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Mapping of the *Hor*-3 Locus Encoding D Hordein in Barley*

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Summary. The hordein storage proteins of barley (*Hordeum vulgare* L.) are of intense interest due to their genetic diversity and prominence and impact on the industrial and agricultural uses of the seed. Two major hordein loci have been previously mapped on chromosome 5 (*Hor*-1 and *Hor*-2 encoding the C and B hordeins, respectively). A third major locus, *Hor*-3, which codes for D hordein, has been located in the centromeric region of chromosome 5, probably on the long arm. Two allelic variants with apparent molecular weights of 83,000 and 91,000 and similar isoelectric points of 8.0 comprise the products of this locus in the barley varieties 'Advance' and 'Triple Awned Lemma'. The D hordein examined is similar in molecular weight and isoelectric point to the high molecular weight (HMW) glutenin proteins encoded by the 1B chromosome of wheat (*Triticum aestivum* L.)

Key words: Storage proteins – Hordein D – Three point test – *Hordeum vulgare*

Introduction

The hordeins comprise somewhat more than half of all the protein found in barley (*Hordeum vulgare*) seed of normal lysine content (Doll and Andersen 1981). Reported values have risen over the past century primarily due to improved methods for hordein extraction. Inclusion of reducing agents (Shewry et al. 1978), modification of solvent constituents (Shewry et al. 1980b) and inclusion of a buffer in the extraction medium (Doll and Andersen 1981) have all improved extraction efficiency. Analytical techniques have improved as well. Discontinuous polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) separates hordeins by molecular weight, and has allowed the genetic characterization of two loci, *Hor*-1 and *Hor*-2, which encode the C and B hordeins, respectively (Oram et al. 1975; Doll and Brown 1979; Shewry et al. 1980a). These loci are located on the short arm of chromosome 5. The *Hor*-2 locus which codes for B hordein has been variously estimated to lie 7 to 17 centimorgans distal with respect to the *Hor*-1 locus (Doll and Brown 1979; Jensen 1981).

Both loci are complex, each encoding several proteins (Holder and Ingversen 1978; Faulks et al. 1981; Schmitt and Svendsen 1980a, b; Shewry et al. 1981). The extremely high level of genetic diversity at these loci led to the development of the concept of selectively neutral genetic variation accumulating within each of these complex gene families (Doll and Brown 1979). Several authors (Shewry et al. 1980a, 1982; Cameron-Mills 1980; Cameron-Mills and von Wettstein 1980; Doll and Andersen 1981; Miflin et al. 1981) have observed a relatively invariant high molecular weight protein in their hordein samples. It has the solubility characteristics of hordeins, and has been located within purified protein bodies (Cameron-Mills 1980; Miflin et al. 1981). The lack of genetic diversity led to little interest in this protein. Recently, Hash and Blake (1981) characterized electrophoretic variants of this high molecular weight hordein.

F_2 populations were developed to determine linkage relationships between the gene(s) encoding these proteins with the *Hor*-1 and *Hor*-2 loci. Isoelectric points and apparent molecular weights of two variants were characterized and their similarities to the high molecular weight glutenin subunits coded by the 1B chromosome (Holt et al. 1981) of wheat (*Triticum aestivum* L.) are discussed.

Materials and Methods

Plant Material

The barley cultivars 'Advance' and 'Triple Awned Lemma' (CI 14747) and F_1 and F_2 populations were grown in the field

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at Washington State University Spillman Agronomy Farm, or in a greenhouse. Triple Awned Lemma seed was supplied through the USDA world barley collection by Dr. Wayne Porter.

One-Dimensional Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Recombination Analysis

Distal half seeds were crushed with pliers into 1.8 ml polypropylene centrifuge tubes. Extraction medium containing 55% 2-propanol, 4 mM dithiothreitol, 0.37 M Tris-Cl pH 8.8 was prepared fresh daily, and 0.5 ml was added to each sample. Samples were incubated at 60 °C for 60 min in a shaking waterbath. Following centrifugation at 12,800 g for 3 min, the supernatants were decanted into fresh centrifuge tubes, heated to 50 °C and alkylated by the addition of 10 µl 0.5 M iodoacetamide (Doll and Andersen 1981). After 15 min, 0.8 ml glass distilled water was added to the samples which were stored at 4 °C overnight. Precipitated hordeins were collected by centrifugation at 12,800 g for 5 min and were dissolved in 200 µl sample buffer containing 0.0625 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 4 mM DTT (Laemmli 1970).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) in 12.5% acrylamide, 0.1% bisacrylamide gels with dimensions of 11 cm × 18 cm × 0.75 mm. A two centimeter stacking gel (3% acrylamide) containing either 10 or 20 sample wells/gel overlaid the resolving gel. Samples were electrophoresed at a constant current of 15 mA per gel until the tracking dye reached the end of the gel (approximately 3 h). Gels were cooled with tap water (approximately 14 °C) throughout the run. Following electrophoresis, gels were stained overnight in 0.1% Coomassie Blue in 20% v/v methanol, 6% acetic acid and destained in 25% methanol, 8% acetic acid. Apparent molecular weights were determined by comparison with the standards lysozyme (14,000), β-lactoglobin (18,000), carbonic anhydrase (32,000), ovalbumin (45,000) and bovine serum albumin (68,000) obtained through Polysciences.

Three hundred F_2 seeds were analyzed for recombination among the three hordein loci. Two replicates per F_2 seed were assessed. Recombination in the F_2 population was analyzed according to the maximum likelihood method as described by Allard (1956) and the approximate and maximum likelihood methods for three point tests described by Søgaard (1974, 1977).

Two-Dimensional Electrophoresis

Isoelectric focusing was in 5% polyacrylamide gels containing 1% pH 3–10 ampholines, 1% Tween-80, and 8 M urea. Gel tubes were 10 cm × 5 mm interior diameter and gels were prefocused at 400 V for 1 h prior to sample application. A 10 gram flour sample of each genotype was ground in a UDY mill to pass a 0.5 mm screen, thrice extracted with 20 ml extraction medium at 60 °C for 1 h, the supernatants bulked and hordeins precipitated by the addition of 2 volumes glass distilled water and overnight storage at 4 °C. These non-alkylated hordeins were collected by centrifugation (10,000 g for 20 min) and redissolved in 6 M urea, 4 mM dithiothreitol, 37 mM Tris-HCl pH 8.8 at room temperature. Samples were diluted to give an approximate protein concentration of 15 mg/ml. Twenty microliters (300 µg protein) of each sample were applied to isofocusing gels and overlaid with 100 µl of either 2% SDS in 0.37% mM Tris-HCl pH 8.8, 10% glycerol or 4% ampholines in 6 M urea.

Gels were photographed under tungsten light without filters. Replica gels were sliced and extracted into glass distilled water to determine the pH gradient.

Results

Electrophoretic Banding Patterns of Hordeins from Seed of Advance, Triple Awned Lemma and F_2 Progeny

The banding patterns of representative genotypes of F_2 seed from the cross Triple Awned Lemma × Advance are shown in Fig. 1. This particular cross was selected for analysis due to the distinctive B, C, and D hordein phenotypes produced by each parental allele. These alleles are expressed codominantly in the triploid endosperm. Differential expression of parental hordeins in heterozygote endosperms has been attributed to a difference in dosage of the parental alleles in the endosperm tissue (Doll and Brown 1979). This may account for the difference in apparent banding pattern between B hordein in lanes 5 and 7 of Fig. 1. Both are from tissue heterozygous at the *Hor*-2 locus (B hordein), but the Advance B complement is expressed much more strongly in the lane 7 extract than in the lane 5 extract. Presumably, the endosperm from which the extract of lane 7 was derived carried two doses of the Advance B hordein allele, while the analogous endosperm for lane 5 carried two doses of the Triple Awned Lemma B hordein allele. Heterozygotes at the *Hor*-1 locus (C hordein) may be characterized by the presence of the most prominent Advance C hordein which has a slightly slower mobility than the prominent group of Triple Awned Lemma C hordein. D hordein phenotypes are quite well defined with homozygotes showing

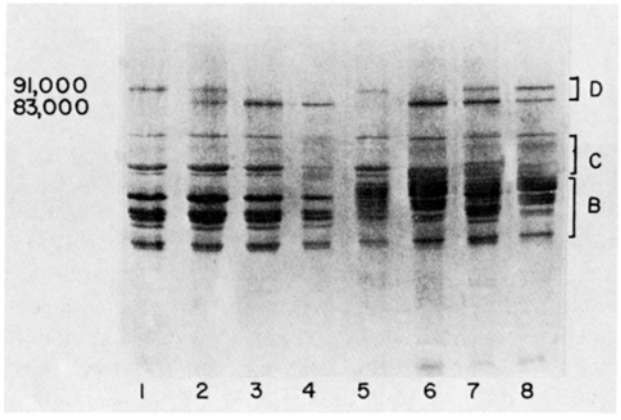

Fig. 1. SDS-PAGE patterns of hordeins from F_2 seeds (Triple Awned Lemma × Advance). Well 1) Advance phenotype for B, C, and D hordeins; 2) Advance B, C, heterozygote D; 3) Advance B, C, Triple Awned Lemma (TAL) D; Advance B, heterozygote C, TAL D; heterozygote B, Advance C, D; 6) TAL B, C, D; 7) heterozygote B, C, D; 8) TAL B, C, heterozygote D

one band in the high molecular weight range and heterozygotes showing two bands. Due to failure of some extracts less than three hundred F_2 seeds produced useable hordein phenotypes for recombination analyses. The method of Allard (1956) provided a maximum likelihood estimate of recombination between each of the three loci (Table 1).

This method provides a measure of recombination through repeated approximation. This analysis located the gene encoding D hordein (*Hor*-3) approximately 30 centimorgans toward the long arm of chromosome 5 from the *Hor*-1 locus, and 38 centimorgans from the *Hor*-2 locus. It estimated the distance between the *Hor*-1 and *Hor*-2 loci to be approximately 11.5 centimorgans, which is within the range reported by previous authors (Doll and Brown 1979; Jensen 1981). The maximum likelihood method of recombination analysis provides more accurate recombination estimates at low recombination frequencies than at high. Therefore, the estimates of recombination descend in accuracy from the *Hor*-1 and *Hor*-2 loci (0.115 ± 0.0098), to the *Hor*-1 and *Hor*-3 loci (0.301 ± 0.021), and to the *Hor*-2 and *Hor*-3 loci (0.382 ± 0.029).

The three point analyses of the data following the methods of Søgaard (1974, 1977) gave similar recombination frequencies (Table 2). They established the assumed order of *Hor*-2, *Hor*-1, *Hor*-3 to be correct. The approximate analysis gave linkage of: *Hor*-2 to *Hor*-1, 12% and *Hor*-1 to *Hor*-3, 28.6%. A positive interference (0.34) was operating in recombination among the three loci.

The computer oriented maximum likelihood method produced linkages of: *Hor*-2 to *Hor*-1, 11.4% and *Hor*-1 to *Hor*-3, 29.5%. The coefficient of coincidence was 0.43 indicating again a positive interference.

SDS-PAGE often fails to uncover much of the diversity within populations of proteins. Therefore, the level of diversity within the D hordein from Advance and Triple Awned Lemma was analyzed by two-dimensional electrophoresis (O'Farrell 1975). Relative to

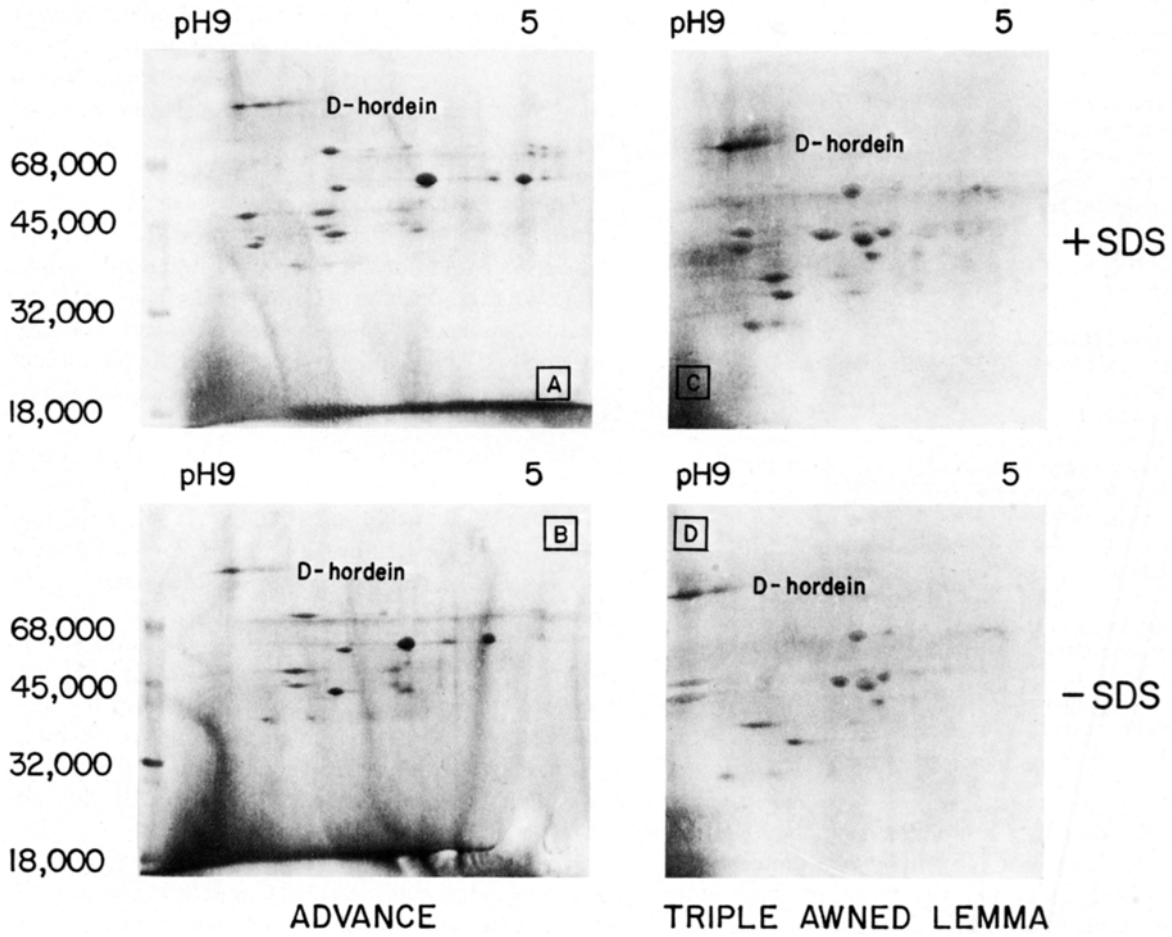

Fig. 2. Two-dimensional separation of hordeins from Advance (gels A, B) and Triple Awned Lemma (gels C, D). Gels A and C were run with a sample overlay containing SDS. Gels B and D were identical, but without the SDS. pH gradients were determined for replica gels, were very similar and were nearly linear with some tailing at both cathode and anode. Molecular weight markers are included at the left of gels A and B

Table 1. Recombination data and analyses for the *Hor*-2 (B hordein), *Hor*-1 (C hordein) and *Hor*-3 (D hordein) loci from Advance × Triple Awned Lemma. Subscripts indicate presence of alleles from either Advance (a) or Triple Awned Lemma (t). A indicates the first locus of each analysis, and B indicates the second locus

Genotype	Genotype frequencies		
	B/C	B/D	C/D
$A_aA_aB_tB_t$	2	19	9
$A_aA_tB_tB_t$	12	37	31
$A_aA_aB_aB_t$	18	30	28
$A_aA_tB_aB_t$ $A_aA_tB_tB_a$	97	62	65
$A_aA_aB_aB_a$	62	29	36
$A_aA_tB_aB_a$	11	19	17
$A_tA_tB_tB_t$	63	32	38
$A_tA_tB_aB_t$	12	30	27
$A_tA_tB_aB_a$	2	9	7
Recombination frequency	0.115	0.382	0.301
Standard error	±0.0098	±0.029	±0.021

Table 2. Distances obtained from estimated frequencies for the hordein F_2 genotypes (from Advance × Triple Awned Lemma) as derived from first principle according to the aproximate method and from computer oriented maximum likelihood method for three point tests

Approximate method	
Total gametes	510
Crossovers region I (*Hor*2-*Hor*1)	61
Crossovers region II (*Hor*1-*Hor*3)	146
Double crossovers	6
Crossover frequency *Hor*2-*Hor*1	12.0%
Crossover frequency*Hor*1-*Hor*3	28.6%
Double crossover frequency expected	0.034
Double crossover frequency observed	0.012
Coefficient of coincidence	0.34
Computer method	
Crossover frequency *Hor*2-*Hor*1	11.4±1.5%
Crossover frequency *Hor*1-*Hor*3	29.5±2.5%
Crossover frequency *Hor*2-*Hor*3	38.0±2.7%
Double crossover frequency expected	0.034
Coefficient of coincidence	0.43

the C and B hordeins, D hordein from these barley varieties yields remarkably simple two-dimensional patterns (Fig. 2). Isofocusing was performed both with and without SDS to dissociate the proteins with substantially similar results, although samples focused slightly farther toward the anode with SDS than without SDS. The D hordein focused as far toward the cathode as the other hordeins, a factor which may prove important in later studies involving its purification. The apparent pI of D hordein from the varieties Advance and Triple Awned Lemma is approximately 8.0 as determined by analysis of gel slices from duplicate gels.

Discussion

Tables 1 and 2 present recombinational analyses which provide unambiguous ordering of the *Hor*-1, *Hor*-2, and *Hor*-3 loci. These data place the *Hor*-3 locus, responsible for the production of D hordein, toward the centromere and approximately 30 centimorgans distant from the *Hor*-1 locus. This would place the *Hor*-3 locus near the centromere according to the composite map of barley chromosome 5 (Jensen 1981). The positive interference figure suggests it may be beyond the centromere and in the long arm. This finding agrees with the location of the *Hor*-3 locus on or near the long arm of chromosome 5 provided by Lawrence and Shepherd (1981). Using a translocation derivative of a barley-wheat substitution line, they showed that the gene encoding the high molecular weight hordein in Betzes barley could be found on their translocated long arm of chromosome 5. Our analysis provides a quantitative estimation of the location of the *Hor*-3 locus.

There is evidence that the *Hor*-3 locus (D hordein) is not under the same genetic control as the other two hordein loci and thus must be an independent and distinct locus. In mutant 1508 of Bomi a gene on chromosome 7 reduces the activity of the *Hor*-2 (B hordein) and *Hor*-1 (C hordein) loci but has no effect on hordein production at the *Hor*-3 (D hordein) locus (Cameron-Mills 1980).

The protein product of the *Hor*-3 locus (D hordein) has the solubility characteristics of a prolamin. Cameron-Mills (1980) and Miflin et al. (1981) suggested that it was a hordein after finding that it is stored in protein bodies, the characteristic storage organs for hordeins in barley.

The apparent molecular weights (83,000 and 91,000) and pIs of D hordein from the varieties Advance and Triple Awned Lemma are similar to those of the high molecular weight subunits of wheat glutenin produced by genes on chromosome 1B (Holt et al. 1981). This may suggest a relationship between the 1B chromosome of wheat and chromosome 5 of barley.

Hordeins, and storage proteins in cereals in general, have long been considered a veritable storehouse for genetic variability (Doll and Brown 1979). Storage proteins were thought to be produced by complex loci which were subject to little selective pressure. D hordein lacks this level complexity, a fact which may

relate to either the structure of the *Hor*-3 locus or to the selective importance of its product, D hordein.

As the D hordein appears to be similar to some of the high molecular weight glutenins from wheat, proteins which are thought to be important factors in gluten extensibility (Burnouf and Bouriquet 1980), an examination of the structural similarities between the HMW glutenins and the D hordein is warranted.

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