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Differential genotoxicity of ethyl methanesulphonate, *N*-ethyl-*N*-nitrosourea and maleic hydrazide in tobacco seedlings based on data of the Comet assay and two recombination assays

Tomáš Gichner*

Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Na Karlovce 1a, 160 00 Prague 6, Czech Republic

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Abstract

The purpose of this study was to determine if mutagen-induced DNA damage is correlated with the frequency of induced recombination events. The alkylating agents ethyl methanesulphonate (EMS) and *N*-ethyl-*N*-nitrosourea (ENU), and the plant growth regulator and herbicide maleic hydrazide (MH) were compared in tobacco seedlings for their ability to induce DNA damage measured by the Comet assay, and recombination activity measured by the GUS gene reactivation assay, and by the somatic twin sectors assay. While EMS and ENU induced a dose-dependent increase in DNA damage in leaf nuclei, MH had no significant effect. By contrast, MH induced a 6-fold higher frequency of homologous recombination as expressed by the GUS assay and a 2.8-fold higher frequency of somatic twin sectors than after EMS treatments.

Keywords: DNA damage; *Nicotiana tabacum*; β-Glucuronidase; Single-cell gel electrophoresis (SCGE)

1. Introduction

Plant genomes contain large amounts of repetitive DNA sequences that provide potential targets for homologous recombination, which may result in alteration of the genome. Somatic recombination in plants is of special biological significance, because plants, unlike animals, do not have a cell line predetermined to produce gametes. Thus, genetic changes occurring during vegetative growth can be transmitted to the progeny [1].

The basic steps of homologous recombination in prokaryotes and lower eukaryotes are reported to include an initiation by a DNA double-strand break and/or single-strand DNA formation, exchange of DNA strands, formation of recombination intermediates (Holliday junctions) and its resolution by the endonuclease *Ruv*C [2]. A similar mechanism may be postulated also for plants [3].

In the work presented here we have treated tobacco seedlings with the monofunctional alkylating agents EMS and ENU, and with the plant growth regulator MH and compared: (1) the DNA damage as measured by the Comet assay, (2) the recombination frequency as measured by the GUS gene reactivation assay and the twin sectors assay. Comparing the data obtained in these three assays could help to elucidate the relationship between recombination events and induced DNA damage expressed by the Comet assay in plants.

Abbreviations: EMS, ethyl methanesulphonate; ENU, *N*-ethyl-*N*-nitrosourea; GUS, β-glucuronidase; MH, maleic hydrazide; TM, tail moment; SCGE, single-cell gel electrophoresis

* Tel.: +420-2-24-310-109; fax: +420-2-24-310-113.
E-mail address: gichner@ueb.cas.cz (T. Gichner).

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The alkaline version of the Comet assay can quantitatively measure DNA damage, including single-strand breaks, double-strand breaks, alkali-labile sites (primarily apurinic and apyrimidinic sites), incomplete excision repair sites and DNA crosslinks [4]. Although this technique has been primarily applied to animal cells, the application of the Comet assay to plant tissues [5–7] significantly extends the utility of plants in basic and applied studies in environmental mutagenesis.

The gene that codes for β-glucuronidase (GUS) in *Escherichia coli* (*uidA*) was introduced as a suitable reporter gene system for plants by Jefferson et al. [8]. Using the disrupted GUS marker gene, the frequency of homologous recombination events was enhanced in tobacco N9 or *Arabidopsis thaliana* seedlings by treatment with various genotoxins [9–11].

The formation of twin sectors in heterozygous plants as in *Glycine max* [12] and *Nicotiana tabacum* [3,13], and the results of in vitro culture of twin spot components in *N. tabacum* [14] were attributed to a somatic crossing-over mechanism.

2. Materials and methods

2.1. Chemicals and media

Ethyl methanesulphonate (EMS, CAS No. 62-50-0), 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt (CAS No. 114162-64-0), *N*-ethyl-*N*-nitrosourea (ENU, CAS No. 759-73-9), maleic hydrazide (MH, CAS No. 123-33-1), the plant growth medium (Phytagel, MS salts), reagents for electrophoresis, normal melting-point (NMP) and low melting-point (LMP) agarose, and general laboratory reagents were purchased from Sigma Chemical Co., St. Louis, MO.

2.2. Plant material

1. The tobacco line N9, derived from *N. tabacum* Petit Havana SR1 [9] containing overlapping sequences of the *uidA* gene in inverted orientation separated by a hygromycin phosphotransferase gene was used for the GUS gene reactivation test and for the Comet assay. The seeds of line N9 were kindly provided by Dr. I. Kovalchuk (University of Lethbridge, Canada).
2. Double heterozygous *N. tabacum* var. xanthi (a_1^+/a_1; a_2^+/a_2) plants [13] were used for the detection of somatic twin sectors, manifested as contiguous dark green and yellow sectors on the pale green leaves.

2.3. Tobacco growth and mutagenic treatment conditions

Tobacco seeds were sterilized by immersion in 70% ethanol for 2 min followed by 20 min in a sterilizing solution (4.5 ml distilled water, 0.5 ml 5.25% sodium hypochlorite, 5 μl 10% Triton X-100). The sterilizing solution was aspirated and the seeds were washed five times in sterile distilled water. Each seed was placed in a vented container that contained 50 ml of sterile, solid growth medium and the plants were grown in a plant growth chamber at 26 °C with a 16 h photo-period until the four to five true leaf stage. At this stage, the roots of seedlings were carefully rinsed in water and immersed in glass vials containing 22 ml of a defined concentration of the tested agents. The plants were treated in the dark at 26 °C for 24 h. A detailed description of plant growth conditions was previously published [15].

2.4. Comet assay

After seedling treatment, individual N9 tobacco leaves were placed in a 60 mm petri dish kept on ice and spread with 250 μl of cold 400 mM Tris buffer, pH 7.5. Using a fresh razor blade, each leaf was gently sliced. The plate was kept tilted in the ice so that the isolated nuclei would collect in the buffer. Regular microscope slides were dipped into a solution of 1% NMP agarose prepared with water at 50 °C, dried overnight at room temperature and kept dry in slide boxes until use. Onto each slide, nuclear suspension (50 μl) and 1% LMP agarose (50 μl) prepared with phosphate-buffered saline were added at 40 °C. The nuclei and the LMP agarose were gently mixed by repeated pipetting using a cut micropipet tip and a coverslip was placed on the mixture. The slide was placed on ice for a minimum of 5 min, the coverslip removed and a final layer of 0.5% LMP agarose (100 μl) was placed on the slide and covered with

a coverslip. The slides were placed in a horizontal gel electrophoresis tank containing freshly prepared cold electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH >13). The nuclei were incubated for 15 min to allow the DNA to unwind prior to electrophoresis at 0.72 V cm^{-1} (26 V, 300 mA) for 30 min at 4 °C. After electrophoresis, the slides were rinsed three times with 400 mM Tris, pH 7.5, stained with 80 μl ethidium bromide (20 μg ml^{-1}) for 5 min, dipped in ice-cold water to remove the excess ethidium bromide and covered with a coverslip. For each slide, 25 randomly chosen nuclei were analyzed using a fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. A computerized image analysis system (Komet version 3.1, Kinetic Imaging Ltd., Liverpool, UK) was employed. The tail moment (integrated value of tail DNA density multiplied by the migration distance) and the percentage of tail DNA were used as the primary measure of DNA damage. Three slides were evaluated per treatment and each treatment was repeated twice. From the repeated experiments, the averaged median tail moment value and the averaged median percentage of tail DNA were calculated for each treatment group from the median tail moment value and the median percentage of tail DNA of each slide [16].

2.5. GUS gene reactivation assay

For each experiment, 14 tobacco N9 seedlings at the stage of four to five leaves were treated with the test mutagens for 24 h and individually cultivated in glass vials with a 50% Hoagland's solution in a growth chamber at 22–26 °C with a 18 h photo-period. After 14 days, the first and after 21 days the second newly formed leaves were histochemically stained [8,10]. The leaves were placed in a beaker with a staining buffer containing 100 mg 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylammonium salt, 150 mg NaN_3, and 300 μl Triton X-100 in 300 ml phosphate buffer adjusted to pH 7.3. To support the penetration of the staining buffer, the leaves were vacuum infiltrated (twice 1 min with a 30 s interval at 100 mbar) and incubated while shaking at 37 °C for 48 h. Then the leaves were incubated in ethanol for 5 h at 65 °C to remove chlorophyll. After enzymatic hydrolysis, the water-soluble indoxyl intermediate undergoes an oxidative dimerization to produce a blue indigo precipitate. The recombination events were scored as blue sectors on the bleached leaves.

2.6. Detection of twin sectors

The *N. tabacum* var. xanthi seedlings were cultivated as described above. For each experiment, eight seedlings at the stage of four to five leaves, treated with the test mutagens for 24 h and individually cultivated in glass vials with a 50% Hoagland's solution in a growth chamber at 22–26 °C with a 18 h photo-period for 2–3 weeks. The green/yellow twin sectors [13] were identified on the pale green leaves newly formed after the treatment (on the first newly formed leaves 14 days and on second leaves 21 days after the treatment). Each experiment was repeated twice.

2.7. Statistical analysis

Data were analyzed using the statistical and graphical functions of SigmaPlot 4.01 and SigmaStat 2.03 (SPSS Inc., Chicago, IL). If in a one-way analysis of variance test a significant F-value of $P < 0.05$ was obtained, a Dunnett's multiple comparison versus the control group analysis was conducted.

3. Results

3.1. Comet assay

After a 24 h treatment period in the dark with the three test agents, nuclei were isolated from leaves of the transgenic *N. tabacum* line N9 and a concentration-response analysis was conducted (Fig. 1). As consensus among investigators as to the most appropriate manner in which to express the DNA damage in the Comet assay has not been obtained [4], the averaged median tail moment values (TM) and the percentage of DNA (T (%)) are given. The values of T (%) in the tail were in most cases slightly higher than the TM values.

With increased EMS doses in the range of 1–4 mM (Fig. 1A), the average median TM values (±S.E.) increased significantly from 5.4 ± 0.3 (negative control) up to 69.1 ± 2.5 μm ($F_{4,29} = 246$; $P < 0.001$).

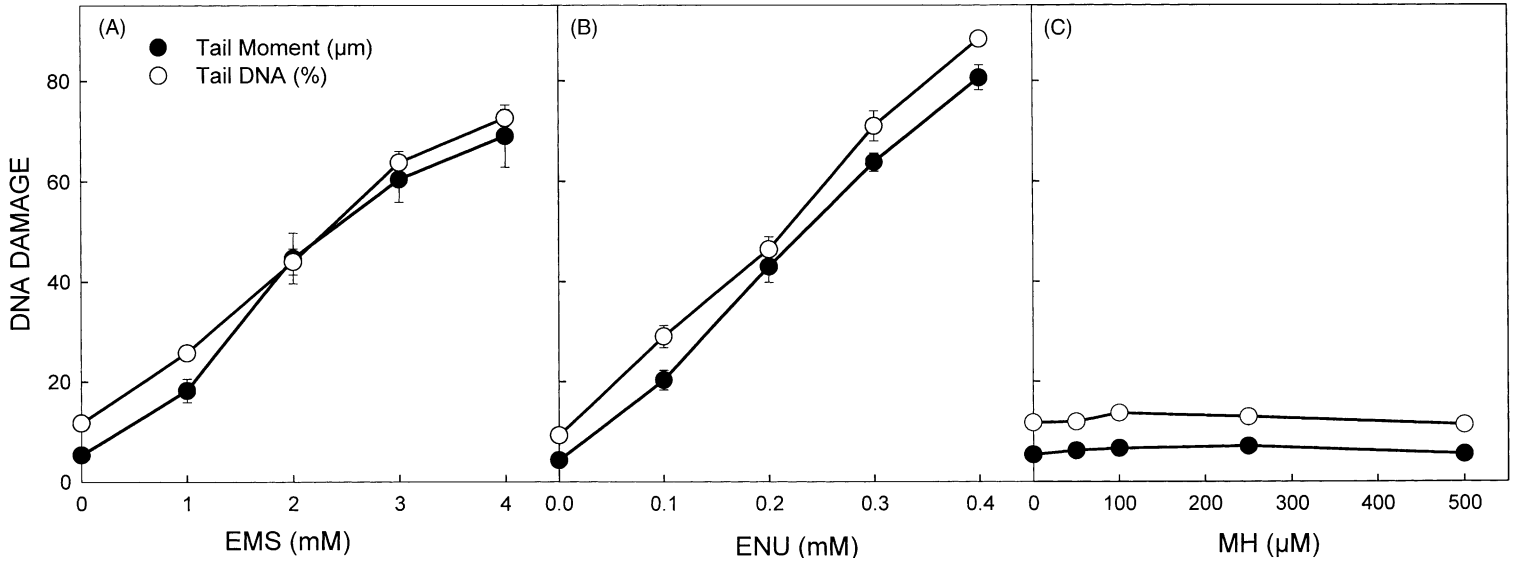

Fig. 1. Dose–response curves of the average median tail moment values (TM) and average median percentage of tail DNA as a function of ethyl methanesulphonate (EMS—A), *N*-ethyl-*N*-nitrosourea (ENU—B) and maleic hydrazide (MH—C) treatment of *N. tabacum* line N9 seedlings for 24 h at 26 °C. The error bars represent the standard error of the mean.

Similarly, increased concentrations of ENU in the range of 0.1–0.4 mM (Fig. 1B) resulted in a significant increase of the average median TM values from 4.3 ± 0.4 (negative control) to 80.7 ± 2.5 μm ($F_{4,29} = 206$, $P = 0.001$).

By contrast, MH doses of 50 to 500 μM (Fig. 1C) did not result in a significant increase in the TM values ($F_{4,29} = 1.5$, $P = 0.217$) compared to the negative control of 5.4 ± 0.3 μm.

3.2. GUS gene reactivation assay

The frequency of blue spots was a measure of homologous recombination after treatment of N9 tobacco seedlings with EMS, ENU and MH (Fig. 2). Concentrations of EMS from 1 to 4 mM significantly increased ($F_{4,49} = 19$, $P < 0.001$) the average frequency of blue spots (±S.E.) that ranged from 1.5 ± 0.6 (negative control) to 11.0 ± 0.6 per first leaf formed after treatment with 3 mM EMS (Fig. 2A). The average frequency of EMS-induced blue spots was significantly lower on the second leaves and increased only from 1.3±0.4 to 5.5±0.7 ($F_{4,49} = 9$, $P < 0.001$).

In Fig. 2B, the data on the recombination activity of 0.1–0.4 mM ENU, applied on N9 tobacco seedlings are presented. Compared with the negative control (1.1 ± 0.2) the frequency of recombination events on the first leaves formed newly after the mutagenic treatment increased significantly to 7.4 ± 0.4 after 0.4 mM ENU ($F_{4,70} = 68$, $P < 0.001$). On the second leaves the frequency of homologous recombination events increased significantly only from 1.1 ± 0.3 (negative control) to 3.1 ± 0.3 after treatment with 0.3 mM ENU ($F_{4,70} = 8$, $P < 0.001$).

The frequency of homologous recombination events after treatment of N9 tobacco with MH was higher than after EMS and ENU treatments (Fig. 2C). Treatments with MH in concentrations ranging from 0 to 500 μM MH significantly increased the average frequency of blue spots from 1.9 ± 0.3 (negative control) to 71.2 ± 7.6 evaluated on the first newly formed leaves ($F_{4.69} = 61$, $P < 0.001$) and from 1.5 ± 0.3 to 30.0±2.8 on the second newly formed leaves ($F_{4.69} = 45$, $P < 0.001$). Thus, MH increased the frequency of recombination events on the first newly formed leaves after treatment 37-fold, while EMS gave only a 7.3-fold and ENU only a 6.4-fold increase compared with the control levels. Higher doses of the test mutagens could not be applied as they inhibited the apical meristem and no new leaves appeared after the mutagenic treatment.

3.3. Twin sectors

After 24 h treatment of seedlings of *N. tabacum* var. xanthi with EMS, ENU and MH, and cultivation of the treated seedlings for 2–3 weeks, the frequency of contiguous dark green/yellow twin sectors was scored on the first and second newly formed leaves. With 0.5–4 mM of EMS (Fig. 3A), the average frequency of twin sectors (±S.E.) on the first leaf increased significantly from 0 (negative control) up to 3.1 ± 0.3 after treatment with 4 mM EMS ($F_{5,95} = 26$, $P < 0.001$) and on the second newly formed leaves only up to 1.8 ± 0.2 per leaf ($F_{5,95} = 14$, $P < 0.001$).

Similar results were obtained with ENU (Fig. 3B). Treatment with 0.1–0.5 mM ENU resulted in a significant increase of the average frequency of twin sectors on the first leaves from 0.06 (negative control) compared with 2.8 ± 0.4 after 0.5 mM ENU ($F_{5,98} = 14$, $P < 0.001$). On the second leaves the average frequency of twin sectors was lower and reached after seedling treatment with 0.5 mM ENU a value of 1.5 ± 0.2 compared with 0 sectors in the controls ($F_{5,98} = 7$, $P < 0.001$).

Seedling treatment with MH resulted in much higher frequencies of twin sectors than after EMS and ENU treatments (Fig. 3C). Treatment with 50–500 μM MH increased the frequency of twin sectors from 0.57 ± 0.3 to 8.7 ± 1.5 per first leaf newly formed after the MH treatment. Compared with the negative control (0.25 ± 0.1), concentrations from 100 μM MH and higher induced a significant response ($F_{5,86} = 17$, $P < 0.001$). In contrast, on the second leaf the average twin sector frequency significantly increased after 500 μM, but only to 5.38 ± 0.8 ($F_{5,86} = 23$, $P < 0.001$).

4. Discussion

The Comet assay performed in the reported experiments detects acute DNA damage in the nuclei of the tobacco somatic leaf cells that do not divide and are arrested at the G0 stage of the cell cycle [17]. By contrast, the DNA lesions that lead to recombination,

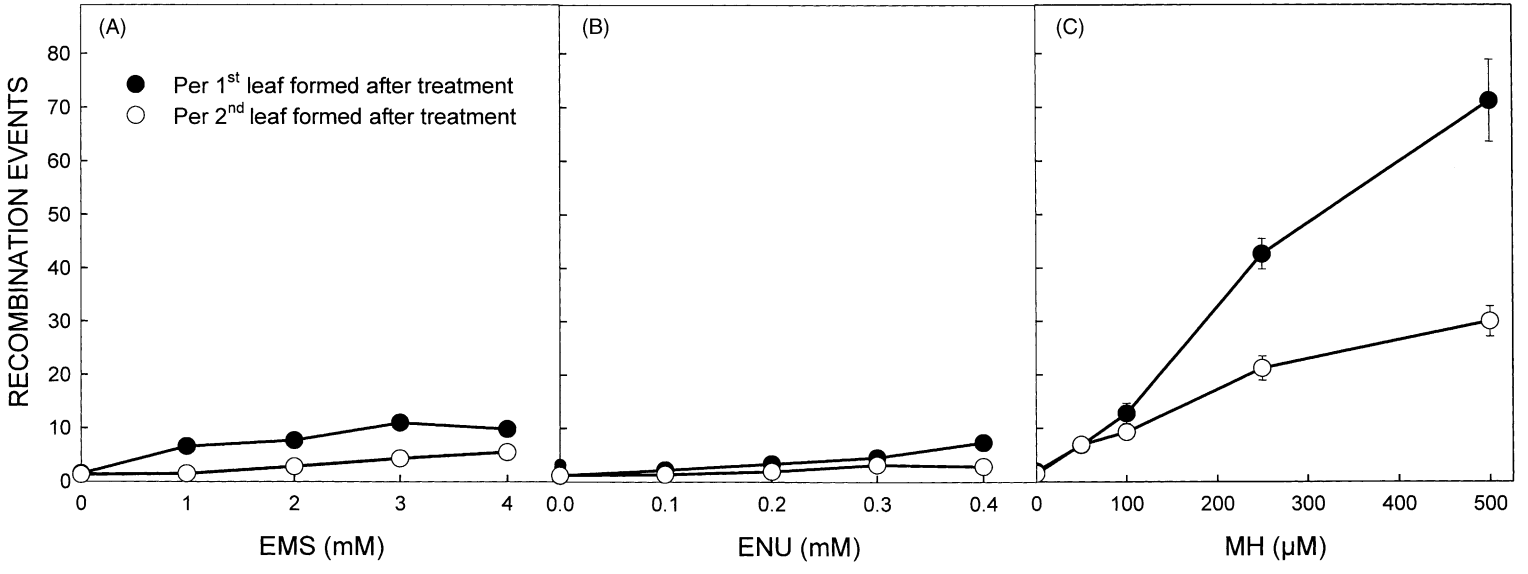

Fig. 2. Dose–response curves of the average frequency of homologous recombination events in the GUS recombination assay on the first and second newly formed leaves after treatment with ethyl methanesulphonate (EMS—A), *N*-ethyl-*N*-nitrosourea (ENU—B) and maleic hydrazide (MH—C) of *N. tabacum* line N9 seedlings for 24 h at 26 °C. The error bars represent the standard error of the mean.

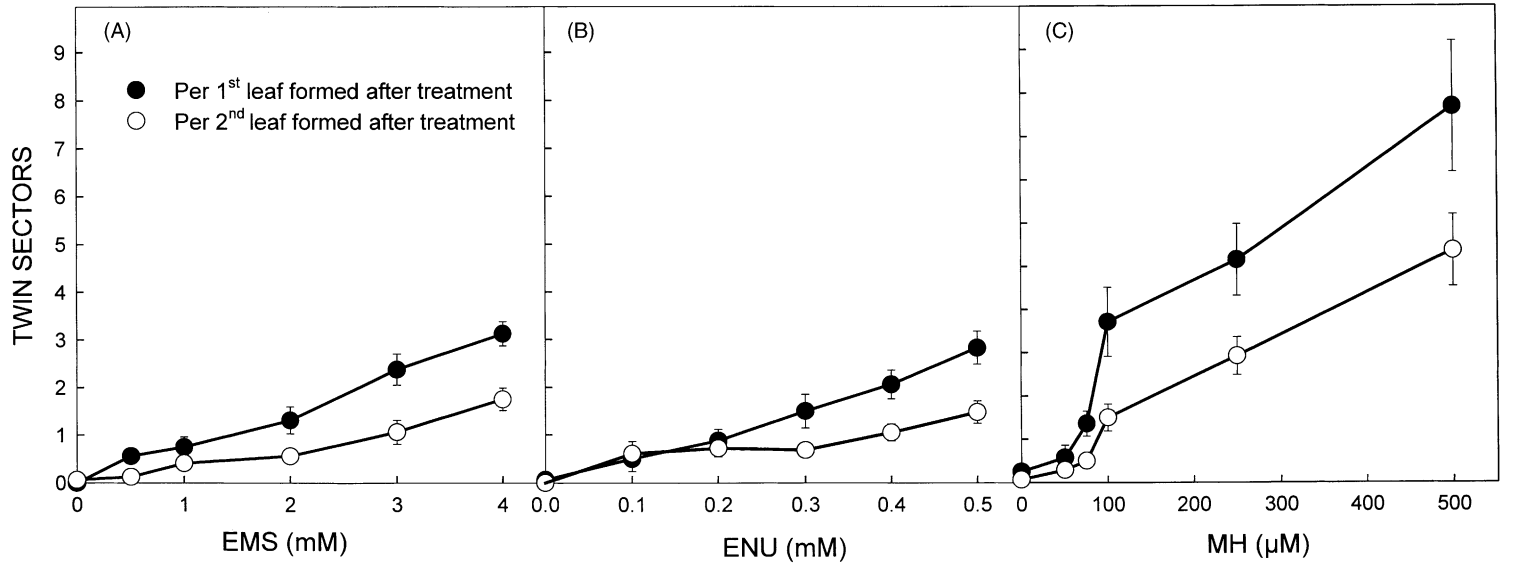

Fig. 3. Dose–response curves of the average frequency of twin sectors on the first and second newly formed leaves after treatment with ethyl methanesulphonate (EMS—A), *N*-ethyl-*N*-nitrosourea (ENU—B) and maleic hydrazide (MH—C) of *N. tabacum* var. xanthi seedlings for 24 h at 26 °C. The error bars represent the standard error of the mean.

have to be induced in the leaf primordia within the apical meristem that will develop into leaves. The mis-repaired DNA lesions are fixed within these cells and as the leaf grows, division of the cells with recombination events leads to a clone of cells that appear on the pale green xanthi tobacco as twin green/yellow sectors, or on the bleached N9 tobacco leaves with the reactivated GUS gene after histochemical staining as blue sectors.

In our previous studies with tobacco (*N. tabacum* var. xanthi) we have demonstrated that the DNA-damaging effect of monofuntional alkylating agents [15], the aromatic amine *o*-phenylenediamine [18] and gamma rays [19] is highly correlated with the frequency of somatic mutations on tobacco leaves. *N*-Methyl-*N*-nitrosourea induced DNA damage detected by the Comet assay [20] and a high frequency of chromosome aberrations in barley [21], and DNA damage [7] and embryonic and chlorophyll mutations in *A. thaliana* [22]. The data presented in this paper demonstrate that the alkylating agents EMS and ENU also induce recombination events. Thus, most genotoxins induce DNA damage detectable by the Comet assay as well as damage measurable by different genetic endpoints. The exception is MH.

The plant growth regulator and herbicide MH (1,2-dihydro-3,6-pyridazinedione, a structural isomer of uracil) is mutagenic in several plant species [23]. In tobacco it induced about 10-fold more somatic mutations than alkylating agents [23] and the data in the present paper demonstrate that MH induced 6-fold more recombination events than EMS. MH also proved to be a S-phase-dependent clastogen in *V. faba* [24] and in barley [21]. As far as the mutagenic activity in bacteria, fungi and animal cells is concerned, MH was negative or exhibited a very low activity [25]. One explanation for the differences in the mutagenic activity of MH in plants and other systems could be its biotransformation in plant tissue to a metabolic product with mutagenic activity.

A question arises why MH, expressing in plants a very high mutagenic, clastogenic and recombination activity, does not induce DNA damage as measured by the Comet assay. Although autoradiographic studies with [^{14}C]MH [26] indicated a preferential labeling of interphase nuclei, nucleoli and mitotic chromosomes, no labeled DNA was found after exposure of plant cells to [^{14}C]MH [27]. In pulse-chase experiments with [^{3}H]thymidine, MH-mediated DNA fragments of replicon size occurred only in labeled DNA [28]. It was therefore speculated that MH induces DNA lesions preferentially or exclusively during replication. If so, MH-induced DNA damage cannot be detected by the Comet assay in the tobacco leaves with non-dividing G0 cells, but DNA lesions resulting in recombination and mutation events can be induced in the dividing apical meristem and then detected in the leaves newly formed after the treatment. However, even a 24 h treatment of *V. faba* root meristems with MH did not result in a dose-dependent increase of DNA migration [23]. We can further speculate that MH might induce the transcription of genes involved in recombination or it might block other pathways, and homologous recombination is used as a substitute.

Mutations as well as recombination events may be the result of mis-repaired DNA lesions. Mis-repaired DNA, leading to severe genetic effects, need not be however detected by the Comet assay, provided the DNA strands involved are not disrupted. This disruption may be caused either by the direct formation of induced DNA breaks or by the conversion of, e.g. alkali-labile sites to DNA breaks during electrophoresis at alkaline conditions (pH 13.5) via β-elimination [29]. If MH does not induce DNA lesions leading to DNA breaks directly or during alkaline electrophoresis, no DNA damage can be detected by the Comet assay.

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