelic form in loci 4, 1, and 17 being, respectively, 0.645, 0.661, and 0.689), the chromosome part that is actually submitted to selection should lie on the other side of (or at) locus 17. Two other chromosome segments showed a segregation distortion, but with a significance level insufficient for this study ($p < 0.05$): other genotypes should be studied to confirm the distortion.

As the studied progeny were not compared to a F_2 progeny, it is not known whether selection took place at a stage common to both modes of reproduction (microspore production and maturation in the anther) or during the different steps specific to DH line production (induction of embryo formation from microspores and green plant development). It should noted that except for locus 16, all the possibly selected allelic forms originated from parental line DH8293, which itself is a DH line obtained by the *Hordeum bulbosum* method. This observation favors the hypothesis that the character submitted to selection is related to DH production, but is not specific to the method of haploid obtainment: e.g., ability to produce barley haploid embryos and, subsequently, plants. Nevertheless, whether the observed selection occurs only during DH line production or also during natural reproduction, the presence of the selected chromosome segment should be advantageous for DH production, at least with the two parental lines studied here; this is currently under investigation. If it is confirmed that the protein markers found in this study actually mark a chromosome segment that is actively selected during haploid production, they could become valuable tools for the breeders to select for improved doubled haploid production.

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Note added in proof

A restriction fragment length polymorphism map of barley developped on the basis of anther culture derived DH lines was recently published (Heun et al. 1991, Genome 34:437–447). Several loci showed also disturbed segregation.