Genet. Res., Camb. (1982), **40**, *pp.* 315–324.
With 2 text-figures
Printed in Great Britain

Genetic basis and natural variation of α-amylase isozymes in barley

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(*Received 5 May 1982*)

SUMMARY

Two physiologically and biochemically distinct groups of α-amylase (E.C. 3.2.1.1) isozymes are synthesized when isolated aleurone layers of barley are incubated with gibberellic acid (GA_3). Isoelectric focusing of the α-amylases showed that the isoelectric points of the isozymes of one group were near pH 5, whereas those of the second group were close to pH 6. Using wheat–barley addition lines, the genes for these groups were located in barley chromosomes 1 and 6 respectively. Joint segregation in the F_2 generation of appropriate crosses indicated that the isozymes within each group were inherited collectively, and were attributed to codominant alleles segregating at two presumably complex loci, *α-Amy 2* and *α-Amy 1*.

The extent of genetic variation at these two loci was examined in 40 lines of *Hordeum spontaneum* (the wild progenitor of barley), and in a complex gene pool representative of *H. vulgare* (composite cross XXI). Variation at the *α-Amy 1* locus was much more extensive than that at the *α-Amy 2* locus. The genetic variation at both α-amylase loci exceeded that at the majority of other allozyme loci. However the α-amylase loci were less variable than the two loci coding for the seed storage protein, hordein. The wild species was found to contain much genetic diversity, which might be useful in modifying α-amylase activity by breeding. Parallels between the genetics and variation of α-amylase in barley and wheat were noted.

1. INTRODUCTION

Aleurone layers of barley synthesize and secrete α-amylase (E.C. 3.2.1.1) in response to the plant hormone gibberellic acid (GA_3). This system has been used extensively to study the action of GA_3 (for reviews see Yomo & Varner, 1971; Jacobsen and Higgins, 1978; Ho, 1980). However, interpretation of these studies is complicated *inter alia* by the existence of multiple forms of α-amylases (Frydenberg & Nielsen, 1966; Jacobsen, Scandalios & Varner, 1970; MacGregor, 1976). Jacobsen & Higgins (1982) have reported immunological and proteolytic evidence that this heterogeneity stems in part from the synthesis of different polypeptides rather than from post-translational modification of a single poly-

peptide. This would indicate that more than one structural gene is responsible for α-amylase activity.

Few formal genetic studies of α-amylase in barley have been reported. Using agar gel electrophoresis, Frydenberg, Nielsen & Sandfaer (1969) have demonstrated an allelic difference involving two of the bands (so-called C and D) of the barley complement and located one α-amylase locus on chromosome 6 (cited in Nielsen & Frydenberg, 1972). In this study using isoelectric focusing, we have defined in *Hordeum* two groups of isozymes which segregate as units. The genes for the groups, designated *α-Amy 2* and *α-Amy 1* were located on chromosomes 1 and 6 respectively. Also, using samples of *Hordeum spontaneum*, the wild progenitor of barley, and various cultivated strains of *H. vulgare* we have compared the level of variation between these two α-amylase loci themselves, between wild and domesticated strains, between the α-amylase loci and other enzyme loci, and between the α-amylase loci and the seed storage proteins (hordeins).

2. MATERIAL AND METHODS

(i) *Seed samples*

(i) Barley (*H. vulgare*) cv. Betzes, wheat (*Triticum aestivum*) cv. Chinese Spring, and the six euplasmic wheat–barley chromosome addition lines developed by Islam, Shepherd & Sparrow (1981).

(ii) Barley (*H. vulgare*) cvs. Clipper, Himalaya and Atlas.

(iii) Lines of *H. spontaneum* from twelve populations in Israel, chosen to include a diverse array of allozyme and hordein genotypes and listed in table V of Doll & Brown (1979), and Table 2 below.

(iv) A late generation (F_{17}) of composite cross XXI of barley (Suneson & Weibe, 1962). This generation has also been studied for allozyme and hordein variation (Doll & Brown, 1979).

(ii) *Preparation and incubation of isolated aleurone layers*

Isolated aleurone layers were prepared and incubated essentially as described by Chrispeels & Varner (1967). Barley grains were cut in halves and the portions containing the embryo and the endosperm were given corresponding numbers. In this way embryos of interest could be identified and grown. Endosperm halves were allowed to imbibe water on moist sand for 3 days at 20 °C under sterile conditions. The aleurone layers were then peeled from the starchy endosperm and incubated under sterile conditions with gibberellic acid (GA_3). Single layers were placed in small vials with 200 μl of a solution containing 10 mM $CaCl_2$, 1 μM GA_3 and 10 μl of chloramphenicol solution (0·5 mg/ml).

The vials were plugged with cotton wool and incubated at 25 °C for 27 h in a shaking water bath. The α-amylases in the incubation medium were separated by isoelectric focusing immediately.

(iii) *Isoelectric focusing of α-amylase and detection of enzyme activity*

α-amylases were subjected to isoelectric focusing (IEF) on ampholine polyacrylamide gel plates (pH range 4–6·5 and 5·0–6·5 obtained from LKB) using an LKB Multiphor apparatus. Gels were prefocused and samples of aleurone incubation medium were applied midway between the electrode strips using two LKB application papers. The two papers absorbed a total of about 50 μl of solution. Gels were focused for a further 2 h to a maximum of 500 V and then the voltage was increased to 1000 V for another 30 min.

When focusing was complete, α-amylase activity was detected using lyosin red-impregnated paper as described by Burdett, Kipps & Whitehead (1976). The paper was prepared using a 1·5 or 2 % solution of lyosin red (Reliable Chemical Co., St Louis, Missouri, U.S.A.) which gave adequate contrast between the pink paper and the white bands of α-amylase activity. The paper was laid on the gel for up to 2 h depending on the amount of enzyme present. When the white bands had developed sufficiently (visible through the gel), the paper with gel attached was soaked in 0·1 N-HCl to stop further enzyme action and then the gel was peeled off the paper and discarded. The paper was blotted to remove excess liquid and allowed to dry overnight on the bench, yielding a permanent record of the amylase zones. Isoelectric points of α-amylase isozymes were determined with the aid of a mixture of proteins of known isoelectric points.

3. RESULTS

(i) *Chromosomal location of isoelectric point groups of α-amylases*

Isoelectric focusing resolved the α-amylase isozymes of all barleys examined into two disjunct groups which we shall designate as the α-amylase 1 group and the α-amylase 2 group. The isoelectric points of the isozymes of the α-amylase 2 group were in the range pH 4·4–5·2. There were 3 to 5 major bands in each sample usually grouped into 1 to 3 separated sets. The α-amylase 1 group was more complex and included forms with isoelectric points in the range pH 5·7–6·2. Two kinds of IEF gel were used to resolve components of group 1. Although resolution of components of both α-amylases 1 and 2 was usually sharper on the broader range IEF gel (pH 4–6·5), the α-amylase 1 isozymes were stacked close to the cathode. Use of a narrower range gel (pH 5–6·5) brought the α-amylase 1 zone nearer the centre of the gel, and spread the component isozymes, although they were not as sharply focused.

In order to attribute the isozymes to particular chromosomes of barley, the α-amylases of the wheat–barley chromosome addition lines and of their parents Chinese Spring wheat and Betzes barley were examined by IEF. Figure 1 shows the resulting α-amylase zymograms. The pattern of enzymes from Chinese Spring was complex, consisting of at least 18 α-amylase isozymes. The pattern of Betzes barley was much simpler, with 7 bands, 4 of which had isoelectric points distinctly different from the wheat bands. Of the six addition lines tested (designated 1, 2,

3, 4, 6, 7 according to which barley chromosome the line carried), four lines (2, 3, 4, 7) were identical to the wheat parent. Line 1 was identical to Chinese Spring in the α–amylase 1 zone but had two additional bands in the α-amylase 2 zone identical to two of the Betzes components, as well as altered relative intensities of some components. From this we infer that the gene(s) for the α-amylase 2 group

Table 1. *Joint F_2 segregation at two α-amylase loci in the cross between two lines of* H. spontaneum [*Eyzariya 32*(≡*B*) × *Tabigha 9*(≡*A*)]

		α-Amy 1			
		AAA	AAB	ABB	BBB
α-Amy 2	AAA	3	7	2	6
	AAB	8	5	3	5
	ABB	2	6	7	6
	BBB	3	3	4	6

Chi-square analysis of two-locus gametic types

	Total	Heterogeneity between sexes
Segregation at *α-Amy 2* locus	0·06	0·00
Segregration at *α-Amy* 1 locus	1·29	0·42
Parental vs recombinant types	0·16	3·80

Each chi-square value has a single degree of freedom. Yates correction was used.

are located on chromosome 1. Line 6 was identical to Chinese Spring in the α-amylase 2 zone, but possessed an additive pattern fo α-amylase 1. Thus the gene(s) for α-amylase 1 are inferred to lie on chromosome 6. This latter result is in accord with the location of an α-amylase locus (*Amy*) on chromosome 6 (Nielsen & Frydenberg, 1972).

(ii) *Joint segregation analysis*

To test the joint independent segregation between α-amylase 1 from α-amylase 2, and the collective inheritance of bands within each group, a cross was made between two dissimilar lines (Eyzariya 32 and Tabigha 9) of *H. spontaneum*. The lines were chosen from a preliminary survey of the lines listed in Table 2 below using agar gel electrophoresis (Jacobsen *et al.* 1970). A total of 76 seeds of the F_2 generation were scored for their α-amylase-1 and -2 complement using IEF. Because of the triploid dosage, each band could be scored in terms of its dosage. Within each of the two zones, there was no evidence of assortment, so that only four different composite banding patterns were apparent. Two of the four patterns corresponded to the parental forms and two were intermediate. The frequencies of these phenotypes (Table 1) conform to a 1 : 1 : 1 : 1 ratio as tested by chi-square.

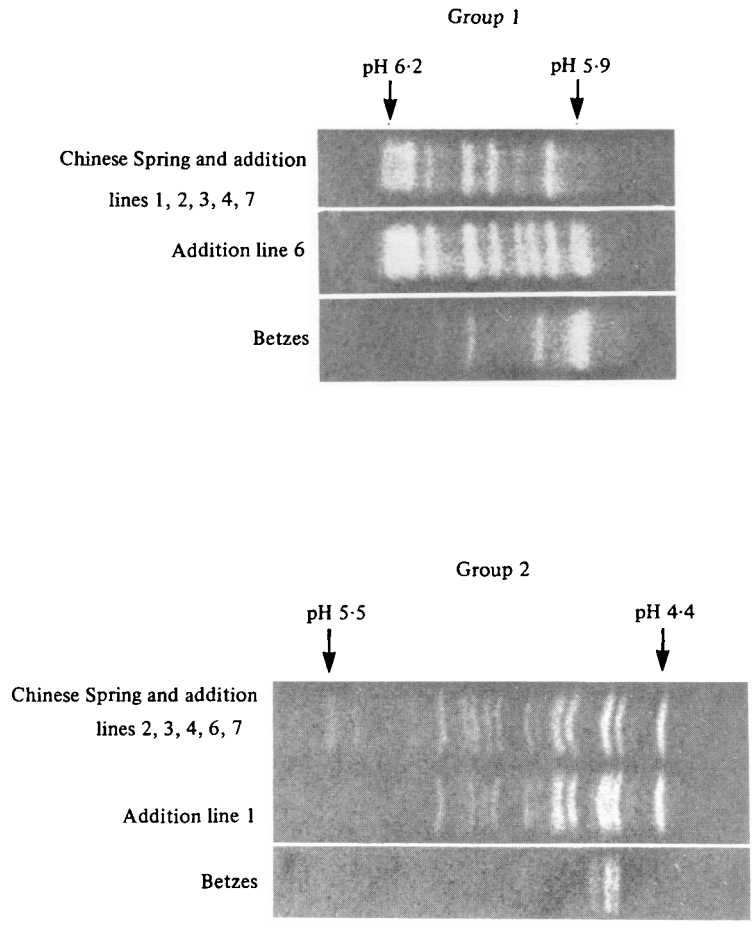

Fig. 1. Zymograms of α-amylase isozymes from Betzes barley, Chinese Spring wheat and Chinese Spring–Betzes chromosome addition lines. Isozymes were separated by isoelectric focusing.

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https://doi.org/10.1017/S0016672300019182 Published online by Cambridge University Press

Thus the composite isozyme patterns behaved as if determined by codominant alleles at a single locus. No intralocus recombinants were observed.

We propose the designation *α-Amy 1* and *α-Amy 2* for the loci specifying the α-amylase 1 and α-amylase 2 groups of isozymes respectively. Using fractionated preparations of the α-amylases of cv. Himalaya, it was shown (Jacobsen, unpublished) that the α-amylase 2 isozymes correspond with bands 1 and 2 detected in agar gel, and belonging to the set previously designated as group A (Jacobsen & Knox, 1973). The α-amylase 1 set includes the bands 3 and 4 of group B.

As the female gamete contributes two alleles to the triploid aleurone, and the male only one, all parental and recombinant male and female gametes can be distinguished in the F_2 4×4 array. Table 1 gives the chi-square analysis of the two-locus (*α-Amy 1* and *α-Amy 2*) gametic types. Overall, the two loci segregated independently as expected from their separate chromosomal location. There was marginally significant heterogeneity ($0{\cdot}05 < P < 0{\cdot}10$) between the sexes in that recombinants were deficient ($\chi^2_1 = 2{\cdot}96$) among the female gametes, but in slight excess ($\chi^2_1 = 1{\cdot}07$) among the male gametes.

(iii) *Variation between* H. spontaneum *lines*

The 40 lines of wild barley chosen for α-amylase analysis were those known to differ from one another for at least one of the two hordein loci. After focusing each sample on gels of both pH ranges, the patterns were compared. At least five distinct phenotypes were found for the α-amylase 2 group, and twelve for the α-amylase 1 group. These phenotypes are diagrammed in Fig. 2. The detailed classification for each line is given in Table 2. In both α-amylase groups there was a single common, and widely occurring phenotype, numbered 1 in Fig. 2, which resembled that of the cultivar Altas. Otherwise the groups differed substantially in their level of variation, with the second group being much more variable. Each phenotype is assumed to represent one allele or segregating unit at the particular α-amylase locus. Table 3 summarizes the variation in terms of three diversity statistics. The statistic H_e is Nei's genic diversity and is computed as one minus the sum of squares of the frequency of each type among the 40 lines. The 'effective number of alleles', n_e, can be thought of as the number of equally frequent genotypes required to yield the observed value for H_e. The actual number of observed phenotypes is symbolized n_a. In the case of the 19-locus genotype, n_a is the number of kinds of composite genotypes, based on all 19 allozyme loci.

The values of these three measures of variation can be compared in Table 3 with those of other allozyme loci, and for the storage protein, hordein. They show that the α-amylases were intermediate in their degree of variation between the other allozymes, and the two highly variable hordein loci. This pattern would be expected if the α-amylase loci are each complex, consisting of a few duplicated copies, as is also indicated by the variation in the number and intensity of component isozymes in each group.

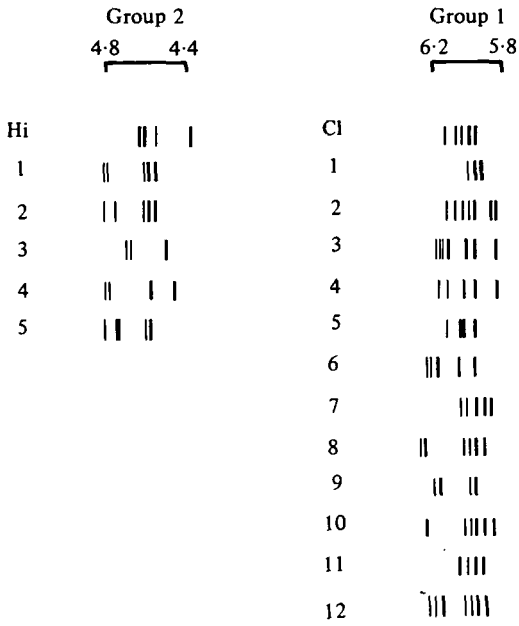

Fig. 2. Diagram of the different group 1 and group 2 α-amylase isozyme patterns obtained from *Hordeum spontaneum* lines, where numbers correspond with the list of patterns in Table 2. Hi = Himalaya, Cl = Clipper. The cultivar Atlas has type 1 for both groups.

(iv) *Comparison of variation in* H. spontaneum *and* H. vulgare

Table 3 also includes the corresponding values of the α-amylase loci in a sample of 30 seeds from the 17th generation of composite cross (CC) XXI. This population has previously been studied for allozymes and hordeins (Doll & Brown, 1977).

Two results for this population which were in accord with similar trends of α-amylase variation in the wild barley strains were:

(i) The *α-Amy 1* locus was much more polymorphic than the *α-Amy 2* locus, which was in fact invariant (for the Atlas phenotype) in this sample from CC XXI.

(ii) The α-amylase loci were more polymorphic than the other allozymes on average but not as polymorphic as the two hordein loci.

Turning to the comparison of genetic variation for α-amylase between the wild lines and CC XXI, the three statistics showed that this generation of the composite was much less diverse than the Israeli lines. This result is similar to that previously found for the hordeins and other enzyme loci.

As the wild lines were selected, the estimates of diversity do not apply directly to the source populations. The extent of the bias can be gauged from comparing the average diversity statistics for allozymes of the twelve natural populations ($H_e = 0{\cdot}15$, $n_e = 1{\cdot}16$, $n_a = 1{\cdot}68$), with the values for the lines themselves in Table 3.

4. DISCUSSION

The present study divides the α-amylases of barley into two genetically defined groups. One group (α-amylase 2) has isoelectric points in the range pH 4·4–5·2 and the other group (α-amylase 1) in the range pH 5·7–6·2. In so far as it was possible to resolve the several Betzes bands within each group when overlaid by the complex Chinese Spring pattern, the evidence from the addition lines indicated

Table 2. *Distribution of 40* H. spontaneum, *lines with respect to site and α-amylase pattern*

Site and line no.		Patterns in α-amylase −2	−1	Site and line no.		Pattern in α-amylase −2	−1
Damon	23	1	1	Talpiyyot	2	1	8
(71284)*	37	1	2	(77144)	11	1	8
Hermon	10	2	3		25	1	1
(77133)							
Tabigha	9	3	1	Eyzariya	19	1	1
(77143)	13	1	1	(77130)	21	2	1
	18	1	1		25	2	7
	19	3	1		32	2	9
	25	1	1		40	2	10
	31	2	1		20	2	9
					41	4	1
Bar	27	1	4	Wadi Qilt	7	1	11
Giyyora	31	1	4	(77135)	31	2	4
(71282)					40	2	7
Atlit	27	1	1		54	4	1
(77129)	50	1	5		55	4	1
	49	1	6				
Caesarea	25	1	5	Mashash	18	5	12
(77132)	38	1	4	(71285)	44	5	11
Mehola	7	1	7	Sede Boker	13	4	12
(77137)	11	1	7	(77141)	29	4	7
	26	1	7		21	4	7
	31	1	7				

* Commonwealth Plant Introduction number.

that all the α-amylase 2 bands are attributable to chromosome 1 whereas all the α-amylase 1 bands are attributable to chromosome 6. The latter group probably corresponds to the enzymes previously studied genetically by Frydenberg *et al.* (1969). The two groups generally differ also in the multiplicity of isozymes within each group (with group 1 usually showing more bands), and in the degree of variation found among cultivated barleys and in the wild progenitor *H. spontaneum* (with group 1 being more variable). Several possible genetic models could account

for the complexity. Each gene complex might be a multigene family or cluster of tightly linked very similar structural genes, whose components may differ in sequence. Differences between the lines, segregating as alleles, might stem from differences in the number of copies, and/or sequence differences. Alternatively, variation in a cis-acting post-transcriptional modification process might explain some of the isozyme diversity within each group.

Table 3. *Genetic variation in* H. spontaneum *and* H. vulgare

	40 *H. spontaneum* lines			CC XXI F_{17}*		
	H_e†	n_e	n_a	H_e	n_e	n_a
Allozymes						
19-locus genotype	0·975	40	40	0·86	7·07	16
Est 2	0·830	5·9	11	0·08	1·08	3
Average per locus	0·312	1·9	3·5	0·10	1·11	1·6
Hordeins						
Hor 1	0·965	27·6	33	0·80	5·2	9
Hor 2	0·973	36·4	38	0·84	6·2	11
α-Amylases						
α-Amy 2	0·646	2·8	5	0	1·0	1
α-Amy 1	0·813	5·3	12	0·47	1·9	3

* Sample sizes for CC XXI were 100 for allozymes, 60 for hordeins and 30 for α-amylases.
† H_e = probability that two random gametes are dissimilar.
$n_e = (1-H_e)^{-1}$ = effective number of alleles.
n_a = actual number of alleles.

The genetic dichotomy among α-amylases described above provides complementary evidence for other biochemical differences found between the two α-amylase groups in *H. vulgare* (Frydenberg & Nielsen, 1966; Jacobsen *et al.* 1970; Jacobsen & Knox, 1973; Bilderback, 1974; Jacobsen & Higgins, 1982). In contrast with the α-amylase 1 group, the isozymes of the α-amylase 2 group are more stable at low pH, more sensitive to sulphydryl reagents and heavy metal ions, and less sensitive to chelating agents. The activities of group 2 isozymes have lower pH optima, and a lesser requirement for Ca^{2+} ions. They appear earlier after GA_3 treatment but they accumulate more slowly than the group 1 isozymes. The two groups of isozymes differ markedly in serological type and in proteolytic fingerprints. The α-amylase components within each group appear to be very similar enzymically and in their induction characteristics although proteolytic fingerprint studies indicate that there may be amino acid sequence differences between them (Jacobsen & Higgins, 1982). In total, this evidence shows that the synthesis of the two different groups of α-amylases in aleurone of barley (*H. vulgare* and *H. spontaneum*) in response to GA_3 results from the expression of two groups of α-amylase genes which occur at different loci, *α-Amy 1* and *α-Amy 2* on different chromosomes.

Remarkable parallels in chromosomal location and isoelectric points exist among the α-amylases of *H. vulgare* and *H. spontaneum*, and those of α-amylases of the wheats and related species. From the isoelectric focusing of ditelocentric lines of Chinese Spring wheat, Nishikawa & Nobuhara (1971) defined two triplicate sets of α-amylase isozymes. The group with higher isoelectric points were attributable to genes on chromosomes 6A, 6B and 6D, except for three prominent bands which were invariant among the lines. Each prominent band of the group with lower isoelectric points was attributable to one of chromosomes 7A, 7B or 7D. Hart (1979) has compared the location of the enzyme loci *Amp 1* and *Got 2* on chromosomes of the homoeologous group 6 of wheat, and 6 of barley, and of the locus *Ep 1* on chromosomes of the homoeologous group 7 of wheat, and chromosome 1 of barley as tentative evidence of their possible homoeology. The location of the two sets of α-amylase loci in wheat and barley thus provides further evidence for the general conservation of linkage relationships in members of the Triticeae, as proposed by Hart (1979).

A third parallel between wheat and barley exists in the comparative genetic variability of the two groups. Nishikawa & Nobuhara (1971) reported that in 43 lines of wheat, 12 distinct phenotypes occurred for the group with higher isoelectric points, and only 1 rare variant was found for the other group. Further, Nishikawa *et al.* (1979) found that among tetraploid wheats, wild strains of *T. dicoccoides* were much more diverse in their α-amylase pattern than cultivated lines of *T. turgidum*, which again resembles our result for barleys. These comparisons can be extended to the diploid *Aegilops squarrosa*. Nishikawa, Furuta & Wada (1980) surveyed Iranian samples of this species for α-amylase patterns and found the group with lower isoelectric points (group 2) had fewer isozyme bands (one as opposed to 3–5 bands) and were markedly less variable than the other group. The actual number of alleles (n_a) for group 2 was 2 (compared with 7 for group 1) and the diversity index (H_e) for the whole collection of strains was 0·32 for group 2 and 0·76 for group 1.

Hence the conclusion from these studies of wheat and its relatives, and our study of *H. vulgare* and *H. spontaneum*, is that natural populations contain considerable genetic variation for α-amylase. In the case of barley, the contrast between the Israeli lines of *H. spontaneum* and CC XXI is particularly striking. It is likely that the early generations of CC XXI were more variable than this one because considerable selection has occurred over the 17 generations of propagation. However this estimate of diversity for α-amylases is similar in magnitude to those derived from the data of both Frydenberg *et al.* (1969), who typed over 100 lines of European barley for α-amylase, and of Fedak & Rajhathy (1971), who typed about 55 lines of North American barley. The diversity estimates from their data are $H_e = 0{\cdot}52$ and $H_e = 0{\cdot}29$ respectively, where $n_a = 3$ in both studies. Thus the wild materials represent a rich source of variation. This variation could be of use in breeding for modified α-amylase activity, which is a character of key economic importance in barley and wheat.

We wish to thank Drs Islam and Shepherd for the gift of seeds of the wheat–barley addition lines. We also wish to thank Rosemary Metcalf and Jack Munday for technical assistance.

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