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DNA staining with the fluorochromes EtBr, DAPI and YOYO-1 in the comet assay with tobacco plants after treatment with ethyl methanesulphonate, hyperthermia and DNase-I

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Abstract

We applied the alkaline version of the single-cell gel electrophoresis (comet) assay to roots and leaves of tobacco (*Nicotiana tabacum* var. *xanthi*) seedlings or isolated leaf nuclei treated with: (1) the alkylating agent ethyl methanesulphonate, (2) necrotic heat treatments at 50 °C, and (3) DNase-I. All three treatments induced a dose-dependent increase in DNA migration, expressed as percentage of tail DNA. A comparison of the fluorochrome DNA dyes ethidium bromide, DAPI and YOYO-1 demonstrated that for the alkaline version of the comet assay in plants, the commonly used fluorescent dye ethidium bromide can be used with the same efficiency as DAPI or YOYO-1.

Keywords: DNA migration; *Nicotiana tabacum*; Single-cell gel electrophoresis

1. Introduction

A variety of fluorochromes have been applied for DNA staining in the comet assay, e.g. acridine orange, DAPI, EtBr, propidium iodide [1–3]. Developments in the synthesis of DNA-binding dyes have led to a new family of asymmetric cyanine dyes with improved fluorescence properties upon binding to DNA (Molecular Probes Inc.). These dyes remain largely non-fluorescent in aqueous solution and display substantial enhancement of their fluorescence quantum yield upon binding to DNA [4,5]. Several of these dyes were tested for DNA-imaging applications and it was reported that YOYO-1 in particular, improves the image quality [6,7].

We applied the comet assay to tobacco seedlings or isolated tobacco leaf nuclei treated with: (1) the genotoxic alkylating agent ethyl methanesulphonate, (2) necrotic heat treatments at 50 °C, and (3) DNase-I, a nuclease that digests DNA to nucleosomal-sized fragments. The objective was to compare the sensitivity of the DNA-binding dyes EtBr, DAPI and YOYO-1 in revealing the DNA-damaging effects of these three different toxic stimuli in the comet assay.

Abbreviations: DAPI, 4′,6-diamidino-2-phenyindole, dilactate; EMS, ethyl methanesulphonate; EtBr, ethidium bromide; YOYO-1, Quinolinium,1,1′-[1,3-propanediylbis[(dimethyliminio)-3,1-propanediyl]]bis[4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]]-tetraiodide

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2. Materials and methods

2.1. Chemicals and media

4′,6-Diamidino-2-phenyindole, dilactate (DAPI, CAS No. 28718-90-3), ethyl methanesulphonate (EMS, CAS No. 62-50-0), ethidium bromide (EtBr, CAS No. 1239-45-8), Phytagel, MS salts, normal and low melting-point agarose and general laboratory reagents were purchased from Sigma Chemical Co., St. Louis, Missouri. The YOYO-1 stain (CAS No.143413-85-8) Quinolinium,1,1′-[1,3-propanediylbis[(dimethyliminio)-3,1-propanediyl]]bis[4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]]-tetraiodide was purchased from Molecular Probes Inc., Eugene, Oregon.

2.2. Tobacco growth

Leaves and roots of heterozygous *Nicotiana tabacum* var. *xanthi* (a_1^+/a_1; a_2^+/a_2) plants [8] were used. Tobacco seeds were sterilized by immersion in 70% ethanol for 2 min followed by a 20 min treatment in a solution containing 4.5 ml of distilled water, 0.5 ml of 5.25% sodium hypochlorite and 5 μl 10% Triton X-100, and washed 5× in sterile distilled water. Each seed was placed in a vented plastic container with 50 ml of sterile, solid growth medium and the plants were grown in a plant growth chamber at 26 °C with a 16 h photoperiod each day. A detailed description of the plant growth conditions was previously published [9].

2.3. Treatment conditions

2.3.1. EMS treatment

At the four to five true-leaf stage, the seedlings were carefully removed from the containers, the roots rinsed in water and immersed in plastic vials containing 22 ml of a defined concentration of EMS. For analysis of root nuclei the seedlings were treated with EMS for 2 h in the dark at 26 °C, for leaf nuclei the treatment was for 24 h. The long 24 h treatment of leaves with EMS (compared with the 2 h treatment of roots) is necessary to enable the mutagenic solution to be sufficiently absorbed through the roots into the leaves.

Nuclei were isolated after treatment and subjected to the comet assay procedure.

2.3.2. Heat treatment

Excised leaves from tobacco seedlings were immersed for 1–10 min in water at 50 °C. Nuclei were isolated immediately after the heat-treatment and subjected to the comet assay procedure.

2.3.3. DNase-I treatment

Agarose slides with nuclei from untreated tobacco leaf cells were prepared as outlined below. Each slide was exposed for 1 min to 800 μl of DNase-I (0–0.5 Units) dissolved in water. After the treatment the slides were dipped in water and subjected to the comet assay procedure.

2.4. Comet assay

For isolation of nuclei, leaf or root tissues, treated or untreated as appropriate, were placed in a 60 mm petri dish containing 250 μl of cold 400 mM Tris–buffer, pH 7.5 and kept on ice. Using a fresh razor blade, leaves were 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% normal melting-point agarose in water at 50 °C, dried overnight at room temperature and kept dry in slide boxes until use. Nuclear suspensions (50 μl) and 1% low melting-point (LMP) agarose (50 μl) in phosphate-buffered saline were gently mixed at 40 °C by repeated pipetting using a cut micropipette tip, added onto each slide. A cover slip was placed on the mixture to obtain a uniform layer. The gel was allowed to solidify by keeping the slide in a steel tray on ice for a minimum period of 3 min, the coverslip was removed and a final layer of 0.5% LMP agarose (100 μl) was placed on the slide and covered with a cover slip. The slides were placed in a horizontal 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 25 min at 4 °C. After electrophoresis, the slides were rinsed three times with 400 mM Tris–buffer, pH 7 and air-dried.

Air-dried slides were immersed for 5 min in cold water and then stained for 5 min with 80 μl EtBr (20 μg/ml), DAPI (2 μg/ml) or YOYO-1 (2 μM). All the dyes were dissolved in water. The slides were rinsed in cold water to remove the excess stain and covered with a cover slip. The nuclei were analyzed by use of a fluorescence microscope. For EtBr a BP 546/10 nm excitation filter and a 590 nm emission filter was used, for DAPI and YOYO-1, the excitation filters were 330–385 and 470–490 nm, and the emission filters were 420 and 520 nm, respectively. A computerized image-analysis system (Komet version 3.1, Kinetic Imaging Ltd., Liverpool, UK) was used. For each slide, 25 randomly chosen nuclei were analyzed, three slides were evaluated per treatment and each treatment was repeated twice. From the repeated experiments, the averaged median percentage of tail DNA as the primary measure of DNA migration was calculated for each treatment group.

2.5. Statistics

Data were analyzed using the statistical and graphical functions of SigmaPlot 8.0 and SigmaStat 3.0 (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 test between the treated and control group was conducted. Differences between two groups were statistically evaluated by a t-test.

3. Results

3.1. DNA migration in root nuclei after treatment with EMS

Fig. 1 illustrates the DNA-damaging effect on the nuclei of tobacco roots of a 2 h treatment with the alkylating agent EMS. After isolation of nuclei, slides with nuclei were processed for the comet assay and stained with one of three fluorescent dyes: EtBr, DAPI and YOYO-1. The percentage tail DNA (±S.E.) after staining with EtBr increased significantly ($P < 0.001$) from 4.3 ± 0.2% (negative control) to 72.5 ± 3.5% with 40 mM EMS. With the same EMS concentration, staining with DAPI resulted in a significant ($P < 0.001$) increase in the % tail DNA from 4.3 ± 0.4% (negative control) to 72.6 ± 2.3%, and with YOYO-1 a significant increase ($P < 0.001$) from 5.0 ± 0.2% (negative control) to 75.8 ± 1.4% was observed. There was no significant difference in the percentage tail DNA between staining with EtBr and DAPI ($P = 0.986$) or with EtBr and YOYO-1 ($P = 0.499$), after treatment with 40 mM EMS.

3.2. DNA migration in leaf nuclei after EMS treatment

Fig. 2 illustrates the DNA-damaging effect on the leaf nuclei from tobacco seedlings treated with EMS for 24 h. With increasing concentrations of EMS the % tail DNA (±S.E.) after staining with EtBr increased significantly ($P < 0.001$) from 8.2 ± 0.4% (negative control) to 90.9 ± 0.4% with 4 mM EMS. Staining with DAPI resulted in a significant ($P < 0.001$) increase in the % tail DNA from 6.8 ± 0.7% (negative control) to 86.3 ± 1.5% and after staining with YOYO-1 it increased significantly ($P < 0.001$) from 13.0 ± 0.9% (negative control) to 94.6 ± 0.7% with 4 mM EMS. There was no significant difference in the % tail DNA between staining with EtBr and DAPI ($P = 0.205$) or between EtBr and YOYO-1 staining ($P = 0.172$) after treatment with 4 mM EMS.

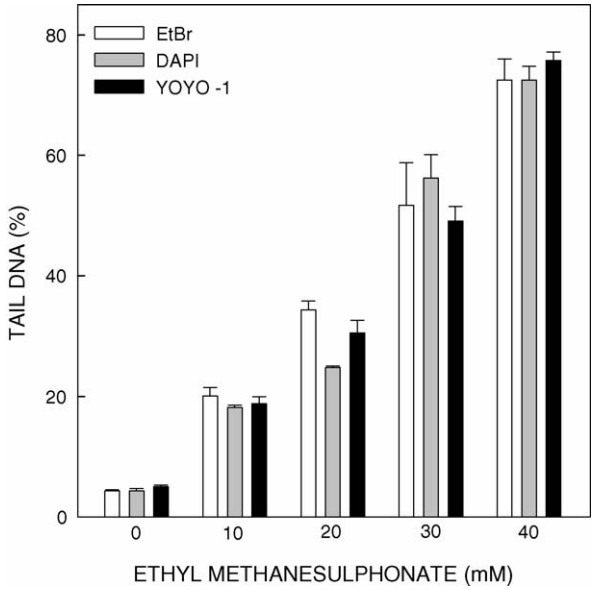

Fig. 1. The average median % tail DNA of root nuclei after a 2 h ethyl methanesulphonate treatment of tobacco seedlings. The nuclei were stained with EtBr, DAPI or YOYO-1. The error bars represent the standard error of the means.

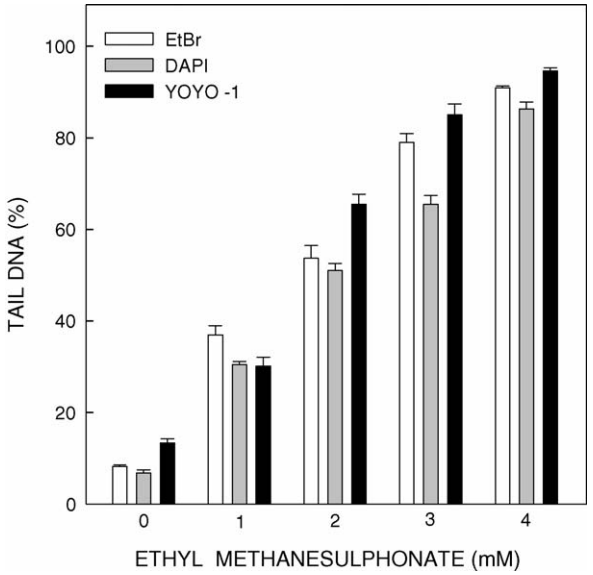

Fig. 2. The average median % tail DNA of leaf nuclei after a 24 h ethyl methanesulphonate treatment of tobacco seedlings. The nuclei were stained with EtBr, DAPI or YOYO-1. The error bars represent the standard error of the means.

3.3. DNA migration in leaf nuclei after heat treatment

To induce necrotic DNA fragmentation, tobacco leaves were incubated in water at 50 °C for 1–10 min. The % tail DNA (±S.E.) of EtBr-stained nuclei increased significantly ($P < 0.001$) from 5.8 ± 0.4% at 0 min to 83.7 ± 2.5% after 10 min of heating (Fig. 3). Staining with DAPI resulted in a significant ($P < 0.001$) increase from 5.1 ± 0.4% (negative control) to 79.4 ± 2.3% (10 min), and in nuclei stained with YOYO-1 there was a significant increase ($P < 0.001$) from 4.5 ± 0.7% (negative control) to 77.3 ± 6.3% (10 min). The % tail DNA after heat treatment at 50 °C for 10 min was not significantly different between EtBr and YOYO-1 ($P = 0.114$) or between EtBr and DAPI ($P = 0.312$) staining.

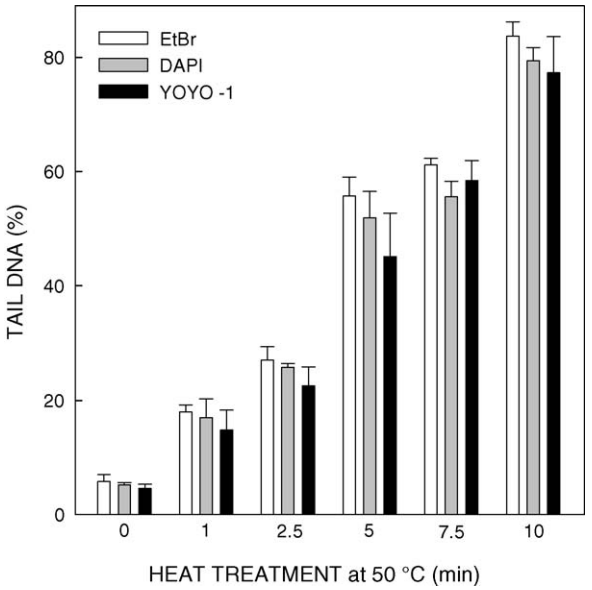

Fig. 3. The average median % tail DNA after heat treatment of tobacco laves at 50 °C for 1–10 min. The nuclei were stained with EtBr, DAPI or YOYO-1. The error bars represent the standard error of the means.

3.4. DNA migration in leaf nuclei after DNase-I treatment

DNase-I digests nuclear DNA to nucleosome-sized DNA fragments [13]. Tobacco nuclei exposed to DNase-I for 1 min were stained with EtBr, DAPI or YOYO-1 (Fig. 4). With increasing amounts of DNase-I (0.075–0.5 Units), the % tail DNA (±S.E.) in EtBr-stained nuclei increased significantly ($P < 0.001$) from $8.3 \pm 0.3\%$ (negative control) to $87.5 \pm 1.5\%$ (0.5 Units). After DAPI staining, a significant ($P < 0.001$) increase from $5.5 \pm 0.8\%$ (negative control) to $90.3\% \pm 2.3\%$ (0.5 Units) was seen, and after YOYO-1 staining a significant increase ($P < 0.001$) from $4.7 \pm 0.5\%$ (negative control) to $84.3 \pm 1.5\%$ (0.5 Units) was observed. The % tail DNA after treatment with 0.5 Units of DNase-I was not significantly different between EtBr and YOYO-1 staining ($P = 0.294$) or between EtBr and DAPI staining ($P = 0.474$).

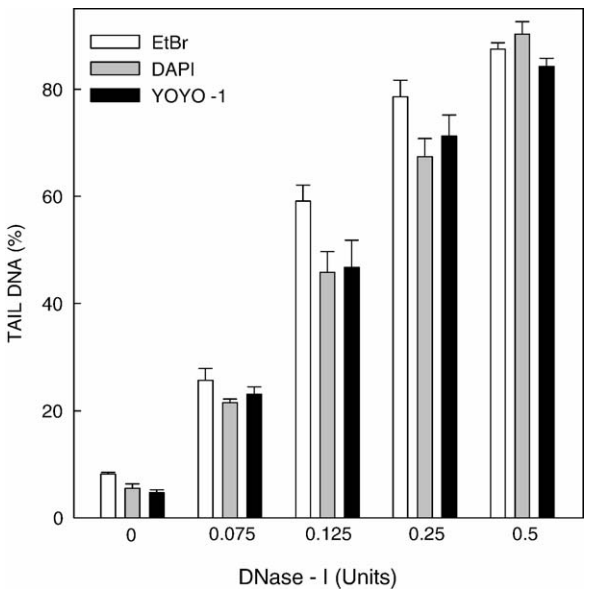

Fig. 4. The average median % tail DNA after treatment of isolated leaf nuclei embedded in agarose for 1 min with 0.075–0.5 Units of DNase-I. The nuclei were stained with EtBr, DAPI or YOYO-1. The error bars represent the standard error of the means.

4. Discussion

The comet assay protocols for plants and animals differ due to the presence of the plant cell wall. Plant cell nuclei cannot be isolated like nuclei from human and animal cells by standard lysing, but they are isolated mechanically by slicing the plant tissue with a razor blade (see Section 2.4); the lysing step can be avoided. In animal and human cells, lysis generates nucleus-like structures in the cell (nucleoids) with presumably a loss of protective histone proteins and non-DNA-associated nuclear components [10].

The three agents applied in this study were: (1) the genotoxic monofunctional alkylating agent ethyl methanesulphonate, known to induce DNA damage, somatic mutations and homologous recombination in tobacco plants [11], (2) necrotic treatment achieved by treating leaves with 50 °C hyperthermia, and (3) DNase-I, a nuclease involved in apoptosis [12], which digests DNA to nucleosome-sized fragments of 180 bp [13]. All three agents induced a dose–response increase in DNA migration in tobacco nuclei, as measured by the comet assay and expressed by the % tail DNA.

From the data presented here we may conclude that genotoxicity (represented by EMS treatment), necrosis (represented by heat treatment) and apoptosis (partly simulated by DNase-I, one of the enzymes involved in the final step of cellular apoptosis) can contribute to the total DNA migration measured by the comet assay.

Of the three fluorochromes used in the experiments reported here, EtBr binds to DNA by intercalation between base pairs of double-stranded DNA and by electrostatic interaction with the phosphate groups in the DNA backbone. It binds more efficiently to double-stranded than to single-stranded DNA [3,14]. DAPI forms a fluorescent complex preferentially by attaching in the minor groove of AT-rich DNA sequences. It

also forms intercalative complexes with double-stranded DNA [15]. YOYO-1 is referred to as a bis-intercalator [4] and was reported to show a 20- to 500-fold higher increase in fluorescence upon binding to DNA compared with EtBr [6,7]. The DNA migration measured by the comet assay in human monocytes after irradiation with gamma rays from ^{137}Cs [16] and in human lymphocytes after treatment with X-rays [7], was slightly higher when YOYO-1 was used as the DNA stain, compared with EtBr staining.

In contrast to human cells, the data of our experiments demonstrate that for the comet assay using plants, there are no significant differences after staining DNA with EtBr, DAPI and YOYO-1, although a dye that binds with higher efficiency to DNA should in theory increase the sensitivity of the comet assay [2]. Recently, no significant differences were reported in the level of DNA damage, as measured by the DNA diffusion assay, in tobacco nuclei after DNA staining with EtBr, DAPI and YOYO-1 [17].

In conclusion, EMS, hyperthermia and DNase-1 treatments induced a dose-dependent increase of DNA migration, measured by the comet assay in tobacco plants. For the alkaline version of the comet assay in plants, the fluorochromes ethidium bromide, DAPI and YOYO-1 can be used for DNA-staining with the same efficiency.

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