Theor Appl Genet (1991) 82:771–776

THEORETICAL AND APPLIED GENETICS

α-Amylase structural genes in rye

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Received March 8, 1990; Accepted March 25, 1991
Communicated by F. Salamini

Summary. Rye α-*Amy1*, α-*Amy2*, and α-*Amy3* genes were studied in the cross between inbred lines using wheat α-amylase cDNA probes. The α-*Amy1* and α-*Amy2* probes uncovered considerable restriction fragment length polymorphism, whereas the α-*Amy3* region was much more conserved. The numbers of restriction fragments found and the F_2 segregation data suggest that there are three α-*Amy1* genes, two or three α-*Amy2* genes, and three α-*Amy3* genes in rye. These conclusions were supported by a simultaneous study of α-amylase isozyme polymorphism. The F_2 data showed the three individual α-*Amy1* genes to span a distance of 3cM at the locus on chromosome *6R*L. The genes were mapped relative to other RFLP markers on *6R*L. On chromosome *7R*L two α-*Amy2* genes were shown to be separated by 5 cM. Linkage data within α-*Amy3* on *5R*L were not obtained since RFLP could be detected at only one of the genes.

Key words: *Secale cereale* – RFLP – α-Amylase – Genetics – Isozymes

Introduction

The breakdown of starch granules in the endosperm of germinating cereal grain is governed mainly by the activity of two groups of α-amylase (α-1,4-glucan-4-glucan hydrolase EC. 3.2.1.1.). The α-AMY1 (high pI) isozymes produced in high concentration during germination are controlled by the α-*Amy1* gene family on group 6 homoelogous chromosomes. The less active enzyme, present in developing grain as well as during germination, is α-AMY2 (low pI), encoded by the α-*Amy2* genes on group 7 chromosomes (Gale et al. 1983; Lazarus et al. 1985; Ainsworth et al. 1987). As many as 12–14 α-*Amy1* and 10–11 α-*Amy2* genes were found in wheat by hybridization to α-amylase cDNA probes (Martienssen 1986). The estimated numbers of α-amylase genes were five or six on *6B* and *6D*, two on *6A*, and three or four at the α-*Amy2* loci on *7A*, *7B*, and *7D*. In barley, three genes have been reported to encode the high pI group of α-amylases (MacGregor and MacGregor 1987). Three structural genes at the α-*Amy1* loci in rye have also been reported from isoenzyme evidence (Masojć 1987).

A third group of α-amylase genes (α-*Amy3*), active only in developing grain, has been reported on the group 5 chromosomes in wheat (Baulcombe et al. 1987). The wheat α-*Amy3* genes are probably present as a single copy on each wheat homoeologue. Hybridization of the wheat α-*Amy3* probe onto rye genomic DNA showed that this species also contains analogous α-*Amy3* genes.

Rye, a diploid, is a highly polymorphic species in which α-amylase isoenzyme polymorphism has been studied previously on polyacrylamide gels containing starch (Lapiński and Masojć 1983; Masojć and Lapiński 1984; Masojć 1987). However, this technique allowed the detection of only the α-AMY1 group of isozymes.

In the present study, both isozyme and restriction fragment length polymorphism (RFLP) analyses have been employed to assess the number of genes encoding each group of rye α-amylase and to investigate intralocus gene relationships.

Materials and methods

Plant material

The genotypes investigated included DS 2 and RXL 10, inbred lines obtained after more than ten selfing generations at Szczecin, and 90 F_2 progeny from a cross between them.

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DNA procedures

The methods for RFLP analysis were essentially as described by Sharp et al. (1988) except that 'Gene Screen Plus' (NEN) nylon membranes were used for alkaline blotting.

The cloned cDNA fragments of wheat *α-Amy1* (AMY-46), *α-Amy2* (AMY 4848), and *α-Amy3* (AMY33) sequences (Baulcombe and Buffard 1983; Baulcombe et al. 1987) were kindly provided by Dr. D. C. Baulcombe and used as probes. Some anonymous cDNA probes (S. Chao and M. D. Gale, unpublished results) were also used for mapping the *α-Amy1* genes within chromosome *6RL*.

Isozyme analysis

The semiliquid starchy endosperms of grain germinating 5 days at room temperature were ground separately in 50 µl of distilled water, heated at 70 °C for 10 min to inactivate β-amylase, and centrifuged for 5 min at 12,000 × *g*. Sample applicators (5 × 2 mm, Pharmacia) were soaked in the supernatants and laid near the anode on prefocussed (500 Vh), flat-bed polyacrylamide gels (0.25 mm thick, 125 mm wide, 5% C, 3% T) containing 10% glycerol and 2% ampholytes (2:1 mixture of Isolyte 6–8 and LKB 5–7 for α-AMY1 or 3:1:1 mixture of Pharmalyte 4.2–4.9, Pharmalyte 5–6, and Isolyte 3–5 for α-AMY2 separation). The electrolytes used were 0.1 *M* NaOH (cathode) and 0.04 *M* glutamic acid (anode). Isoelectric focussing (IEF) was performed at constant power (1 W cm^{-1}) for 4,300 Vh. The gels were then immersed in 2% w/v starch solution for 7 min and stained with iodine, as described by Sargeant and Walker (1978).

The polyacrylamide gel electrophoresis (PAGE) of α-AMY1 isozymes was carried out as described by Lapiński and Masojć (1983) except that the incubation buffer contained no calcium chloride.

Results

RFLP between inbred lines

DS 2 and RXL 10, the parents of a cross applied earlier in the genetic study of α-AMY1 isozymes (Masojć 1987), was used to analyze RFLPs detected by the α-amylase probes. The parental lines were screened for RFLPs with wheat *α-Amy1*, *α-Amy2*, and *α-Amy3* probes and with the eight restriction enzymes (REs) listed in Table 1. Each of the probes cross-hybridized to some extent with genes for the other α-amylases (Fig. 1). Nevertheless, it was possible to identify separate fragments for each group based on the degree of hybridization, and confirmation of the identity of hybridizing band showing RFLP was obtained later from the linkage analysis. The hybridization patterns and the number of hybridizing bands for each gene group varied (Table 1). Depending on the RE used, the number of *α-Amy1* fragments ranged from two with *Eco*RV to six with *Apa*I, but most frequently three were observed (*Dra*I, *Eco*RI, *Hin*dIII, and *Xba*I). Similarly, most REs produced two *α-Amy2* and three *α-Amy3* fragments. Differences between the parental lines were revealed by all eight (*α-Amy1*), six (*α-Amy2*), or two (*α-Amy3*) REs. Only *Dra*I, *Bgl*II, *and Xba*I digests were employed in the study of F_2 segregations.

Segregation between α-Amy genes

α-Amy1 genes. Five *α-Amy1* fragments of different length with strong hybridization segregated in *Dra*I digests (Fig. 2A). DS 2-type homozygotes produced 7.7-kb (strong signal), 11.6-kb, and 13.5-kb fragments, whereas RXL 10-type homozygotes produced 7.7-kb (faint signal), 15.5-kb, and 18.2-kb fragments. In addition to the parental-type homozygotes and heterozygotes, three different types of recombinant were found among F_2 progeny (Fig. 2A; R_1, R_2, and R_3). Examination of the segregation (Table 2) showed that the 11.6- and 18.2-kb fragments represent two alleles at one sublocus (*α-Amy1-1*), while the 13.5- and 15.5-kb fragments represent alleles at the second sublocus (*α-Amy1-2*), with recombination between them at a frequency of 3% ± 1%. It was also found that strong and faint signals of a 7.7-kb fragment segregated in agreement with a 3:1 ratio. Furthermore, the strong signal was always associated with the presence of the 11.6-kb fragment. This cosegregation suggests that the *α-Amy1-1* sublocus contains two genes represented by the 7.7-kb fragment (*α-Amy1-1-1*) and the allelic 11.6- and 18.2-kb fragments (*α-Amy1-1-2*).

In order to examine the relationship between individual restriction fragments and α-AMY1 isozymes, the endosperms of germinating parental and F_2 seedlings used in the RFLP study were analyzed for IEF and PAGE

Table 1. Numbers of restriction fragments and RFLP (+ or −) observed between DS 2 and RXL 10 after hybridization with *α-Amy1*, *α-Amy2*, and *α-Amy3* cDNA probes

Restriction enzyme	*α-Amy1*			*α-Amy2*			*α-Amy3*		
	DS 2	RXL	RFLP	DS 2	RXL	RFLP	DS 2	RXL	RFLP
*Apa*I	6	4	+	2	2	−	4	4	+
*Bam*HI	4	4	+	4	3	+	3	3	−
*Bgl*II	4	4	+	3	2	+	3	3	−
*Dra*I	3	3	+	2	2	+	3	3	−
*Eco*RI	3	3	+	1	1	−	3	3	−
*Eco*RV	2	2	+	2	2	+	3	3	−
*Hin*dIII	3	3	+	2	2	+	3	3	−
*Xba*I	3	3	+	2	2	+	3	3	+

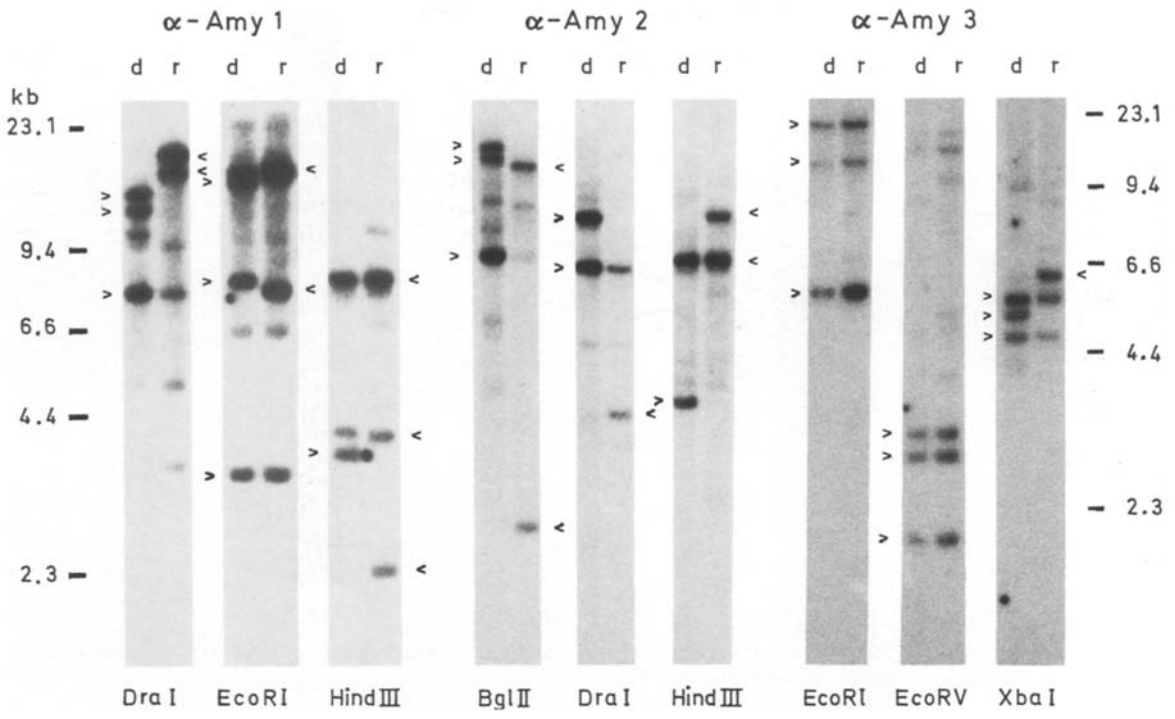

Fig. 1. RFLPs detected in DS 2 (*d*) and RXL 10 (*r*) rye lines by means of wheat α-*Amy1*, α-*Amy2*, α-*Am3* probes with several restriction enzyme digests. The *arrows* indicate fragments homologous to the probe used

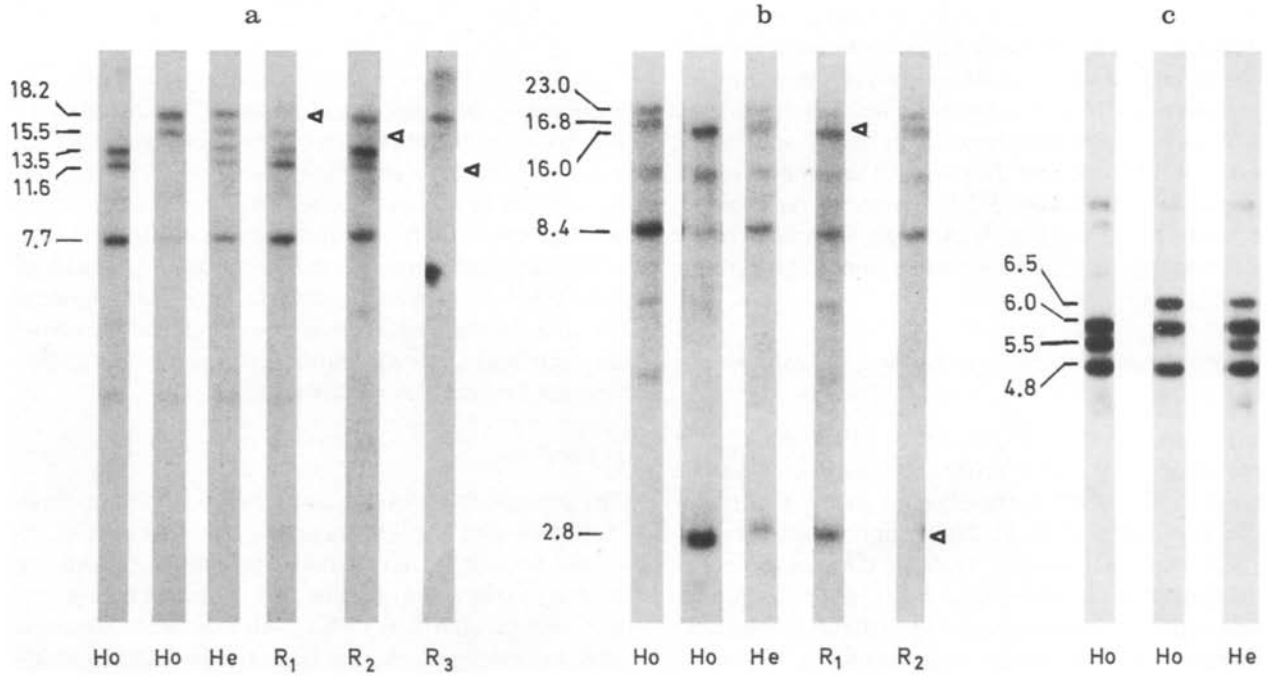

Fig. 2. RFLPs detected in F_2 progeny of the DS 2 × RXL 10 cross using α-*Amy1*/*Dra*I (**A**), α-*Amy2*/*Bgl*II (**B**), and α-*Amy3*/*Xba*I (**C**) probe restriction enzyme combinations. The lengths of fragments found in parental-type homozygotes (Ho), heterozygotes (He), and recombinants (R) are given in kb on the *left*. The *arrows* indicate absent fragments in the recombinant genotypes

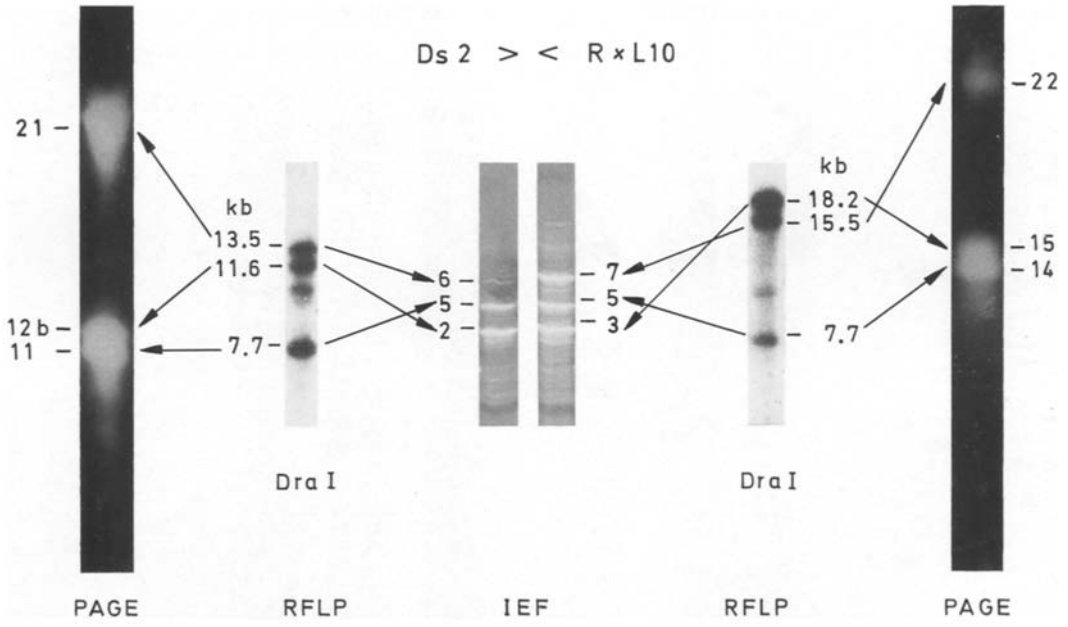

Fig. 3. RFLP, PAGE, and IEF patterns of α-*Amy1* in DS 2 and RXL 10. The corresponding fragments and bands are connected by *arrows* (see text). The PAGE bands are numbered according to Masojć (1987)

α-amylase patterns. The IEF pattern of DS 2 consists of two strong bands (2 and 5) and a single faint band (6), whereas that of RXL 10 includes three strong (3, 4, and 7) and a number of faint bands (Fig. 3). The three main bands are also present on the PAGE zymogram of each line (Fig. 3). The comparison of the F_2 segregations for both isozymes and RFLP showed that IEF band 6 and PAGE band 21 correspond to the 13-5-kb fragment and IEF band 7 and PAGE band 22, to the 15.5-kb fragment at the α-*Amy1-2* sublocus. Because no recombinants between α-*Amy1-1-1* and α-*Amy1-1-2* were observed, only the whole group of IEF bands 2, 3, and 5, and PAGE bands 11, 12b, 14, and 15 could be attributed to the group of 7.7-, 11.6-, and 18.2-kb fragments representing the α-*Amy1-1* locus (Fig. 3). A single isozyme – single restriction fragment correspondence among these groups is shown in Fig. 3.

Intrachromosomal mapping of α-Amy1 on chromosome 6RL

The F_2 progenies were also scored for RFLPs detected by three other wheat cDNA probes previously shown to be located at the wheat homoeologous group 6 chromosomes (S. Chao and M. D. Gale, unpublished data).

The map obtained (Fig. 4) shows the α-*Amy1* genes to be flanked by the *Xpsr149* ad *Xpsr154* loci. The orientation of this linkage group relative to the centromere has been derived by comparison with the map of the barley homoeoloci reported by Kleinhofs et al. (1988). The relocation of *Xpsr106* distal to *Xpsr154* was unexpected since, in wheat and barley, PSR154 detects a single copy

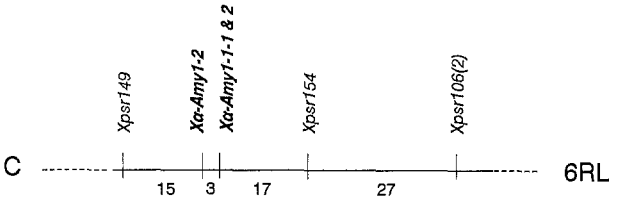

Fig. 4. Genetic map of chromosome *6RL* in the α-*Amy1* region

sequence on the short arm of group 6 homoeologues. In rye, however, the probe detects two independent RFLP loci. There are two possible explanations. The mapped locus could be a duplicated sequence removed from the short to the long arm of *6R*, as shown in Fig. 4. On the other hand, the *Xpsr149* – α-*Amy1* – *Xpsr154* region of *6RL* could be inverted relative to barley chromosome *6H*, and the mapped *Xpsr106* locus could still lie across the centromere on *6RS*. Further data is required to distinguish between these alternatives.

α-Amy2 genes

The genomic DNA of DS 2 was cut by *Bg1*II into three (8.4-, 16.8- and 23.0-kb) fragments, and that of RXL 10 - into two (2.8- and 16.0-kb) fragments hybridizing strongly to the α-*Amy2* probe (Fig. 2B). The F_2 segregation showed that 8.4- and 2.8-kb fragments represent alleles at one locus whereas 16.8- and 16.0-kb fragments represent alleles at another locus. The linkage analysis showed that these loci, designated α-*Amy2-1* and α-*Amy2-2*, respectively, are 5 cM apart (Table 2).

Table 2. RFLP segregation among α-*Amy1* and α-*Amy2* genes in F_2 progeny of the cross DS 2 × RXL 10 (linkage analysis according to Allard 1956)

Genes	Parental phenotype (kb)		No. F_2s	Hom-ozyg.	Het-erozyg.	Rec.	Linkage	
	DS 2	RXL 10					*P*	SE
α-*Amy1-1*	11.6	18.2	87	39	43	5	0.03 ± 0.01	
α-*Amy1-2*	13.5	15.5						
α-*Amy2-1*	8.4	2.5	85	34	43	8	0.05 ± 0.02	
α-*Amy2-2*	16.8	16.0						

The third (23-kb) fragment found in DS 2 segregated at a 3 (present):1 (absent) ratio. It was found together with the 16.8-kb fragment in 80 and alone in 5 F_2 plants. This observation excludes the possibility that 16.8- and 23.0-kb fragments represent the same gene cut at the internal *Bgl*II site. A more likely explanation is that the 23.0-kb fragment contains a third linked α-*Amy2* gene. Unlike α-*Amy1*, the α-*Amy2* RFLPs between parental lines are not reflected in the IEF isozyme pattern, where both lines have a phenotype of one strong and two faint bands.

α-Amy3 genes

Two common (4.8- and 6.0-kb) and two different (5-5- and 6.5-kb) fragments hybridizing to the α-*Amy3* probe were revealed by *Xba*I digests of DS 2 and RXL 10 (Fig. 2C). The F_2 segregation was consistent with a 1:2:1 ratio, indicating that the 5-5- and 6.5-kb fragments represented different alleles at a single locus. The apparent homogeneity of the cross with respect to the two remaining fragments precluded further analysis.

The genes encoding rye α-amylase isozymes have been previously assigned the symbols α-*Amy1*, α-*Amy2*, and α-*Amy3* (Masojć 1987), which are currently attributed to the three groups of α-amylase genes. Therefore, in this paper, we propose to denote the respective genes from α-*Amy1* group as α-*Amy1-1-1*, α-*Amy1-1-2*, and α-*Amy1-2*, in line with nomenclature systems used in wheat for compound loci (Hart and Gale 1988).

Discussion

α-Amy1 genes

The results of simultaneous PAGE, IEF, and RFLP studies of the rye α-*Amy1* gene system each lead to similar conclusions. PAGE separations revealed three α-AMY1 isozymes in inbred lines. These same lines showed the presence of three major IEF bands. Moreover, three *Dra*I, *Eco*RI, *Hin*dIII, and *Xba*I fragments were detected in DS 2 and RXL 10 genomic DNAs probed with a wheat α-*Amy1* cDNA. In wheat, the number of *Eco*RI fragments revealed by the α-*Amy1* probe is thought to be equivalent to α-amylase gene numbers (Martienssen 1986) because no *Eco*RI sites internal to α-*Amy1* genes have been found. It might be expected that this is also true for rye α-*Amy1* fragments uncovered by *Eco*RI or *Dra*I. Thus, both isozyme and RFLP studies seem to show that three structural genes encode for rye high pI group α-amylases. This number is equal to that suggested for barley α-AMY1 isozymes (MacGregor and MacGregor 1987) and is similar to the mean number on each of the wheat group 6 chromosomes (Martienssen 1986).

Although there is no recombination evidence that the α-*Amy1* genes are separable on chromosome *6A* or *6D* in wheat (Gale et al. 1983) or *6H* in barley (Kleinhofs et al. 1988), there is growing evidence that cereal α-amylase gene copies are spatially separated along the chromosomes. Nishikawa et al. (1981) described two α-*Amy1* loci on wheat chromosome *6B* separated by 20% recombination. More recently, Cheung et al. (1991) have demonstrated by pulsed field gel analysis and long-range restriction mapping that five α-*Amy1* genes on *6B*L are dispersed over at least 4 Mbp and are separable by 1% recombination. The results obtained here provide an intermediate result in rye with 3% recombination separating three genes.

α-Amy2 genes

Both RFLP and IEF data presented here suggest that two α-*Amy2* loci are located on chromosome *7R*. This is probably the first evidence for recombination within an α-*Amy2* locus on a single chromosome in cereals. In wheat and barley, no recombinants among genes encoding the low pI group of isozymes have been reported (Gale et al. 1983; Brown and Jacobsen 1982). We propose that the alleles represented by 8.4- and 2.8-kb *Bgl*II fragments be assigned the symbols α-*Amy2-1 a* and α-*Amy2-1 b*, whereas the second pair of 16.8- and 16.0-kb fragments he assigned the symbols α-*Amy2-2 a* and α-*Amy2-2 b*, respectively. The remaining 23.0-kb fragment may represent a third α-*Amy2* gene; however, the evidence for this is not yet convincing.

α-Amy3 genes

The third group of α-amylase genes, observed only as mRNAs in developing seeds (Baulcombe et al. 1987), exhibits the lowest polymorphism among the three multigene families, as judged on the basis of the detected RFLPs. Only four out of ten REs uncovered RFLPs between parental lines. Observation of three α-*Amy3* fragments with each of eight restriction digests strongly suggests that there are three α-*Amy3* genes in rye genome, compared to the single copy per genome observed in

hexaploid wheat (Baulcombe et al. 1987). A definitive answer concerning the number and linkage relationships between α-*Amy3* genes in rye, however must await further studies. At the moment we suggest that the segregating *Xba*I alleles be assigned the symbols α-*Amy3-1 a* (5.5 kb) and α-*Amy3-1 b* (6.5 kb), whereas the remaining two *Xba*I fragments be assigned to different α-*Amy3-2* (6.0-kb) and α-*Amy3-3* (4.8-kb) genes.

Acknowledgements. The senior author was a visiting scientist at the Cambridge Laboratory during the course of this study. He sincerely thanks the CL personnel for all facilities and support in this work and the British Council for financial support of his stay in UK.

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