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The inheritance of genetic markers in microspore-derived plants of barley *Hordeum vulgare* L.

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Summary. Biochemical, molecular and morphological markers have been used to monitor the segregation of alleles at major gene loci in microspore-derived lines of four spring barley crosses and their parents. Significant deviations from the expected Mendelian ratios were observed for four of the ten markers studied in the cross. Distorted ratios were associated with loci located on chromosomes 4H and 6H. The differential transmission of alleles was in favour of the responsive parent (Blenheim) used in the anther culture studies. For the *α-Amy-1* locus on chromosome 6H, the preferential transmission of Blenheim alleles was most pronounced in the haploid regenerants that were colchicine treated. These results are discussed in relation to the genetic control of androgenetic response in barley and with respect to the exploitation of another culture in barley improvement.

Key words: Doubled haploids – Microspore – Isozymes – RFLPs – Barley

Introduction

Haploidisation and subsequent chromosome doubling provides an opportunity to produce very quickly desired gene combinations from segregating material in the homozygous state. The increase in selection efficiency associated with doubled haploidy (DH) indicates that DH procedures offer considerable promise in plant breeding. Snape et al. (1986) have identified three criteria for the successful and cost-effective incorporation of DH into breeding programmes. These criteria are: the production of large numbers of DH of all genotypes should be easy and consistent, DH should be genetically normal and stable and the DH population should contain a random sample of the parental gametes.

Regeneration of plants from microspores is one of the methods available for haploid production in barley, *Hordeum vulgare* (Choo et al. 1985). Refinements in anther culture procedures, which include the substitution of sucrose by maltose in the culture medium (C. P. Hunter, personal communication; Finnie et al. 1989; Powell 1990), have resulted in a dramatic improvement in the efficiency of green plantlet regeneration from microspores. In addition, on maltose-based culture media, the mode of regeneration of plantlets tends to be via an embryogenic route rather than via a callus phase. The absence of an intermediate callus phase will contribute to the genetic stability of microspore-derived plantlets of barley. Two of the three criteria identified by Snape et al. (1986) for the inclusion of DH procedures in barley breeding therefore appear to have been satisfied. The third criterion relating to the utilisation of barley anther culture involves determining whether DH derived by anther culture are an unbiased sample of the parental gametes. A random sample of gametes is desirable if the potential genetic variation within a cross is to be fully exploited.

The segregation of alleles at specific loci may be used to test for the random assortment of genetic information in DH extracted from F_1 hybrids. Genetic analysis of morphological and disease response traits has been carried out in early studies of anther-derived plants of *Nicotiana tabacum* (Nakata 1971; Nakata and Kurihara 1972) and *Hyoscyamus niger* L. (Corduan 1975). In *N. tabacum*, the phenotypic ratios were found to be in

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agreement with the expected (1:1) segregation ratios, whereas in studies with *H. niger*, significant deviations from the expected ratios were observed.

Plants recovered from microspore culture may be skewed in the direction of one of the parents by selective regeneration of certain genotypes. Deviations from the expected gametic ratio among microspore-derived plants of broccoli were reported by Orton and Browers (1985). Powell et al. (1986) compared barley DH populations produced by anther culture and the *H. bulbosum* technique, and observed deviations from the expected gametic frequency for three of five loci in the anther-culture-derived DHs and for one locus among the *H. bulbosum* DHs. *Japonica* rice cultivars are more responsive in anther culture than *Indica* cultivars, and a skewed distribution for 4 out of 12 isozyme markers was observed by Guiderdoni et al. (1989) in microspore-derived plants from a *Japonica* × *Indica* rice hybrid. These results emphasise the need to monitor the segregation of alleles in DH populations derived from F_1 hybrids.

In this study the inheritance of ten marker genes located on five of the seven pairs of barley chromosomes in populations of microspore-derived lines derived from two pairs of reciprocal F_1 hybrids have been analysed. Both colchicine doubled haploid and spontaneous diploid lines have been included in the analysis.

Materials and methods

Plant material

Microspore-derived plants were regenerated from reciprocal crosses between the spring barley cultivar, Blenheim, and two breeding lines, TS264 and E224. The parents were chosen to complement the good malting quality characteristics of Blenheim with improved disease resistance genes from TS264 and E224. Methods for plantlet regeneration from microspores have been described in detail previously (Finnie et al. 1989).

Detection of polymorphism in parental material

Restriction fragment length polymorphism (RFLP). Leaf material was harvested from field-grown plots of anther culture lines derived from crosses between Blenheim × E224 and E224 × Blenheim. Total plant DNA was extracted from powdered, freeze-dried leaves by a modification of the CTAB extraction procedure (Murray and Thompson 1980). Following restriction endonuclease digestion (10 µg DNA), the resulting DNA fragments were fractionated on 1% agarose/TAE gels and transferred to HYBOND N+ charged nylon membrane (Amersham) by alkaline blotting (Reed and Mann 1985). Probe labelling and hybridisation conditions were as described by Sharp et al. (1988). The sources of clones used are given in Table 1.

Protein polymorphism. The isoelectric focusing (IEF) methods used have been described by Thompson et al. (1990). Polymorphic systems are given in Table 2 together with the chromosomal location of their structural genes.

Morphological markers. The microspore-derived lines, together with the three parents, were grown in replicated field experiments wherein they were treated with fungicide to control foliar pathogens. Eight weeks after sowing, the material was scored for juvenile growth habit. Blenheim possesses the "denso" dwarfing gene, which confers a semiprostrate juvenile growth habit. E224 possesses the alternative allele and, hence, doubled haploids derived from F_1 hybrids may be classified by visual observation for the alternative allele at this locus.

Table 1. Genetic markers used in the segregation analysis

Genetic marker	Chromosomal location (reference)	Method of detection	Parents exhibiting polymorphism
Hordein-1	1HS(1)	RFLP: pB11(6)	Blenheim/E224
Hordein-2	1HS(1)	RFLP: pCp387(6)	Blenheim/E224
rDNA	5HS(2)	RFLP: pBG35(7)	Blenheim/E224
Leaf esterase-1	3HL(3)	IEF	Blenheim/E224/TS264
Leaf esterase-2	3HL(3)	IEF	Blenheim/E224
β-Amylase-1	4HL(4)	IEF	Blenheim/TS264
Water-soluble protein-3	4H(5)	IEF	Blenheim/TS264
α-Amylase-1	6HL(3)	IEF	Blenheim/E224/TS264
Water-soluble protein-2	Unknown	IEF	Blenheim/TS264
Juvenile growth habit	Unknown	Morphological	Blenheim/E224

References – 1: Oram et al. 1975; 2: Saghai-Maroof et al. 1984; 3: Nielsen and Frydenberg 1971; 4: Powling et al. 1981; 5: Liu and Gale 1989 (WSP=1BF); 6: Forde et al. 1985; 7: Ellis et al. 1983

Results

The protein and RFLP profiles for the three parents are given in Fig. 1. The segregation ratios of the parental phenotypes for each marker are presented in Table 2. Segregation of alleles at the hordein loci on chromosome 1H does not deviate significantly from the expected 1:1 ratio. Similarly, alleles at the rDNA locus do not deviate significantly from the expected ratio. The polymorphism detected in the Blenheim × E224 cross and its reciprocal corresponds to the *Nor-H3* (*Rnr2*) locus, which segregates for the 6.2-kb fragment located to chromosome 5H (Saghai-Maroof et al. 1984). The leaf esterase loci, *Est-1* and *Est-2*, which are located on chromosome 3H, are polymorphic for the three parental genotypes. Segregation of alleles at these two loci does not differ significantly from the expected ratio (Table 2). Two isozyme loci located on chromosome 4H were used to characterise microspore-derived lines from the Blenheim × TS264 cross and its reciprocal. Alleles at both the *β-Amy-1* and *Wsp-3* locus do not deviate from the expected ratio in the Blenheim × TS264 cross, although there is an apparent

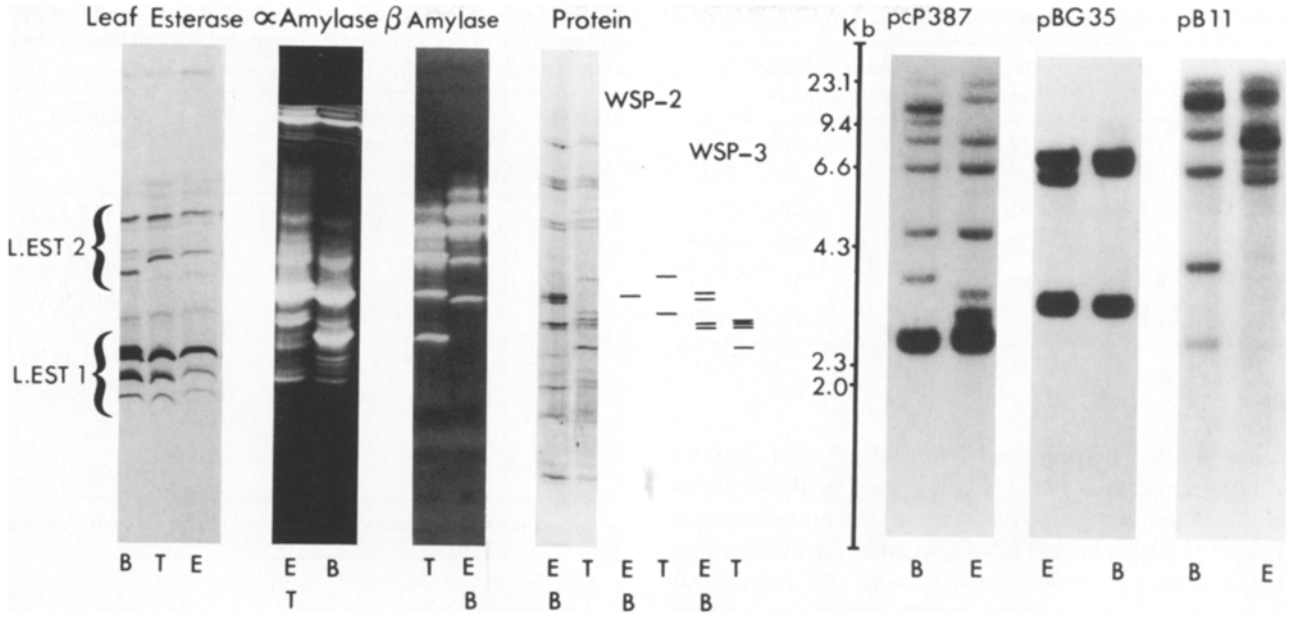

Fig. 1 Protein and RFLP phenotypes of parents B (Blenheim), E (E224) and T (TS264)

Table 2. Segregation of RFLP and isozyme markers in colchicine-doubled [AC(X)] and spontaneously doubled [AC(2X)], microspore-derived lines from Blenheim (B) × E224 and E224 × Blenheim

(a)		*PBG35 DNA*		Hordein-1		Hordein-1		Leaf EST-1		Leaf EST-2		α-AMY-1		JGH	
Cross	Group	B	E224	B	E224	B	E224	B	E224	B	E224	B	E224	B	E224
B × E224	AC (X)	14	13	14	14	–	–	19	19	5	6	27	9**	22	11
	AC (2X)	13	10	10	14	–	–	9	19	9	19	16	13	16	7
	Total	27	23	24	28	–	–	28	38	14	25	43	22**	38	18**
E224 × B	AC (X)	14	15	17	19	20	18	23	24	21	26	31	7***	23	15
	AC (2X)	3	4	7	9	7	5	8	9	8	10	10	5	13	3*
	Total	17	19	24	28	27	23	31	33	29	36	41	12***	36	18*

(b)		Leaf EST-1		Leaf EST-2		β-AMY-1		WSP-3		α-AMY-1		WSP-2	
Cross	Group	B	TS264	B	TS264	B	TS264	B	TS264	B	TS264	B	TS264
B × TS264	AX (X)	13	16	13	16	24	14	24	13	21	16	20	17
	AC (2X)	2	6	3	5	4	4	4	4	2	6	4	4
	Total	15	22	16	21	28	18	28	17	23	22		
TS264 × B	AC (X)	19	10	18	11	28	7***	27	7***	26	11*	22	12
	AC (2X)	5	7	7	5	8	3	6	5	4	7	5	6
	Total	24	17	25	16	36	10***	33	12***	30	18	27	18

* $P<0.05$, >0.01
** $P<0.01$, >0.001
*** $P<0.0001$
– Not determined

excess of Blenheim alleles. However, in the reciprocal cross (39), segregation of alleles at these two loci deviates significantly from the 1 : 1 ratio in the colchicine-treated, microspore-derived lines. Thus, for both loci there is an excess of the Blenheim phenotypes. With the exception of Blenheim (B) × TS264, the segregation of parental phenotypes for α-AMY-1 deviates significantly from the expected 1 : 1 ratio in the colchicine doubled haploid lines of all the crosses. There is an excess of Blenheim phenotypes in each case and the deviation is greater in Blenheim × E224 and its reciprocal.

The parental lines Blenheim and TS264 were found to be polymorphic for protein bands that have not as yet been located to a chromosome. These bands have been

Table 3. Segregation of biochemical markers in F_2 populations

Marker	Cross	Blenheim phenotype	Hetero-zygote	TS264/ E224	χ^2
β-AMY-1	TS264 × B	14		49	0.26
	B × TS264	76		178	3.3
L.EST-1	E224 × B	65		29	1.7
L.EST-2	E224 × B	23	46	24	0.05
WSP-3	B × TS264	93		33	0.10
WSP-2	TS264	84		42	4.67*

* $P<0.05$

called WSP-2 and segregate independently from WSP-3 (Thompson et al. 1990). The segregation of *Wsp-3* alleles in the anther-culture-derived lines occurs in the expected 1:1 ratio. Blenheim and E224 also differ for the morphological character juvenile growth habit, and the segregation of this character in the anther-culture-derived lines has been analysed. There is a tendency towards distorted segregation with an excess of Blenheim phenotypes, the greatest deviation occurring in the progeny derived by spontaneous chromosome doubling of haploids.

There was no evidence of reciprocal difference in the segregation patterns of alleles at the loci tested. A heterogeneity Chi-square analysis (summed over reciprocal crosses) for the *β-Amy-1* locus (B1 × TS264) indicated that both groups, i.e. the spontaneously doubled and the colchicine-treated haploids, agree in showing an excess of Blenheim phenotypes. However, when we consider the *α-Amy-1* locus (B1 × E224), there are significant differences ($P<0.05$) between the colchicine-treated and spontaneously doubled haploids for *α-Amy-1*.

In order to compare the transmission of alleles in selfed progenies, F_2 populations were analysed for the segregation of alleles at five protein loci (Table 3). For *β-Amy-1*, *L.Est-1*, *Wsp-3* and *Wsp-2* it was not possible to distinguish heterozygotes, and these loci were tested for deviations from a 3:1 ratio. With the exception of *Wsp-2* (which deviated only at the 5% level of significance), the segregation of alleles at these loci did not deviate significantly from the expected Mendelian ratios (Table 3), although *β-Amy-1* and *Wsp-3* deviated in the microspore-derived lines.

A number of the loci examined in the microspore-derived lines are located on the same chromosome. It is of interest to calculate the intrachromosomal map distance for linked loci and to compare the estimates obtained from the microspore lines with estimates from the F_2 populations and published data (Table 4). Where disturbed ratios were obtained in the microspore samples, the product method (Mather 1953) was used to detect and estimate linkage. For the hordein loci on chromosome 1H the recombination fraction is not significantly different from published data. No recombinant genotypes were detected in the microspore-derived lines for the esterase loci, and this is reflected in the low number of recombinants obtained in the F_2 generation. The recombination values for *β-Amy-1* and *Wsp-3* on chromosome 4H are not significantly different in the F_2 and the microspore-derived lines.

Table 4. Intrachromosomal linkage estimate obtained from F_2 and microspore-derived samples compared with published data (where available)

Marker	Chromo-somal location	Microspore samples	F_2	Published data
Hordein-1 and *-2*	1H	0.204 ± 0.041	–	0.138 ± 0.043 (Shewry et al. 1980)
Leaf esterases	3H	+	0.040 ± 0.020	0.006 ± 0.001 (Kahler and Allard 1970)
β-amylase and water-soluble protein-2	4H	0.023 ± 0.023	0.111 ± 0.022	–

+ No recombinant genotypes identified in the microspore samples

Discussion and conclusions

Markers on five of the seven pairs of barley chromosomes were used to monitor the segregation of alleles in the microspore-derived populations. Six of the ten genetic markers did not differ significantly from the expected Mendelian ratios in the progeny from F_1 barley hybrids. Differential survival of gametes is specific to those containing Blenheim alleles of *β-Amy-1* and *Wsp-3* on chromosome 4H and *α-Amy-1* on 6H. In addition there was an excess of Blenheim alleles at the juvenile growth habit locus in the cross between Blenheim and E224. The segregation of *Wsp-3* and *β-Amy-1* alleles in conventional, sexually derived F_2 progeny provides little evidence of differential survival of gametes specific to alleles at these loci.

Distortions in the segregation of marker alleles in anther-culture-derived populations have been reported. In barley, one report dealt with only a small population size (Powell et al. 1986); in another, the genetics of the disease response character being measured was not well understood, giving inconclusive results (Foroughi-Wehr and Friedt 1984). Hayward et al. (1990) detected disturbed segregation ratios for two isozyme loci, phosphoglucoisomerase (PGI) and glucamate-oxaloacetate transaminase (GOT), in androgenetic progeny from two

genotypes of *Lolium perenne*. In rice, 2 of 12 isozyme markers were shown to give distorted segregation, specifically in anther-culture-derived progeny (Guiderdoni et al. 1989). However, the progeny was derived from a *Japonica* × *Indica* cross and the distorted segregation effect may be similar to the non-Mendelian segregation that has been noted in interspecific crosses or crosses between distantly related material (Zamir and Tadmor 1986; McCouch et al. 1988).

In winter wheat, significantly distorted segregation of *Gpi-1* alleles was observed in anther-culture-derived progeny of hybrids of the anther-culture-responsive cultivar Slejpner, which carries the 1RS/1BL translocation, and seven other winter wheat cultivars with normal karyotype. Although the segregation of *Gpi-1* alleles was 1:1 among albino plants, the segregation was distorted among green regenerants in favour of the Slejpner allele (I. K. Due, personal communication). Wheat cultivars that carry the 1RS/1BL translocation have been shown to have a high anther culture regenerative ability (Agache et al. 1989), and it was suggested that the distortion in *Gpi-1* allele segregation was due to selection for the 1RS/1BL translocated chromosome.

It has been shown that response to anther culture that is strongly genotypically dependent. This has been illustrated for barley (Knudsen et al. 1989; Finnie et al. 1989; Powell 1988) and for a number of other crops, including wheat (Bullock et al. 1982), rice (Miah et al. 1985) and broccoli (Orton and Browers 1985). In the present study, four of the genetic markers showed distorted segregation in favour of the Blenheim alleles, and Finnie et al. (1989) have shown that the cultivar Blenheim is highly responsive to anther culture, with an average of 3.5 green plants being produced per spike cultured, whereas the lines TS264 and E224 were less responsive. All four F_1 hybrids involving the three parental genotypes were very responsive to anther culture, suggesting that the genetic response factors from Blenheim were dominant in the F_1. The results presented here may indicate that some of these genetic factors are linked to *β-Amy-1* and *α-Amy-1* loci and are therefore located on chromosomes 4H and 6H, respectively. Lazar et al. (1987), working with wheat/rye chromosome addition lines, demonstrated that rye chromosome 4 contains factors that significantly enhance anther culture response. Alternatively, the amylase alleles may be involved in the mobilisation of carbohydrates and, hence, may be the subject of selection, as suggested by Pedersen (1988).

The segregation pattern for alleles at the *a-Amy-1* locus on chromosome 6H differs significantly between the two groups, i.e. between the colchicine-treated and the spontaneously derived diploids, with the colchicine-treated group containing a significant excess of Blenheim alleles. The anther culture methodology used to derive the lines studied here produced significantly more haploids than spontaneous diploids (Finnie et al. 1989). There is evidence from studies with *Brassica napus* (Siebel and Pauls 1989) that the proportion of haploids to spontaneous diploids decreases with increasing time in culture. The high number of haploids produced and analysed in the present study may reflect the sampling of highly responsive gametes containing alleles conditioning androgenetic response. Since Blenheim is the more responsive parent, an excess of Blenheim alleles would be predicted. Furthermore, the responsive gametes would be expected to respond most rapidly and regenerate viable green plants. These gametes would be in culture for the least length of time and would have less opportunity for chromosome doubling to occur.

The availability of biochemical and molecular markers has made it possible to monitor the segregation of alleles at major gene loci in microspore-derived lines. It will be important to investigate the consequences of distorted segregation on the agronomic performance of these populations. For example, allelic variation at the *α-Amy-1* locus made a large contribution to the genetic variation for single-plant yield in barley (Powell et al. 1990). The differential transmission of specific regions of the barley genome may therefore have beneficial effects on traits of economic and commercial importance.

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