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Intrachromosomal mapping of chromatid aberrations induced by restriction endonucleases in barley

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Abstract The intrachromosomal distribution patterns of chromatid aberrations induced by the restriction endonucleases *Hpa*II, *Msp*I and *Hae*III in a multi-reconstructed barley karyotype were analysed. All of these endonucleases, which differ with respect to their DNA recognition sequences but produce only one type of DNA damage, showed nearly the same pattern of localized chromosome breakage. The most pronounced aberration hot spots proved to be the loci of transcriptionally active and condensed, inactive, rDNA. Possible mechanisms involved in the specific distribution of induced aberrations along the distinct chromosomal regions are discussed.

Key words Restriction endonucleases · Chromatid aberrations · Intrachromosomal distribution · rDNA · *Hordeum vulgare*

Introduction

Non-random patterns of the intrachromosomal distribution of chromatid aberrations have been established for a variety of mutagenic factors, including ionizing radiation (Holmberg and Johansson 1973; Schubert and Rieger 1976), biotic agents (Kato 1967), and substances belonging to different chemical classes (cf. Kihlman 1966; Rieger et al. 1975; Schubert and Rieger 1977).

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In spite of the abundant literature on the subject, the mechanisms underlying this phenomenon, which is known as the regional specificity of mutagens, are far from being understood. Nevertheless, there is clear evidence that mutagens with a non-delayed effect generally show much less-pronounced aberration hot spots than those with a delayed mode of action (Schubert and Rieger 1977). Moreover, such mutagens as maleic hydrazide and mitomycin C, which belong to different chemical classes, have produced similar patterns of aberration distribution (Rieger et al. 1977; Gecheff 1991).

It is generally accepted that chromosomal rearrangements arise as a result of misrepair processes of initially induced DNA lesions, the latter being mainly double-strand breaks. If this is so, the question as to whether a specific distribution of the initial chromosomal lesions along the chromosomes, and/or the heterogeneity of their repair among the chromosomal regions involved, is responsible for the regional specificity of the mutagens seems to be of primary importance. Although the non-random distribution of damage and repair in different chromatin domains has been unequivocally established (Bohr et al. 1987; Terieth et al. 1991; Boulikas 1992; Natarajan et al. 1994), the relationship between the phenomenon mentioned above and the specific clastogenic activity of mutagens along the chromosomes remains obscure at present.

It has already been established that restriction endonucleases (REs) are efficient inducers of chromosomal aberrations both in mammalian (for a review see Bryant 1988; Bryant and Johnston 1993) and plant cells (Stoilov et al. 1996). REs could be effectively utilized in elucidating the mechanisms underlying the regional specificity of mutagens, since they recognize defined DNA sequences and produce only one type of initial DNA damage, namely double-strand breaks. Surprisingly, the available data on this point are rather scarce. Recently a clear association has been found between the occurrence of aberrations and intercalary telomeric

repeat sequences in Chinese hamster cells treated with REs (Balajee et al. 1994).

The present study was aimed at investigating the intrachromosomal distribution patterns of the chromatid aberrations induced by three restriction endonucleases in a reconstructed karyotype of barley.

Materials and methods

A multi-reconstructed barley karyotype, PK-88, with complete cytological marking of all the chromosomes, was used throughout (Gecheff 1989 a).

All procedures concerning the germination of seeds, the permeabilization of the cells with Driselase (Fluka), treatment with the restriction enzymes *Hpa*II, *Msp*I and *Hae*III (New England Biolabs), and recovery times were essentially the same as previously described (Stoilov et al. 1996). Details of the metaphase block, the fixation of the material, the preparation of Feulgen-stained squashes and the scoring of chromatid aberrations are published elsewhere (Gecheff 1989 b). For analyzing the regional specificity of aberration induction the metaphase chromosomes we subdivided into 53 segments of nearly equal sizes. The centromeres and secondary constrictions are designated individually. The segments are numbered respective to their position in the standard karyotype. All experiments were performed with at least two repeats and the data pooled.

The effectivity of DNA double-strand break (dsb) induction after restrictase treatment was analyzed either on isolated nuclei or dissected root tips. The nuclear isolation was performed according to the method of Van't Hof (1975). The resulting material was centrifuged (5000 RPM, 5 min), washed with 20 µl of the digestion buffers for *Hpa*II, *Hae*III and *Msp*I, and re-suspended so as to have about 2×10^5 nuclei (2 µg of DNA) in a 25-µl volume. The samples were treated with 50 U of each enzyme for 2 h at 37°C. After digestion, the material was lysed for 2 h at 37°C with 0.5% SDS and 500 µg/ml of proteinase K. Respectively, about 25 manually dissected root tips, yielding approximately the same number of nuclei, were subjected to restrictase digestion (50 U in a 100-µl vol, 2 h, 37°C). Immediately after treatment the nuclei were isolated and processed further as already described.

The resulting material was electrophoretically separated in 0.7% neutral agarose gels, run overnight at 15 V with TBE buffer containing ethidium bromide, and photographed under UV-illumination (Maniatis et al. 1982).

Results

DNA breakage induced by restriction endonucleases

Electrophoretic patterns obtained after *Msp*I-treatment of nuclei and *Hae*III-digestion of root tips are shown in Fig. 1 (panels a and b, respectively). The data are also representative of the effectivity of *Hpa*II (data not shown). As can be seen, a wide spectrum of DNA fragments was generated in both cases, forming a smear through almost the entire gel length, with the share of the high-molecular-weight DNA being substantially reduced. The mode of action of the enzymes resembled their previously tested efficiency on naked DNA (Stoilov et al. 1996), although the treatment of the roots tips appeared to be relatively less effective. It is also apparent that some of the material remains on the top of the gel, due most probably to the fact that we were loading partially disagregated nuclear chromatin and not pure DNA.

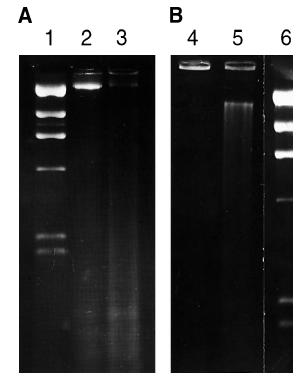

Fig. 1 Electrophoretic behavior of the digestion products obtained after treatment of isolated nuclei with *Msp*I (panel **A**) and root tips with *Hae*III (panel **B**). *Lanes 1 and 6*: *Hin*dIII-digested lambda DNA; *lanes 2 and 4*: untreated nuclei; *lanes 3 and 5*: *Msp*I- and *Hae*III-digested samples respectively

Intrachromosomal distribution of aberrations induced after treatment with *Hpa*II

Amongst the different aberration types induced by REs only four are amenable to chromosomal localization of the breakpoints: isolocus breaks, intercalary deletions, duplication-deletions and chromatid translocations. Figure 2 shows the non-random pattern of the intrachromosomal distribution of these aberrations induced by *Hpa*II. Segments 38 and 46 [nucleolar organizing regions (NORs) of chromosome 6^i and 7^5, respectively], as well as segment 48 (residing in the short arm of chromosome 7^5) appeared to be the most pronounced hot spots. It can be seen in Table 1 that the involvement of these segments in aberrations surpasses nearly twice the upper confidence limits. While the increased sensitivity of segments 38 and 46 proved to be due to their preferential involvement in both isolocus breaks and chromatid translocations, the pronounced hot-spot character of segment 48 was related only to its increased predilection for the formation of isolocus breaks. Other segments, such as 4 (centromere of chromosome 1^2), 11 (centromere of chromosome 2^1) and 47 (distal part of the short arm of chromosome 7^5) show a very low expressivity of their aberration hot spots, i.e. their involvement in aberrations just surpasses the upper confidence limit (1% level) for random distribution (Table 1).

Intrachromosomal distribution of aberrations induced after treatment with *Msp*I

The intrachromosomal distribution pattern of *Msp*I-induced chromatid aberrations is presented in Fig. 3. It can be seen that nearly the same chromosome segments as those affected by *Hpa*II appeared to be the aberration hot spots. Again NORs of chromosomes 6^i and

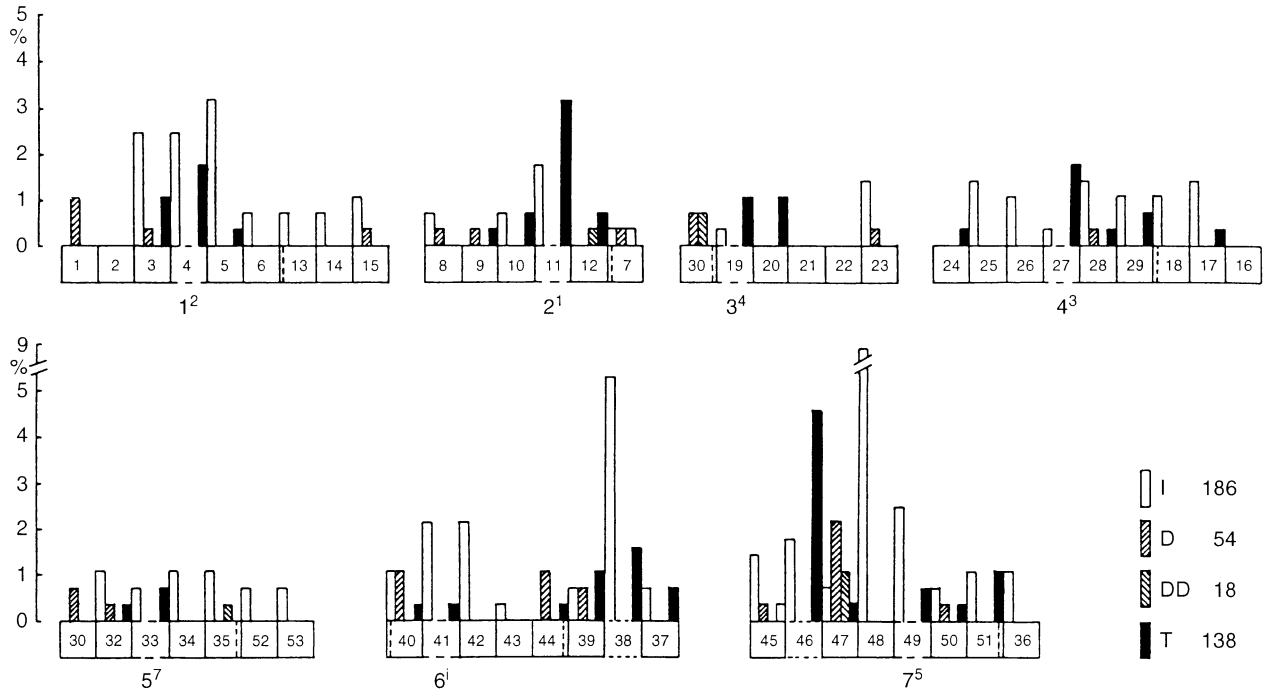

Fig. 2 The distribution of *Hpa*II-induced chromatid aberrations in individual chromosomal segments. *I* = isolocus breaks; *D* = intercalary deletions; *DD* = duplication-deletions; *T* = chromatid translocations

7^5 (segments 38 and 46) showed the most pronounced expressivity. However, some quantitative differences were observed. Thus, segment 11 is no longer an aberration hot spot. Instead, segment 41 (centromere region of chromosome 6^i) increases its involvement in isolocus breaks and chromatid translocations. Moreover, as is evident from Table 1, the hot-spot expressivity of segment 47 is much more pronounced mainly due to it increased predilection for involvement in isolocus breaks and intercalary deletions. Again segment 48 shows a preferential involvement in isolocus breaks, although not as much pronounced as after *Hpa*II treatment.

Intrachromosomal distribution of aberrations induced after treatment with *Hae*III

Surprisingly, the pattern of intrachromosomal distribution of *Hae*III-induced chromatid aberrations (Fig. 4) does not differ essentially from those obtained after treatment with *Hpa*II (Fig. 2) and *Msp*I (Fig. 3). The most pronounced aberration hot spots proved again to be segments 38, 46 and 47. It can be seen in Table 1 that the involvement of all these segments in aberrations surpasses more than twice the upper confidence limit for random distribution. Segment 48 is also a clearly pronounced aberration hot spot, again mainly due to its increased predilection for the formation of isolocus breaks.

Table 1 Statistical evaluation of "hot-spot" chromosomal segments after treatment with *Hpa*II, *Msp*I and *Hae*III

Treatment and segment no.	Total number of chromatid aberrations			
	Theoretically expected random distribution	Lower confidence limit	Upper confidence limit	Observed values
*Hpa*II				
4	5.4	1.0	11.80	12
11	5.4	1.0	11.80	15
38	5.4	1.0	11.80	20
46	5.4	1.0	11.80	18
47	4.8	1.40	11.0	12
48	4.8	1.40	11.0	25
*Msp*I				
4	7.6	0.02	15.24	17
38	7.6	0.02	15.24	36
41	7.6	0.02	15.24	18
46	7.6	0.02	15.24	27
47	6.9	0.37	14.20	29
48	6.9	0.37	14.20	17
*Hae*III				
38	8.4	0.43	16.40	37
41	8.4	0.43	16.40	19
46	8.4	0.43	16.40	32
47	7.6	0.10	15.30	31
48	7.6	0.10	15.30	26

Discussion

DNA double-strand breaks (dsbs) induced by the restriction endonucleases employed were revealed by constant-field gel electrophoresis under non-denaturating

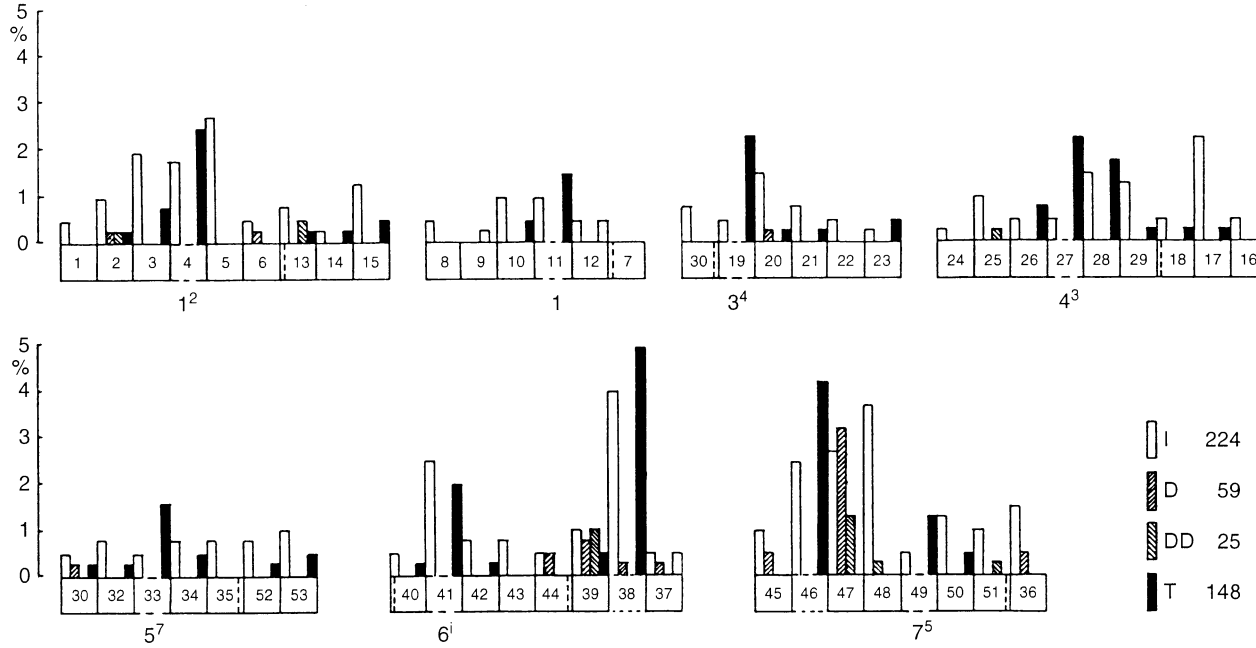

Fig. 3 The distribution of chromatid aberrations induced by *Msp*I in individual chromosomal segments (the abbreviations are the same as in Fig. 2)

conditions. The generation of a broad range of DNA fragments is an indication that a distinct number of initial dsbs are formed. Our assay is relevant mainly for dsbs, which is not the case for some of the earlier studies on the subject, where the techniques employed (alkaline unwinding or nucleoid sedimentation) does not distinguish double-strand from single-strand breaks in DNA (Bryant 1984; Natarajan et al. 1985). Although the actual frequency of cleavage can not be derived from such data, it is clear that a substantial number of dsbs are induced in barley nuclear DNA under our experimental conditions.

Both standard and reconstructed karyotypes of barley have been widely explored in investigating the regional specificity of mutagenic agents (Nicoloff and Gecheff 1968; Nicoloff et al. 1975; Gecheff 1972, 1989 a, 1991). The main conclusions to be drawn from these studies are as follows:

(1) centromere regions of chromosomes prove to be the most pronounced hot spots for all alkylating agents tested, the effect being partly mutagen-specific;
(2) a characteristic feature of other agents with delayed effect, such as maleic hydrazide, is a very pronounced clustering of aberrations in segments 39 and 47 containing condensed, non-transcribed, ribosomal DNA (rDNA) (Gecheff et al. 1994);
(3) a nearly random pattern of aberration distribution is observed after the action of mutagens with a non-delayed effect.

The most essential and interesting finding observed in the present study is that all REs tested show nearly the same pattern of intrachromosomal distribution of induced chromatid aberrations, irrespective of their recognition sequence – CCGG for *Hpa*II and *Msp*I, and GGCC for *Hae*III. This differs from both of the patterns observed after the action of mutagens with delayed and non-delayed effects. It was previously established on the same plant material (Stoilov et al. 1996) that *Msp*I, *Hpa*II and *Hae*III induce chromosomal rearrangements independently of DNA replication (S-independent or non-delayed effect). Since there is a clear difference in the patterns of localized breakage induced by S-independent agents and REs it seems obvious that reasons other than the specific induction of primary lesions at different stages of interphase (G_1, S, G_2) underlie the preferential distribution of chromatid aberrations produced by REs.

Based on the available data it might be assumed that the regional specificity of mutagenic factors results from the non-random induction of primary lesions along chromosomal DNA and/or the heterogeneous repair of DNA in different chromatin domains. Clear evidence has been provided that both these processes occur in the eukaryotic genome. It has been established that euchromatic and heterochromatic regions differ in their accessibility for the induction of primary lesions and the effectivity of repair processes (Terleth et al. 1991; Boulikas 1992; Natarajan et al. 1994). It is very noticeable that the most pronounced hot-spot segments after the action of all the REs applied prove to be the sites of actively transcribed (segments 38 and 46) and non-transcribed, condensed, (segment 47) rDNA repeats. It may turn out to be the case that different mechanisms are responsible for the preferential

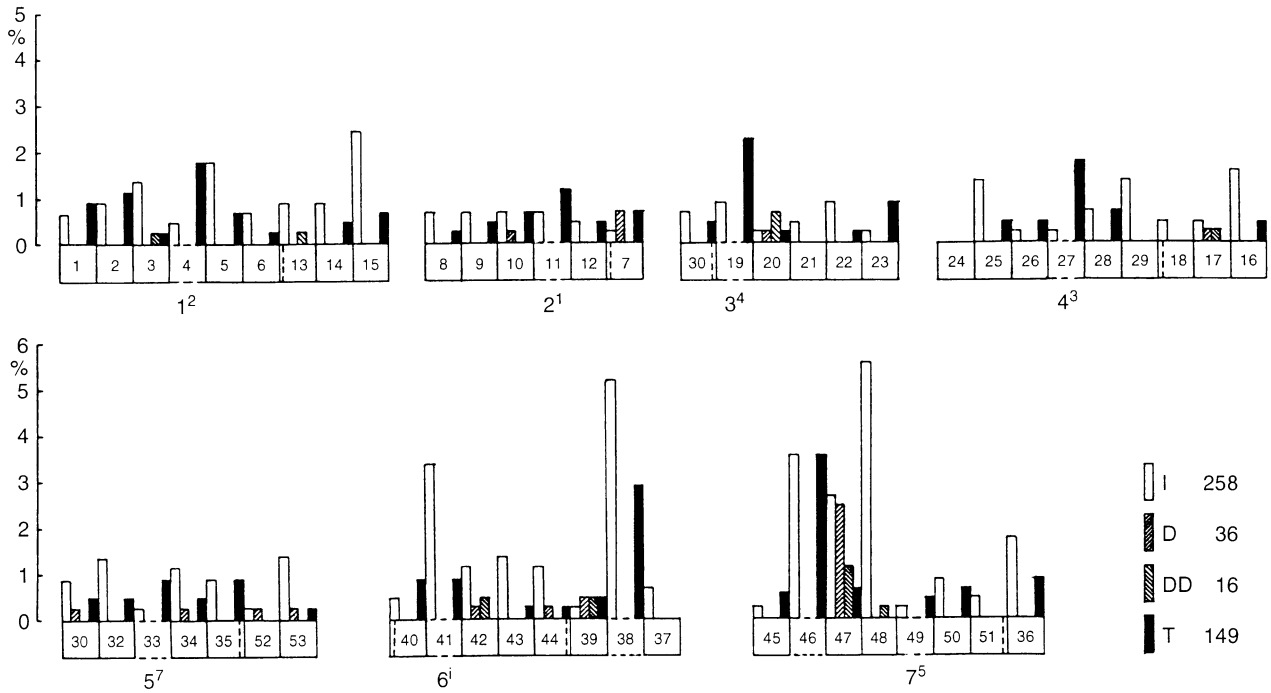

Fig. 4 The distribution of *Hae*III-induced chromatid aberrations in individual chromosomal segments (the abbreviations are the same as in Fig. 2)

involvement on the one hand of segments 38 and 46 in aberrations and of segment 47 on the other. The increased sensitivity of the actively transcribed NORs could be due to their higher accessibility to the action of REs while the pronounced aberration clustering in segment 47 might be related to the hampered repair of heterochromatin. One could suppose therefore that, being nearly identical with respect to their sequence specificity, these segments contain a great number of recognition sites for REs which has resulted in the clustering of DNA primary lesions, most probably dsbs, in these segments. In fact, this was proved to be the case for the CCGG tetranucleotide (recognizable with both *Hpa*II and *Msp*I) in wheat rDNA repeats (Flavel et al. 1988). The different hot-spot expressivity of segment 47 after treatment with *Hpa*II and *Msp*I could be accounted for by the methylation-specific action of these REs.

Segment 48 was the only heterochromatin-containing segment in karyotype PK 88 which appeared to be a clearly pronounced hot spot in all RE treatments. For a long time it was commonly accepted that, due to their structural and functional peculiarities, heterochromatic regions are preferentially involved in the chromosomal aberrations induced by different clastogens (Natarajan and Ahnstrom 1972). However, based on cytological techniques with higher resolving power it was recently established that in plants the most sensitive regions to clastogenic factors are the boundaries between eu- and hetero-chromatin (Fernandez et al. 1990).

It is remarkable, but difficult to explain, that in all our treatments with REs, the increased hot-spot expressivity of segment 48 was due to its preferential involvement in one particular type of aberration, namely isolocus breaks. As far as the other heterochromatin-bearing segments of this karyotype were concerned (Gecheff 1989 b), they were not found to be predisposed to aberration clustering, which is in agreement with the observed damage resistance of condensed chromatin in mammalian chromosomes (Natarajan et al. 1994).

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