Oxidative damage to plant DNA in relation to growth conditions

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In this study we investigated the level of 8-oxo-2'-deoxyguanosine (8-oxodG) in DNA of *Cardamine pratensis* plants subjected to different growth conditions trying to answer the question whether factors like light and water accessibility or low temperature may have an impact on the total DNA oxidative damage. The level of this modified nucleoside was determined using HPLC coupled to UV absorbance and electrochemical detection (HPLC-UV-EC). We did not observe any statistically significant differences in 8-oxodG level between DNA of etiolated and light exposed plants as well as between DNA of regularly watered and drought-subjected plants. In contrast, we have shown that chilling (1°C for 28 h) brings about the increase of 8-oxodG level in DNA.

Oxygen-centered free radicals generated during aerobic metabolism and oxidative stress have been implicated in the formation of different kinds of DNA lesions including oxidized DNA bases [1, 2]. Nucleobase damage appears to be important class of lesions since some of them exhibit mutagenic properties and may play an important role in carcinogenesis [3–6]. 8-Oxo-2'-deoxyguanosine (8-oxodG) is the most abundant lesion of this type that causes GC → TA transversion mutations due to mispairing properties of this nucleoside [7, 8]. Availability of the sensitive detection method made 8-oxodG widely accepted sensitive biomarker of oxidative DNA damage.

The last decade was a period of growing interest in oxygen free radicals involvement in plant physiology and pathology. The increased production of free radicals has been

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Abbreviations: APX, ascorbate peroxidase; HPLC/UV-EC, HPLC with UV absorbance and electrochemical detection; 8-oxodG, 8-oxo-2'-deoxyguanosine; ROS, reactive oxygen species; SOD, superoxide dismutase.

implicated in aging, fruit ripening, plant response to wounding and pathogen attack, damage of plants by xenobiotics and stress conditions [9]. Despite many studies devoted to investigation of free radical involvement in damaging different plant cellular components almost nothing is known about free radical modification of DNA. To our knowledge the only study dealing with this subject is the work of Floyd et al. [10] who investigated 8-oxodG content in DNA of chloroplasts treated with ozone.

Water and light accessibility as well as temperature are the factors most commonly associated with plant growth. Interestingly, all these factors have some influence on the increased production of free radicals. Therefore, in this study we decided to use HPLC with UV absorbance detection and electrochemical detection (HPLC/UV-EC) to test whether these factors may influence the level of mutagenic, free radical-derived, modified nucleoside (8-oxodG) in DNA of *Cardamine pratensis*.

MATERIALS AND METHODS

Plant material. Lady's-smock plants (*Cardamine pratensis*) have been chosen as the biological material for investigations. If not indicated otherwise, plant growth conditions (here defined as 'standard conditions') were as follow: seeds were grown in Petri dishes (around 400 plants per dish) covered with lignin layer moistened every 12 h with 50 ml tap water. Air temperature and relative humidity were 24°C and 70%, respectively. Typically, plants were exposed to 40 W/m^2 white light at 10 h/14 h light/dark photoperiod. After completion of experiments 1/3 upper part of plants from particular Petri dishes were cut off and frozen at −80°C. Plant material from one dish was pooled for further nucleic acids

isolation. Each experimental group consisted of three dishes of the plants subjected to particular environmental factor and three dishes of the control plants grown under 'standard conditions'. Control groups for different stress experiments are not intercomparable because of different growth periods.

Isolation of nucleic acids from plant material. Plant tissue (5–10 g) was disrupted in the mortar in the presence of liquid nitrogen and nucleic acids were isolated according to the method of Rogers & Bendich [11] except phenol was not used for protein precipitation and RNA was not digested. The concentration of nucleic acids in the samples was determined spectrophotometrically at 260 nm.

Enzymatic hydrolysis of nucleic acids. Nucleic acids (200–700 μg) were hydrolyzed to nucleosides according to the method described by Kasai et al. [12]. All DNA hydrolyzates were ultrafiltered using Ultrafree-MC filter units (cut off 5 kDa).

Determination of 8-oxo-2'-deoxyguanosine and 2'-deoxyguanosine by HPLC-UV-EC. The HPLC system consisted of 2150 LKB pump, Rheodyne 7125 sample injector (20 μl loop), 250 mm × 4.6 mm Spherisorb ODS-2 column and two detectors working in series: 2140 UV Rapid Spectral Detector (LKB) and Coulochem II 5200A electrochemical detector (ESA Inc.) equipped with 5010 standard analytical cell. Nucleoside preparations were chromatographed isocratically using 30 mM ammonium acetate, pH 5.0/methanol (85:15, v:v). Detection of dG was carried out at 290 nm by the UV detector. 8-OxodG was determined with the coulometric electrochemical detector: guard cell +450 mV, working electrode 1: +130 mV (as the screening electrode), working electrode 2: +400 mV (sensitivity − 50 nA/V). The amount of 8-oxodG in DNA was calculated as the number of 8-oxodG molecules per 10^5 unmodified deoxyguanosine (dG) molecules.

RESULTS

### Notes on the 8-oxo-2'-deoxyguanosine determination

The applied procedure of nucleic acid isolation results in a mixture of RNA and DNA. We decided to omit the enzymatic RNA removal and to maximally shorten the time of sample processing in order to avoid the risk of artifactual 8-oxodG formation in the isolated DNA. The chromatographic method presented here ensures a satisfactory resolution of dG from other nucleosides present in RNA/DNA hydrolyzates (Fig. 1B). Homogeneity of the dG peak was supported by peak spectral analysis (data not shown). Applied conditions of electrochemical detection also secure the selectivity and sensitivity of 8-oxodG determination (Fig. 1A). The presented method is suitable even for nucleic acid preparations with great predominance of RNA (Fig. 1).

Light accessibility and 8-oxodG level in DNA

The seeds were sown in six Petri dishes. Three of them were immediately placed under absolute dark and the remaining plants were grown under standard conditions defined in the Materials and Methods section. With the exception of light exposure both groups of plants were subjected to the same growth conditions. After 5 days of growth all plants were harvested and 8-oxodG in DNA was determined. The 8-oxodG level in DNA of etiolated plants was 8.10 ($\pm$1.93 S.D.) versus 7.26 ($\pm$2.1 S.D.) in control plants (Fig. 2).

Water deficit stress (drought) and DNA 8-oxodG level

Seeds were sown in six Petri dishes and grown under standard conditions for 3 days. Starting from the 4th day of the experiment water delivery to plants growing on 3 dishes was discontinued. These plants tended to
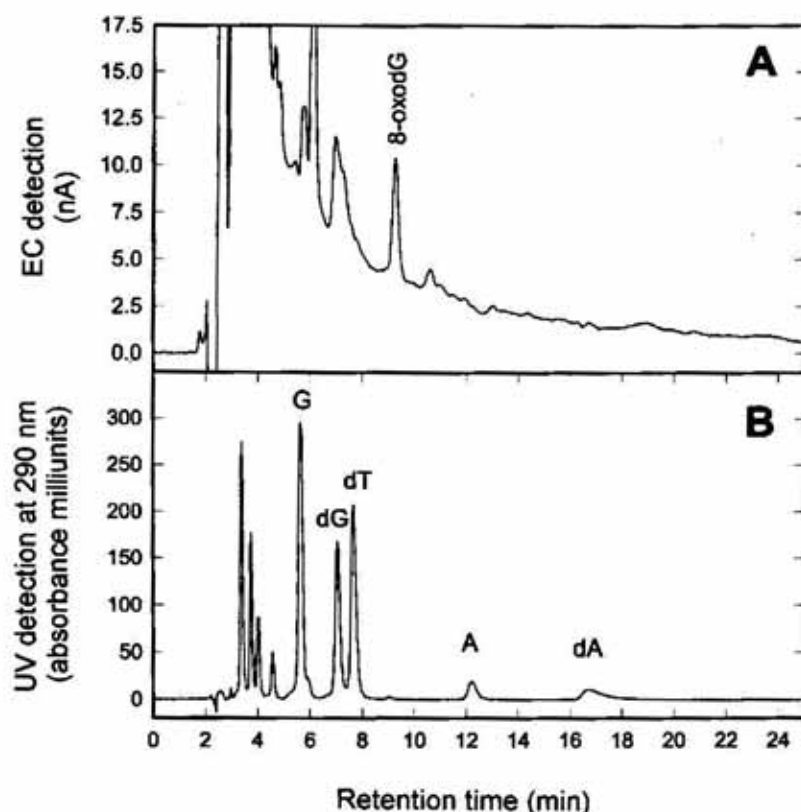
Figure 1. Reversed phase HPLC separation of nucleosides resulting from the hydrolysis of plant nucleic acid preparation.

A. Electrochemical detector response. B. UV-Absorbance detector response. The peaks are marked as follows: 8-oxodG, 8-oxo-2'-deoxyguanosine; G, guanosine; dG, deoxyguanosine; dT, deoxythymidine; A, adenosine; dA, deoxyadenosine.
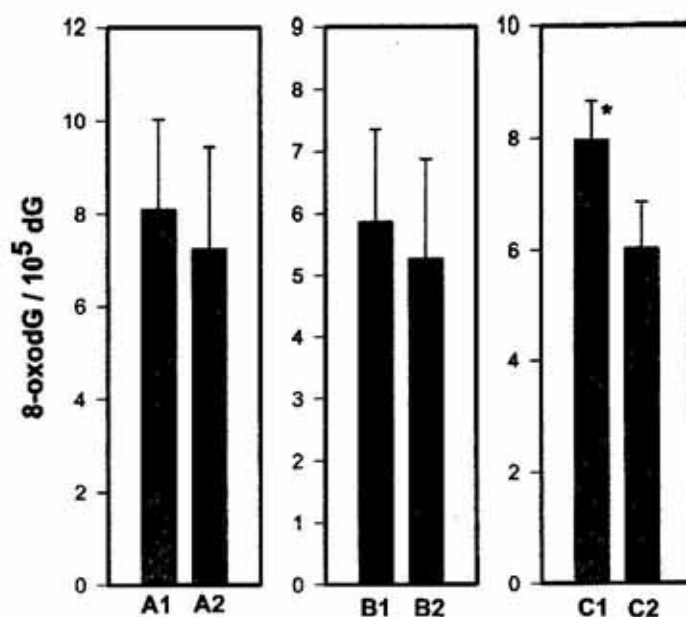
Figure 2. The level of 8-oxo-2'-deoxyguanosine in DNA of *Cardamine pratensis* plants subjected to different growth conditions in comparison to respective control plants grown in 'standard conditions' (see Materials and Methods).

The plants grown in absolute darkness (A1) in comparison to plants grown under illumination (40 W/m^2, 12 h/12 h dark/light photoperiod) (A2). Drought subjected plants (4 days of water shortage) (B1) in comparison to control plants watered every 12 h (B2). Plants subjected to low temperature (1°C) for 28 h (C1) in comparison to control plants grown at 24°C (C2). Error bars represent standard deviation (n = 6; each of 3 separate nucleic acid preparations chromatographed in duplicate). *Statistically different from control value at $P < 0.05$ (Student's *t*-test).

loose the turgor in the 5th day and appeared to be intensely dehydrated in the 7th day, the last day of the experiment. The control and water stressed plants were harvested for nucleic acid isolation and 8-oxodG determination. The mean level of 8-oxodG in DNA of drought-subjected plants was 5.87 (±1.60 S.D.) molecules of 8-oxodG per 10^5 molecules of dG *versus* 5.27 (±1.49 S.D.) for plants grown under standard conditions (Fig. 2).

Low temperature (chilling) and DNA 8-oxodG level

In this experiment the influence of low temperature on DNA oxidative damage of *Cardamine pratensis* was investigated. The plants were grown on six Petri dishes in standard conditions for 4 days. Thereafter, three dishes were transferred to glass cooling chamber (1°C) for 28 h. Except of the temperature, all other growth conditions were the

same in both control and chilled plants. After the chilling period the plants of both groups were harvested and stored for nucleic acid isolation. The mean level of 8-oxodG in DNA of chilled plants was 7.96 (±0.77 S.D.) molecules/10^5 dG molecules and 6.03 (±0.82 S.D.) for corresponding control plants (Fig. 2).

DISCUSSION

In this study we have measured the level of 8-oxo-2'-deoxyguanosine in total DNA. Considering that nuclear DNA constitutes a vast majority of isolated total DNA of the cell, investigated 8-oxodG level reflects mainly a status of nuclear DNA oxidation.

Light is one of the best recognized oxidative stress factors for plant cells. However, our results do not show any significant differences in DNA oxidation between green and etiolated plants. It is well known that chloroplasts

contain an effective protection system against oxidative stress consisting of enzymes and antioxidants. All known forms of superoxide dismutase (SOD), namely Cu,Zn-SOD, Mn-SOD, Fe-SOD are present in chloroplasts of different plant species. Hydrogen peroxide formed by superoxide anion dismutation is sufficiently decomposed in chloroplasts by ascorbate peroxidases (APX) which co-operate with other enzymes of the so called ascorbate-glutathione cycle: monodehydroascorbate reductase (NAD(P)H dependent), dehydroascorbate reductase (glutathione dependent) and glutathione reductase (NADPH dependent) [13]. Mishra *et al.* [14] demonstrated important increases in SOD and APX activities in wheat seedlings upon exposure to strong light. Besides ascorbate and glutathione which are antioxidants, the enzymatic defense of chloroplasts is reinforced by other antioxidants like α-tocopherol and carotenoids, well known as oxygen free radical scavengers and singlet oxygen quenchers. All these facts indicate that the plant cell is well equipped to face the excessive reactive oxygen species (ROS) formation during photosynthetic activity and that antioxidant systems can effectively preserve DNA from effects of light operation.

Recent publications regarding water stress in plants provide the evidence that drought may be a cause of excessive ROS formation and can affect antioxidative systems of the plant cell, but the data are inconsistent. Moran *et al.* [15] subjected pea plants to drought and found a pronounced decrease in the activities of catalase, dehydroascorbate reductase, glutathione reductase and a slight decrease in monodehydroascorbate reductase and ascorbate peroxidase activities. The activity of superoxide dismutase was elevated. The levels of antioxidants were also influenced (decline of reduced glutathione concentration, increase in α-tocopherol level). On the contrary, Mittler and Zilinskas [16] reported a rise of activities of cytosolic ascorbate peroxidase, cytosolic Cu,Zn-superoxide dismu-

tase, chloroplastic Cu,Zn-superoxide dismutase and catalase in pea plants exposed to drought. We did not observe any statistically significant increase in 8-oxodG level in DNA of drought-stressed plants. We assumed that the lack of any changes is connected with an efficient functioning of the antioxidative protection of *Cardamine pratensis* DNA. It is also possible that the drying rate of so small plants was too rapid to result in the manifestation of all stress symptoms.

In contrast to the above described experiments, statistically significant changes of oxidative DNA damage in plants subjected to the low temperature stress were observed. We found higher 8-oxodG level in DNA of plants stressed at 1°C for 28 h as compared to the control plants grown under standard conditions. Chilling is generally well recognized as a process inducing ROS formation, especially in chilling-sensitive plants [17]. Low temperature damages plant cell mainly through mitochondria dysfunction resulting from overproduction of hydrogen peroxide which is a consequence of depressing the respiratory activity, the cytochrome pathway of electron transport and F_1-ATPase activity [18]. Hydrogen peroxide can permeate cell membranes. It could potentially enter the nucleus and form hydroxyl radicals by the reaction with transition metal ions associated with negatively charged DNA. Experiments carried out on different rice cultivars indicate that the tolerance to chilling injury is closely linked to the cold stability of catalase (CAT) and APX [19]. We hypothesize that chilling applied in our experiment resulted in oxidative stress which overwhelmed the antioxidative defense of *Cardamine pratensis* plants and caused increased level of 8-oxodG in DNA.

To our knowledge it is the first work regarding influence of light, decreased water content and low temperature on the level of 8-oxodG in DNA of the plant and the second as far as 8-oxodG determination in plant DNA is concerned. Floyd *et al.* [10] investigated the 8-oxodG content in DNA of pea and bean plants

treated with ozone. They did not observe any significant changes of 8-oxodG content in total DNA even after treatment of the plants with acute doses of ozone. A significant increase in 8-oxodG level was restricted to chloroplast DNA which constitutes only a small fraction of total DNA. This suggests that many stress factors of moderate intensity may result in remarkable oxidative damage only to those cell structures which are subjected to their action, like chloroplasts, mitochondria, peroxysomes and glyoxysomes. They are separated from the nucleus and possess their own antioxidative systems. Thus, it should be considered that the local increase of ROS formation in these compartments does not necessarily influence nuclear DNA. Further research concerning oxidative damage to chloroplastic and mitochondrial DNA in plants subjected to different stress factors may confirm this hypothesis.

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