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The effects of gamma irradiation on growth and expression of genes encoding DNA repair-related proteins in Lombardy poplar (*Populus nigra* var. *italica*)

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ABSTRACT

In this study, to elucidate the mechanisms of adaptation and tolerance to ionizing radiation in woody plants, we investigated the various biological effects of γ-rays on the Lombardy poplar (*Populus nigra* L. var. *italica* Du Roi). We detected abnormal leaf shape and color, fusion, distorted venation, shortened internode, fasciation and increased axillary shoots in γ-irradiated poplar plants. Acute γ-irradiation with a dose of 100 Gy greatly reduced the height, stem diameter and biomass of poplar plantlets. After receiving doses of 200 and 300 Gy, all the plantlets stopped growing, and then most of them withered after 4–10 weeks of γ-irradiation. Comet assays showed that nuclear DNA in suspension-cultured poplar cells had been damaged by γ-rays. To determine whether DNA repair-related proteins are involved in the response to γ-rays in Lombardy poplars, we cloned the *PnRAD51*, *PnLIG4*, *PnKU70*, *PnXRCC4*, *PnPCNA* and *PnOGG1* cDNAs and investigated their mRNA expression. The *PnRAD51*, *PnLIG4*, *PnKU70*, *PnXRCC4* and *PnPCNA* mRNAs were increased by γ-rays, but the *PnOGG1* mRNA was decreased. Moreover, the expression of *PnLIG4*, *PnKU70* and *PnRAD51* was also up-regulated by Zeocin known as a DNA cleavage agent. These observations suggest that the morphogenesis, growth and protective gene expression in Lombardy poplars are severely affected by the DNA damage and unknown cellular events caused by γ-irradiation.

1. Introduction

Ionizing radiation has been well known to deleteriously affect the growth and reproduction of herbaceous and woody plants. Early studies on the biological effects of ionizing radiation enabled, the radiosensitivity of various herbaceous and woody plants to gamma (γ) rays to be determined in detail (Capella and Conger, 1967; Sparrow et al., 1968, 1970; Sparrow and Sparrow, 1965). These studies revealed that woody gymnosperms are generally more sensitive to ionizing radiation than woody angiosperms, and that the radiosensitivity of plant species is relevant to each interphase chromosome volume. Following the accident at the Chernobyl atomic power plant in 1986, many important studies on the harmful effects of ionizing radiation and fallout on forest trees were performed and reported (Arkhipov et al., 1994; Kal'chenko and Fedotov, 2001; Kovalchuk et al., 2003; Tulik and Rusin, 2005). Conversely, ionizing radiation has also been used as a tool for plant breeding. A total of 2252 mutant varieties including woody plant species were recorded in the FAO/IAEA Mutant Varieties Database by the end of 2000, and in fact 1411 mutant varieties of this total were obtained with the use of ionizing radiation as the mutagen (Maluszynski et al., 2000).

Ionizing radiation is considered as an environmental stressor as well as light, temperature or water. In fact, X- or γ-rays were reported as causing DNA damage in plants such as *Vicia faba* L. (Koppen and Angelis, 1998) and *Nicotiana tabacum* L. (Gichner et al., 2000). Ionizing radiation not only arises from human actions but also exists as natural background radiation including cosmic and terrestrial radiation. Almost all terrestrial plants continue to be exposed to natural background radiation during their growing and dormant periods. Although natural background radiation is usually negligible, it is sufficiently capable of ionizing water and biological molecules such as DNA and proteins in cells. If the free radicals and abnormal molecules generated by ionization increase and accumulate in such cells, the cells may be damaged and die. In particular, perennial woody plants are probably subjected to natural background radiation for an extended period due to their longevity and are thus likely to have developed the mechanisms of adaptation and tolerance to ionizing radiation for their survival.

As one of the mechanisms of radiation resistance, versatile DNA repair systems are mentioned. A great variety of DNA repair-related proteins for detection and elimination of DNA damage and for DNA

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synthesis are supposed to play an important role, and thus contribute to maintaining genome stability. Activation of the genes that encode each DNA repair-related protein is considered a key event in the mechanism for tolerating ionizing radiation. The genes encoding the DNA repair-related proteins were isolated from plants such as *Arabidopsis thaliana* (L.) Heynh. (Bleuyard et al., 2006), rice (Kimura and Sakaguchi, 2006) and moss (Ayora et al., 2002). The knock-out of DNA repair-related genes partially reduced the tolerance of the knock-out plants to γ-irradiation (Osakabe et al., 2005; Riha et al., 2002). However, to the best of our knowledge, DNA repair-related proteins in woody plants have not been studied in detail.

In the present study, to elucidate the mechanisms of adaptation and tolerance to ionizing radiation in woody plants, we acutely exposed the Lombardy poplar (*Populus nigra* L. var. *italica* Du Roi) to γ-rays. Species in the *Populus* genus have been studied as good models for woody plant biology, due to their rapid growth, ease of clonal propagation, the use of in vitro cultured-plants and cells, and abundant molecular biological information such as ESTs and genome sequences (Tuskan et al., 2006). We have also gained information on genes in various stress responses and the flowering of the Lombardy poplar to date (Igasaki et al., 2008; Nanjo et al., 2007; Nishiguchi et al., 2002). However, the radiation effects on *Populus* species have not been sufficiently investigated (Scandalios, 1964; Stettler and Guries, 1976). Here, we report that exposure of the Lombardy poplar to acute γ-irradiation at various doses causes morphological change, growth delay, withering, nuclear DNA breaks, and that γ-rays or a DNA cleavage agent affect the gene expression of DNA repair-related proteins, the cDNAs of which were newly isolated from the Lombardy poplar.

2. Materials and methods

2.1. Plant materials

Young branches of the Lombardy poplar, approximately 10 cm in length, were cut off and rooted in moist vermiculite. The rooted branches were then grown in a phytotron at 25 °C and with 70% relative humidity under metal halide lamps (500 μmol m^{-2} s^{-1} of photosynthetically active radiation, 16:8 h light:dark photoperiod).

2.2. Gamma irradiation

Poplar plantlets approximately 15 cm in height (6 weeks after cutting) in a Wagner pot were used for the γ-irradiation experiments. The plantlets in the pot were concomitantly exposed to γ-rays at doses of 10, 20, 50, 100, 200 and 300 Gy respectively (dose rates ranged from 0.5 to 15 Gy h^{-1}) for 20 h in the cobalt-60 (^{60}Co) gamma room at the Institute of Radiation Breeding (Hitachiohmiya, Japan). After γ-exposure, the irradiated plants were returned to the phytotron. For RNA isolation, shoots were harvested from each plantlet at 1, 6 and 24 h after the end of γ-irradiation and frozen in liquid nitrogen. The other irradiated plants were allowed to continue growing in the phytotron to observe and measure their growth. The γ-irradiation experiments were repeated twice.

2.3. Measurement of growth

The plant height and stem diameter at the ground level of the control (non-irradiated) and γ-irradiated poplar plants were measured immediately and at weekly intervals for 10 weeks after γ-irradiation. The roots, stems and leaves were also harvested to measure the dry mass at 10 weeks after γ-irradiation or when the whole plant had turned brown. Each sample was dried at 60 °C to a constant weight and then measured.

2.4. Treatment of suspension-cultured poplar cells with γ-rays and a DNA cleavage agent

Suspension-cultured poplar cells were derived from sterile callus (Nishiguchi et al., 2002) of the Lombardy poplar in a liquid medium containing 20 mM Mes-KOH (pH 5.8), Murashige and Skoog medium (Murashige and Skoog, 1962), 3% (w/v) sucrose and 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid. The suspension-cultured cells were cultivated in 100-ml Erlenmeyer flasks on orbital shakers (120 rpm) in darkness. One milliliter of the cell suspension was subcultured every 14 days in 25 ml of the same fresh medium. For γ-irradiation experiments, seven days after subculturing, the cells were transferred into a thin-walled plastic flask and exposed to γ-rays for 20 h. The irradiated cells were collected 1 h after the end of γ-irradiation and cooled on ice for the Comet assay. To treat the cells with a DNA cleavage agent, Zeocin (Invitrogen, Carlsbad, CA) was added to the medium at a concentrations of 10, 50 and 250 μg ml^{-1} seven days after subculturing. After cultivation for 1, 6 and 24 h with Zeocin, the cells were collected and either cooled on ice for the Comet assay or frozen in liquid nitrogen for RNA preparation.

2.5. Comet assay

Nuclei were isolated from the suspension-cultured poplar cells using a previously reported method with modifications (Ptáček et al., 2001). The cells were harvested using cell strainers with 40-μm pores (BD Falcon, Bedford, MA) and sliced with a razor blade in a 50 mM sodium phosphate buffer (pH 6.8) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% (v/v) dimethyl sulfoxide on a Petri dish kept on ice. The released nuclei were separated from the cell debris using the cell strainer. A Comet assay was performed using the CometAssay Kit (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions. Following alkaline electrophoresis, the nuclei were stained with SYBR Green I and observed by fluorescence microscopy. Images of the nuclear DNA were taken by digital camera and analyzed using the CASP program (Comet Assay Software Project, http://casp.sourceforge.net/) for semi-quantification of the DNA damage based on the amount of DNA in the tail.

2.6. RNA preparation

Total RNA was prepared from each frozen sample as described by Shinohara and Murakami (Shinohara and Murakami, 1996) with the following modifications. Samples were ground in liquid nitrogen, and subsequently mixed with 10 volumes (v/w) of a lysis solution of 100 mM Tris–HCl (pH 9.5), 20 mM EDTA, 1.4 M NaCl, 2% (w/v) hexadecyltrimethylammonium bromide and 2% (v/v) 2-mercaptoethanol. This mixture was then heated at 65 °C for 10 min. After extraction with chloroform:isoamyl alcohol (24:1) and centrifugation, the supernatant was mixed with one quarter the volume of ice-cold 10 M LiCl and stored at −20 °C for 2 h to overnight. The precipitated RNA was recovered by centrifugation and dissolved in an SV RNA lysis buffer of the SV Total RNA Isolation System (Promega, Madison, WI). The RNA was further purified to remove contaminated DNA and polysaccharides according to the manufacturer's instructions. Quantification of RNA was performed using the Quant-iT RiboGreen RNA Assay Kit (Invitrogen).

2.7. cDNA cloning of DNA repair-related genes

PnRAD51, *PnKU70* and *PnLIG4* cDNAs were cloned by reverse transcription PCR using the total RNA from apical buds of the Lombardy poplar. Reverse transcription was performed using Transcriptor Reverse Transcriptase (Roche Applied Science, Penzberg, Germany) and oligo$(dT)_{18}$ primers. We designed specific primer sets