

Received 15 April 2002
Accepted 30 May 2002

STUDIES ON CHANGES IN SPECIFIC RYE GENOME REGIONS DUE TO SEED AGING AND REGENERATION

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Abstract: The aim of this study was to identify the genetic changes in rye seeds induced by natural aging during long-term storage and successive regeneration cycles under gene bank conditions. Genomic DNA from four rye samples (cv. Dańkowskie Złote), varying in their initial viability and having gone through one or three reproduction cycles, were analysed using specific PCR targeting of a secalin locus, and various repetitive fragments defined by the R173 sequence. A statistical analysis of the band frequencies for both secalin and R173.3 primer pairs revealed no changes in their frequencies. Similar data on R173.1 demonstrated significant changes between samples of different initial viability showing a lack of a band of the expected length (987 bp) in progeny originating from low viability seeds lots. These changes were inherited even after three regeneration cycles. Our results may indicate that long-term storage that leads to loss of viability also generates heritable changes in the preserved germplasm. However, it remains to be discovered where these changes occur and whether they are connected with coding or with non-coding DNA regions.

Key Words: Rye, *Secale cereale* L., Seed Aging, Molecular Markers, Specific-PCR

INTRODUCTION

It is known that the quality or integrity of the DNA within a seed can vary depending on the physiological state or quality of the seed itself. Samples of aged seed are known to generate more morphological variability than those from highly viable seeds. Genetic changes induced by seed aging fall into two general groups: spontaneous genetic changes [1], such as point mutation or chromosome rearrangements; and selection effects, such as genetic shift or drift. In general, point mutations have a more lasting effect on germplasm than chromosomal

aberrations, since the latter are less likely to be transmitted through the germline to the next generation. Although a case can be made for the induction, inheritance, and possible accumulation of point mutations in seed accessions as a direct result of seed deterioration, selection within accessions during storage and regeneration has far greater potential impact on the genetic structure of the *ex situ* population than induced genetic changes do [2-4]. We hypothesized that changes induced by aging can be more frequently accumulated in non-functional regions of the genome than in coding DNA regions, which are more likely to be eliminated by natural selection. To verify this hypothesis, we designed two kinds of primer pairs specific to fragments of coding DNA regions (controlling the expression of ω - and γ -secalins), and two that are specific to repetitive motifs (R173.3 and R173.1). The secalins are prolamin, a class of seed storage alcohol-soluble proteins, which are found in the grain of many cereals including wheat, barley and rye [5]. The R173 sequence belongs to a family of rye-specific repeated DNA sequences, defined by its hybridisation to the probe pAW173. [6]. The copy number of these elements is about 15,000 per diploid rye genome, distributed over all seven chromosomes in a dispersed manner [7, 8]. The range between 3 kbp and 5 kbp in size generally occurred in single units, often flanked by other repeated sequences.

MATERIALS AND METHODS

Plant material

Four rye samples of cv. Dańkowskie Złote (DZ) progenies of the initial sample DZ1.0 were used in the experiments: seeds stored for 14 years under seed bank conditions (viability about 5%) followed by one reproduction cycle in the field, marked as sample DZ1.1; seeds stored as above but followed by three reproductive cycles, marked DZ1.3; and seeds with an initial viability of more than 91%, followed by one or three reproductive cycles, marked as samples DZ4.1 and DZ4.3, respectively (Tab. 1).

Tab. 1 Rye samples used for investigation

Code of sample	Period of storage (years)	Germination ability (%)	Numbers of reproduction cycles
DZ4.1	4	91	1
DZ4.3	4	91	3
DZ1.1	14	5	1
DZ1.3	14	5	3

Seeds of each treatment (44 individuals in each sample) were germinated in sterile, watered sand at 20°C in germination chambers. Leaf samples were taken from seven-day old seedlings.

DNA extraction

DNA was extracted from about 100 mg of fresh leaf tissue ground in liquid nitrogen according to the Qiagen kit procedure (DNeasy Plant Mini Handbook for DNA isolation from plant tissue). DNA integrity was analyzed on 1.4% agarose gel containing 1^x TBE buffer and ethidium bromide (0.5 µg/1 ml) under 20 V/cm. The quantity was determined spectrophotometrically (GeneQuanta, Pharmacia LKB). For routine purposes standard dilutions of 10 µg/ml were used.

Oligonucleotides

A pair of primers specific to sequences of rye DNA was used to amplify DNA fragments of expected length. Primer pairs were designed based on GenBank and EMBL data.

Amplification (PCR)

The polymerase chain reactions were carried out in a total volume of 12.5 µl containing: 10 x reaction buffer (Qiagen), 2.5 mM MgCl₂ (Qiagen), 1.0 mM dNTP (Sigma), 0.08 mM selective primer U, 0.08 mM selective primer L, 0.25 uTaq DNA polymerase (Qiagen), 30 ng of DNA template.

In order to make the PCR reaction conditions sufficiently stringent to avoid non-specific amplifications, PCR cycle parameters were modified until only the product of expected length was generated.

Electrophoresis

Amplification products were separated on 1.4% agarose gel containing 1 x TBE buffer and ethidium bromide (0.5 µg/1 ml) under 20 V/cm during two hours. Additionally, for R173.1 one primer of the pair was labelled with ³²P. Radioactive PCR products were separated on 5% PAGE and exposed to X-ray film (FOTON-XC) at -80°C overnight.

Band scoring and statistics

Bands were scored for presence or absence. The distribution of the appropriate DNA fragments between samples was compared by means of a chi square test at df=1 (Tab. 3).

RESULTS

Primer pairs targeted at the ω - and γ -secalin genes and two regions of the R173 rye macrosatellite were designed based on the data available in the EMBL or GenBank data bases, and were complementary to the appropriate DNA fragments as indicated in Table 2. Although all the primer pairs generated clearly visible amplification products of the expected lengths on agarose gels, it was necessary to optimise reaction conditions. The most important factor here was annealing temperature. On the one hand, decreasing temperature led to the

appearance of additional signals and resulted in a lower quality of fingerprints or even led to the misdetection of the appropriate amplification products (SCSECAB, R173.1). On the other hand, a reasonable increase in the annealing temperature improved specificity and image quality. The optimal temperatures are listed in Table 2.

Tab. 2 The data on primer pairs and amplified products

Primer code Fragment type	Total length (bp)	Primer position	Amplifi- cation product (bp)	Ann- ealing temp.	Primer sequences 5' → 3'
R173.1-L R173.1-U Macrosatellite	6676	1022- 1042 56-76	987	65	GCAACGGCGCCAGAAATAGC ATGCTGGCCGGGTCCGCACT
R173.3-L R173.3-U Macrosatellite	4959	1078- 1098 193-213	906	65	TGGGTTCTGACTCGTTATGA TGTTTGGTATTTGCTTCTTG
SCSEC1AB-L SCSEC1AB-U ω-secalin	1832	1717- 1739 99-125	1640	69	CCAGCAATGTCCTTGTGACA AGATGTAGAGCATCACAAAC TGAATTCC
SCSEC1GR-L SCSEC1GR-U γ-secalin	714	685-710 118-139	592	67	TTGAAGTAGAACTGGTCTGG GTCACAACAACCGTCGATTC AGCTATC

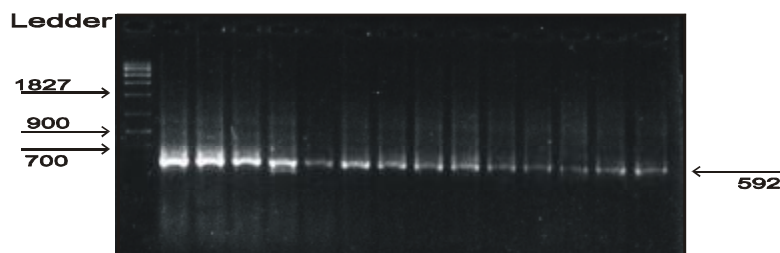


Fig. 1. Image of the specific PCR products for a fragment of the γ -secalin gene. The specific product, which is 592 bp, is indicated by an arrow in the right-hand part of the figure.

To amplify the parts of the secalin coding regions SCACAB U/L (ω -secalin) and SCRECGR U/L (γ -secalin), primers were used. The first pair amplified a DNA fragment of 1640 bp, the second one of 592bp. Non-coding regions were amplified by means of R173.1 U/L and R173.3 U/L primers generating fragments of length 987 bp and 906 bp, respectively. In all cases except for R173.1, a single PCR product was detected on the agarose gels and this was present in all, or nearly all individuals from DZ1.1, DZ1.3, DZ4.1 and DZ4.3.

The R173.1 U/L primer pair generated DNA fragments in DZ4.1 and DZ4.3, but in DZ1.1 and DZ1.3 they were randomly distributed among individuals and occurred in about half of them. No signals were detected even if radioactive labelled amplified products were separated on acrylamide gels.

In addition to the main product, all the primer combinations amplified some shorter fragments. Some of these were artefacts, but others were either monomorphic or the changes of their frequencies evaluated among samples were not statistically significant, and thus they were not considered in the subsequent statistical analysis.

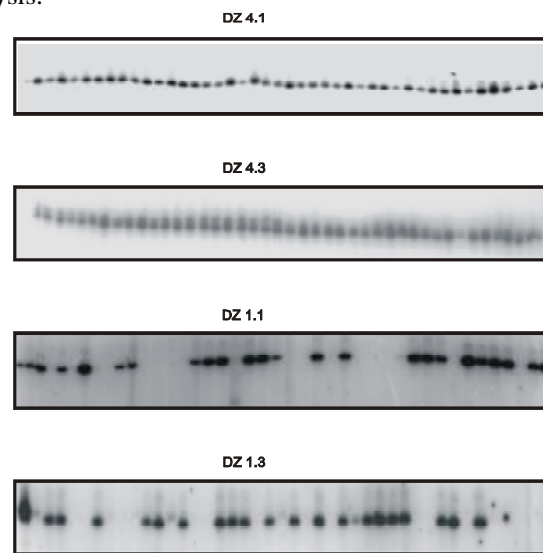


Fig. 2. Amplification products (987 bp) of a fragment of the R173.1 rye macrosatellite DNA; amplification performed on four samples

Tab. 3. Independent chi square test for all primer pairs and samples at $df=1$; n.s. – not significant

Primer Code	χ^2			
	DZ1.1-DZ4.1	DZ1.3-DZ4.3	DZ1.1-1.3	DZ4.1-DZ4.3
R173.1-L	25.88	27.58	0.045	n.s.
R173.1-U				
R173.3-L	n.s.	n.s.	n.s.	n.s.
R173.3-U				
SCSEC1AB-L	n.s.	n.s.	n.s.	n.s.
SCSEC1AB-U				
SCSEC1GR-L	n.s.	n.s.	n.s.	n.s.
SCSEC1GR-U				

The results of a statistical analysis of the coding and non-coding fragments is presented in Table 3. No significant changes were observed in case of the fragments amplified from secalin genes. However such changes were clearly visible in case of R173.1. The chi square test was statistically significant at $\alpha=0.05$ (df=1) in the case of DZ1.1-DZ4.1 and DZ1.3-DZ4.3 ($\chi^2=3.841$), but revealed no differences between DZ1.1-DZ1.3 and DZ4.1-DZ4.3.

DISCUSSION

Natural seed aging and regeneration may lead to changes in the genetic structure of the germplasm preserved in a gene bank [9, 10]. As demonstrated by Puchalski [11], these changes may result from a genetic shift of allozymes. Molecular studies based on RAPD [12, 13] profiling of the same samples revealed statistically significant changes in many DNA bands. Similar results were obtained in our preliminary studies with the AFLP technique [14]. These data suggested that both long-term seed storage causing very low viability and successive regenerations evidently influenced the genetic structure of the preserved rye samples. The question is in which part of genome they appear. This information is important for efficient genetic diversity conservation.

It is rather difficult, without extra effort, to link DNA fragments amplified by RAPD or AFLP to specific regions of genome. To avoid such a problem these results were obtained based on the amplification of rye-specific sequences that were available from GenBank and EMBL data. Primers were designed to amplify coding and non-coding regions.

Gamma- and omega- secalin sequences were chosen as representatives of coding regions. These seed storage proteins have no metabolic function, so little selection pressure was expected. On this assumption much variation should be identified for the amplified fragments. However, this was not the case. This is probably the result of the secalin gene organisation in the form of a single multigenic locus [5, 15]. Thus, even if any changes occurred within this region, another copy will be amplified. This may confirm the results presented by Stoyanova [16, 17], who has not identified changes in the prolamin profiles induced by artificial aging. However, we cannot exclude the possibility that these regions still remain under selection pressure and thus, any changes are eliminated, or that the multigenic nature of these genes hinders existing variability that could be identified under our experimental conditions.

By contrast, changes in band frequencies among the analysed samples were observed in case of one of the amplified fragments of the R173 macrosatellite. The R173.1 U/L primer pair revealed differences which appeared between samples with initially low and high viability (DZ1.1 and DZ4.1). This was not changed even after three regeneration cycles (DZ1.3 and DZ4.3) reflecting a lack of selection pressure. This result was a bit surprising since this macrosatellite is known to be present in many copies in the rye genome. So, any changes that occur in one R173 copy should be hindered by the other copy and

no polymorphisms should be identified by PCR. However, our primers were designed based on the sequence available in the EMBL and it is probable that they are highly specific to only one copy of the macrosatellite allowing the identification of variability within their annealing regions. The identified variation may somehow reflect the so-called "hot-spots" [18] – the DNA regions that are considered to be more susceptible to mutations.

Summing up, our results may indicate that long-term storage of seeds in gene bank conditions that led to loss of viability also generates heritable changes in the preserved germplasm. However, it remains to be seen where these changes occur and whether they are connected with coding or mostly with non-coding DNA regions.

Acknowledgements. The authors wish to thank to Dr R. M. D. Koebner for his highly important comments.

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