

Genetic regulation of alpha-amylase synthesis in rye (*Secale cereale* L.) grain

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Abstract. Genetic analysis of two rye interline crosses and a set of wheat/rye chromosomal addition lines was performed to reveal the mechanism underlying wide variation range of alpha-amylase activity in sound grain. The long arm of chromosome 6R was found to be responsible for increased enzyme synthesis during late stages of triticale grain maturation. Only nuclear genes seemed to control alpha-amylase activity, as reciprocal crosses between rye lines showed no maternal effects. Low enzyme activity showed complete dominance over high level of its synthesis. Segregation ratios, observed in F₂ and BC₁ crosses, indicated that recessive alleles at two independent duplicative loci underlie intensive alpha-amylase production.

Key words: addition lines, alpha-amylase, duplicative genes, *Secale cereale*, sprouting resistance.

Introduction

Induction of alpha-amylase synthesis in maturing grain usually results in a reduced quality of flour (MARES, MRVA 1993). High levels of late maturity alpha-amylase were reported in wheat (MARES, GALE 1990, MARES et al. 1994), rye (MASOJC, LARSSON-RAŻNIKIEWICZ 1991a, b) and triticale (MARES, OETTLER 1991) varieties, even in the absence of any visible signs of sprouting. The enzyme is synthesised in response to weather conditions (low temperatures and high air humidity) that delay grain maturation (MARES, MRVA 1993, KETTLEWELL, COOPER 1993). Weather-affected, erratic expression of pre-harvest alpha-amylase production has precluded systematic study of the genetic

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mechanisms underlying this process. Although structural genes encoding three groups of polymorphic alpha-amylase isozymes were identified and mapped to cereal chromosomes (MASOJĆ, GALE 1991), little is known about regulatory genes that control variation of the enzyme levels.

MARES and GALE (1990) suggested that one recessive gene might be responsible for high enzyme activity in the wheat cv. Spica. In triticale, the chromosome arm 6RL was shown to control "de novo" synthesis of alpha-amylase in aleurone layer (GALE et al. 1990). A study of rye inbred lines and their crosses allowed to postulate two recessive duplicative genes to be a genetic cause of high level of enzyme synthesis (MASOJĆ, LARSSON-RAŻNIKIEWICZ 1991b). Possible role of endogenous alpha-amylase inhibitor (ISA-1) in reducing starch degradation was also investigated (MACGREGOR et al. 1986, ZAWISTOWSKA et al. 1988). However, its concentration in grain seems to be insufficient to hinder the effects of intensive enzyme synthesis during sprouting (MASOJĆ 1993).

Recent reports considering vivipary (*vp1*) gene in maize (McCARTY et al. 1989) and in other cereals (CADLE et al. 1993), together with growing evidence on the GA- and ABA-responsive promoters of alpha-amylase structural genes (GUBLER, JACOBSEN 1992, ROGERS, ROGERS 1992) show that several loci might affect the enzyme level in cereal grain. Identification and chromosomal localization of such loci should facilitate breeding for sprouting resistant cultivars.

In the present investigation we examine the inheritance mode and chromosomal location of genes regulating alpha-amylase synthesis in rye grain.

Material and methods

Two inbred winter rye lines Ot1-3 and RXL10 having low alpha-amylase activity were reciprocally crossed with line 541 characterized by high enzyme levels. Each line was self-pollinated for more than 20 years. The grain representing F₁, F₂, F₃, BC₁/F₁ (F₁ progeny of BC₁) and BC₁/F₂ (F₂ progeny of BC₁) generations of both crosses were used for the study. Plants were grown in 1992/1993, on the experimental plot of the Agricultural University in Szczecin. Ears of each plant were bagged to avoid cross-pollination. The phenotypes of F₂ and BC₁ plants were characterized by studying a sample of 20 individual kernels from the main ear.

Wheat/rye chromosomal addition and ditelosomic addition lines developed by Danuta MIAZGA, were used to investigate chromosomal location of alpha-amylase regulatory genes. These lines contain individual chromosomes or

chromosome arms of the rye cv. Dańkowskie Żłote introduced to the complete chromosome set of the wheat cv. Grana. Three and ten kernels of each line harvested in 1993 and 1994, respectively, were analysed.

Alpha-amylase activity was assayed in sound grain by the previously described method of radial diffusion in agarose gel containing beta-limit dextrin (DAUSSANT et al. 1980, MASOJC, LARSSON-RAŻNIKIEWICZ, 1991a). Each kernel was ground separately in a micro-hammer mill and soaked in 150 μ l of 20 mM acetate buffer, pH 5.5 with 1 mM CaCl_2 . The slurry was centrifuged at 10 000 g for 5 min. Twenty microlitres of the resultant supernatant was pipetted into radial wells (diameter 5 mm) punched out in agarose gel.

The diameter of the diffusion circle, developed in the gel after 22 h at 6°C and visualised with iodine solution, was measured to an accuracy of 0.5 mm. The diffusion circle diameter (d) in a linear fashion corresponds to the logarithm of alpha-amylase activity (A) given in U/ml.

The regression equation $\log_4 A = 0.49 d - 6.41$ describes a calibration curve obtained empirically by developing diffusion circles of six subsequent four-folds dilutions of concentrated (128 U/ml) purified barley malt alpha-amylase (Sigma) solution. Activity unit (1U) is defined as the enzyme amount releasing 1 mg of maltose in 3 min at pH 6.9 and at 20°C. Because of the exponential mode of alpha-amylase activity variation, the assay results were presented in a logarithmic scale, as a diameter (mm) of diffusion circles.

Results

Wheat/rye chromosomal addition and ditelosomic addition lines showed significant variation in grain alpha-amylase activity (Fig. 1). Examination of sound grain harvested in 1993 revealed that two lines, Grana/Dańkowskie Żłote 6R and Grana/Dańkowskie Żłote 6RL, contained several folds higher enzyme concentration than the rest of lines and the parental wheat cv. Grana. The highest activity was found in amphiploid 8x, which all the lines were derived from. Also, the rye cv. Dańkowskie Żłote showed a high activity level.

Since the sound grain, harvested in 1994, had generally low levels of alpha-amylase, the activity study was carried out after 5-day imbibition of dormant seeds in water. Ten kernels of each line showing no signs of germination were allowed to dry before the assay. It is apparent that all genotypes, except the line Grana/Dańkowskie Żłote 1R, showed a higher alpha-amylase activity than in the previous year. Again, the lines Grana/Dańkowskie Żłote 6R and Grana/Dańkowskie Żłote 6RL responded most strongly to the provoking conditions,

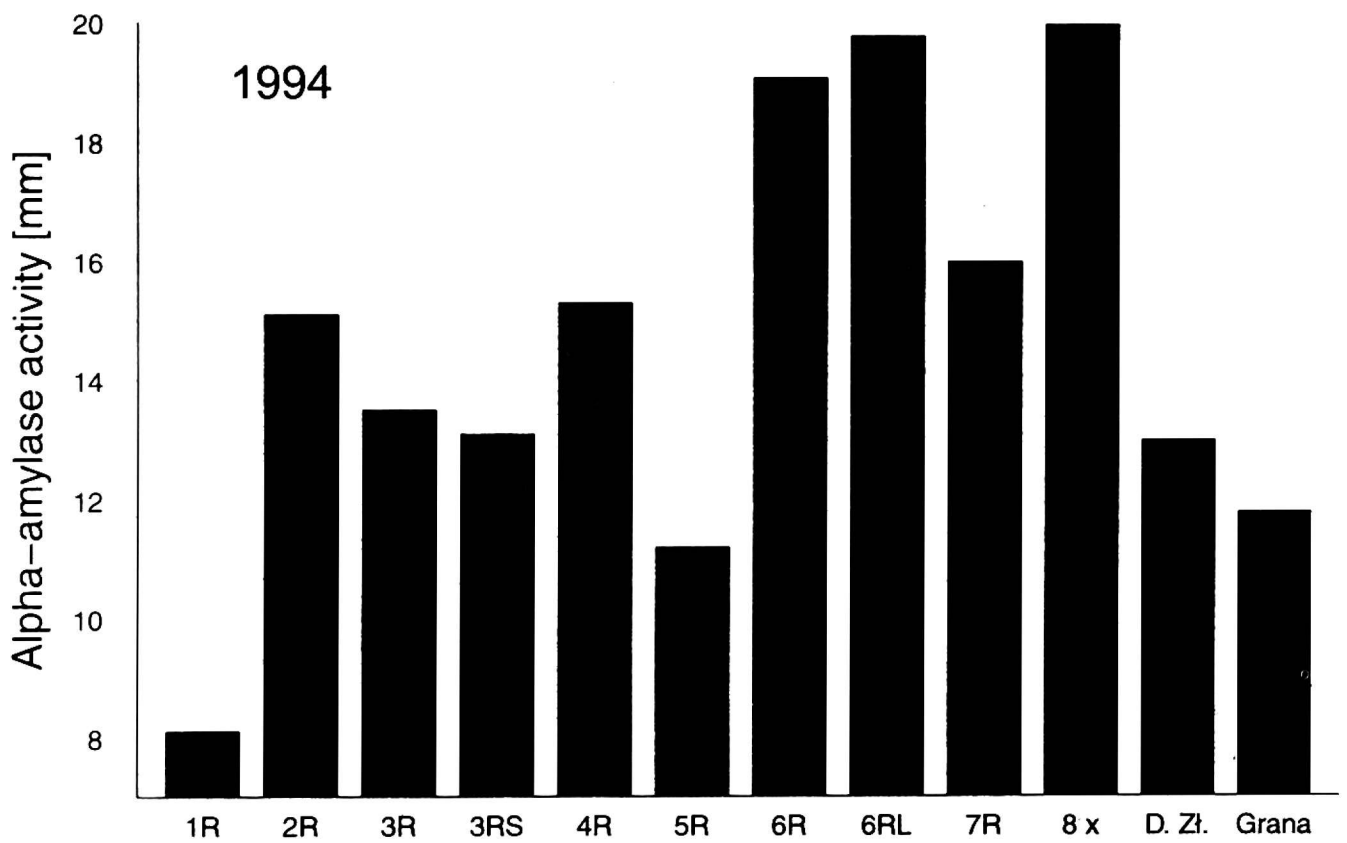
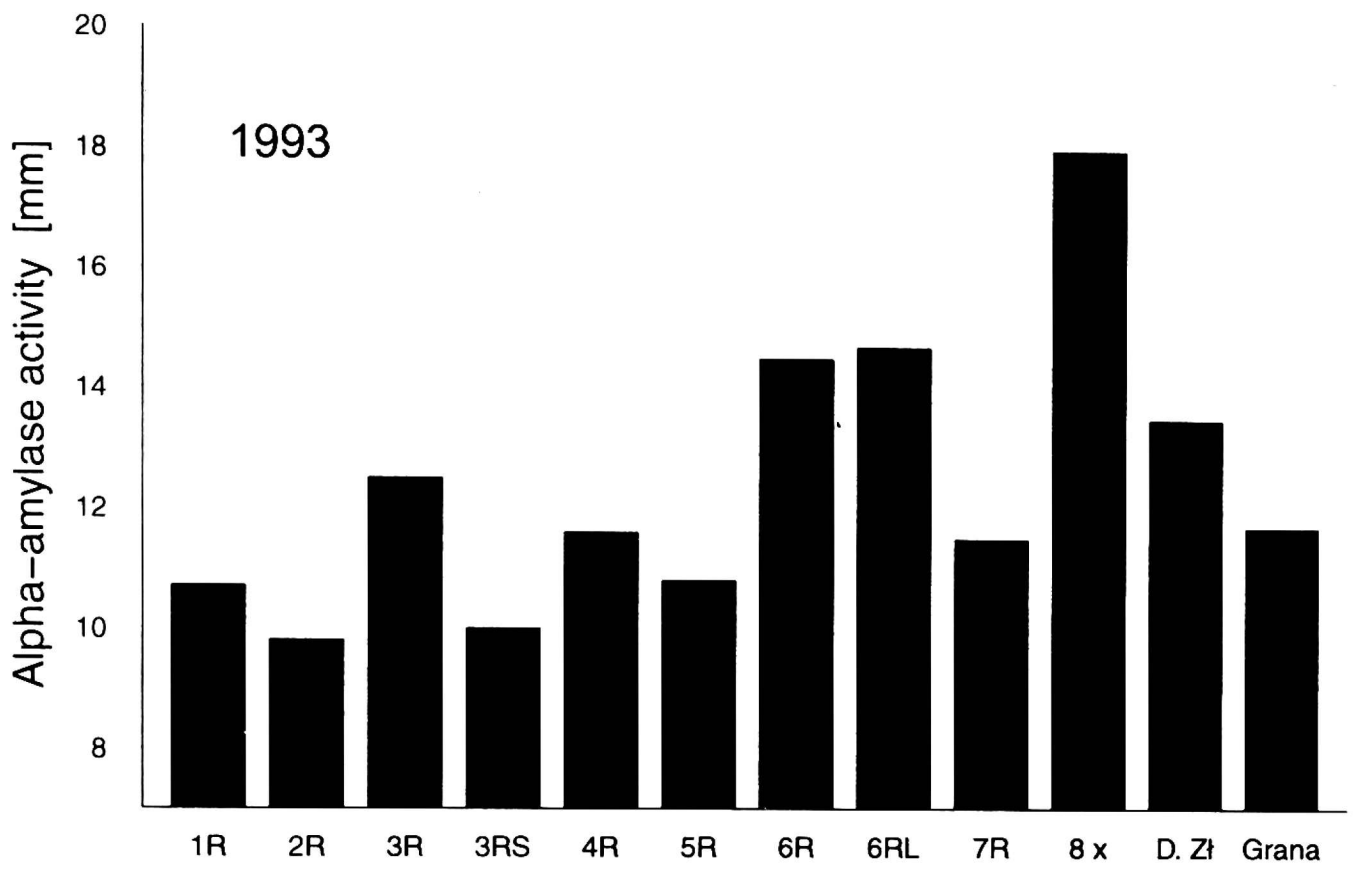


Fig. 1. Alpha-amylase activity in grain of wheat (cv. Grana)/rye (cv. Dańkowskie Złote) chromosomal addition and ditelosomic addition lines, original *AABBDDRR* hybrid (8x), Dańkowskie Złote and Grana strains

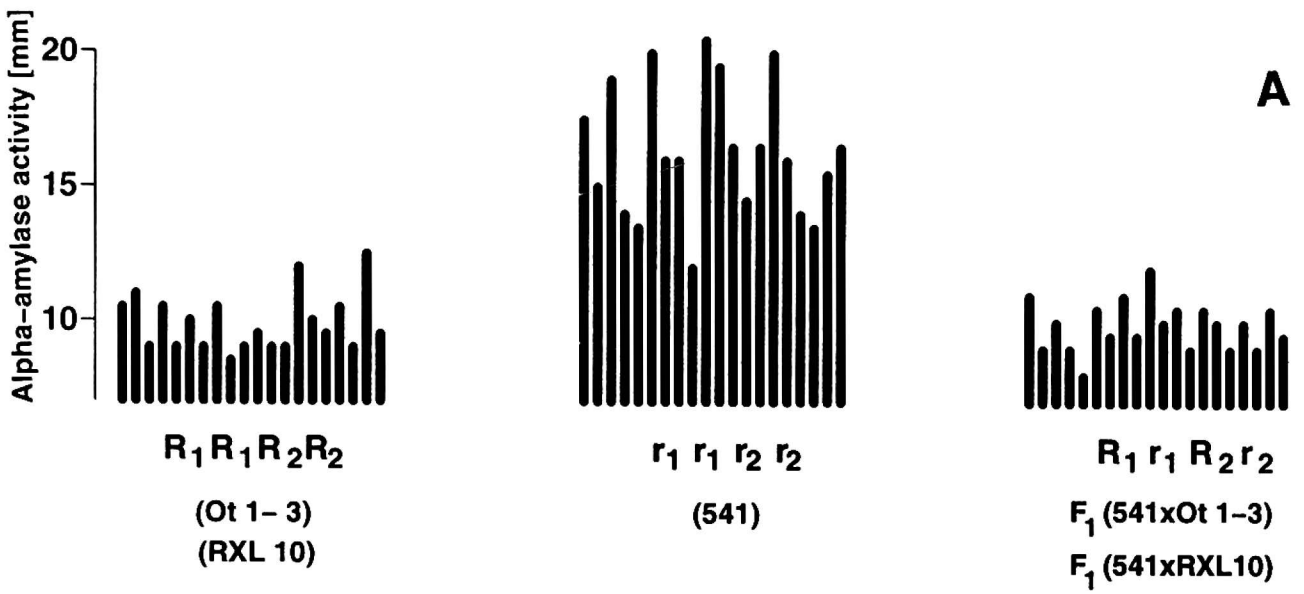
similarly to their octoploid parent. Interestingly, alpha-amylase levels found in rye grain were much lower.

A comparison of alpha-amylase activity in individual kernels of three parental rye inbred lines showed a low activity and a narrow variation range in Ot1-3 and RXL10 as compared to the sprouting susceptible line 541 (Table 1, Fig. 2). Although the majority of the studied kernels of the latter line had

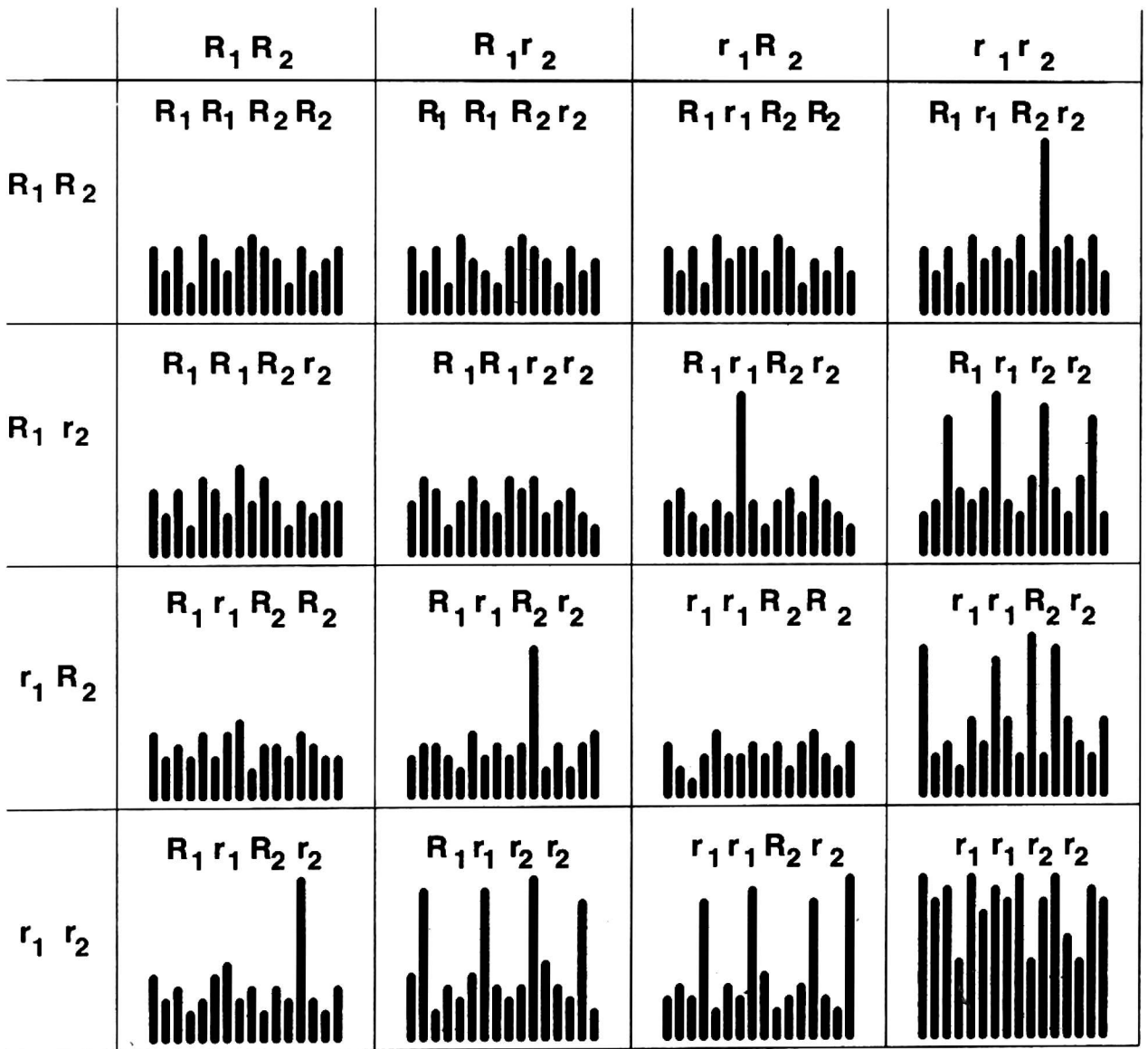
Table 1. Alpha-amylase activity in mature, not sprouted grain of inbred rye lines and their crosses evaluated by the agarose gel diffusion method

Line, cross and seed generation	Number of kernels studied	Alpha-amylase activity/kernel		
		mean (mm)	standard deviation	variation range (mm)
Ot 1-3	30.0	10.3	1.5	8.0-14.0
541.0	30.0	16.3	2.4	12.0-20.5
RXL10	30.0	10.2	1.5	8.0-14.0
F ₁ (541 × Ot 1-3)	30.0	9.9	1.3	8.0-12.0
F ₁ (Ot 1-3 × 541)	30.0	9.6	1.1	8.5-13.0
F ₂ (541 × Ot 1-3)	40.0	9.9	1.9	8.0-16.5
BC ₁ /F ₁ [(541 × Ot 1-3) × 541]	30.0	10.4	2.4	8.0-17.0
BC ₁ F ₂ [(541 × Ot 1-3) × 541]	740.0	11.6	2.2	8.0-20.5
F ₃ (541 × Ot 1-3)	1500.0	10.8	2.1	7.0-21.0
F ₁ (541 × RXL10)	30.0	10.5	1.2	8.5-12.5
F ₁ (RXL10 × 541)	30.0	9.6	1.4	8.5-13.5
F ₂ (541 × RXL10)	40.0	10.0	1.8	7.0-17.5
BC ₁ F ₁ [(541 × RXL10) × 541]	30.0	10.5	1.8	8.0-14.0
BC ₁ F ₂ [(541 × RXL10) × 541]	1100.0	12.8	2.2	7.0-22.0
F ₃ (541 × RXL10)	1340.0	12.2	2.1	8.0-22.0

alpha-amylase activity exceeding much the variation range found in Ot1-3 and RXL10, few seeds exhibited values which could be also attributed to the both sprouting resistant lines. Nevertheless, the enzyme activity distribution in individual kernels (Fig. 2) showed clear difference between sensitive and resistant lines. Such activity patterns characterising the phenotype of the parental line, F₁ generation or F₂ plant seem to be more informative than the mean alpha-amylase activity in the bulk grain sample alone.



F_2



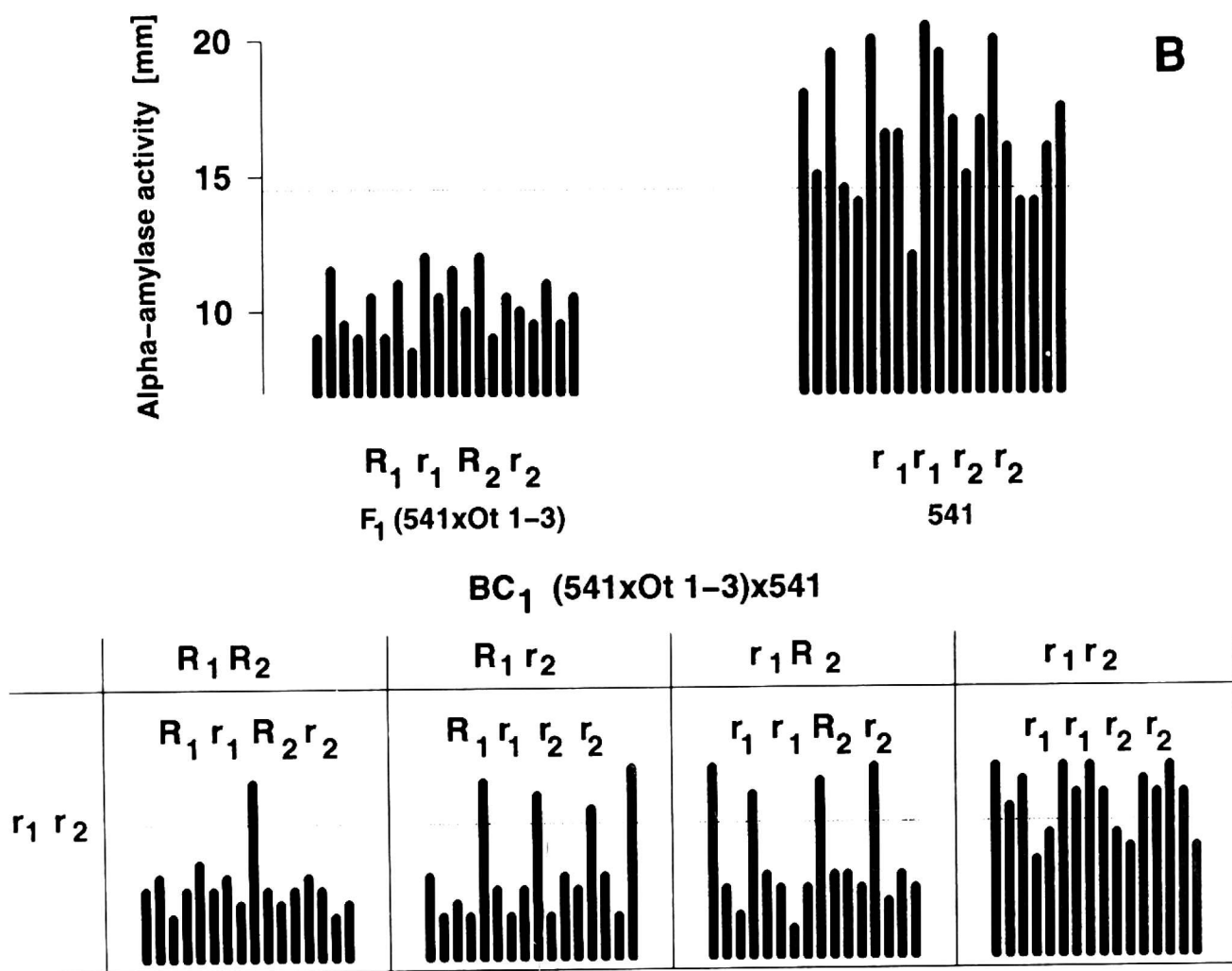


Fig. 2. Expected phenotypic segregation among F_2 (A) and BC_1 (B) plants of the inter-line crosses assuming that the late maturity alpha-amylase synthesis is controlled by two duplicative recessive genes. A pattern of alpha-amylase activities in individual grains collected from F_2 or BC_1 plant represents its phenotype

The mean values and variation range of F_1 reciprocals did not show apparent maternal effects, for they were very similar in both crosses and did not differ significantly from those of sprouting resistant parents (Table 1). Low mean activities found in grain representing F_2 , F_3 and BC generations gave additional evidence for the dominance of low and recessiveness of high alpha-amylase activity. However, wide ranges of variation, observed in these progenies, indicated segregation of genes affecting the enzyme synthesis.

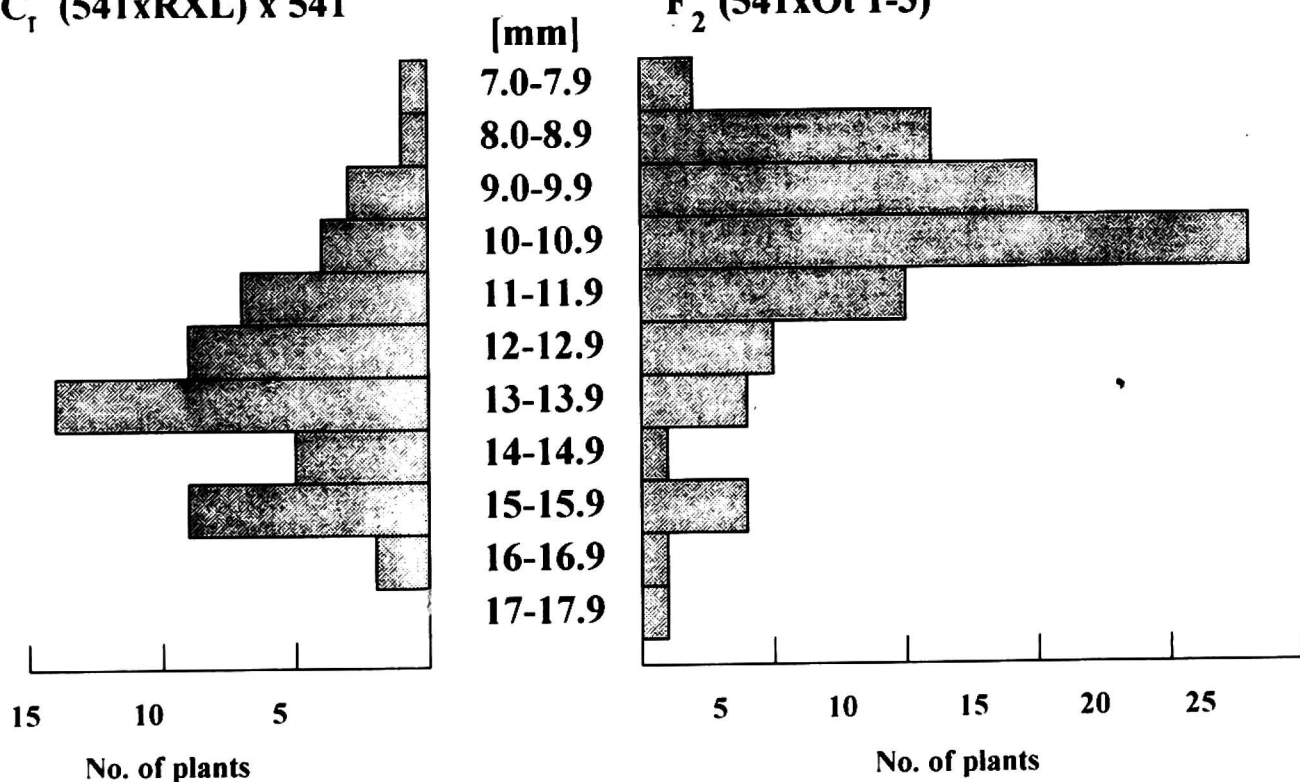
Earlier, preliminary results (MASOJĆ, LARSSON-RAŻNIKIEWICZ 1991b) suggested that two independent genes control the intensity of alpha-amylase synthesis in rye. To check the validity of this hypothesis, individual kernels developed on self-pollinated F_2 and BC_1 plants were examined. The genes under study were temporarily assigned with the R_1 and R_2 symbols. Expected segregation of genotypes and respective activity patterns in a progeny grain are shown in Fig. 2.

Table 2. Genetic analysis of variation in alpha-amylase activity in rye kernels collected from BC₁ and F₂ plants

Cross	Phenotypic class*			Expected segregation	χ^2	P
	a	b + c	d			
BC ₁ (541 × RXL) × 541		43	12	3 : 1	0.30	0.8-0.9
BC ₁ (541 × Ot 1-3) × 541		30	7	3 : 1	0.73	0.6-0.7
F ₂ (541 × Ot 1-3)	33	36	6	7 : 8 : 1	0.42	0.8-0.9
F ₂ (541 × RXL)	24	37	6	7 : 8 : 1	2.10	0.3-0.4

*kernels of alpha-amylase activity: a – below 15 mm, b – equal or higher than 15 mm, c – exceeding 15 mm, d – high, similarly as in the parental line 541.

Since the variation range observed in Ot1-3 and RXL10 lines did not reach a 15-mm level, the F₃ kernel showing 15-mm or larger diameter of the diffusion circle indicates the existence of $r_1r_1r_2r_2$ segregants in the progeny of a given F₂ plant. Four alpha-amylase activity patterns (phenotypic classes) should therefore be expected among F₂ plants: a – all kernels having activity below 15 mm level, b – 1/16 of the kernels with activity equal or higher than 15 mm, c – 1/4 of kernels exceeding 15 mm and d – the majority of kernels showing high enzyme levels, similarly as in the parental line 541. The expected phenotypic ratio of a : b : c : d should be 7 : 4 : 4 : 1, or 7 : 8 : 1 if the b and c phenotypes are pooled together, assuming independent segregation of the two

BC₁ (541xRXL) x 541**F₂ (541xOt 1-3)****Fig. 3.** Distribution of the mean values of alpha-amylase activities in grain collected from ears of F₂ and BC₁ plants

genes as shown in Fig. 2A. Accordingly, high alpha-amylase homozygous recessive genotypes versus heterozygous are expected in 1 : 3 ratio among BC₁ plants (Fig. 2B).

The examination of 20 individual kernels from each F₂ and BC₁ plants allowed to distinguish three phenotypic classes equivalent to a, b + c and d, respectively (Table 2). All plants within the first class had low alpha-amylase activities in each kernel studied. Only few (usually 1-6) kernels per plant in the middle class exhibited elevated alpha-amylase levels. The third class comprised plants with the majority (11-19) of high alpha-amylase activity kernels. The observed segregation ratios agree with 3 : 1 expectation for BC₁ and with 7 : 8 : 1 expectation for F₂ cross, confirming the two-gene inheritance mode presented in Fig. 2. In addition, mean activity values of the F₂ and BC₁ plants showed distributions allowing to disrupt the population into two groups (Fig. 3). The ratios between plants with high activity alpha-amylase (range 15.0-17.9) and those within the activity range of 7.0-14.9 were close to 1 : 15 in F₂ and 1 : 3 in BC₁ generation, as expected for two duplicative loci with dominant alleles.

Discussion

The analysis of Grana/Dańkowskie Żłote chromosome addition and ditelosomic lines suggested that the long arm of the chromosome 6R is a site of genes enhancing alpha-amylase synthesis in triticale grain. This result agrees with earlier study (GALE et al. 1990) performed on genetically different set of wheat/rye addition lines (Holdfast/KingII). Also in wheat, the chromosome 6B was shown to control development of the late maturity alpha-amylase (MARES, GALE 1990). The long arm of homoeologous chromosomes 6 in cereals is known to carry a family of linked genes encoding high-pI alpha-amylase isozymes – a predominant group synthesised "de novo" in sprouting grain (MASOJĆ, GALE 1991). A promoter sequence of these genes has recently been shown to be a site of GA- and ABA-mediated regulation of alpha-amylase synthesis (GUBLER, JACOBSEN 1992, ROGERS, ROGERS 1992). Incompatibility between regulatory proteins encoded in A and B genomes with rye α -Amy1 promoter sequences might be the first explanation for the 6RL chromosome enhancing effect on alpha-amylase synthesis in triticale.

The presence of Amy1 structural genes itself on the chromosome 6RL seems to be the second possible explanation for the increased enzyme levels in the wheat/rye 6R and 6RL lines. However, a several fold higher activity as compared to other chromosomal addition lines suggests, that there might be more genes on the chromosome arm 6RL or on other chromosomes which control transcription at alpha-amylase structural loci. Such hypothesis is sup-

ported by a two-gene inheritance mode confirmed in this study. It may be speculated that one of the regulatory genes is linked with a cluster of alpha-amylase structural genes, positioned near centromere, while the second one is located further towards the distal end of the chromosome arm. Interestingly, the RFLP map of the chromosome 6 RL reveals the segment homologous to the fragment of the wheat chromosome 3 L (DEVOS et al. 1993). This segment contains RFLP markers flanking the gene for red grain coat colour (FLINTHAM, HUMPHRAY 1993) and most likely the red coat gene itself (MELTZ et al. 1992). Selection for dominant alleles of this gene is one of the strategies to reduce sprouting damage in wheat (FLINTHAM 1993).

Another candidate for r_1 or r_2 gene might be rye homoeolog of $vp1$ recessive mutation found in maize which causes ABA-insensitivity of grain and vivipary (McCARTY et al. 1989). In fact, the line 541 is prone to sprouting in the ear, being significantly less responsive to exogenous ABA than sprouting resistant lines (MASOJĆ et al. 1995).

A set of genes known to decrease alpha-amylase levels are GA-insensitivity genes (Gai) located on the chromosomes 5 R and 7 R (BÖRNER 1991, BÖRNER et al. 1992). These, however, are recessive towards GA-responsive alleles and, therefore, cannot account for alpha-amylase variation described in this paper. On the other hand, there are also reports on dominant genes for GA-insensitivity (JLIBENE, GUSTAFSON 1992), which relationship with $R_1 R_2$ alleles is not excluded.

The dominance of low alpha-amylase activity means, that in heterogeneous rye population varieties it is difficult to reduce frequency of unfavourable recessive alleles by conventional selection. As even a small fraction (ca 1%) of sprouted kernels in a grain sample might significantly reduce breadmaking quality of flour (MUNCK 1987), sprouting resistant cultivars should be practically free from recessive $r_1 r_2$ alleles. The method of genotype determination by analysing individual kernels from the major ear, applied in this study, enabled differentiation between heterozygous $R_1 r_1 r_2 r_2$ or $R_1 r_1 R_2 r_2$ and homozygous $R_1 R_1 R_2 R_2$ genotypes. However, selection of $R_1 R_1 r_2 r_2$ or $r_1 r_1 R_2 R_2$ from $R_1 R_1 R_2 R_2$ genotypes cannot be achieved. This last conclusion explains difficulties in selection against r_1 and r_2 alleles in rye varieties in practical breeding for sprouting resistance.

A considerable recent progress in understanding genetic and biochemical mechanisms controlling alpha-amylase synthesis in maturing grain will eventually result in development of efficient selection strategies. Since both, hidden and visible sprouting are highly dependent on weather, molecular marker-assisted selection methods would be the best solution (ANDERSON et al. 1993). With the development of RFLP maps of cereal chromosomes (DEVOS et al. 1993, DEVOS, GALE 1993), this task should be accomplished in the near future.

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