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Original article

Multicolour FISH in an analysis of chromosome aberrations induced by N-nitroso-N-methylurea and maleic hydrazide in barley cells

Jolanta Juchimiuk, Barbara Hering, Jolanta Maluszynska

Department of Plant Anatomy and Cytology, University of Silesia, Katowice, Poland

Abstract. The present study is a rare example of a detailed characterization of chromosomal aberrations by identification of individual chromosomes (or chromosome arms) involved in their formation in plant cells by using fluorescent *in situ* hybridization (FISH). In addition, the first application of more than 2 DNA probes in FISH experiments in order to analyse chromosomal aberrations in plant cells is presented. Simultaneous FISH with 5S and 25S rDNA and, after reprobing of preparations, telomeric and centromeric DNA sequences as probes, were used to compare the cytogenetic effects of 2 chemical mutagens: N-nitroso-N-methylurea (MNU) and maleic hydrazide (MH) on root tip meristem cells of *Hordeum vulgare* (2n = 14). The micronucleus (MN) test combined with FISH allowed the quantitative analysis of the involvement of specific chromosome fragments in micronuclei formation and thus enabled the possible origin of mutagen-induced micronuclei to be explained. Terminal deletions were most frequently caused by MH and MNU. The analysis of the frequency of micronuclei with signals of the investigated DNA probes showed differences between the frequency of MH- and MNU-induced micronuclei with specific signals. The micronuclei with 2 signals, telomeric DNA and rDNA (5S and/or 25S rDNA), were the most frequently observed in the case of both mutagens, but with a higher frequency after treatment with MH (46%) than MNU (37%). Also, 10% of MH-induced micronuclei were characterized by the presence of only telomere DNA sequences, whereas there were almost 3-fold more in the case of MNU-induced micronuclei (28%). Additionally, by using FISH with the same probes, an attempt was made to identify the origin of chromosome fragments in mitotic anaphase.

Keywords: barley, centromere, FISH, micronuclei, rDNA, telomere.

Introduction

Among various bioassays, the micronucleus (MN) test is widely recommended for the evaluation of genotoxic effects of chemical and physical agents. The MN test seems to be less time-consuming and easier to perform than a chromosomal aberration (CA) assay. The unquestionable importance of the MN test has made the standardization of its protocol the subject of a few studies. Fluorescent staining of chromosomes, instead of less accurate traditional methods, such as the Feulgen method and acetoorcein staining, is recommended in MN analysis (Dias et al. 2005; Sugihara et al. 2000).

Although chromosomal aberrations can be detected by using simple cytogenetic methods, physical mapping technologies and especially fluorescent *in situ* hybridization (FISH), provide new tools for their analysis. The identification of individual chromosomes or chromosome arms is very helpful in the detection of small chromosome rearrangements, as well as their detailed characterization. An additional advantage of the FISH technique is the possibility of detection of chromosomes or chromosome fragments in non-dividing cells. FISH, with specific DNA probes, can improve the existing MN test and give the possibility of better understanding of the composition of the micronuclei (Maluszynska et al. 2003).

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Correspondence: J. Juchimiuk, Department of Plant Anatomy and Cytology, University of Silesia, Jagiellońska 28, 40-032 Katowice, Poland; e-mail: juchimiu@us.edu.pl

A complete identification of all chromosome fragments in micronuclei by using FISH is still not possible in plant cells because of the lack of chromosome-specific DNA sequences. Nevertheless, application of region-specific DNA probes (e.g. telomere- and centromere-specific) enables the analysis of the chromosomal breakpoints leading to micronuclei. Therefore, FISH with these sequences as probes, gives the possibility to check whether whole chromosomes or chromosome fragment(s) are involved in micronucleus formation.

Until now, FISH has not been widely applied in plant mutagenesis for the detection and identification of chromosome aberrations, because DNA probes required for chromosomes of particular plant species are very limited. Nevertheless, there are some examples where FISH has been helpful in the analysis of chromosomal aberrations in plant species, both with small and large chromosomes. FISH has proved to be the only method for the detection of translocations in *Arabidopsis thaliana* (Weiss and Maluszynska 2000) and *Secale cereale* (Hasterok et al. 2002a), as well as in the analysis of cell ploidy level in interphase nuclei of *A. thaliana* (Weiss and Maluszynska 2001). FISH with 25S rDNA and bacterial artificial chromosomes (BACs) as probes, was also used to demonstrate the participation of specific chromosomes of *A. thaliana* in the formation of anaphase bridges (Siroky et al. 2003). The application of telomere- and centromere-specific FISH probes combined with the MN test enabled elucidation of the origin of the micronuclei induced by N-methyl-N-nitrosourea (MNU) in barley cells (*Hordeum vulgare*), and to compare the frequency of various types of chromatid aberrations analysed by a traditional chromosome staining method, and of micronuclei by using FISH (Jovtchev et al. 2002). The use of a few DNA probes for FISH, if chromosome preparations are reprobed after double target probing, is well known (Heslop-Harrison et al. 1992). Although the reuse of slides to localize a few DNA probes has found wide application in plant cytogenetics, it has not been used in mutagenesis so far.

In contrast, the combination of multiple DNA probes labelled with various fluorochromes makes FISH a powerful tool in human cytogenetics, as well as in carcinogenicity studies in mammals (Marshal et al. 1996; Natarajan et al. 2003).

In the present paper, we quantitatively analyse micronuclei induced by N-nitroso-N-methylurea (MNU) and maleic hydrazide (MH) in order to compare the possible origin of the micronuclei induced by these mutagens, characterized by different mechanisms of action. MH is a clastogenic agent that can lead to chromosome breaks, as well as cause spindle fibre defects. MNU is an alkylating agent that mainly induces gene mutations, but it can also lead to chromosomal aberrations. The applied mutagens act in different phases of the cell cycle: MH in the S phase and MNU in the G2 phase (Maluszynska and Maluszynski 1983). FISH with multiple probes (5S, 25S rDNA, and telomere- and centromere-specific DNA sequences, including reprobing of preparations) was used to identify the specific chromosomes or chromosome fragments involved in the micronuclei and chromosome aberrations. Due to its large chromosomes and the possibility of distinguishing the majority of chromosomes by the presence and specific localization of rDNA, barley was used as a model plant in this study.

Materials and methods

Plant material and treatment

Seeds of barley (*Hordeum vulgare* 'Start' variety, 2n=14) were presoaked in distilled water for 8 hours and then treated for 3 hours with N-nitroso-N-methylurea (MNU; 2 mM or 3 mM; Sigma, CAS 684-93-5) or maleic hydrazide (MH; 3 mM or 4 mM; Sigma, CAS 123-3301). After the treatment, the seeds were washed 3 times in distilled water and then germinated in Petri dishes. The material was fixed in ethanol : glacial acetic acid (3:1) 36 h, 48 h and 60 h after treatment. Roots of M_1 seedlings were used as the source of meristems for investigations of micronuclei.

Chromosome preparations

For chromosome preparations the material was washed with 0.01 mM sodium citrate buffer (pH 4.8) for 30 min, and digested with an enzyme mixture containing 2% cellulase (w/v, Onozuka, Serva) and 20% pectinase (v/v, Sigma) for 2 hours at 37°C. After digestion the material was washed again with sodium citrate buffer for 30 min. Squash preparations were made in a drop of 45% acetic acid. After freezing and coverslip removal, the slides were dried.

Fluorescence *in situ* hybridization (FISH)

FISH was applied according to the method described by Maluszynska and Heslop-Harisson

(1991) with some minor modifications. Four DNA probes were used: (1) HT100.3 - Arabidopsis-type telomeric repeats ($(TTTAGGG)_n$) labelled with rhodamine-4-dUTP by PCR (Roche); (2) CCS1 – centromere DNA isolated from *Brachypodium sylvaticum* (Aragon-Alcaide et al. 1996), labelled with digoxygenin-11-dUTP (Roche); (3) 5S rDNA isolated from *Triticum aestivum* - pTa 794 (Gerlach and Dyer 1980; Hasterok et al. 2002b) labelled with rhodamine-4-dUTP using PCR labelling kit (Amersham Life Sciences); and (4) 25S rDNA isolated from *Arabidopsis thaliana* (Unfriend and Gruendler 1990), labelled with digoxygenin-11-dUTP by nick translation (Roche). HT100.3 and CCS1 were used as probes in the first FISH experiment, whereas 25S and 5S rDNA in the second, after slides reprobing.

Prior to FISH, the chromosome preparations were pretreated with RNase (1 mg mL^{-1}) for 60 min at 37°C and washed 3 times for 5 min in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) at room temperature, dehydrated in a methanol series and air-dried.

The hybridization mixture, containing 2.5 μg mL^{-1} of labelled DNA, 50% (v/v) formamide, 10% (w/v) dextran sulphate, and 0.1 mg μL^{-1} salmon testes DNA in 2 × SSC, was denatured at 75°C for 10 min and immediately placed on ice for a few minutes. A sample of the mixture (38 μL) was added to the chromosome preparations and covered with a plastic coverslip. The chromosomes and DNA probes were denatured for 5 min at 70°C on a hot plate (Hybaid Thermal Cycler PCR – in situ). Hybridization was carried out at 37°C in a moist chamber for 20 h. Slides, after hybridization, were subsequently washed for 4 min in 2 × SSC at 42°C, 2 × 4 min in 0.1 × SSC at 42°C, 3 × 3 min in 2 × SSC at 42°C, 3× 3 min in 2 × SSC at room temperature, and for 5 min in 0.2% Tween in 4 × SSC at room temperature.

The digoxigenin-labelled probe was detected by using FITC-conjugated anti-digoxigenin antibodies, and then the signal was amplified by a FITC-conjugated secondary antibody. After 3 washes for 8 min in 0.2% Tween in 4 × SSC at 37°C and dehydration in the ethanol series, the slides were mounted in a Vectashield medium (Vector) containing 6 μg mL^{-1} DAPI. After capturing of the images of the first probes, the slides were incubated for 30 min at 37°C, and coverslips were removed. Slides were subsequently washed 2 × 30 min in 4 × SSC with 0.2% Tween at 37°C, and 2 × 15 min in 2 × SSC at 37°C. Then preparations were dehydrated in the methanol series and air-dried. The hybridization mixture with rDNAs, hybridization conditions, post-hybridization washing, and detection, were as described above.

Preparations were examined with an OLYMPUS PROVIS epifluorescence microscope by using the proper filter set. Images were captured by using a CCD camera Hamamatsu C5810 and processed in Adobe Photoshop 4.0. Frequencies of micronuclei with specific DNA signals and without signals were calculated. For each experimental group, 50 cells with micronuclei on 3 slides, each made from 3 meristems, were evaluated. Results of the analyses were pooled for all concentrations of MH and MNU and postincubation times. The total frequencies of micronuclei was estimated on the same slides – 2000 cells for each experimental group were analysed.

Results and discussion

In the present study, by using fluorescence *in situ* hybridization (FISH) with centromere- and telomere-specific DNA, and rDNAs as probes, the analysis of signals present in micronuclei and anaphase fragments allowed the examination of the possible origin of micronuclei induced by MH and MNU.

Before FISH, the slides were stained with DAPI and the frequency of micronuclei in barley root meristematic cells (control and treated ones) was estimated. The frequency of micronuclei after

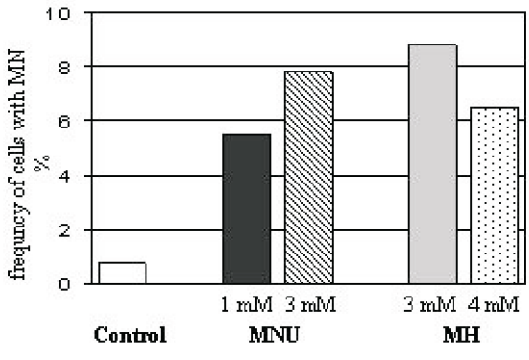

Figure 1. Frequency of MH- and MNU-induced micronuclei in meristematic cells of barley roots, 48 h after treatment

treatment with MH or MNU varied from 5.5% to 8.8% (Figure 1). The highest frequency of cells with micronuclei was observed 48 h after treatment (the frequencies at 36 h and 60 h after treatment are not shown). The treatment with MNU

induced micronuclei in 5.5% (1 mM) or 7.7% of cells (3 mM). 3 mM MH was the strongest inducer of micronuclei (8.8%), but the higher concentration of the mutagen (4 mM) caused a decrease in the frequency of micronuclei. It appears that the concentration of 4 mM MH may be too high for barley cells and may reduce their mitotic activity. Similar frequencies of micronuclei in barley cells induced by MNU were shown by Jovtchev et al. (2002), but an accurate comparison is not possible due to the different doses of the mutagen used in their study, as well as differences in other conditions of the experiments. No published data on frequency of MH-induced micronuclei in barley root cells have been found.

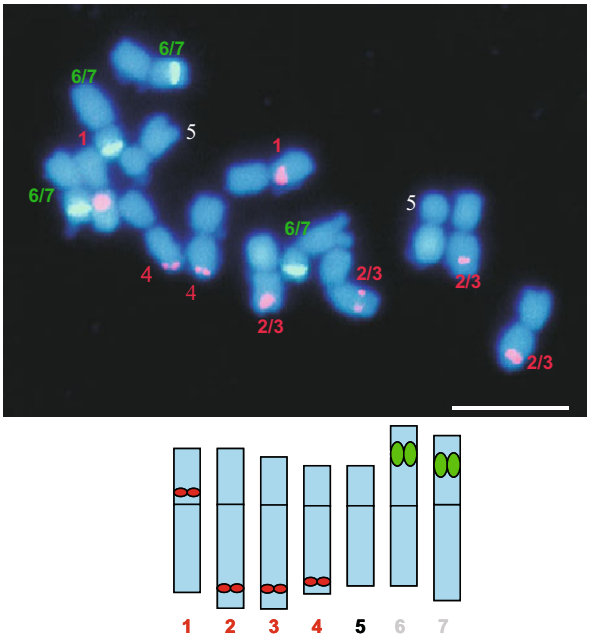

Figure 2. Karyotype and idiogram of barley (*Hordeum vulgare* 'Start'), showing distribution of rRNA genes and chromosome numbering. 5S rDNA = red; 25S rDNA = green; DAPI staining = blue. Bar represents 10 µm.

Due to its large chromosomes and many rDNA sites on the chromosomes, barley was used as the experimental model in this study. The previous cytogenetic study of physical location of 5S and 18S-25S rDNA sequences in a few *Hordeum* species proved that rDNA provides useful chromosome landmarks (Leich and Heslop-Harrison 1993). As the high polymorphism in the number and location of 18S-25S and 5S rDNA was shown for a few wild *Hordeum* species and their cytotypes (Taketa et al. 1999), the location of both rDNAs in *Hordeum vulgare* 'Start' variety used in this study was analysed. Physical mapping of 25S and 5S rDNA in this variety also showed a large number of rDNA sites. Only one chromosome pair

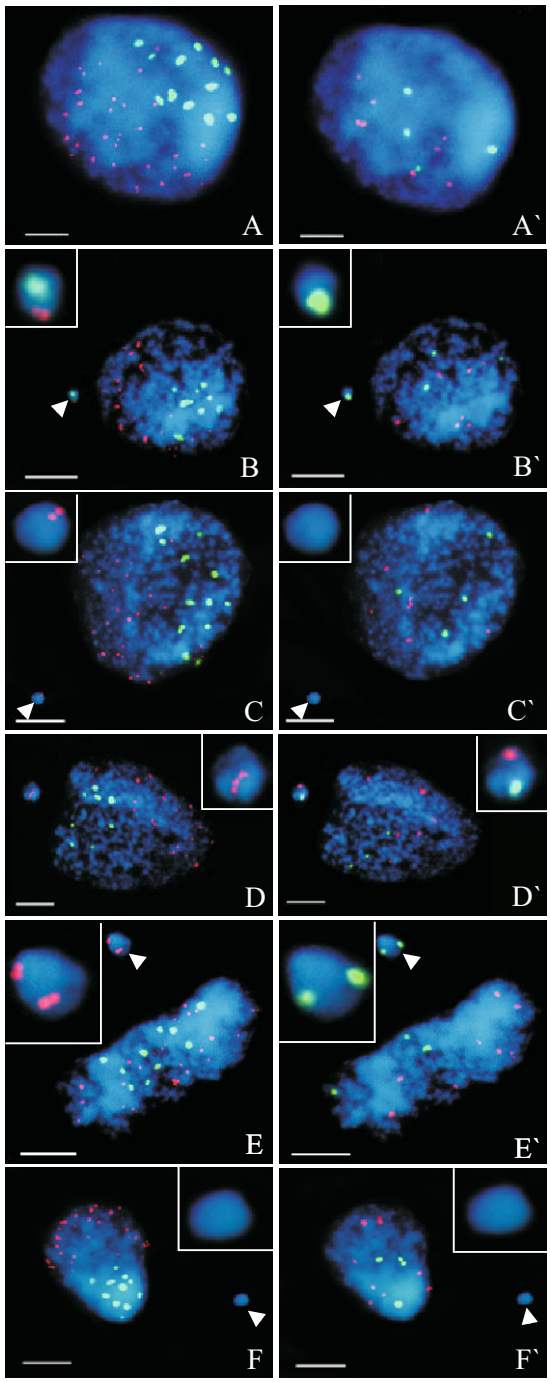

Figure 3. Distribution of telomeric and centromeric DNA sequences – results of the first FISH experiment (A, B, C, D, E) and rRNA genes – second FISH, after slides reprobing (A`, B`, C`, D`, E`) in interphase nuclei of Hordeum vulgare cells in (A, A`) control cells and (B, B`-E, E`) after treatment with MH and MNU. Telomeric sequences and 5S rDNA- red, centromeric sequences and 25S rDNA – green, DAPI staining- blue. Micronucleus with: B, B` - two signals of FISH with telomeric DNA, one with centromeric DNA and one signal of 25S rDNA; C, C` - two signals of FISH with telomeric DNA and lack of rDNA signals; D, D` - four signals of FISH with telomeric DNA and one signal of 5S and 25S rDNA; E, E` - two signals of FISH with telomeric DNA, two green signals of 25S rDNA. One additional signal of 25S rDNA in nucleus is observed. F, F` - lack of signals; Bar represents 10 µm.

is characterized by the lack of rDNA signals. Due to the large number and specific localization of rDNA loci, 3 out of 7 chromosome pairs could be easily distinguished (chromosomes 1, 4 and 5, Figure 2).

The use of rDNA enables the analysis of the involvement of specific chromosomes with 5S or 25S rDNA in the micronuclei formation. Moreover, the use of centromeric and telomeric probes in this study enabled the explanation of whether chromosome fragments or whole chromosomes are involved in micronuclei formation. This is the first example of use of reprobing of *in situ* hybridization preparations due to analysis of chromosomal aberrations in plant cells. We found that the barley chromosome slides did not suffer from high background or chromatin loss, and the signals in the micronuclei were easily distinguished after reprobing. In this study, micronuclei with various FISH signals were observed after treatment with the applied mutagens – 8 categories of micronuclei were distinguished (see Figure 4). Micronuclei with the same signals of applied probes were observed after MH and MNU treatment. Examples of control interphase cells and cells with micronuclei with signals of different DNA sequences induced by MH and MNU treatment are presented in Figure 3.

The micronucleus in Figure 3B, B` contains telomeric and centromeric signals (Figure 3 B) and a signal of 25S rDNA (Figure 3B`), which indicates its origin from a complete chromosome 6 or 7. It is also probable that it could have come from 2 or more fragments from different chromosomes, providing that one of them was a 25S rDNA-bearing chromosome. The micronucleus in Figure 3C, C`, having only telomere-specific DNA, is an example of the involvement of acentric chromosome fragments in its formation. Due to the lack of rDNA signals in this micronucleus, the examination of its accurate origin is difficult. By contrast, micronucleus in Figure 3E, E` probably originated from 2 acentric fragments of 25S rDNA-bearing chromosomes. Furthermore, due to one additional locus of 25S rDNA in this cell, a break involving the 25S rDNA locus or duplication took place. The presence of 5S and 25S rDNA signals and 4 signals of telomere-specific DNA in the micronucleus in Figure 3D, D` clearly proved its origin from 2 distal acentric fragments: one from chromosome 6 or 7 and the other from 5S rDNA-bearing chromosome (no. 1, 2, 3 or 4). Beside the micronuclei with signals of probes used for *in situ* hybridization in this study, some micronuclei without any signals were also observed (Figure 3F, F`), indicating the involvement of the interstitial chromosome region in micronuclei formation, but their accurate origin remains unknown.

As MH- and MNU-induced micronuclei characterized by different composition (i.e. containing different chromosome fragments or whole chromosomes) were observed, our findings confirm the earlier studies that pointed out that there are only a few genotoxic agents that induce solely one type of micronuclei (Schuler et al. 1997).

The applied concentrations of mutagens, as well as the postincubation times used in this study, did not influence the frequency of micronuclei with a particular type of signals; thus all obtained data were pooled for MH and MNU. Figure 4 shows the total results of the analysis for both applied mutagens. Interestingly, differences between the frequency of MH- and MNU-induced micronuclei with specific signals were revealed. The micronuclei with 2 signals, telomeric DNA

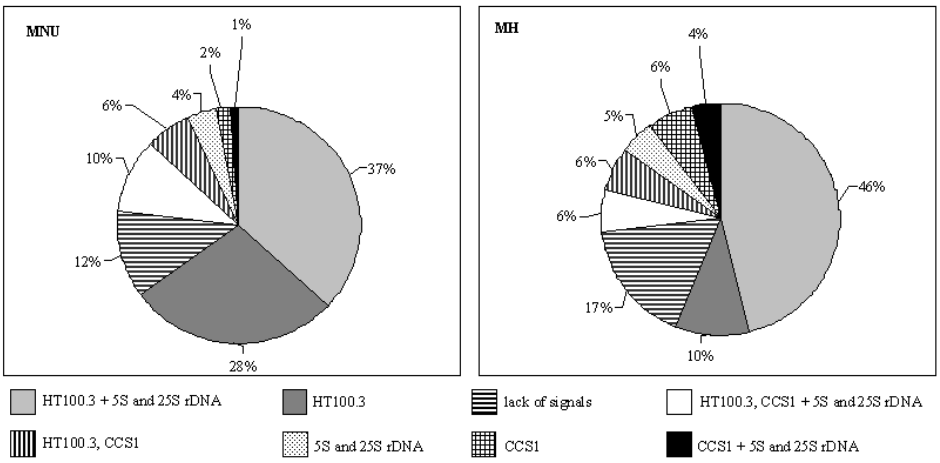

Figure 4. Comparison of the frequency of micronuclei with specific DNA sequences in barley cells after treatment with MNU and MH

and rDNA (5S and 25S rDNA), were the most frequently observed in the case of both mutagens, but with a higher frequency after MH treatment (46%) than MNU (37%). Moreover, 10% of MH-induced micronuclei are characterized by the presence of only telomere DNA sequences, whereas there were almost 3-fold more in the case of MNU-induced micronuclei (28%). These data could indicate that in the case of MNU, a higher number of small distal acentric fragments (which do not include rDNA loci) are involved in micronuclei formation. By contrast, MH leads to large acentric chromosome fragments including rDNA loci, which are located in the interstitial regions or near the centromere.

Relatively high frequencies of micronuclei including both centromeric and telomeric signals after hybridization were observed: – 12% (MH-induced micronuclei) and 16% (MNU-induced micronuclei) was observed. The presence of rDNA signals in such micronuclei confirms that they contain a complete chromosome or chromosome arm with a centromere – one out of 6 chromosomes, as chromosome 5 does not have any rDNA markers.

Very similar results were reported by Jovtchev et al. (2002), who used FISH with telomere- and centromere-specific sequences as probes, and showed that most MNU-induced micronuclei revealed telomere-specific signals. However, the frequency of micronuclei with only centromere-specific signals was lower (1%) than in the presented study. Unfortunately, we do not know any other similar analyses of MH-induced micronuclei, and any comparisons of the composition of micronuclei produced by other mutagens.

As micronuclei are formed as a result of acentric fragments and multicentric chromosomes, the double FISH experiments (including slide reprobing) with 5S, 25S rDNA and telomeric, centromeric DNA sequences as probes were used to identify the origin of chromosome fragments in the mitotic anaphase. Figure 5 shows control cells (A, A`) and examples of anaphase with fragments after MH or MNU treatment (B, B`- E, E`). The presence of 2 telomeric signals, a centromeric signal and a 5S rDNA signal in the fragment, indicates that a complete chromatid of chromosome 1, 2, 3 or 4 is included in it (Figure 5B, B`). In the case of anaphase in Figure 5C, C`, 4 small acentric interstitial chromosome fragments without telomeric signals were observed. The presence of two 25S rDNA signals could indicate that both fragments derived from chromosomes 6 and 7. Interestingly, only 2 rDNA signals are present in each sister nucleus, so this indicates that fragments in Figure 5C, C` represent chromosome-type, not chromatid-type aberrations.

The fragment with a single telomeric signal and 5S rDNA after hybridization indicates that it is an acentric distal fragment of a 5S rDNA-bearing chromosome (Figure 5D, D`). At the same time, a signal of 5S rDNA is missing in one sister nucleus. Additionally, a very small fragment of unknown origin can be seen.

Figure 5E, E` shows cells representing the fusion of 2 fragments – the presence of 2 signals of telomeric DNA and a single 25S rDNA signal means that a distal acentric fragment from chromosome 6 or 7 and an acentric fragment of unknown origin are involved in this aberration. The lack of only one 25S rDNA signal in a sister nucleus indicates that 25S rDNA-bearing fragment represents a chromatid-type aberration and precludes its origin from an isochromosome.

As micronuclei can derive from acentric fragments as a result of chromosome breakage or whole chromosomes lagging behind in anaphase due to damaged kinetochores or spindle fibre defects, the FISH with MN assay was previously used to detect both the clastogenic and aneugenic effects of mutagenic treatment (Kirsch-Volders et al. 1997). In this study the presence of micronuclei revealing centromeric and telomeric signals could indicate aneugenic action of the applied mutagens, which fits the expectations in the case of MH, but was not expected for MNU. This is related to the mechanism of action, namely MH can cause spindle fibre defects (Mann et al. 1975; Jovtchev et al. 2001; Marcano et al. 2004).

The centromere- and telomere-specific DNA probes used in this study are especially preferable in the quantification of micronuclei in plants (Schubert et al. 1998; Jovtchev et al. 2002) as well as in humans (for review see Norppa and Falck 2003). As in the majority of organisms, telomere sequences are localized at the end of chromosomes, with few exceptions (Schubert 1992; Adams et al. 2000; Fajkus et al. 2005), the terminal deletions especially can easily be detected by using telomere-specific DNA sequences as a FISH probe. Also the application of a subtelomeric tandem repeat HvT01 as a FISH probe, allowed the detection of deletion in individual barley chromosomes and translocations between barley and wheat chromosomes (Schubert et al. 1998). The combined application of these probes and rDNA increases the accuracy of the analysis of micronucleus origin. FISH with rDNA sequences as probes for the distinction between micronuclei

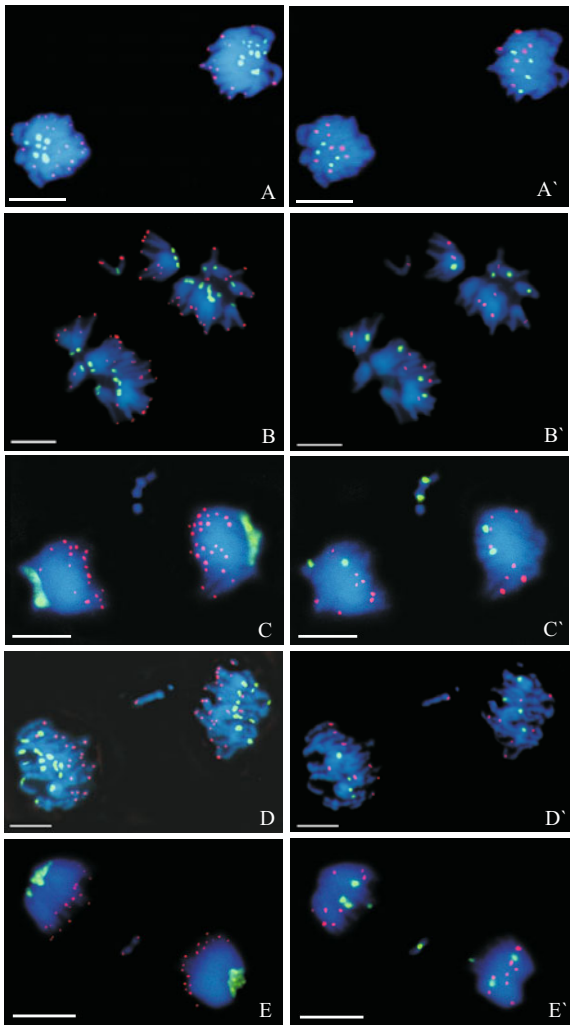

Figure 5. Anaphases in *Hordeum vulgare* cells hybridized with: telomeric and centromeric DNA sequences (A, B, C, D); 5S, and 25S rDNA (A`, B`, C`, D`) in (A, A`) control cells and (B, B`– E, E`) after treatment with MH and MNU. Telomeric sequences and 5S rDNA – red, centromeric sequences and 25S rDNA – green, DAPI staining – blue. B, B` – one complete chromatid of chromosome no. 1, 2, 3 or 4 - two telomere signals at the distal regions, and 5S rDNA signal. C, C` – four small acentric interstitial chromosome fragments without telomeric signals. The presence of two 25S rDNA signals indicate that two fragments derived from chromosome 6 and 7. D, D` – one acentric distal fragments of one of 5S rDNA –bearing chromosome and small interstitial fragment. E, E`– two acentric chromosome fragments with telomeric sequences. One 25S rDNA signal present in one of the fragments indicates it origin from chromosome no. 6 or 7. Bar represents 10 μm.

of various origin and detailed characterization of other chromosomal aberrations could be applied in some plant species, such as barley or Arabidopsis, in which rDNA marks each chromosome. The low number and unfavourable location of rDNA loci in the majority of plant species makes such an analysis impossible.

As the quantification of micronuclei in plant cells is not very common, the presented results are an example of such a possibility in plant genotoxicity studies, especially in order to understand the mode of action of MN inducers. These results, which show differences in the frequency of MH- and MNU-induced micronuclei with specific signals, could indicate that the mechanisms of the action of the mutagen could influence the breakpoints in barley chromosomes, leading then to differences in the composition of micronuclei. This suggestion does not confirm the hypothesis of the presence of 'hot spots' for induction of chromosomal aberrations in the plant genome (Andersson and Kilhman 1987; Kanaya et al. 1994). Nevertheless, the results of this study revealed that the distribution of chromosomal aberrations is not random. MH (an S-phase- dependent mutagen) leads to large acentric fragments, whereas MNU (acting in G2) leads to small terminal chromosome fragments. So regarding the pericentromeric location of heterochromatin in barley chromosomes, the results of this study are consistent with the hypothesis that heterochromatin regions represent 'hot spots' of aberration formation induced by S-phase-dependent mutagens (Schubert et al. 1998, 2004).

Conclusions

FISH with centromere- and telomere-specific DNA, 5S, and 25S rDNA as probes, proved to be a promising technique for evaluation of the origin of the micronuclei in barley cells and also for analyses of the action of mutagens. In this study, differences in the frequency of MH- and MNU-induced micronuclei with various probes were shown. Moreover, the application of the reprobing of the DNA-DNA *in situ* hybridization preparations used in this study, can provide new possibilities for the detailed analysis of chromosomal aberrations. In relation to this, even though the availability of plant DNA probes is not wide, the MN test combined with FISH can improve the effectiveness of genotoxicity assessment in plants.

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