Theor. Appl. Genet. 64, 309–316 (1983)

Genetic Control of α-Amylase Production in Wheat

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Summary. An analysis of the α-amylase isozymes in GA-treated endosperm of wheat nullisomic-tetrasomics shows that there is more variation at the *α-Amy-1* and *α-Amy-2* homoeoallelic loci than was previously thought. Among the 16 isozymes produced by genes on the group 7 chromosomes, most could be definitely established as products of a single homoeoallele.

Inter-varietal allelic differences would be expected at such loci and clear variation was found in isozymes produced by chromosomes 6B and 7B. The latter allele, *α-Amy-B2b* carried by the variety 'Hope', was used to locate the enzyme structural gene within chromosome 7B relative to the centromere and five other gene markers.

The nature of the *α-Amy-B2b* phenotype and the rare non-parental isozyme patterns found among the recombinant lines indicates that the locus is large and compound, probably involving some degree of intra-locus gene duplication.

Key words: Wheat – α-Amylase – Isozymes

Introduction

In hexaploid wheat, *Triticum aestivum* (2n = 6x = 42), a number of enzyme systems have been analysed in aneuploid lines. These genotypes, mainly nullisomic-tetrasomics, lacking both chromosomes of a pair of homologues but compensated by an additional chromosome pair in the same homoeologous group, and ditelocentrics, lacking individual chromosome arms, have been instrumental in the chromosomal location of structural genes in 19 enzyme systems (Hart 1979).

Amongst the systems studied α-amylase has received much attention, in evolutionary studies (Nishikawa et al. 1975) and because of its adverse effects on breadmaking quality following its production during preharvest sprouting (Kruger 1980). Nishikawa and Nobuhara (1971) noted that some of the isozymes produced in germinating grain were controlled by genes on the long arms of the group 6 [assigned the symbol *α-Amy-1* by Hart (1979)] and group 7 chromosomes (*α-Amy-2*). In a recent developmental study, Marchylo et al. (1980) have identified three groups of α-amylase isozymes, GI and GII being present mainly during grain development and GIII during germination.

Nullisomic-tetrasomic and ditelocentric analyses permit the chromosomal location of genes and provide information about their structural complexity. However, in order that these genes can be exploited as genetic markers, allelic variation, expressed as an observable difference in the gene products, must be found.

This paper describes a further aneuploid analysis of α-amylase isozymes in the variety 'Chinese Spring'. Two major allelic differences are described and one of these, located on chromosome 7B, has been mapped against five other gene markers and the centromere.

Genotypes

1 Nullisomic Analysis

Eleven nullisomic-tetrasomic (NT) lines for the group 6 (N6B-T6D unavailable) and group 7 chromosomes, were employed to establish the chromosomes carrying the genes controlling isozyme formation. Ditelocentric lines, lacking entire chromosome arms, were used to identify the chromosome arm involved.

2 Chromosome Control of Allelic Variation

Twelve hexaploid varieties, listed in Results, section 2, were screened for isozyme variation. Intervarietal chromosome substitution lines involving some of these varieties were employed to identify the chromosomal control of isozymes not produced by 'Chinese Spring' (CS). These were the series of substitutions of 'Hope' chromosomes into CS, developed by Professor E. R. Sears at Missouri, and those of *Triticum spelta* and 'Lutescens 62' into CS developed by Dr. C. N. Law and A. J. Worland at the Plant Breeding Institute.

3 Intra-chromosomal Recombinant Lines

By hybridising an intervarietal chromosome substitution line with its recipient variety it is possible, using appropriate

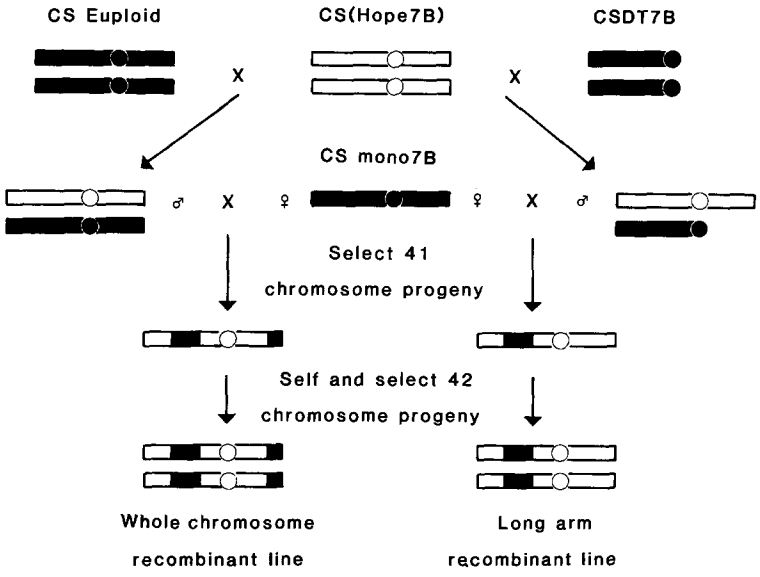

Fig. 1. Methods used in producing disomic recombinant lines between chromosome 7B of 'Chinese Spring' and 'Hope'. *Note:* Only monosomic progeny having complete chromosomes were selected from the cross of the hybrid CS('Hope 7B') × CS DT7B with CS mono 7B

cytological and crossing procedures, to produce a series of lines in which the products of a single round of recombination between a single pair of homologues are fixed as homozygotes. This method was applied by Law (1966) to the study of the differences between chromosome 7B of 'Hope' and CS using the substitution line CS ('Hope 7B') crossed to CS and was extended to involve crosses of the substitution line with the long-arm and short-arm telocentric lines of CS 7B. In these lines recombination is restricted to one or other of the arms of chromosome 7B, thereby permitting the mapping of genes in relation to the centromere. The methods are illustrated in Fig. 1.

Altogether 68 whole chromosome, 23 long-arm and 30 short-arm recombinant lines were used in the present study. These lines had previously been classified for allelic variation at the *Pm5* locus for powdery mildew (Law and Wolfe 1966), the leaf rust resistance loci, *Lr14a* and *Lrm* (Law and Johnson 1967), the purple-culm locus, *Pc* and the locus for ear-emergence time, *Vrn5* (Law 1966).

Methods

1 α-Amylase Induction

For each sample, three distal halves of mature dry grain were sterilised in 2% sodium hypochlorite for 15 min and washed prior to incubation for 72 h at 25 °C on a slow shaker in 2 ml medium 10 mM sodium acetate buffer (pH 4.8), calcium chloride 10 mM, gibberellic acid (GA_3), 1×10^{-5} M and two drops of streptomycin. The half grains were homogenised with the incubate using a Polytron (Kinamatica, GMBH) and spun at 3,000 rpm for 10 min and the supernatant stored at −10 °C.

2 Iso-electric Focussing

The supernatant was incubated at 70 °C for 10 min to remove β-amylase activity prior to recentrifuging. 20–60 µl samples, equivalent to 20–60 mU α-amylase activity (as measured using Phadebas (Pharmacia, Uppsala) dyebound starch tablets), were applied to the cathodal sides of pH 6–6.5 and pH 5–8.5 range gels (LKB) and run at constant power of 1 watt per cm gel width at 4 °C for 2.5 h. Isoelectric points (pI) were ascertained by measuring pH with a surface electrode before refocusing for 10 min and visualising the zymogram by immersion in starch solution for 10 min and staining with iodine solution as described by Sargeant and Walker (1978).

Gene and Enzyme Nomenclature

The symbols for the α-amylase structural genes are derived using the following system:

i) *α-Amy-1* and *α-Amy-2* are the triplicate homoeoallelic series of structural loci on the group 6 and group 7 chromosomes respectively, ii) individual loci within a triplicate series are referred to by genome, e.g. *α-Amy-B1* being the *α-Amy-1* locus on chromosome 6B, iii) alleles at individual loci are identified by postscripts, e.g. *α-Amy-B1b* is a specific variant at the 6B locus relative to CS (which always carries the 'a' allele at all loci).

The isozymes produced by these loci are similarly classified, but in upper case, e.g. α-AMY1, refers to all the malt isozymes produced by the genes on the group 6 chromosomes.

Results

1 Nullisomic Analysis

a) Group 7 Chromosomes. The α-AMY2 enzyme, which comprises the group of isozymes with more acidic PIs, produced by 'Chinese Spring' and the six CS NT genotypes for this group are shown in Plate 1.

In all, sixteen bands, with pI between pH 4.9 and 6.0, can be identified in α-AMY2. The samples were applied at high activities (about 60 mU per track) to display the more minor bands.

It is clear that the removal of either 7A, 7B or 7D in the NTs removes the genes controlling the production of most of the isozymes: chromosome 7A, five bands; 7B, six bands; 7D, three bands. The minor bands 3 and 4 cannot be clearly allocated to control by a single

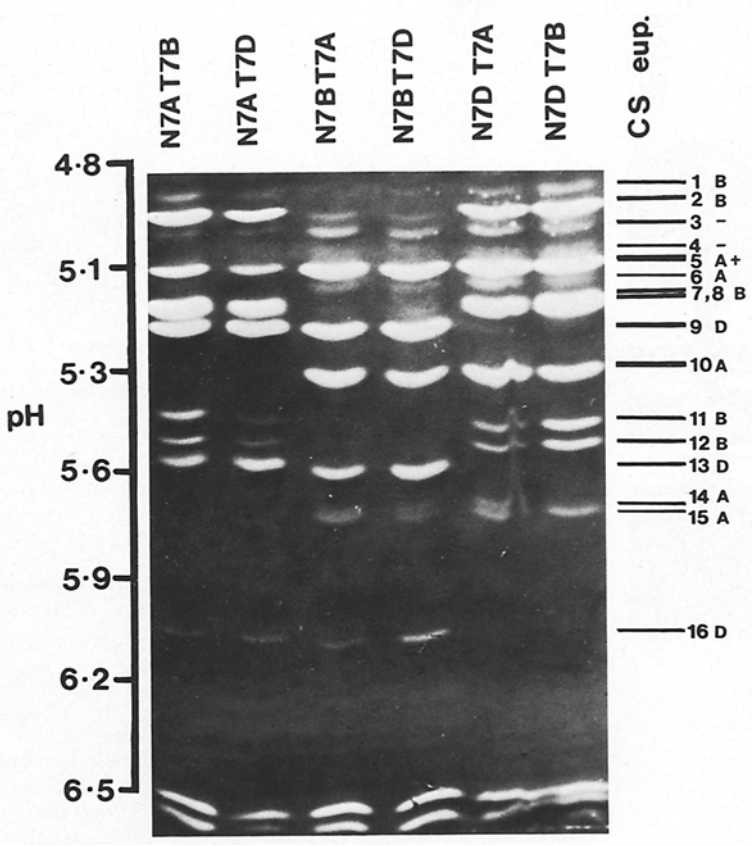

Plate 1. The α-AMY2 group of α-amylase isozymes produced in GA-treated half grains of 'Chinese Spring' euploid and the group 7 nullitetrasomics of 'Chinese Spring'. The group 7 chromosome encoding for each of the isozyme bands is shown on the right e.g. CS band 1 is encoded by chromosome 7B

chromosome and band 5 appears to be present in all genotypes but in reduced amounts when chromosome 7A is absent. Analysis of the entire set of CS NTs did not indicate that any chromosomes other than those in group 7 were involved in the control of these isozymes, so it is probable that the major band 5 is composite and includes more than one isozyme for which control is located on more than one group 7 chromosome. Analysis of the group 7 ditelocentric lines confirmed the location of *α-Amy-2* on the long arms of chromosomes 7A, 7B and 7D.

The available group 6NT lines were similarly analysed for control of α-AMY1, the more basic group of isozymes with pI between 6.2 and 6.8 (Plate 2). Variation between homoeologous chromosomes is not so evident for *α-Amy-1*. However, 11 major bands are resolved of which 6 may be shown to be the products of genes on single chromosomes. Ditelocentric analysis confirmed the location of *α-Amy-1* of the long arms of the group 6 chromosomes.

2 Varietal Differences for α-Amylase Isozymes

The very common variant in the α-AMY2 isozyme group described below as *α-Amy-B2b*, was characterised by a strong band focussing between CS α-AMY2 bands 3 and 4. Of the 12 varieties studied (*T. spelta*, 'Timstein', 'Hope', 'Cappelle-Desprez', 'Ciano 67', 'Hybride du Joncquois', 'Lutescens 62', 'Poros', 'Bezostaya I', 'Mara', 'Koga II' and 'CS') all displayed this band except CS.

Analysis of the group 7 substitutions of 'CS/Lutescens 62' (Plate 3) showed the band (B1) to be controlled by chromosome 7B. In addition, the extra band was associated with the absence of CS band 11.

A second common variant is characterised by the presence of 2 bands at lower pI than the main group of α-AMY1 isozymes. Among the varieties studied *T. spelta*, 'Timstein', 'Lutescens 62', 'Bezostaya I', 'Mara' and 'Hybride du Joncquois' produced these bands. Plate 4 shows the band patterns produced by the group 6 substitution lines of 'CS/*T. spelta*', indicating clearly that the extra bands α-AMY1-B1a and α-AMY1-B1b are controlled by chromosome 6B. Analysis of the 'CS/Lutescens 62' substitution lines confirmed this result. Further variation involving chromosomes 6A and 6D can also be seen among the higher pI isozymes in Plate 4.

3 Intrachromosomal Location of α-Amy-B2

The different α-AMY2 phenotypes of lines carrying either the 7B chromosome of 'Hope' or CS allowed the

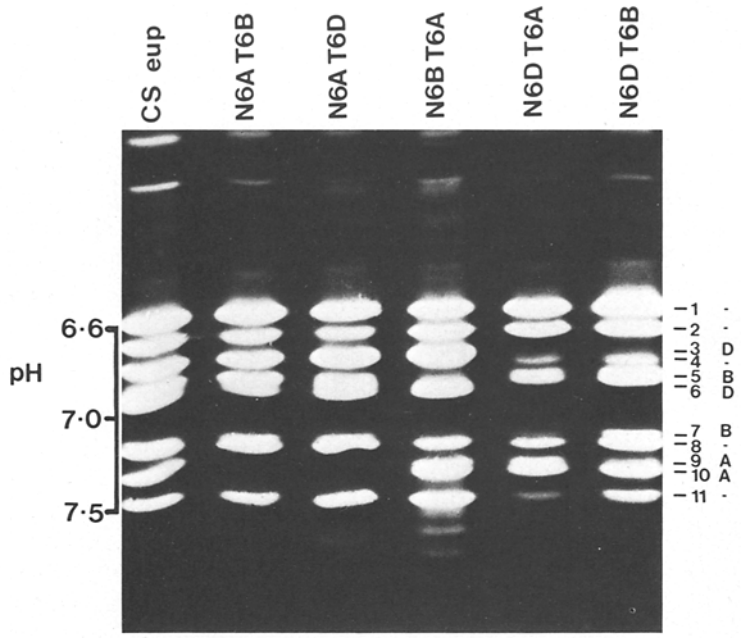

Plate 2. The α-AMY1 group of α-amylase isozymes produced in GA-treated half grains of 'Chinese Spring' and the group 6 nulli-tetrasomics

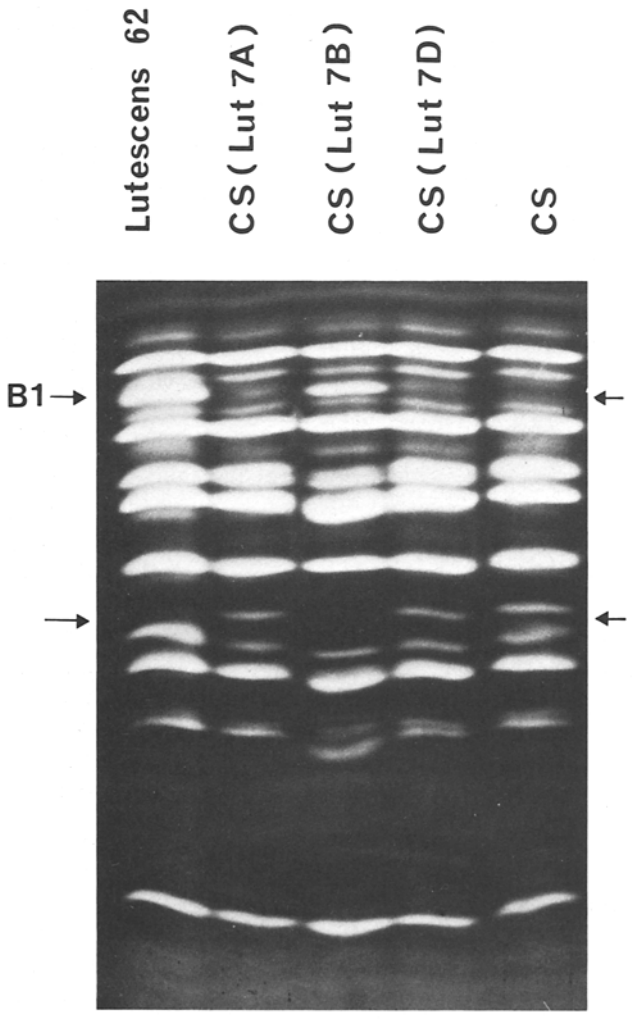

Plate 3. The α-AMY2 group of α-amylase isozymes produced by 'Chinese Spring', 'Lutescens 62' and the CS ('Lutescens 62') group 7 chromosome substitutions. The uppermost arrow indicates the isozyme characterising *α-Amy-B2b,* the lower ◀ arrow indicates an associated polymorphism in 'Lutescens 62'

Plate 4. The α-AMY1 group of α-amylase isozymes produced by 'Chinese Spring', *Triticum spelta* and the intervarietal substitution lines of the *T. spelta* group 6 chromosomes in 'Chinese Spring'. The arrows indicate the isozymes characteristic of *α-Amy-B1b*

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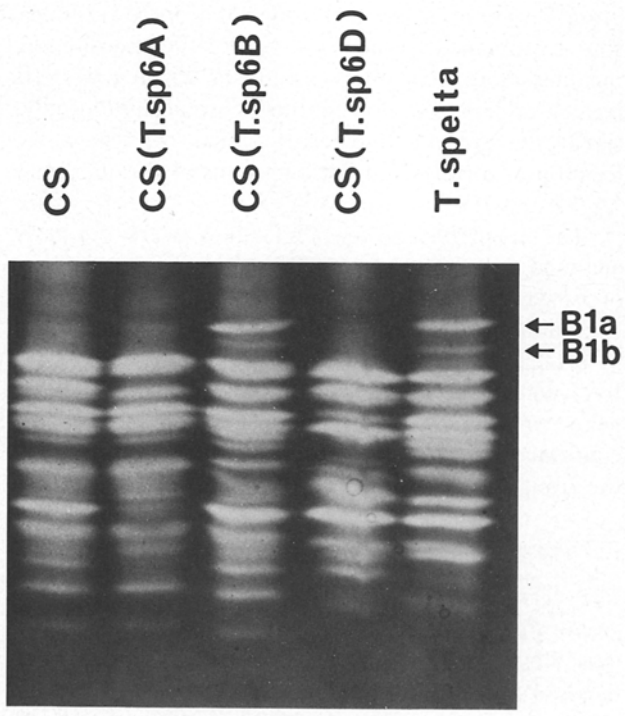

gene(s) controlling this difference to be mapped on this chromosome. The 121 'CS/Hope 7B' recombinant lines, already classified for *Lr14a, Lrm, Pm5, Vrn5* and *Pc* alleles, were assayed for the presence or absence of the α-AMY2 bands B1, 7 and 11.

All the 30 short arm (S lines) and all but one of the long arm recombinants (L lines) showed the 'Hope' phenotype confirming the location of *α-Amy-B2* on the long arm of 7B and indicating close linkage to the centromere. Of the 68 whole chromosome recombinants (W lines), 28 produced the 'Hope' B1 band, 39 lacked the band and one line produced none of the α-AMY2 bands controlled by 7B and was discarded as a probable nullisomic.

Single chromosome recombinant lines are particularly useful in mapping since all the lines are homozygous and can, as a consequence, be extensively replicated as required. Complete classification should usually therefore be obtainable. However in this case only the lines carrying *Lr14a* allele can be classified for the modifier allele at the *Lrm/lrm* locus. In addition about 10% more lines were scored for leaf rust resistance, vernalization requirement and culm colour than were available for classification of the *α-Amy-B2* phenotype. A method of analysis was thus required to a) combine all the available information in estimating the recombinant fractions, b) provide an objective test of possible gene orders and c) provide tests of homogeneity in combining information from different experimental sources. This last provision is important where recombination might have been restricted in some of the experiments, as for instance by the use of telocentrics. In these cases it is necessary to test whether the recombination frequencies are identical to those obtained where recombination is not restricted.

The method developed consists of contructing an overall likelihood function for the phenotypes of all lines based on the six recombination frequencies between six segregating loci and the centromere, and deriving joint estimates of the frequencies which maximise that likelihood. In general these maximum likelihood estimates cannot be expressed explicity in terms of the data values so they were derived using a general optimising routine (Ross 1980). Using the gene order model *Lr14, Pm5, α-Amy-B2,* centromere, *Pc, Lrm, Vrn5* the recombination estimates and standard errors are shown in Table 1. Correlations between estimates were close to zero, apart from y_3 and y_4, which were mildly negatively correlated because of their pooled estimation in the W lines.

These estimates rely on the choice of gene order, and an alternative model in which the positions of *Vrn5* and *Pc* on the short arm were reversed was also considered. This was the original model proposed by Law and Johnson (1967) but later withdrawn when information from the S lines became available (Law 1971). The maximum log likelihood for this model, $L = 164.0$, could be compared with $L = 179.2$, with *Pc* closer to the centromere, confirming that the latter model is more likely to be correct.

The joint likelihood method also allows a test of homogeneity of the recombination frequencies estimated from the W, L and S lines separately (Table 1). To examine whether the goodness of fit of the model is significantly improved when frequencies are estimated separately for each group we adopt a likelihood ratio test, when groups are homogenous, twice the increase in log-likelihood will follow an approximate χ^2 distribution with degrees of freedom equal to the number of additional parameters fitted. In this case L increased by 2.8 from 179.2 to 182.0 and five additional recombination frequencies were fitted (Table 1). Hence the test statistic χ^2_5 is 5.6 ($P > 0.30$) and there is no evidence of heterogeneity in these results, although the fact that

Table 1. Recombination frequencies obtained from the whole chromosome, short arm and long arm recombinant lines for chromosome 7B from 'Hope' and 'Chinese Spring'

Locus	Recombination frequency	Whole chromosome	Long arm	Short arm	Combined data
Lr14a	y_1	31.5	26.7	–	30.1±4.5
Pm5	y_2	46.0	39.9	–	44.4±5.1
α-Amy-2B	y_3	30.5	4.0	–	5.9±5.5
Centromere	y_4		–	16.2	22.8±5.3
Pc	y_5	12.8	–	10.8	10.5±3.7
Lrm	y_6	17.4	–	35.1	25.5±4.6
Vrn5					
log likelihood		54.7	46.7	80.5	
			182.0		179.2

the sum of y_3 and y_4 obtained from the S and L lines is lower than the joint estimate obtained from the W lines is consistent with reduced proximal crossing over in telocentrics found by Sears (1972).

4 Allelic Variation

Almost all (the two exceptions are discussed below) the recombinant lines displayed either the complete Hope (having B1, and lacking band 11) or 'Chinese Spring' (having band 11 but lacking B1) phenotypes. The 'Hope' allele at this locus is assigned the symbol *α-Amy-B2b,* the 'Chinese Spring' allele being, by convention, *α-Amy-B2a.*

Similarly, the major variant at the locus on chromosome 6B (Plate 4, bands B1a and B1b) has been assigned the symbol *α-Amy-B1b.*

Discussion

It is now clear that the α-amylase isozymes of the grain comprise two groups which are genetically distinct and appear at different times during grain development. The first group, probably the most significant in terms of preharvest sprouting damage in wheat, has been described as the 'malt' type II by Daussant et al. (1981), Group I by Sargeant (1980) and GIII by Kruger (1980). The isozymes in this group appear about 48 h after the commencement of germination in whole grains (Sargeant 1980) but much more rapidly (after about 24 h) in the GA-induced distal half grain system used here. These enzymes, described here as α-AMY1, can be shown to be the products of the *α-Amy-1* triplicate series of genes on the long arms of chromosomes 6A, 6B and 6D.

The second group of isozymes, α-AMY2, all have more acidic pIs than α-AMY1 and are those prevalent during early grain development. They are produced again during whole grain germination after four or five days (Sargeant 1980), much later than α-AMY1. In the system used here the α-AMY2 isozymes can be detected in the incubation medium at about the same time as α-AMY1. These enzymes are quite active with soluble starch as a substrate but are much less readily, adsorbed onto wheat starch granules (Sargeant and Walker 1978) and thus may be less important as a factor in wheat breadmaking quality. Almost all the isozymes in the α-AMY2 group can be shown to be the products of the *α-Amy-2* loci on the long arms of the group 7 chromosomes and have been described as 'green' type, Group II and GI by the authors above.

A third group, GII, has been described by Marchyllo et al. (1980) as isozymes present during grain development with pI intermediate between the main bodies of the α-AMY2 isozymes. The 'Chinese Spring' α-AMY2 isozymes 14, 15, 16, (Plate 1) would fall into this classification but are clearly products of the *α-Amy-2* genes. The position of these bands is similar to those shown as GII by Marchyllo and coworkers and it is possible that they are simply those with the highest pI produced by the genes on the group 7 chromosomes.

For many of the triplicate structural gene series in wheat that are known to encode for monomeric enzymes like α-amylase, the individual gene products appear on gels as single isozyme bands [e.g. aminopeptidase, *Amp-1* (Hart 1972), alcohol dehydrogenases, *Adh-1* and *Adh-2* (Hart and Langston 1977; Jaaska 1978)]. The previous analysis of 'Chinese Spring' α-amylases by Nishikawa and Kobuhara (1971) showed a similar situation for the *α-Amy-1* and *α-Amy-2* genes where although disc-isoelectric focussing showed the presence of 15 stable bands only 6 could be allocated to particular chromosomes, i.e. one per gene. The present analysis shows a different situation, possibly because of the improved resolution by flat-bed isoelectric focussing. Although 27 isozymes were identified, almost all, especially those isozymes encoded by *α-Amy-2,* could be assigned to control by one or other of the 6 chromosomes in group 6 and 7. For example, the removal of chromosome 7B removed 6 isozymes from the gel pattern. Most of the products of *α-Amy-A2, B2* and *D2* are distinct and can be completely separated. This is not the case for the *α-Amy-1* isozymes where 5 of the 11 bands could not be assigned to control by a single chromosome. Analysis of all the nullisomic-tetrasomics of 'Chinese Spring' did not indicate control of these bands by a further set of *α-Amy* loci. Therefore they could represent identical products of two or more of the *α-Amy-1* loci or, more likely, they may not have been completely resolved by the separation techniques employed here.

One prediction concerning triplicate loci that encode for several products and display almost complete homoeoallelic dissimilarity is that allelic variation might be expected. Surprisingly little such variation has been described in wheat and in only a few cases has this variation been used to map the loci concerned intrachromosomally viz. *Got-E3* (Hart et al. 1976), *β-Amy-A2* (Gale et al. 1982) and *Gpi-D1* (Chojecki et al. 1983).

The variants described here as *α-Amy-B1b* and *α-Amy-B2b* probably represent only a fraction of the variation that could be found in a larger sample of varieties. The mapping of the chromosome was easily accomplished using the recombinant lines already available. Figure 2 shows the complete map of chromosome 7B, including *Sr17* mapped in two segregating populations by McIntosh et al. (1967).

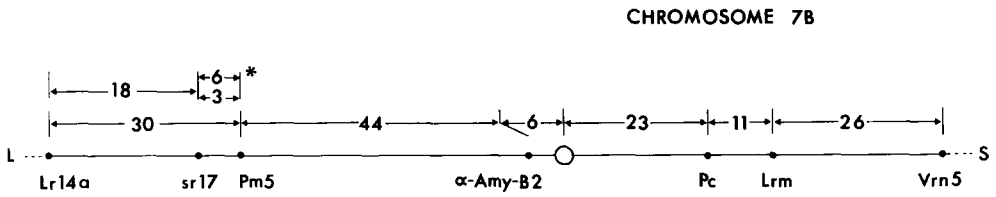

Fig. 2. The genetic map of chromosome 7B
(*, sr17 data from McIntosh, Luig and Baker 1967)

Critical observation of CS ('Hope 7B') recombinant lines also revealed some clues to the nature of enzyme structural genes that encode for several isozymes. Most of the 90 recombinants for the long arm of 7B (67W and 23L lines) produced parental *α-Amy-B2* phenotypes, however two lines produced patterns which appear to be explainable only as intra-locus recombinants (W3 and W42, Plate 5). W3 shows the 'Hope', *α-Amy-B2b* phenotype but, in addition, lacks bands 7 and 12 which are present in both parents, while W42 shows the 'Chinese Spring' phenotype but lacks band 11. One model that would accommodate the recovery of these band patterns is that of a compound locus evolved by extensive duplication and mutation, both to produce segments which encode for different isozymes and to produce non-productive, or null, segments within the locus. Band 12 could then be lost by recombination within a segment of chromosome containing productive and null copies in reciprocal homologous positions. Band 11 could be lost in an otherwise *α-Amy-B2b* phenotype by simple recombination within a complex locus.

The frequency of non-parental types would indicate that the *α-Amy-B2* complex gene is rather large but of the same order as a series of esterase genes, EA, EB and EC, in barley which recombine with frequencies of 0.23% and 0.48% (Kahler and Allard 1970).

It seems probable that many more allelic differences could be found at the impressive array of loci controlling enzyme production already identified in wheat. The intra-chromosomal mapping of these genes could provide the backbone of a map for wheat comparable to those already available in organisms like barley and maize. This information would be valuable for identifying or confirming chromosomes transferred within wheat by aneuploid techniques, in the same way that several alien chromosome additions and substitutions have been identified (review Hart 1979). In addition the markers could be used to manipulate closely linked genes of agronomic importance which are difficult to identify in single plants in, for example, a backcross breeding programme. The amount of alien chromosomal material, transferred from wild relatives of wheat and carrying useful genes, could be identified and minimised through the use of markers. Eventually such maps may be useful in the positioning of genes transferred to wheat by recombinant DNA techniques.

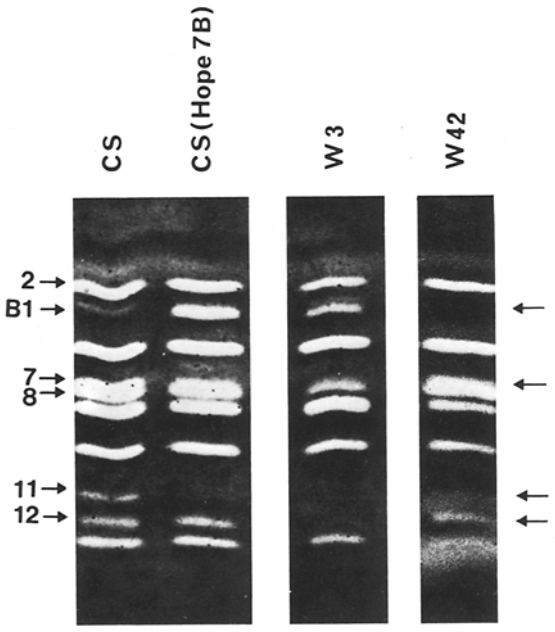

Plate 5. The *α-Amy-2* isozymes produced by 'Chinese Spring' and CS ('Hope 7B') and two possible intra-*α-Amy-B2* recombinants. For explanation see text

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Received November 22, 1982
Communicated by R. Riley

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