Frequency changes in storage protein genes in a hybrid bulk population of barley

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Changes of frequency at three hordein loci were investigated in Composite Cross Five (CCV) of barley using SDS-PAGE of endosperm proteins. The frequency of individual banding patterns, as well as pattern combinations, showed directional trends in successive generations. This suggests that selection has favoured certain allelic combinations at two hordein loci, *Hor1* and *Hor2*. We argue that the selective advantage of resistance to powdery mildew, the inbreeding nature of barley, and linkage between some major mildew resistance genes and the hordein loci could have led to the observed shifts.

Keywords: barley, composite cross, hordein, powdery mildew, selection.

Introduction

Biochemical markers, especially allozymes, have been extensively studied in various composite cross populations of barley and used to demonstrate the effects of natural selection (Allard *et al.*, 1972; Edwards, 1975; Clegg *et al.*, 1978), the hitchhiking of neutral alleles with genes that are targets of selection (Luckett, 1982; Luckett & Edwards, 1986), the multilocus structure of populations of predominantly inbreeding species (Weir *et al.*, 1972, 1974), as well as for quantifying genetic diversity (Brown, 1978; Knight, 1991) and estimating levels of outcrossing in barley (Kahler *et al.*, 1975; Luckett, 1982; see also Allard, 1988).

The B hordeins are encoded by a single structural gene, *Hor2*, located on the short arm of chromosome 5, 7–8 cM distal to the *Hor1* gene which codes for the C hordeins (Jensen *et al.*, 1980; Shewry *et al.*, 1981). The *Hor3* locus which encodes the D hordeins is located on the long arm of chromosome 5 (Shewry *et al.*, 1983). The estimated map distance between *Hor1* and *Hor3* is 65 cM and the gene order is *Hor2-Hor1-Hor3* (Shewry *et al.*, 1984).

Hordein fractions separated from a range of cultivars using SDS-PAGE show extensive variation in the banding pattern of polypeptides within each of the B and C hordein groups. In contrast, the D

hordein of European cultivars of barley consists of a single component polypeptide, but two components have been reported in other cultivars (Field *et al.*, 1982; Shewry *et al.*, 1982). The polypeptide banding patterns of B and C hordeins have been used as markers for varietal identification (Shewry *et al.*, 1978a; Doll & Brown, 1979). Because *Hor1* and *Hor2* are closely linked to loci for mildew (*Mla*, *Mlat*, *MIga*, *Mlk*, *Mlnn* and *Mlra*) and yellow rust (*Yr4*) resistances, a number of hordein patterns in European cultivars of barley are associated with specific resistance alleles (Shewry *et al.*, 1981; Jensen *et al.*, 1980; Kreis *et al.*, 1985; Jørgensen, 1994).

In a previous study (Ibrahim & Barrett, 1991), we showed that three subpopulations of a composite cross population of barley, Cambridge CCV, have evolved towards significantly higher resistance to powdery mildew caused by *Erysiphe graminis* f. sp. *hordei*.

Materials and methods

Plant material

Composite Cross Five (CCV) was developed between 1937 and 1940 by hierarchical crossing of 30 diverse accessions from the USDA collection of barley (Suneson, 1956). The bulked hybrid seeds were designated F₁ and subsequent generations have been grown at Davis, California since 1941. The material has been maintained as discrete generations in large populations, with no conscious selection (Jain & Allard, 1960). In 1974, large samples of seed from the F₁₀, F₂₀ and F₃₀ generations of Californian

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CCV were used to establish three parallel populations in Cambridge, U.K. — Populations 1, 2 and 3 of Cambridge CCV, respectively (Luckett, 1982; Luckett & Sharif, 1987; Ibrahim, 1989; Ibrahim & Barrett, 1991).

The plant material used in this study consisted of the 100 family lines derived from each of the F_{12} , F_{15} , F_{18} and F_{21} generations of Population 1, Cambridge CCV, as described by Ibrahim & Barrett (1991).

Hordein extraction and separation

Individual seeds were cut transversely and the part distal to the embryo was crushed using a pair of pliers the serrated jaws of which had been machined to a smooth surface. A single solvent mixture containing 2-mercaptoethanol, as described by Smith & Payne (1984), was used to extract the endosperm proteins from the crushed sample. During the extraction, the proteins are reduced and denatured allowing individual subunits to be resolved by gel electrophoresis.

The polypeptide units were separated in 17.5 per cent acrylamide gels (3 per cent stacking gel) and stained with Coomassie Blue R, as described by Smith & Payne (1984). The number, width and migration distance of the different bands were scored on each gel. Following the convention of Shewry *et al.* (1978a,b), the band patterns of the B hordeins were given numbers and those of the C and D hordeins were assigned letter codes.

Mildew resistance

Reaction to natural infection from the air spora was scored on the parent plants from which the 400 family lines were derived; the plants were scored on a scale of 0 (highly resistant) to 5 (highly susceptible) (for details see Ibrahim & Barrett, 1991) and regrouped into two categories 'resistant' (0–2) and 'susceptible' (3–5) for analysis. In addition, seedlings from 99 family lines from the F_{12} and F_{21} generations were grown in a disease-free environment and tested for resistance to four mildew isolates with known virulence spectra (for details, see Ibrahim & Barrett, 1991).

Results

Hordeins

The frequencies of the B and C hordein patterns (Table 1) show that there is a gradual decrease in

Table 1 Frequency of hordein band patterns in 400 family lines from four generations of barley Population 1, Cambridge CCV

Hordeins	Pattern code	Generations			
		F_{12}	F_{15}	F_{18}	F_{21}
B	1	31	25	22	19
	2	28	9	7	5
	3	5	7	3	5
	4	8	12	7	3
	5	11	17	1	3
	6	8	19	53	59
	7†	3	1	0	0
	8†	3	2	5	1
	9†	3	8	2	5
C	A	14	22	65	70
	A1	40	27	13	11
	B	13	18	9	9
	B1	5	17	3	4
	C	20	12	8	6
	D†	2	2	1	0
	E†	2	2	1	0
D	G†	4	0	0	0
	S	95	84	94	95
	F	5	16	6	5

Tests of association between these hordein patterns and the four generations gave χ^2 values of 111.28 (d.f. = 18, $P < 0.001$), 118.46 (d.f. = 15, $P < 0.001$) and 9.32 (d.f. = 3, $0.01 < P < 0.05$) for B, C and D hordeins, respectively (see Table 3b). † indicates the pattern classes that were pooled to avoid small expected values.

the frequency of all but one of the patterns of both hordein groups. For the B hordeins, pattern 6 has become the predominant type by F_{18} , although it was one of the least common patterns in F_{12} . Similarly, pattern A of the C hordeins which was present in 14 per cent of the lines in F_{12} marked 70 per cent of the lines in F_{21} . There is also a reduction in the total number of patterns in successive generations. Nine B hordein patterns were detected in the F_{12} and F_{15} lines, whereas in F_{18} and F_{21} there were only eight. Similarly, eight, seven, seven and five distinct C hordein patterns were detected in the F_{12} , F_{15} , F_{18} and F_{21} lines, respectively. It is clear also that there has been a directional shift towards increased frequency of specific patterns of both hordein groups in successive generations. The two D hordein patterns, S and F, showed frequency fluctuations in successive generations, but no directional trend was evident.

B and C hordein pattern combinations might be expected to show a high level of association because of the tight linkage between their respective loci, *Hor2* and *Hor1*. With nine and eight variants, respectively, the number of possible pattern combinations between the B and C hordeins is 72. The actual number observed in the total sample (i.e. before pooling for analysis) was 36. There were 28,

25, 19 and 15 distinct pattern combinations in F_{12} , F_{15} , F_{18} and F_{21} , respectively, again clearly indicating a gradual loss of some genetic diversity. Table 2 shows the frequency of pattern combinations in successive generations after combining B and C hordein patterns present at less than 5 per cent into the pooled classes, 0 and P, respectively. This reduced the number of B and C hordein patterns to

Table 2 The frequency of hordein pattern combinations in four generations of Cambridge CCV of barley

B/C hordeins	Generations				Total	
	F_{12}	F_{15}	F_{18}	F_{21}	R	S
1/A	3	5	4	9	11	11
1/A1	14	2	5	2	6	17
1/B	5	1	6	4	4	12
1/B1	4	15	3	4	5	21
1/C	3	0	2	0	1	4
1/P	2	2	1	0	0	5
2/A	2	1	2	1	3	3
2/A1	19	6	4	4	7	26
2/C	5	2	1	0	0	8
2/P	2	0	0	0	0	2
3/B	0	0	0	1	0	1
3/C	5	6	3	4	7	11
3/P	0	1	0	0	0	1
4/A	1	0	1	0	2	0
4/A1	2	8	3	3	4	12
4/B	0	1	0	0	0	1
4/B1	1	1	0	0	0	2
4/C	2	2	2	0	1	5
4/P	2	0	1	0	1	2
5/A	0	3	0	0	2	1
5/A1	2	4	0	1	3	4
5/B	5	8	1	0	1	13
5/C	4	2	0	2	1	7
6/A	5	13	53	59	109	21
6/A1	2	4	0	0	3	3
6/B1	0	1	0	0	0	1
6/C	1	0	0	0	0	1
6/P	0	1	0	0	0	1
0/A	3	0	4	1	4	4
0/A	1	3	1	1	1	5
0/B	3	8	2	4	4	13
0/P	2	0	0	0	0	2
No. of lines	100	100	100	100	180	220
B/C combinations	26	24	19	15	21	31
H' (diversity)	2.91	2.82	1.97	1.66	1.77	3.02
Maximum H'	3.26	3.18	2.94	2.71	3.04	3.43
Rel. diversity	0.89	0.89	0.67	0.61	0.58	0.88

The Total columns show the patterns of the lines resistant (R) or susceptible (S) to powdery mildew summed over all generations. Patterns '0' and 'P' are the pooled categories for the B and C hordeins, respectively. Relative diversity = $H'/\text{maximum } H'$ (see text).

seven and six, respectively, and hence there were 42 possible pattern combinations of which only 32 were detected in the total sample.

Six combinations (eight individuals) that were not present in F_{12} were detected in the later generations. Five of these (seven individuals) were in F_{15} , whereas the remaining one was detected in the F_{21} lines. These new combinations were all present at frequencies less than 5 per cent. It is possible that they were present in F_{12} but were not detected because of the limited sample size. It is also possible that they originated from segregating families arising from residual heterozygosity (from the crossing stages) or the rare outcrossing (less than 1 per cent) reported in the Cambridge CCV populations (Luckett, 1982).

A crude indication of the relative decline in multi-locus diversity can be shown using H' , the Shannon-Weaver diversity index (Shannon & Weaver, 1964) which integrates the changes in the numbers of genotypes and their frequencies. For an infinite population, $H' = -\sum p_i \ln p_i$ where p_i is the frequency of genotype i ; the bias in H' because of finite sample size is small enough to be ignored in most applications (Peet, 1974). Table 2 shows the values of H' and Relative Diversity ($=H'/\text{maximum } H'$) for each of the generations; maximum H' is estimated by $\ln N$ where N is the number of genotypes. For consistency, both indices have been estimated using the pooled categories; the use of all genotypes, without pooling, makes little difference to either index. The decline in absolute and relative diversity in successive generations seen for each of the hordein loci is also evident in the pattern combinations.

There is also clear evidence of linkage disequilibrium between variants at the *Hor1* and *Hor2* loci. Single combinations of the two always account for more than 40 per cent of the lines showing a particular B pattern. For example, 67.35 per cent of all lines marked by B pattern 2 had the A1 pattern of the C hordeins. Similarly 3/C, 4/A1, 5/B, 6/A and 0/B combinations accounted for 90 per cent, 53 per cent, 44 per cent, 94 per cent and 52 per cent of all the lines showing the respective B patterns. Pattern 1 of the B hordeins appears to have 'recombined' freely with four C patterns, A, A1, B and B1. A striking aspect of the data in Table 2 is the marked increase in the frequency of the 6A pattern combination in F_{18} and F_{21} at the expense of nearly all other pattern combinations. The frequency of lines marked by the 6A combination increased from 5 per cent in F_{12} to 59 per cent in F_{21} .

Hordeins and mildew resistance

The frequency changes observed in the hordein banding patterns of the successive generations of Cambridge CCV were comparable to those previously reported for reaction to powdery mildew (Ibrahim & Barrett, 1991). Because both characters were studied on the same family lines, it was possible to test for association between them, and to investigate whether the shifts in hordein banding patterns represent a correlated response to selection for mildew resistance.

Table 3a lists the numbers of the family lines which showed the different hordein banding patterns and their reaction to natural infection. Patterns occurring at frequencies less than 5 per cent were pooled, as described above. Taking each hordein locus in turn, the data were analysed as three-dimensional contingency tables, following the procedures for log-linear models described by Bishop *et al.* (1975) and Sokal & Rohlf (1981). To avoid problems with cells containing zero, 0.5 was added to all cell entries before analysis. The sequence of fitting in each case was to test for the three-way interaction (Generations (G) $\times$ Disease Reaction (R) $\times$ Hordein Pattern (P)) and then to remove sequentially the two-way interactions for Hordein Pattern $\times$ Disease Reaction, Hordein Pattern $\times$ Generations and finally Disease Reaction $\times$ Generations (see Table 3b). Analyses of five-way (*HorB* $\times$ *HorC* $\times$ *HorD* $\times$ Disease Reaction $\times$ Generations) and four-way (omitting *HorD*) tables were not justified because of the small number of observed three-locus and two-locus genotype combinations, relative to the possible number of combinations (see previous section and Table 2). To reduce the chances of Type I error, tests yielding probability values greater than 1 per cent were considered to be 'not significant'. There was no three-way interaction for any of the three loci, indicating that the form of association between disease reaction and hordein phenotype does not vary among generations. In the case of the B and C hordeins, all two-way interactions were highly significant. These indicate that both disease reaction and hordein pattern frequencies were, individually, changing with time and that there was a strong association between disease reaction and hordein pattern at these two loci. In the case of the D hordeins, the only strong effect was the change in disease reaction with time, which is to be expected because the data for disease reaction in each generation are the same for all three loci.

In the total sample of 400 family lines, 180 were resistant to natural infection, and, of these, 112 (62.2

Table 3a The hordein banding pattern and the reaction to natural infection (R = resistant, S = susceptible) of the 400 family lines from the four generations of barley Population 1, Cambridge CCV

Band pattern	Generation									
	F ₁₂		F ₁₅		F ₁₈		F ₂₁		Total	
	R	S	R	S	R	S	R	S	R	S
B hordeins										
1	5	26	2	23	7	15	13	6	27	70
2	6	22	0	9	1	6	3	2	10	39
3	2	3	1	6	1	2	3	2	7	13
4	3	5	1	11	2	5	2	1	8	22
5	2	9	3	14	0	1	2	1	7	25
6	4	4	10	9	49	4	49	10	112	27
0	0	9	0	11	5	2	4	2	9	24
C hordeins										
A	7	7	11	11	56	9	57	13	131	40
A1	10	30	5	22	3	10	6	5	24	67
B	1	12	0	18	2	7	6	3	9	40
B1	0	5	0	17	2	1	3	1	5	24
C	3	17	1	11	2	6	4	2	10	36
P	1	7	0	4	0	2	0	0	1	13
D hordeins										
F	0	5	0	16	3	3	4	1	7	25
S	22	73	17	67	62	32	72	23	173	195
Total	22	78	17	83	65	35	76	24	180	220

Table 3b Summary of the fully hierarchical log-linear analyses for independence of pattern (P), disease reaction (R) and generations (G) for each hordein type (data from Table 3(a))

Interaction term removed and (goodness-of-fit) after removal	B hordein pattern		C hordein pattern		D hordein pattern	
	d.f.	χ^2	d.f.	χ^2	d.f.	χ^2
Pattern $\times$ reaction $\times$ generations (Goodness-of-fit)	18	20.41 NS	15	16.02 NS	3	2.09 NS
Pattern $\times$ reaction (Goodness-of-fit)	6	55.22***	5	57.07***	1	3.61 NS
independence of P and R at each level of G	(24	75.63***)	(20	73.08***)	(4	5.70 NS)
Pattern $\times$ generations (Goodness-of-fit)	18	111.28***	15	118.46***	3	9.32*
independence of G and combinations of P and R	(42	186.91***)	(35	191.54***)	(7	15.02*)
Reaction $\times$ generations (Goodness-of-fit)	3	105.76***	3	106.89***	3	111.68***
complete independence	(45	292.68***)	(38	298.43***)	(10	126.69***)

per cent) showed B pattern number six. These make up 80.6 per cent of all of the lines that had this pattern. Pattern A of the C hordeins marked 72.8 per cent of the resistant lines and these accounted for 76.6 per cent of the lines showing this pattern. Among D hordein patterns nearly all resistant lines (96.1 per cent) appeared to have the slow band (S), but a substantial proportion of the susceptible plants (88.6 per cent) also showed this pattern.

We have already shown that the number of B/C pattern combinations detected decreased in successive generations. The distribution of pattern combinations in the mildew reaction classes provides an explanation for the reduction of diversity over generations (Table 2). There were 21 pattern combinations in the resistant lines, the most frequent being 6/A which accounted for 60.6 per cent of the resistant lines. The second most common pattern combination was 1/A and it accounted for only 6.1 per cent of the resistant lines. In contrast, 31 of the 32 pattern combinations detected in the total sample were present in the susceptible lines; the most frequent pattern in this class, 2/A1 accounted for only 11.8 per cent of the lines. Hence selection for resistance to powdery mildew may have led to a reduction in diversity of pattern combinations among resistant lines. This is supported by the observation that the hordein pattern diversity in the susceptible lines, as measured by the Shannon-Weaver index, was higher than the diversity in the resistant lines (Table 2).

The fully hierarchical log-linear analyses of the results for the tests of 99 family lines against four powdery mildew isolates with known virulence spectra were carried out separately for each hordein locus. There was a highly significant three-way interaction between hordein pattern, mildew isolate and disease reaction in the analyses of the B hordeins ($\chi^2_9 = 63.79^{***}$) and C hordeins ($\chi^2_9 = 40.52^{***}$) but not for the D hordein patterns ($\chi^2_3 = 1.59$ NS); application of Williams's Correction (Williams, 1976) on the significant three-way interactions did not remove the significance (B hordein patterns, $\chi^2_9 = 56.64^{***}$; C hordein patterns, $\chi^2_9 = 35.72^{***}$). This implies that the degree of association between these hordein patterns and disease reaction differs for different isolates. In this case, tests of association between hordein phenotype and mildew reaction are only useful within each mildew isolate level (these tests are shown in Table 4). For the D hordeins, the test for a three-way interaction was not significant ($\chi^2_1 = 1.59$ NS). However, there were highly significant two-way interactions between hordein pattern and disease reaction ($\chi^2_1 = 17.09$; see also Table 4)

and, as might be expected, between mildew isolate and disease reaction ($\chi^2_3 = 39.60$).

Reactions to isolates CC/1, CC/99 and CC/128 were found to be significantly associated with the B and C hordein pattern variants, whereas D patterns only showed a significant association in their reaction to CC/1 (Table 4). Reaction to the fourth isolate CC/124, which did not vary between generations (Ibrahim & Barrett, 1991), showed no significant association with the hordein variants (Table 4). Where significant association between reaction to mildew isolates and hordein variants was present, the pattern was similar to that observed between the reaction to natural infection and hordein patterns. Pattern 6 of B hordein and pattern A of C hordein were by far the most common in the lines resistant to the three isolates CC/1, CC/99 and CC/128 (Table 4).

Discussion

Repeated tests of the family lines have shown that the different B and C hordein patterns are both replicable and distinct from one another. Nevertheless, two-dimensional electrophoresis has shown that differences may exist between patterns which are apparently identical when using one-dimensional electrophoresis (Shewry *et al.*, 1978b). Hence, the separation procedure adopted here may not have resolved all of the pattern variants, and 'identical' band patterns may not be the product of the same allele. The data presented therefore provide a conservative estimate of the level of polymorphism at the three hordein loci.

Because of the tight linkage between the *Hor1* and *Hor2* loci, linkage equilibrium between these two loci may not have been achieved during the initial crossing phase, and, because of the inbreeding nature of barley, any initial linkage disequilibrium will persist in the absence of selection. In the presence of selection on one or both of these loci, or on a locus (or loci) with which they are also in linkage disequilibrium, the initial linkage disequilibrium will change over the generations because of the hitch-hiking effect. The frequency shifts of the patterns at each locus, individually, and of the multi-locus combinations suggest that selection has favoured certain alleles at one or both of these loci, or other loci in linkage disequilibrium with the *Hor1* and *Hor2* loci. The 6 A pattern combination, in particular, appears to have marked plants that have been favoured by selection.

When the genetic composition of populations of inbreeding species shows consistent directional

Table 4 The hordein banding patterns and reaction to four mildew isolates (R = resistant, S = susceptible) of 99 family lines derived from F₁₂ and F₂₁ of barley Population 1, Cambridge CCV

Isolate		Hordein pattern													
		B							C					D	
		0†	1	2	3†	4†	5†	6	A	A1	B	C†	D†	F	S
CC/1	R	1	9	2	0	0	0	36	38	7	2	0	1	0	48
	S	7	22	7	3	4	8	0	4	14	21	9	3	8	43
		(63.37) $P < 10^{-6}$							(54.82) $P < 10^{-6}$					(8.19) $P = 0.004$	
CC/99	R	0	3	2	0	1	0	35	36	2	2	1	0	1	40
	S	8	28	7	3	3	8	1	6	19	21	8	4	7	51
		(73.47) $P < 10^{-6}$							(59.02) $P < 10^{-6}$					(3.00) $P = 0.083$	
CC/124	R	0	6	3	0	0	0	2	4	5	2	0	0	0	11
	S	8	25	6	3	4	8	34	38	16	21	9	4	8	80
		(10.63) $P = 0.016$							(5.30) $P = 0.151$					(1.09) $P = 0.296$	
CC/128	R	0	5	1	0	0	0	35	38	2	1	0	0	0	41
	S	8	26	8	3	4	8	1	4	19	22	9	4	8	50
		(74.05) $P < 10^{-6}$							(72.68) $P < 10^{-6}$					(6.15) $P = 0.013$	

Figures in brackets for each subtable are the χ^2 values for tests of association between the patterns at individual hordein loci and their reaction to each isolate; d.f. = 3 for all tests involving B hordeins and C hordeins and d.f. = 1 for all tests involving D hordeins. Columns marked with † indicate patterns that were pooled at each locus to avoid small expected values.

change over time because of natural selection, it is difficult to identify a particular character as the target of selection. In a previous study (Ibrahim & Barrett, 1991), we have argued that natural infection by mildew could be a major selective force responsible for the changes observed in Cambridge CCV. Here we argue that the linkage between some hordein and mildew resistance loci (Jensen *et al.*, 1980; Shewry *et al.*, 1981) could have led to directional changes in the frequencies of alleles at the hordein loci.

In spite of the magnitude of the shifts favouring particular genotypes, a substantial amount of genetic variation is still retained in Cambridge CCV. This is evident from the fact that, despite the limited sample size, we were able to detect in F₂₁ all but four rare patterns (frequency less than 5 per cent in F₁₂) of the 19 band patterns observed in F₁₂ (Table 1). The mean expected panmictic heterozygosity

($H_e = \sum p_i(1-p_i)$ where p_i is the frequency of the i th allele) averaged over the three hordein loci was 0.5497, 0.6364, 0.4383 and 0.3953 in F₁₂, F₁₅, F₁₈ and F₂₁, respectively (Brown, 1978; Asins & Carbonell, 1987); this indicates that genetic diversity at the hordein loci has not been seriously eroded when measured on a per-locus basis.

However, in terms of multilocus genotypes in the population, there has been a demonstrable decline in genetic diversity over 10 generations (Table 2). Although it seems reasonably obvious that bulk populations can have a role in genetic conservation (Ford-Lloyd & Jackson, 1986; Allard, 1990), different strategies might have to be adopted in the case of inbreeding species, like barley, depending on whether the objective is to conserve multilocus genetic structure or genetic diversity locus-by-locus. This is because linkage disequilibria caused by both drift and selection will lead to a higher rate of loss

for multilocus genotypes than for alleles at individual loci.

Acknowledgements

We wish to thank Professor M. S. Wolfe for providing laboratory facilities and guidance for the mildew isolate tests, and Mr John Spencer for technical assistance and helpful discussions.

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