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DNA damage induced by indirect and direct acting mutagens in catalase-deficient transgenic tobacco Cellular and acellular Comet assays

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Abstract

We have measured the level of DNA damage induced by treating roots (cellular Comet assay) and isolated root nuclei (acellular Comet assay) of catalase-deficient (CAT1AS) and wild-type (SR1) tobacco with the promutagen *o*-phenylenediamine (*o*-PDA) and the direct acting genotoxic agents hydrogen peroxide and ethyl methanesulphonate (EMS). The roots of CAT1AS have about 60% less catalase activity compared to the roots of SR1. The promutagen *o*-PDA applied on tobacco roots induced significantly higher levels of DNA damage in the CAT1AS transgenic line than in SR1, while after application of *o*-PDA on isolated root nuclei, no DNA damage could be detected. In the catalase-deficient line CAT1AS about six-fold lower concentrations of H_2O_2 are sufficient to induce the same levels of DNA damage as in SR1. By contrast, after treatment of isolated root nuclei with H_2O_2 no difference in the induced levels of DNA damage was observed between CAT1AS and SR1. The DNA damaging effect of EMS was not affected by the presence of catalase in the tobacco roots and the levels of DNA damage measured by the cellular and acellular assay were similar.

Comparing the effects of genotoxic agents in both the cellular and acellular Comet assays may help to elucidate their mechanism of action. Differences in both systems may reveal the participation of scavengers and of repair and metabolic enzymes on the activity of the genotoxic agent and the role of the cell wall in preventing the agent from reacting with nuclear DNA.

Keywords: Hydrogen peroxide; Single-cell gel electrophoresis; *Nicotiana tabacum*; *o*-Phenylenediamine; Ethyl methanesulphonate

1. Introduction

DNA damage induced by genotoxic agents depends on their transport across cellular/nuclear membranes, activating and deactivating intracellular enzymatic processes, levels of radical scavengers and the repair competency of the target cell population. To determine the effect of these cellular processes on the amount of induced DNA damage, the acellular (sub-cellular, cell-free) Comet assay was developed [1–5]. In the acellular assay using human and animal cells, slides with gels prepared from untreated cells are exposed after lysis to the test agents and then processed according to the standard Comet assay protocol. However, in plants having a cell wall, the nuclei cannot be isolated by standard lysis, but by slicing the plant

Abbreviations: CAT1AS, catalase-deficient tobacco; EMS, ethyl methanesulphonate; *o*-PDA, *o*-phenylenediamine; TM, tail moment; SCGE, single-cell gel electrophoresis; SR1, wild-type tobacco

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tissue with a razor blade [6,7] or by agitating plant cells in suspension with sea sand [8]. Thus, in the acellular assay with plants, slides with gels prepared with untreated isolated nuclei are exposed to the test agents, and processed according to the Comet assay protocol where the lysis step can be avoided [9,10].

In the work presented here, we have compared the DNA-damaging effect of three genotoxic agents in the catalase-deficient and wild-type tobacco as measured by the cellular and acellular Comet assays: (1) the promutagen *o*-phenylenediamine (*o*-PDA), metabolized by peroxidases in tobacco plant cells to product(s) inducing DNA damage [11]; (2) hydrogen peroxide, a direct acting genotoxic agent with DNA-damaging activity dependent on the level of cell catalase [10]; and (3) ethyl methanesulphonate (EMS), a direct acting monofunctional alkylating agent having a strong DNA-damaging effect in tobacco [7,12]. By comparing the data obtained in the cellular and acellular Comet assays, the role of the cell wall and the cytosol on the level of DNA damage induced by the genotoxic agents tested can be determined.

2. Material and methods

2.1. Chemicals and media

Ethyl methanesulphonate (EMS, CAS No. 62-50-0), *o*-phenylenediamine (*o*-PDA, CAS No. 95-54-5), reagents for electrophoresis, catalase assay, the plant growth medium (Phytagel, MS salts) and general laboratory reagents were purchased from Sigma. Hydrogen peroxide (H_2O_2, CAS No. 7722-84) was purchased from Lachema, Neratovice (Czech Republic). The mutagens were dissolved in distilled water.

2.2. Plants, plant growth and mutagenic treatment conditions

Nicotiana tabacum var. Petit Havana SR1-wild-type and *N. tabacum* CAT1AS (a transgenic line that expresses only 10% of wild-type catalase activity in the leaves because of the antisense expression of the *cat1* gene). The latter was derived from *N. tabacum* Petit Havana SR1 [13]. The seeds of the transgenic tobacco line were kindly provided by Dr. F. Van Breusegem (Gent, Belgium).

SR1 and CAT1AS seedlings of tobacco were cultivated on solid growth medium [14] in sterile conditions in a plant growth chamber at 26 °C with a 16-h photoperiod until the 5–6 leaf stage [11,12].

2.3. Cellular Comet assay

The roots of seedlings were immersed in glass vials containing 22 ml of the test agents for 2 h at 26 °C in the dark. After seedling treatment, excised tobacco roots were rinsed in water and 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, the roots were gently sliced. The plate was kept tilted in the ice so that the isolated root nuclei would collect in the buffer. All operations were conducted under dim or yellow light.

Regular microscope slides were dipped into a solution of 1% normal melting point (NMP) agarose prepared with water at 50 °C, dried overnight at room temperature and kept dry in slide boxes until use. Onto each slide, a nuclear suspension (50 μl) and 1% low melting point (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. Next, the coverslip was removed and a final layer of 0.5% LMP agarose (100 μl) was placed on the slide. A coverslip was placed on the LMP agarose and the slide kept at 4 °C for 5 min. 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 3 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 (TM, integrated value of tail DNA

density multiplied by the migration distance) was used as the primary measure of DNA damage. Three slides were evaluated per treatment and each treatment was repeated at least twice. From the repeated experiments, the averaged median tail moment value was calculated for each treatment group from the median tail moment value of each slide [15].

2.4. Acellular assay

SCGE slides with nuclei from untreated SR1 and CAT1AS root cells were prepared as outlined above and the slides were immersed in solutions of 400 mM Tris–HCl buffer, pH 7.5 containing different concentrations of the test agents for 2 h at 26 °C. After the treatment period, the slides were rinsed three times for 5 min by immersion in cold distilled water, and electrophoresed and analyzed as described earlier.

2.5. Catalase assay

Catalase activity was determined by measuring the initial decomposition of H_2O_2 at 240 nm (ε: 0.036 $mM^{-1}\,cm^{-1}$) [16]. Spectrophotometric analyses were conducted on a Hitachi U-3300 spectrophotometer. Leaves and roots of tobacco, stored at −70 °C were homogenized in cold extraction buffer (100 mM phosphate buffer, 1 mM DL-dithiothreitol, 1 mM EDTA, pH 7.3) at concentration of 1 g of leaves or roots (fresh weight) to 5 ml buffer and centrifuged at 14 000 × g at 2 °C for 10 min. The supernatant fluid was recovered, and stored at −70 °C. The reaction mixture consisted of 1 ml (roots) or 0.1 ml (leaves) supernatant, 0.5 ml of 100 mM H_2O_2 and 100 mM phosphate buffer (pH 7.3) for a final volume of 3 ml.

Catalase activity was expressed in units (U) mg^{-1} protein (1 U = μmol min^{-1} substrate degradation). The protein content of each supernatant was determined using the Bio-Rad protein assay according to manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA, USA).

2.6. Statistical analysis

Data were analyzed using the statistical and graphical functions of SigmaPlot 4.01 and SigmaStat 2.03 (SPSS Inc., Chicago, IL, USA). The median tail moment values were used in a one-way analysis of variance test. If a significant F-value of $P \leq 0.05$ was obtained, a Dunnett's multiple comparison versus the control group analysis was conducted. Differences between two groups were statistically evaluated by the Paired t-test.

3. Results

3.1. Concentration–response SCGE analysis of o-phenylenediamine

After 2 h treatment of roots of the CAT1AS and SR1 tobacco with *o*-PDA, nuclei were isolated and a concentration–response analysis in the cellular Comet assay was conducted (Fig. 1). In CAT1AS tobacco, with increased concentrations of *o*-PDA the average median TM values increased significantly from 4.9±0.5 (negative control) to 56.8±2.7 μm after treatment with 8 mM *o*-PDA ($F_{5,35} = 65$, $P < 0.001$). Treatment of roots of the wild-type tobacco SR1 with concentrations of 2–8 mM *o*-PDA also resulted in a significant increase of the TM values compared to

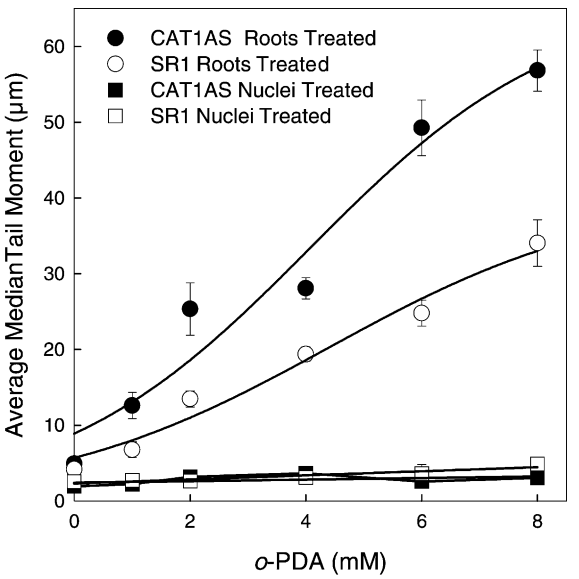

Fig. 1. Dose–response curves of the average median tail moment values (TM) as a function of *o*-phenylenediamine (*o*-PDA) treatment for 2 h at 26 °C of roots (A) or isolated root nuclei (B) of catalase-deficient (CAT1AS) or wild-type (SR1) tobacco. The error bars represent the standard error of the mean.

the negative control ($F_{5,34} = 51$, $P < 0.001$), however the DNA damage was lower than induced in the CAT1AS tobacco. With 8 mM *o*-PDA the TM value in CAT1AS reached a value of 56.8 ± 2.7 whereas in SR1 the TM value was only 34.0 ± 3.1 μm and was significantly lower ($P = 0.004$).

To determine the direct effect of the promutagen *o*-PDA on DNA, we have applied the acellular Comet assay, in which DNA is no longer held under the regulation of any metabolic pathway or cell wall barrier (Fig. 1). By contrast to the root treatments with *o*-PDA, treatments of isolated root nuclei did not result in a significant increase in the TM values, either in the CAT1AS ($F_{5,35} = 1.5$, $P < 0.226$) or SR1 ($F_{5,35} = 1.6$, $P < 0.178$) tobacco.

3.2. Concentration–response SCGE analysis of hydrogen peroxide

We compared the DNA damaging effect of H_2O_2 after treating roots of CAT1AS and SR1 tobacco (Fig. 2A). In CAT1AS tobacco, increased concentrations of H_2O_2 in the range from 0.6 to 3.0 mM, induced TM values (±S.E.) that ranged from 9.7 ± 0.5 to 79.1 ± 6.9 μm. Compared to the negative control (3.2 ± 0.3), concentrations from 1.2 mM induced a significant DNA damaging response ($F_{5,35} = 83$, $P < 0.001$). In contrast, in SR1 tobacco treated with 4–20 mM H_2O_2, concentrations of 8 mM or higher resulted in a significant increase ($F_{5,35} = 298$, $P < 0.001$) in the TM values compared to the negative control (1.8 ± 0.4). Thus, in the cellular Comet assay, the DNA damaging effect of H_2O_2 is much higher in the catalase-deficient tobacco CAT1AS than in the wild-type tobacco SR1.

In the acellular Comet assay (2B), where isolated root nuclei were treated with H_2O_2 in concentrations ranging from 0 to 1.2 mM, the treatment resulted in a significant increase in the TM values ranging from 3.3 ± 0.5 to 62.0 ± 1.8 μm in CAT1AS ($F_{5,35} = 81$, $P < 0.001$) and from 4.4 ± 0.5 to 68.0 ± 2.8 μm in SR1 tobacco ($F_{5,35} = 179$, $P < 0.001$). The DNA damage induced in SR1 tobacco was slightly higher compared to the damage induced in CAT1AS, but the differences were not significant ($P = 0.142$, for 1.2 mM H_2O_2).

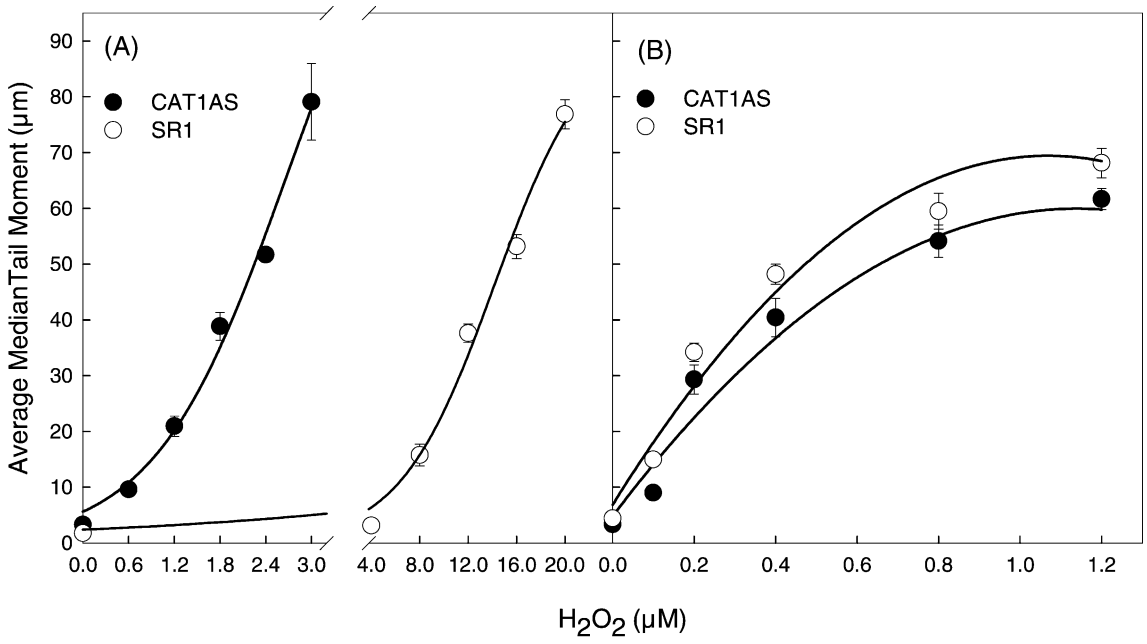

Fig. 2. Dose–response curves of the average median tail moment values (TM) as a function of H_2O_2 treatment for 2 h at 26 °C of roots (A) or isolated root nuclei (B) of catalase-deficient (CAT1AS) or wild-type (SR1) tobacco. The error bars represent the standard error of the mean.

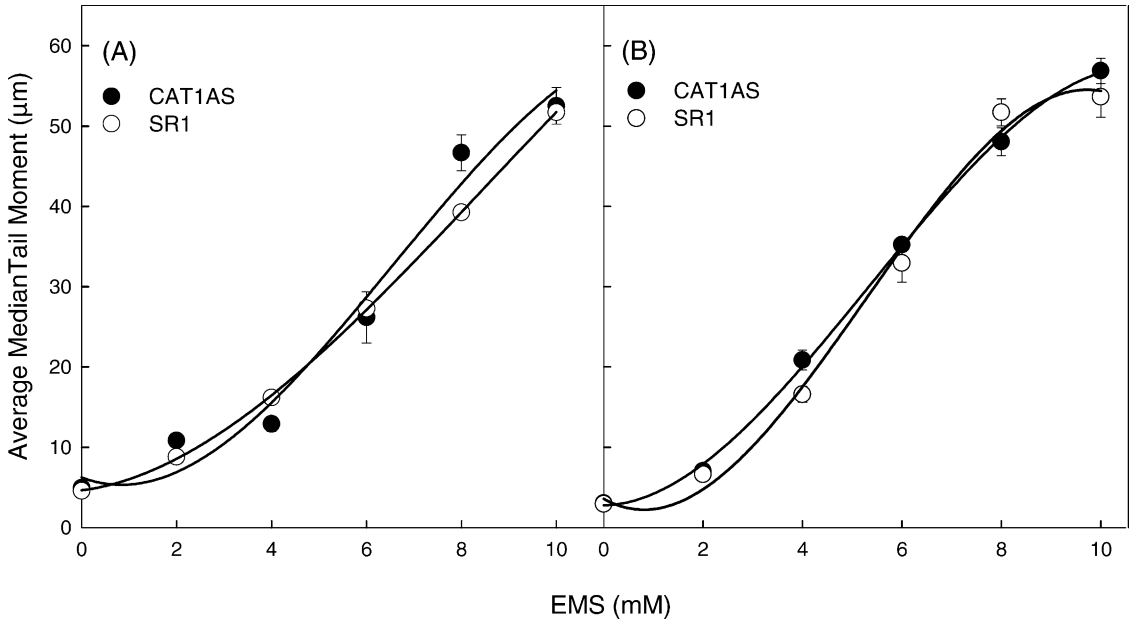

Fig. 3. Dose–response curves of the average median tail moment values (TM) as a function of ethyl methanesulphonate (EMS) treatment for 2 h at 26 °C of roots (A) or isolated root nuclei (B) of catalase-deficient (CAT1AS) or wild-type (SR1) tobacco. The error bars represent the standard error of the mean.

3.3. Concentration–response SCGE analysis of ethyl methanesulphonate

Fig. 3A illustrates the DNA-damaging effect of the direct acting monofunctional alkylating agent EMS applied for 2 h on roots of the CAT1AS and SR1 tobacco. The data of the cellular Comet assay clearly demonstrate that with increasing concentrations of EMS there is a significant increase in the TM values both in CAT1AS ($F_{5,33} = 103$, $P < 0.001$) and in SR1 ($F_{5,34} = 179$, $P < 0.001$) and the TM values after treatment with 10 mM EMS are in both tobacco plants not significantly different ($P = 0.965$).

Similarly, in the acellular Comet assay, the treatment of isolated root nuclei with 2–10 mM EMS (Fig. 3B) resulted in a significant increase in the TM values ($F_{5,35} = 341$, $P < 0.001$) in CAT1AS from 3.1 ± 0.4 (negative control) to $56.9 \pm 1.6\,\mu m$ and in SR1 tobacco from 3.0 ± 0.1 to $53.7 \pm 2.5\,\mu m$ ($F_{5,35} = 182$, $P < 0.001$). TM values after treatment with 10 mM EMS were in CAT1AS and SR1 tobacco not significantly different ($P = 0.447$).

3.4. Catalase activity in CAT1AS and SR1 tobacco leaves and roots

The catalase activity in leaves of CAT1AS was 7.6 ± 0.4 compared to the value of $99.0 \pm 5.3\,U\,mg^{-1}$ protein in the leaves of SR1 tobacco. Thus, the leaves of the catalase-deficient transgenic tobacco expressed only about 8% of the catalase activity found in the wild-type tobacco. Similarly, the catalase activity in CAT1AS roots was reduced to 1.3 ± 0.3 compared to the value of $3.3 \pm 0.2\,U\,mg^{-1}$ protein in the roots of the SR1. Roots of the catalase-deficient tobacco have thus about 40% of catalase activity of the wild-type tobacco (Fig. 4).

4. Discussion

4.1. DNA damage in catalase-deficient tobacco

Hydrogen peroxide is produced by endogenous metabolic and catabolic processes in cells. Although direct effects of H_2O_2 to nuclei were shown, it is possible that H_2O_2 triggers a signal-transduction cascade

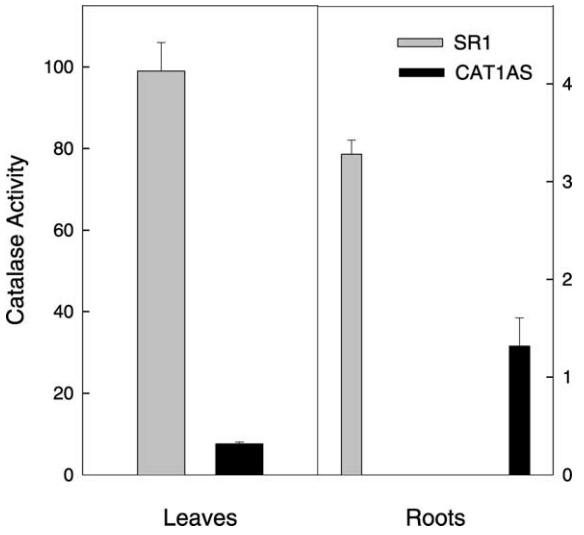

Fig. 4. A comparison between the catalase activity (units mg^{-1} protein) in leaves and roots of the catalase-deficient (CAT1AS) or wild-type (SR1) tobacco. The error bars represent the standard error of the mean.

leading to nuclear damage [17]. Catalases are the principal H_2O_2-scavenging enzymes in plants and function as a cellular sink H_2O_2 [18]. The transgenic catalase-deficient tobacco CAT1AS was reported to express only 10% catalase activity in the leaves compared to wild-type *N. tabacum* Petit Havana SR1 [13]. Our measurements (Fig. 4) demonstrate that under the cultivation conditions used in our experiments, the leaves and roots of CAT1AS expressed about 8 and 40%, respectively, of the wild-type SR1 tobacco catalase activity. The higher DNA-damaging effect of H_2O_2 in CAT1AS compared to SR1 tobacco roots (Fig. 2A) may thus be associated with the higher catalase activity, scavenging H_2O_2 before it can react with DNA in the nuclei. The approximately 13-fold higher activity of catalases in leaves compared with their activity in roots may explain why H_2O_2 induces DNA damage in roots but not in wild-type tobacco leaves (unpublished data).

The promutagenic *o*-PDA is an arylamine that is metabolized in plants preferentially by peroxidases, and the peroxidative reaction requires hydrogen peroxide [19]. The higher content of H_2O_2 due to reduced catalase activity in CAT1AS compared with SR1 tobacco roots results in a more active metabolic conversion of the promutagen *o*-PDA to a genotoxic product, thus inducing more DNA damage (Fig. 1A).

4.2. Cellular and acellular Comet assay

Kasamatsu et al. 1996 [1] reported on treating lysed murine leukemia cells on SCGE slides with various mutagens. The direct acting mutagens *N*-methyl-*N*-nitrosourea, H_2O_2 and bleomycin induced DNA damage in both the cellular and acellular systems. Agents that require metabolic activation, including 4-nitroquinoline-1-oxide, 5-fluorouracil and methotrexate, increased DNA damage only in the cellular system. With the help of the cell-free version of the Comet assay it was demonstrated that despite being present as a scavenger in the cell at millimolar concentrations, the dietary antioxidant glutathione can act on isolated nuclei as a DNA-damaging pro-oxidant [3].

In tobacco TX1 cells in suspension, isolated nuclei were more sensitive to the DNA-damaging effect of H_2O_2 than intact TX1 cells [10]. In tobacco, isolated leaf nuclei were more sensitive to gamma-radiation than nuclei isolated from irradiated excised leaves and roots [9]. The higher response of isolated nuclei to H_2O_2 and gamma-radiation was explained by the absence of the cell cytosol where catalases, repair enzymes, and scavengers are located. By contrast, EMS as a direct-acting alkylating agent, induced DNA damage in tobacco TX1 cells in suspension at approximately the same level in both the cellular and acellular systems [8]. Similar results were reported in Fig. 3 of the present paper after treatment of intact roots and isolated root nuclei of tobacco with EMS.

In conclusion, the level of genomic damage induced by H_2O_2 and the promutagen *o*-PDA, as measured by the Comet assay, was higher in catalase-deficient compared with wild-type tobacco. In contrast, the DNA damage induced by the direct-acting alkylating agent ethyl methanesulphonate was the same regardless of the cellular catalase activity.

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