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Characterization of cDNA clones for rye endosperm β-amylase and analysis of β-amylase deficiency in rye mutant lines

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Summary. In order to characterize a β-amylase deficiency in the endosperm of mutant rye lines, homologous cDNA probes were prepared. A rye cDNA library was constructed from a normal line and screened with a barley β-amylase probe. Three partial cDNA clones specific for endosperm β-amylase in rye were isolated and characterized. The largest of these clones was used to investigate the expression of endosperm β-amylase in mutant and normal lines by Northern hybridization. These experiments, as well as *in vitro* translation experiments, demonstrate the absence of endosperm β-amylase mRNA in mutant lines. Sequencing of three different cDNA clones revealed a single nucleotide difference, which suggests that two genes encoding endosperm β-amylase genes might exist in rye. From Southern blots we anticipate that these two genes are tightly linked. Results of these experiments and previous data indicating that the mutation was located within the β-amylase locus on chromosome 5 are consistent with the hypothesis that the mutation results from a deletion simultaneously affecting the two genes. However, due to extensive polymorphism within normal lines used as control, additional experiments will be required to further substantiate this conclusion. The deduced amino acid sequence reveals the occurrence of three short glycine-rich repeats containing 11 or 12 residues close to the carboxyl terminus of the protein. A comparison of the nucleotide sequence between rye and previously described barley cDNA clones revealed ca. 90% homology at the amino acid level, except in this C-terminal repeated part, where it drops to 45%.

Key words: Rye – β-Amylase – cDNA – Mutants

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Introduction

β-Amylase (1,4-α-D-glucan maltohydrolase; EC 3.2.1.2) catalyzes the liberation of α-maltose from the nonreducing ends of starch-related 1,4-α-glucans (Robyt and Whelon 1968). In cereals, β-amylase takes part in the mobilization of starch in germinating kernels. It is synthesized at late stages of seed development and, at maturation, it accounts for 1–2% of endosperm proteins (Hejgaard and Boisen 1980). Although most studies deal with the mature seed form of the enzyme, β-amylase is also present in vegetative tissues such as leaves and roots (Levi and Preiss 1978; Shewry et al. 1988). Recent studies in cereals indicate that there are two forms of the enzyme which correspond to different isozymic groups encoded by separate loci. A single locus on chromosome 5 in rye encodes mature-grain β-amylase (form II), and β-amylase located in shoots, roots, and in pericarp of developing seeds (form I) is controlled by another locus on chromosome 2 (Sharp et al. 1988; Daussant et al. 1991).

A number of studies have shown that the β-amylase of mature kernels exists in multiple isoenzymatic forms. In rye, endosperm β-amylase can be separated by isoelectric focusing into at least six isoenzymes with different isoelectric points (Sadowski et al. 1988). The whole isoenzymatic pattern behaves as if it were encoded by a single locus, *β-amy-1* (Sadowski and Daussant 1989), which is located on the long arm of chromosome 5 (Artyeomova 1982).

Some inbred lines of rye display a drastically reduced β-amylase activity in mature kernels (Daussant et al. 1981). The deficiency of β-amylase activity in these lines results from a lack of synthesis of the β-amylase forms typical of seed endosperm. Further studies at genetical and molecular levels are necessary to clarify the nature of this mutation. For instance, it is not known whether

the deficiency results from synthesis of an unstable abnormal protein, from a defect in mRNA stability, from the absence of transcription of the gene, or from a default in the gene itself.

Our current studies are aimed at understanding the genetic and molecular aspects of the β-amylase mutation in two inbred lines. As a first step, we isolated and characterized several cDNA clones coding for rye β-amylase using a barley heterologous probe (Kreis et al. 1987). These cDNAs have been used to study the expression and organization of β-amylase genes in normal and mutant lines of rye.

Materials and methods

Plant material

Rye inbred normal (Kazimierskie B4, Rogalinskie Pa, Rogalinskie F, Zeelandzkie E) and mutant (Kazimierskie H, Wegierskie 1) lines originating from 22 to 25 generations of inbreeding were obtained from the Laboratory of Rye Genetics, Academy of Agriculture, Cracow, Poland. Plants were grown in a glasshouse in Poznan. Developing endosperms were isolated 22–25 days after anthesis, frozen in liquid nitrogen, and stored at −80 °C. Etiolated shoots were grown for six days at 25 °C.

Protein extraction and analysis

Extraction, isoelectric focusing, and β-amylase characterization after IEF were carried out as previously described (Sadowski et al. 1988). Immunoprecipitation of β-amylase from the total translation products was carried out according to a published procedure (Martin and Northcote 1983), with antibodies against the wheat β-amylase used in previous studies (Daussant et al. 1981; Daussant and Laurière 1990). Immunoprecipitates were analyzed by SDS-PAGE and visualized by fluorography.

Isolation of polysomal RNA and in vitro translation

Membrane-bound polysomes from 22- to 25-day-old seeds were isolated as described previously (Forde et al. 1981). Poly(A)-rich RNA from membrane-bound polysomes was separated on an oligo (dT) cellulose column. Translation of the poly(A)-rich RNA was carried out in a reticulocyte lysate system (N.90, Amersham) containing ^{35}S-methionine. The translation products were separated by SDS-PAGE on 12% gels and visualized by fluorography.

Construction of a cDNA library

The poly(A)-rich RNA from membrane-bound polysomes of Rogalinskie Pa was used as a template for cDNA synthesis. The cDNA library was constructed in plasmid pBR322 according to described procedures (Gubler and Hoffman 1983; Laroche-Raynal and Delseny 1986). Plasmid DNA was cut with *Pst*I and tailed with dG. The cDNA was tailed with dC and annealed to the vector. *E. coli* strain RR1 was transformed by the calcium chloride method. Approximately 4,000 recombinants were recovered from 100 ng cDNA. Colonies were ordered on NEN GeneScreen nylon membranes. The cDNA library was screened by hybridization of bacterial colonies (Grunstein and Hogness 1975), using a nick-translated ^{32}P-labelled *Eco*RI-*Hin*dIII fragment of a barley β-amylase cDNA insert as a probe. The barley β-amylase full-length cDNA clone pcβC51 was kindly given by Dr. M. Kreis (Kreis et al. 1987). Hybridization was carried out at 42 °C in 50% formamide, 2 × SSC, 10 × Denhard's solution, 1% SDS, 100 μg/ml calf thymus DNA, and 20 ng/ml probe. The filters were washed at 65 °C in 2 × SSC and 0.1% SDS.

Further identification of β-amylase cDNA clones

Plasmid DNA was prepared from recombinants selected by colony hybridization with barley pcβC51 insert, digested with *Pst*I, and separated on a 1.4% agarose gel, then transferred to nitro-cellulose filters (Southern 1975). Both the *Eco*RI-*Hin*dIII fragment of the pcβC51 insert and the longest rye β-amylase insert pcβ1 were used as a ^{32}P-labelled probe. Hybridization was carried out under two stringency conditions. Low-stringency conditions were at 60 °C in 5 × Denhardt's solution, 5 × SSC, 0.5% SDS, 100 μg/ml calf thymus DNA, and 20 ng/ml probe. Washing of the filters was in 2 × SSC, 0.1% SDS at room temperature. For high stringency, hybridization was carried out at 69 °C in 2 × SSC, 5 × Denhardt's solution, 0.5% SDS; 100 μ/ml calf thymus DNA, and 20 ng/ml probe. Washing was at 65 °C in 2 × SSC, 0.1% SDS and in 0.1 × SSC, 0.1% SDS at room temperature.

Following this screening, positive clones were further characterized by DNA sequencing. Inserts of approx. 400 bp were transferred into pUC 18 and sequenced directly. The insert pcβ1 of ca. 800 bp was mapped, and restriction fragments were subcloned into pUC 18. The nucleotide sequence of rye cDNA inserts was determined using the double-stranded sequencing method (Chen and Seeburg 1985).

Northern hybridization

Poly(A)-rich RNA from membrane-bound polysomes derived from normal and mutant lines was denatured using formaldehyde and dimethylsulphoxide (Lehrach et al. 1977), separated on 1% agarose slab gel, and transferred to Amersham Hybond-N membrane. The *Pst*I-*Hin*dII fragment of the pcβ1 insert distal to the termination codon was used as a probe. Hybridization was at 42 °C in 50% formamide, 5 × SSC, 10 × Denhardt's solution, 50 m*M* Na_2HPO_4, 0.5% SDS, 200 μg/ml calf thymus DNA, and 20 ng/ml probe. Washing was at 65 °C in 0.1 × SDS and 0.1% SDS.

Southern hybridization

DNA was extracted from frozen leaves and purified on an ethidium bromide-CsCl gradient, as previously reported (Grellet et al. 1986). A total of 10 μg of DNA was digested with restriction enzymes. To ensure that DNA digestion was complete, 5 units/μg of DNA was used. The DNA restriction fragments were separated by 1% agarose gel electrophoresis and transferred to a Hybond-N membrane by alkali transfer, as recommended by the manufacturer. The *Pst*I-*Hin*dII fragment of the pcβ1 insert distal to the termination codon was used as a ^{32}P-labelled probe. The probe was labelled using the Amersham Multiprime DNA Labelling System (RPN 1600Z). Hybridization was carried out at 42 °C for 20 h in 50% formamide, 0.4 × SSC, 0.1% SDS, 10 m*M* Na-phosphate (pH 7.0), 1 m*M* EDTA (pH 8.0), 10 × Denhardt's solution, 10% dextran sulphate, 50 μg/ml calf thymus DNA and 2 ng/ml probe. Washing was in 2 × SSC, 0.1% SDS at room temperature, followed by 1 × SSC, 0.1% SDS at 65 °C and 0.1 × SSC, 0.1% SDS at 65 °C.

Results

Genetic analysis of the β-amylase mutation

The expression of β-amylase coding genes was analyzed in F_1 kernels derived from two reciprocal crosses:

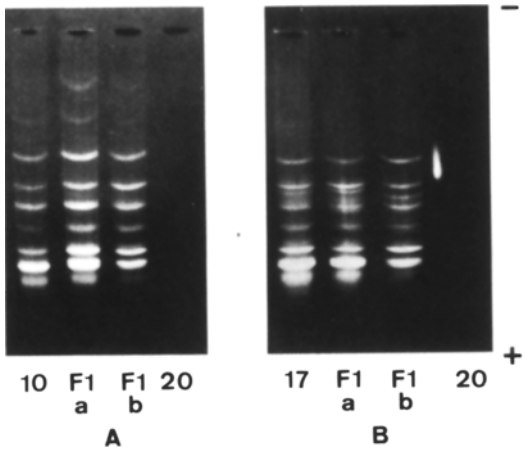

Fig. 1 A and B. IEF test of activation of mutant β-amylase genes in F_1 kernels. **A** β-Amylase zymogram for Rogalinskie F normal line (*10*), Rogalinskie F × Wegierskie 1 [F_1 (*a*)], Wegierskie 1 × Rogalinskie F [F_1 (*b*)], Wegierskie 1 mutant line (*20*). **B** β-Amylase zymogram for Zeelandzkie E normal line (*17*), Zeelandzkie E × Wegierskie 1 [F_1 (*a*)], Wegierskie 1 × Zeelandzkie E [F_1 (*b*)], Wegierskie 1 mutant line (*20*)

Wegierskie 1 (mutant line) × Rogalinskie F (normal line) and Wegierskie 1 (mutant line) × Zeelandzkie E (normal line). The selected normal lines used in the crosses had different IEF patterns (Sadowski et al. 1988; Daussant and Laurière 1990). If the mutation was affecting a *trans* regulatory element in the Wegierskie line, at least one or several additional bands would have appeared in either one of the two F_1s, as a result of complementation by the normal genome. Indeed, an identical IEF phenotype of the enzyme was found for the F_1 and corresponding normal lines in each cross (Fig. 1). No additional bands could be observed, indicating that the mutation does not alter a transacting regulatory gene. This is confirmed by the analysis of the F_2 progeny (not presented), which showed that the normal and mutant β-amylase phenotypes segregated 3:1, indicating that the mutation is at the *β-amy-1* locus on chromosome 5 (Sadowski and Daussant 1989).

Identification of rye β-amylase in the total in vitro translation products

Antibodies specific against wheat β-amylase (Daussant and Laurière 1990) were used to identify rye β-amylase in the total translation products of poly(A)-rich RNA derived from developing seeds of normal and mutant inbred rye lines. The immunoprecipitates were separated by SDS-PAGE. Two protein bands of approx. 60 and 41 kDa are visible on the fluorogram in the normal line (Fig. 2, lane 4). The upper band of 60 kDa comigrates with the purified rye β-amylase isolated from dry seeds, is absent in the mutant line (Fig. 2, lane 9), and most likely corresponds to β-amylase. The lack of a β-amylase band in immunoprecipitated, cell-free translation products derived from the mutant line is in agreement with previous results, which showed that the mutant rye line does not deposit β-amylase in endosperm (Daussant et al. 1991). It demonstrates that the absence of β-amylase results from the absence of a corresponding translatable mRNA in the poly(A)-rich RNA fraction.

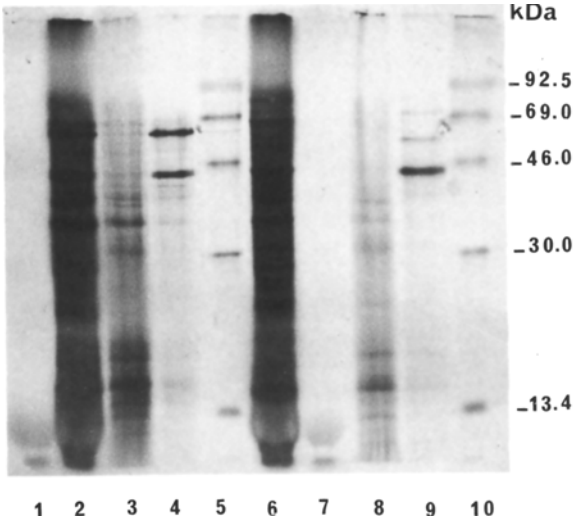

Fig. 2. SDS-PAGE of in vitro tanslation products of poly(A)-rich RNA prepared from 24-day-old endosperms of Rogalinskie Pa (normal line) and Wegierskie 1 (mutant line). Labelled products were separated on a polyacrylamide gel and fluorographed, before and after immunoprecipitation, with β-amylase antibodies. Lanes *1*, *7*: no mRNA; lanes *2*, *6*: total translation products of mRNA from Rogalinskie Pa (*2*) and Wegierskie 1 (*6*); lanes *3*, *8*: same as lanes *2* and *6*, following precipitation with nonimmune serum; lanes *4*, *9*: immunoprecipitates of translation products from Rogalinskie Pa (*4*) and Wegierskie 1 (*9*) mRNA with antibodies against β-amylase; lanes *5*, *10*: molecular weight markers

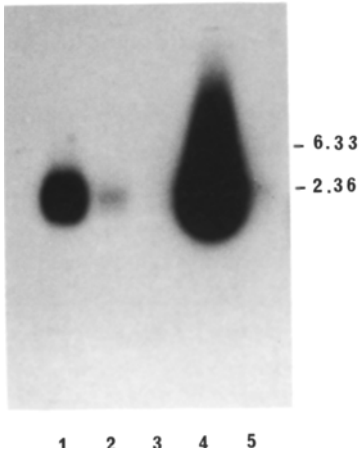

Fig. 3. Northern blot analysis of poly(A)-rich RNA derived from membrane-bound polysomes isolated from developing seeds of Rogalinskie Pa and Wegierskie 1. Lanes *1*, *2*, *3*, *4*: mRNA from Rogalinskie Pa, 1.5 µg, 150 ng, 30 ng, and 4.5 µg, respectively; lane *5*: mRNA from Wegierskie 1, 3–10 µg

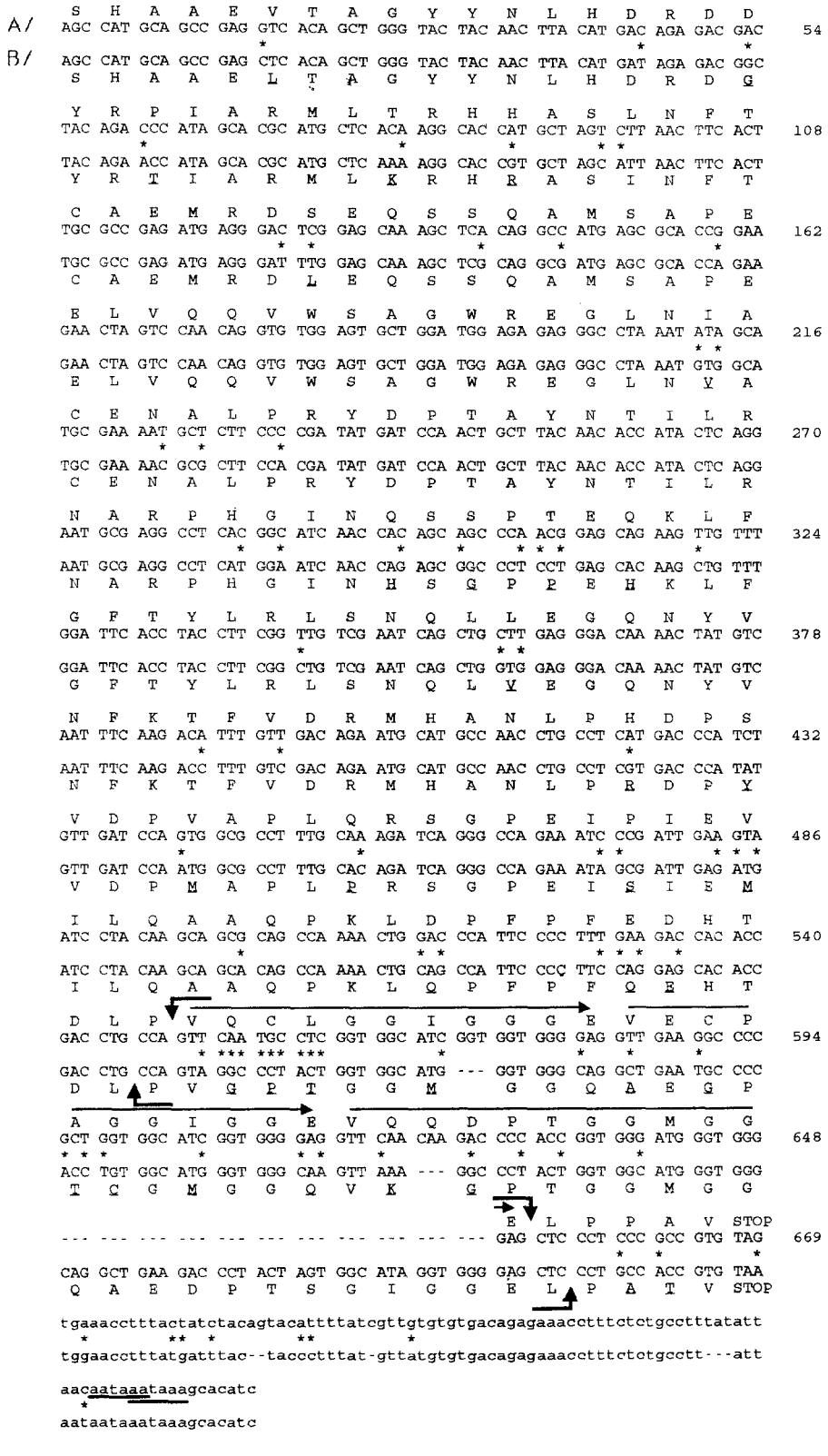

Fig. 4. Comparison of the nucleotide sequence of the partial rye β-amylase clone pcβ1 (*A*) with the corresponding region of barley β-amylase cDNA clone pcβC51 (*B*). The β-amylase coding sequence of the clones was arranged to obtain maximum homology. Nucleotide substitutions are indicated by *asterisks* and amino acid changed are *underlined*. The limits of the C-terminal amino acid repeats are indicated by *right-angled* arrows. The three rye repeats are shown by *arrows above* the rye sequence. Polyadenylation signals are *underlined*

The lower band of 41 kDa, on the other hand, is present in both the normal and mutant lines and does not correspond to any known β-amylase. It might correspond to a contaminant antigen present in the initial preparation used to prepare the immune serum.

Identification of β-amylase cDNA clones and detection of β-amylase mRNA

In order to assay directly for β-amylase mRNA we prepared cDNA clones beforehand. Poly(A)-rich RNA from developing seeds of Rogalinskie Pa was used as a template for cDNA synthesis. Approximately 4,000 recombinants were recovered. The rye cDNA library was screened for β-amylase-specific clones by colony hybridization, with a heterologous probe consisting of the *Eco*RI-*Hin*dIII fragment derived from the insert of barley β-amylase cDNA clone pcβC51 (Kreis et al. 1987). Ten clones were selected under moderately stringent conditions and four, called pcβ1, pcβ2, pcβ3, and pcβ4, were further characterized as β-amylase clones by Southern blot hybridization under the same conditions. These results were confirmed when rye pcβ1 insert was used as a probe with high-stringency conditions. These hybridization data (not shown) indicate that there is a very high degree of homology among all the rye cDNA clones, but that the extent of homology between the rye and barley β-amylase sequence is lower. The longest rye β-amylase cDNA insert, pcβ1, is approx. 800 bp and the others are approx. 400 bp.

Northern blot analysis of poly(A)-rich RNA from normal rye lines displays an RNA band of approx. 2 kb (Fig. 3). Therefore, the isolated cDNA clones are not full-length copies of the mRNA. This RNA is sufficient to encode a protein of 60 kDa. In the mutant line there is a faint signal representing about 2% of β-amylase mRNA compared with the normal line. However, this mRNA seems to be slightly longer than that of the normal line and might therefore be the product of another gene at the *β-amy-2* locus (Fig. 3, lane 5). The results correlate well with the decreased deposition of β-amylase in mutant seeds compared with the normal line. The deficiency in β-amylase synthesis in the mutant line therefore results either from a transcriptional deficiency or from instability of the mRNA.

Sequence analysis of the β-amylase cDNA

The inserts of pcβ1, pcβ2, and pcβ3 clones were sequenced on both strands. The longest insert, pcβ1, was subcloned and two *Hin*dII fragments were sequenced directly. The *Hin*dII site of pcβ1 was verified by sequencing shorter clones pcβ2 and pcβ3. The nucleotide and deduced amino acid sequences of the longest insert are shown in Fig. 4, where they are compared to the corresponding sequences of the barley cDNA clone pcβ51. The nucleotide sequence has been entered in the EMBL data bank under accession no. X 56785. The 763-bp insert, excluding the poly(A) tail, covers the 3′ end of rye β-amylase mRNA. The first nucleotide of pcβ1 corresponds to nucleotide 968 of pcβC51 (Kreis et al. 1987). The open reading frame of this partial clone codes for 223 amino acids. In the 91-nucleotide 3′ untranslated region there are two nested polyadenylation consensus sequences (AATAAA). The last one is located nine nucleotides upstream from the poly(A) tail. The nucleotide sequences of cDNA clones pcβ2 and pcβ3 are identical to that of pcβ1, except for a single nucleotide substitution at position 556 of the pcβ1 insert. The substitution converts a glycine to a cysteine in the deduced amino acid sequence. Close to the C-terminus of the deduced protein there are three imperfect repeats. Two of them are 11 and the other is 12 amino acid residues long. These repeats are glycine-rich as observed in barley (Kreis et al. 1987). The single nucleotide substitution is located in one of these repeats.

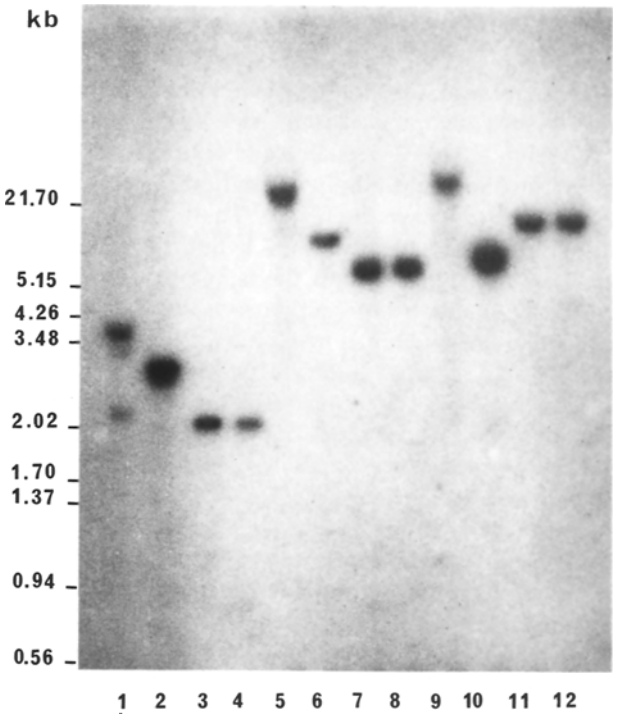

Fig. 5. Southern blot analysis of genomic DNA from normal and mutant lines of rye. Genomic DNA of Kazimierskie B4 normal line (lanes *1*, *5*, *9*), Rogalinksie Pa normal line (lanes *2*, *6*, *10*), Wegierskie mutant line (lanes *3*, *7*, *11*), and Kazimierskie H mutant line (lanes *4*, *8*, *12*) was digested with *Eco*RI (lanes *1–4*), *Bam*HI (lanes *5–8*), and *Hin*dIII (lanes *9–12*). The probe was a ^{32}P-labelled *Pst*I-*Hin*dII fragment of pcβ1 insert. *Eco*RI-*Hin*dIII-digested lambda phage DNA was included as size marker

Organization of the structural genes encoding β-amylase in normal and mutant rye lines

The organization of the β-amylase structural genes was investigated in normal (Kazimierskie B4 and Rogalinskie Pa) and mutant (Wegierskie 1 and Kazimierskie H) rye lines using Southern blot hybridization and three different restriction enzymes (Fig. 5). The two normal lines display distinct phenotypes of hybridizing fragments. The two mutant lines show the same phenotype, which suggests a similar locus organization. Most of the digests contain a single hybridizing fragment. *Eco*RI digests of the DNA of normal lines are the only ones to show several bands, a major one and one or several faint bands. As the labelled probe does not contain an internal *Eco*RI site and corresponds to DNA sequences from Rogalinskie Pa, this suggests that the β-amylase locus might be composed of at least two coding units or, more likely, that an *Eco*RI site is present in an intron. With one exception (lane 10), fragments from the mutant lines are much shorter than fragments from normal lines. The discrepancy might be explained by the presence of an additional *Hin*dIII site in the Rogalinskie Pa line yielding two comigrating fragments.

Discussion

We have previously identified rye inbred lines that are deficient for β-amylase in the endosperm. Genetic analysis of the deficiency indicated that the mutation is located within the β-amylase locus on chromosome 5 (Sadowski and Daussant 1989). In this report, we have presented new evidence which demonstrates that the blocking in endosperm β-amylase synthesis is at the level of mRA synthesis. Neither *in vitro* protein synthesis programmed with the mutant line mRNA, nor direct assay by Northern blot hybridization detected β-amylase mRNA in the mutant lines. These results are consistent with a block in transcription of the gene or a very high instability of the mRNA in the mutants. The later hypothesis is not very likely with respect to the genetic analysis, which indicates that the gene itself or a *cis*-acting element is altered. On Northern blots of mutant lines, a faint band corresponding to a mRNA slightly larger than the endosperm β-amylase mRNA is detected only at a very high RNA concentration. This band might correspond to the product of the other β-amylase locus (Sharp et al. 1988), which is located on rye chromosome 2. Experiments in our group have shown that this product corresponds to β-amylase form I, which accumulates in pericarp tissues (Daussant et al. 1991). The very low intensity of this band might be accounted for by a low amount of this mRNA, due to the relatively low proportion of pericarp cells in the immature grain, or by divergence between its sequence and that of the endosperm β-amylase probe.

As a further attempt to characterize the mutation, Southern blot patterns with three different restriction enzymes were analyzed. In most cases, a single restriction fragment hybridizes with the probe in stringent conditions, except for the *Eco*RI digestion of normal lines. This indicates that the protein is most likely coded by a single gene or two closely linked genes. With all three enzymes the two mutant lines exhibit the same pattern. When less stringent conditions are used, a limited number of additional fragments appear, presumably corresponding to the second β-amylase locus. Another observation is that, most frequently, the fragments derived from mutant lines are smaller than the fragments in normal lines. This observation is consistent with a deletion hypothesis. However, because there is extensive restriction fragment length polymorphism between the two normal lines used as controls, it is not possible to draw a definitive conclusion.

In order to achieve these experiments, homologous cDNA clones were isolated and characterized. Although none of these cDNA is full length, analysis of their sequence provided some interesting information. The rye sequence described here is the second sequence, after that of barley (Kreis et al. 1987), available for a cereal endosperm β-amylase. As expected from cross-antigenicity and cross-hybridization, the two sequences have a very high level of homology in the regions that can be compared. As previously observed in barley, there is a series of glycine-rich repeats in the C-terminal part of the protein, but there are only three such repeats in rye instead of four, as in barley. At the nucleotide level this repeated region is the most divergent part when the two species are compared. In the remaining portion of the compared sequence there are 51 nucleotide substitutions out of 600 aligned positions. Only 30 result in an amino acid change, which most often is neutral. The 3′ nontranslated sequence is also unusually remarkably conserved between rye and barley.

Another puzzling observation is that one of the sequenced cDNA clones has a single nucleotide difference with the others two. This difference has been very carefully controlled and this region has been repeatedly and reproducibly sequenced on both strands. The change results in an amino acid change in the position. A similar limited divergence has been noticed in sequencing several barley cDNA clones (Kreis et al. 1987). Since the line used in this experiment has been inbred for more than 20 generations, it is not very likely that the difference results from allelic variation and heterozygosity. Therefore, we have to consider the possibility that there are two genes encoding the endosperm β-amylase in rye. Because a single fragment is observed with several restriction enzymes, these two genes should be tightly linked. If this prediction is right, then we have to explain how two genes can be inactivated with a single mutation mapping within the

locus. As mentioned earlier, our Southern blot results are consistent with a deletion event; this deletion can affect part of each gene, accounting for simultaneous inactivation of both of them. Demonstration of this hypothesis will require cloning and analysis of genomic DNA from mutant lines. Alternatively, for some unknown reason, limited heterozygosity might be maintained, even after many selfings, and the mutation would also be easily accounted for by a deletion event.

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