

Oxidative stress as DNA damage in different transgenic tobacco plants

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Abstract

One of the important mechanisms by which plant cells are damaged during adverse environmental conditions is the excessive production of reactive oxygen species (ROS). Major enzymatic ROS scavenging mechanisms of plants include superoxide dismutase (SOD) and catalase (CAT). The aim of this work was to assess leaf cell sensitivity to oxidative stress by measuring DNA damage (Comet assay) in *Nicotiana tabacum* wild type plants and transgenic lines with deregulated levels of CAT and MnSOD; DNA migration, as DNA damage measure, and nuclei with completely disintegrated head region (hedgehogs), as toxicity parameter, were recorded. A general time dependent increase of DNA migration values was observed within 3 h after hydrogen peroxide treatment, followed by an increase of hedgehog percentage. We showed that hydrogen peroxide treatments trigger different degrees of both DNA migration and hedgehog induction in the transgenic lines. There was only weak difference between the transgenic lines with increased MnSOD levels and wild type plants; but within CAT and MnSOD deficient plants, both levels of DNA migration and hedgehogs were significantly altered.

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1. Introduction

Reactive oxygen species (ROS) are very transient species with high chemical reactivity that leads to lipid peroxidation and oxidation of DNA and protein [1]. Increased lipid peroxidation and decreased antioxidant protection frequently occurs: epoxides may spontaneously react with nucleophilic centres in the cell and thereby covalently bind to DNA, RNA and protein [2]. Single strand breaks, much less abundant double strand breaks, alkali labile sites and various species of oxidized purines and pyrimidines are evidenced in cells exposed to oxidative insult [3–6].

Every year environmental stress causes considerable loss in crop quality and productivity. The excessive production of ROS, such as hydroxyl radicals, superoxide anion and hydrogen peroxide, is one of the main mechanisms by which plants are damaged during adverse environmental conditions, even if they have evolved non-enzymic and enzymic protection mechanisms that efficiently scavenge them.

Such oxidative stress has been shown to occur in plants exposed to high and low temperatures, particularly in combina-

tion with high light intensities, drought, exposure to air pollutants (e.g. ozone or sulphur dioxide), ultraviolet light and herbicides [7,8]. Genes encoding enzymes of known antioxidant pathways have been characterized in some detail and cloned. Some evidence now suggests that the manipulation of antioxidant capacities is a valuable way of obtaining stress tolerant plants [9]. However, such attempts are still limited and there is no information available on the DNA damage response in plant mutants exhibiting enhanced sensitivity or tolerance to oxidative stress conditions.

Major ROS scavenging mechanisms of plants include superoxide dismutase (SOD) and catalase (CAT) [8]. The balance between SOD and CAT activities in cells is crucial in determining the steady state level of superoxide radicals and hydrogen peroxide [10,11].

SODs destroy the free radical superoxide by converting it to peroxide that can in turn be destroyed by CAT [10]. The SOD isoforms in tobacco are all nuclear encoded, but the gene products are located in different subcellular compartments. MnSOD is present in the mitochondria, FeSOD in the chloroplasts and Cu/ZnSOD both in the cytosol and chloroplasts [11].

In plants there are three main CAT isoforms: CAT1, CAT2 and CAT3. *Nicotiana plumbaginifolia* contains three expressed CAT genes CAT1, CAT2 and CAT3. CAT1 is the most abundant

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isoform in leaves while CAT3 is mainly found in seeds. From their expression, it is inferred that CAT1 is primarily involved in removing H_2O_2 produced during photorespiration in leaf peroxisomes, whereas CAT3 scavenges H_2O_2 formed in glyoxisomes during fatty acid degradation. In total leaf extracts, CAT2 is much less abundant than CAT1, which represents ~80% of leaf CAT activity [12].

Molecular biology and biotechnology offer a new horizon for understanding and altering the properties of all organisms, including plants, on which humans depend; recombinant DNA technology certainly provides opportunities to develop new products in agriculture and the agro-food area.

In this study, we compared the sensitivity to oxidative stress in leaf cells of *Nicotiana tabacum* wt line SR1 and transgenic derivative lines, CAT1AS (CAT1), SOD+ and SOD–. CAT1 is an antisense line for the *N. plumbaginifolia* CAT isoform 1 and is reported to contain approximately 10% of normal CAT activity with respect to SR1 [12]. SOD+ is an overexpressing line for the *N. plumbaginifolia* mitochondrial MnSOD isoform, i.e. enzyme activity about twice as high as in untransformed plant [11,13]. SOD– transgenic lines have drastically reduced MnSOD protein and activity levels resulting in increased oxidative stress sensitivity. Hydrogen peroxide induced DNA damage was detected in leaf cells by the single cell gel electrophoresis (SCGE) or Comet assay, previously satisfactorily adopted in our laboratory for the assessment of oxidant insults in *N. tabacum* [14]. Furthermore, the high sensitivity of roots to the DNA damaging activity of hydrogen peroxide and cadmium has previously been shown in transgenic tobacco line CAT1 by the Comet assay [15,16].

2. Materials and methods

2.1. Chemicals, media and seeds

Ethidium bromide was obtained from Fluka; Tris from ICN Biochemicals; reagents for electrophoresis, normal and low melting point agarose and general laboratory chemicals from Sigma; plant growth medium (MS; cat. #M0222) from Duchefa. Dr. Van Breusegem (Ghent University, Belgium) has provided *N. tabacum* seeds (SR1 plant and CAT1, SOD+ and SOD– derivative lines).

2.2. Plant growth conditions

Tobacco seeds were sterilized by immersion (20 min) in a sterilizing solution (950 μl distilled water, 50 μl 5.25% sodium hypochlorite, 1 μl 0.5% Tween 20). The sterilizing solution was discarded and the seeds washed five times in sterile distilled water. The seeds were placed in a sterile Petri dish that contained 30 ml of sterile solid growth medium (MS, 2% sucrose, 0.8% agar). Both germination and growth were performed in a plant growth chamber at 25 °C with an 8/16 h dark/light photoperiod. Two weeks after germination, the seedlings were placed in plastic pots ($\varnothing$ = 10 cm) containing vermiculite and irrigated with diluted (1/10) MS medium.

2.3. Catalase assay

CAT activity was determined by measuring the initial decomposition of H_2O_2 at 240 nm (extinction coefficient $0.036 \text{ mM}^{-1} \text{ cm}^{-1}$) (Havir and McHale, 1987). Spectrophotometric analyses were conducted on a spectrophotometer (Cary 219, Varian). Leaves of tobacco, stored at –70 °C were homogenized in cold extraction buffer (100 mM phosphate buffer, 1 mM DL-dithiothreitol, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.3) at concentration of 1 g of leaves (fresh weight) to 5 ml buffer. Supernatant fluid was recovered by centrifugation ($14,000 \times g$, 2 °C, 10 min). The reaction mixture consisted of 0.1 ml 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. CAT activity was expressed in units (U), mg^{-1} fresh weight ($1 \text{ U} = \mu\text{mol min}^{-1}$ substrate degradation).

2.4. Treatment conditions

The seventh leaf of 70-day-old plants (from germination) was harvested and quickly cut with a fresh razor blade on a hard surface to obtain five to six leaf explants (surface about 5 cm^2). Each explant was dipped with the abaxial side facing upwards in a glass tube containing 10 ml of a known concentration (0, 20, 50 or 80 mM) of H_2O_2 in distilled water. A microscope slide was inserted into the tube in order to keep the explant submerged at the bottom. The tubes were transferred into a glass desiccator and maintained in a vacuum (–80 mBar, 1 min) to allow a uniform infiltration of leaf tissue. The samples were maintained in a plant growth chamber at 25 °C for 2, 3 or 4 h in the dark. Finally, each explant was rinsed in water, lightly blotted with paper and placed on ice prior to the isolation of nuclei.

2.5. Isolation of nuclei

The isolation of nuclei was performed according to Gichner and Plewa [17]. The leaf explants were placed in a 60 mm Petri dish containing 300 μl of cold modified Sørensen buffer (50 mM sodium phosphate pH 6.8, 0.1 mM EDTA, 0.5% dimethyl sulfoxide (DMSO)) and kept on ice. Using a fresh razor blade, each leaf explant was sliced so as to form a fringe across most of the tissue. The Petri plate was tilted so that the buffer collected on the side and the leaf fringe was dipped and gently agitated in it. The plate was kept tilted in the ice so that the nuclei would collect in the buffer. All operations were conducted under a yellow light.

2.6. Single cell gel electrophoresis

Low melting point agarose (LMA, 50 μl 1% in PBS) was added onto each microscope slide, previously coated with 1% normal melting point agarose in PBS and placed on a warm surface (37 °C). Nuclear suspension (50 μl) was then added using a cut plastic pipet tip and gently mixed to LMA by repeated pipetting. A cover slip was placed on the mixture and the slide cooled in a steel tray on ice for a minimum of 5 min. Finally,

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