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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.