Kinetic Analysis of Double-Strand Break Rejoining Reveals the DNA Reparability of γ-Irradiated Tobacco Cultured Cells

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DSB rejoining kinetics/Tobacco BY-2 cells/DNA fragment-size analysis.

The rejoining efficiency of double-strand breaks (DSBs) was quantified by a DNA fragment-size analysis in tobacco protoplasts and CHO-K1 cells following γ-ray irradiation in order to compare DNA reparability of higher plants with mammals. Results showed that the DSB rejoining efficiency of tobacco protoplasts is dependent on the temperature of post-irradiation cultivation and that it reaches a maximum at 27°C, which represents the most suitable temperature for protoplast cultivation. The DSB rejoining kinetics of tobacco protoplasts were well represented by a biphasic-exponential equation: half of initial-induced DSBs were rejoined for 1 h and the others were almost rejoined within 4 h. We found that the DSB rejoining kinetics of tobacco protoplasts at 27°C are the same as those of CHO-K1 cells at 37°C. These findings indicate that the DSB rejoining efficiency of tobacco protoplasts and CHO-K1 cells are comparable at their respective cell cultivation temperatures, suggesting that DSB rejoining efficiency is little responsible for the higher radiation-tolerance of tobacco protoplasts.

INTRODUCTION

Mammals are sensitive to ionizing radiation, which is reflected by the fact that 50% lethal dose (LD50) for acute irradiation does not exceed 10 Gy. In contrast, many types of higher plants possess greater tolerance to radiation at the individual level (LD50 ≤ 1,000 Gy). Although this difference in the radiation sensitivity between mammals and plants is an issue of long standing, the mechanism underlying the radiation tolerance of plants are poorly understood.

In the previous study, we reported that the 10% survival dose of tobacco protoplasts was 47.2 Gy for γ rays, which is almost 10 times higher than that observed for mammalian cultured cells. As a first step in elucidating the radiation tolerance mechanism in tobacco cells, we investigated the initial yields of DNA double-strand breaks (DSBs) in γ-irradiated tobacco protoplasts and Chinese hamster CHO-K1 cells by the fraction activity released (FAR) assay. The results demonstrated that the initial DSB yields (bp DNA−1 Gy−1) were 3 times less in tobacco protoplasts than in CHO-K1 cells, which certainly contributes to a part of the radiation tolerance. Additionally, the results also indicated that the number of DSBs (cell−1 mean lethal dose−1) was 5 times more in tobacco protoplasts than in CHO-K1 cells, providing the indirect evidence that tobacco cells possess an efficient mechanism against DSBs to survive. The simplest hypothesis that accounts for this observation is that the DSB repair system of tobacco cells including DSB rejoining efficiency, fidelity, DNA damage checkpoint and the others is superior to that of mammalian cells. However, the whole body of DSB repair machinery is not yet understood in plant cells.

A great amount of evidences has been accumulated on the DSB repair machinery of mammals. In addition, the DSB rejoining kinetics of mammalian cells have been quantified using the pulsed-field gel electrophoresis (PFGE) technology. Recent advances in understanding of the DSB repair machinery of plants have also revealed that a model plant Arabidopsis thaliana repairs DSBs via non-homologous end joining and homologous recombination pathways as mammalian do. However, the DSB rejoining kinetics of plant cells have never been quantified.

Therefore, in the present study, we quantified the DSB rejoining kinetics in γ-irradiated tobacco protoplasts and...
CHO-K1 cells by a DNA fragment-size analysis and compared them each other.

**MATERIALS AND METHODS**

**Preparation of cell samples**
BY-2 cell line originated from tobacco (*Nicotiana tabacum* L.) seedlings was maintained in modified Linsmaier & Skoog medium and subcultured weekly. Protoplasts (single cells without cell wall) were prepared on day 6 post-subculture using the protoplast isolation buffer and embedded in 0.75% agarose GB (Takara Bio Inc., Otsu, Japan) plugs, which were then transferred to plastic tubes filled with protoplast culture medium. Chinese hamster CHO-K1 cells were cultured in Ham’s F12 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS and 0.1 mg/ml kanamycin.

**Irradiation and post-irradiation cultivation**
Tobacco protoplasts embedded in agarose plugs and confluent cell monolayer of CHO-K1 cells were irradiated with $^{60}$Co $\gamma$-rays. Dose rates ranged from 6.6 to 20 Gy/ min, with a constant irradiation time of 30 min. Cell samples were kept on ice during irradiation to inhibit any repair processes. Following irradiation, the conditioned media were replaced with fresh ones and the cell samples were incubated at 27°C under normal atmospheric conditions (tobacco protoplasts) or at 37°C in a humidified atmosphere of 5% CO$_2$ / 95% air (CHO-K1 cells) unless otherwise noted.

**PFGE**
Following post-irradiation cultivation, CHO-K1 cells were collected from culture dishes using 0.05% trypsin-EDTA and then embedded in agarose plugs. The agarose plugs containing tobacco protoplasts or CHO-K1 cells were incubated in proteinase buffer for cell lysis. Following incubation, agarose plugs were washed 4 times in 0.5 × TBE buffer for 30 min each and then embedded in 1% PFC agarose gel (Bio-Rad Laboratories, Hercules, CA) for electrophoresis. PFGE was performed using a CHEF DR-III system (Bio-Rad) under conditions suitable for the separation of DNA fragments ranging from 220 kbp to 1.6 Mbp. *Saccharomyces cerevisiae* chromosomal DNA (Takara Bio) was used as a size standard. Following PFGE, the gel was dried and rehydrated for improving the quantitative capability of fluorescence signal detection and then stained with 1/10,000-diluted SYBR Green I solution (Takara Bio) for 3 h at 50°C. The stained gel was set on a 302-nm UV transilluminator, and the electrophoresis patterns of DNA fragments were recorded using an image analyzer (FluorChem system, Alpha Innotech, San Leandro, CA).

**DNA fragment-size analysis**
The detailed protocol of DNA fragment-size analysis was previously described. In brief, the recorded image was segmented into 7 parts according to the DNA size standard: 0 (end of a gel)-360 kbp, 360–550 kbp, 550–680 kbp, 680–820 kbp, 820–950 kbp, 950 kbp–1.09 Mbp and 1.09–1.6 Mbp. The fluorescence intensity was measured in each segment and corrected by subtracting the background noise, and the corrected intensity was then divided by the total intensity of the lane to estimate the fraction of DNA content in each segment. The number of DNA fragments was obtained by dividing the fraction by the mean size of the segment, and total number of DSBs was calculated by summing up the fragments shorter than 1.6 Mbp. The fragmentation frequency was obtained by dividing the number of fragments by the range of the segment. The total number of DSBs and fragmentation frequency were corrected by subtracting the background DSB level in sham-irradiated cells. For comparing the experimentally-determined fragmentation frequency with a theoretical model, the following formulas were used: frequency of $k$ bp fragments = $(F_{<k+1} - F_{<k})/k$, $F_{<k} = 1 - \exp(-\rho k)$, $\rho k = 1 + k ln(1 + k/\rho)$, $\rho = 0.05$ was considered to be significant.

**RESULTS AND DISCUSSION**
There was no information of the effect of cell cultivation temperature on DSB rejoining. Therefore, we firstly investigated the rejoining efficiency of tobacco protoplasts at several temperatures (4, 15 and 37°C) along with the temperature for cell cultivation (27°C). Induced DSBs were decreased to 62 ± 1, 29 ± 7, 5 ± 2 and 19 ± 2% for 4 h post-irradiation cultivation at 4, 15, 27 and 37°C, respectively. The rejoining efficiency was dependent on the temperature during post-irradiation cultivation and became the maximum at 27°C. Thus, the rejoining efficiency of tobacco protoplasts was estimated at 27°C in the following experiments. A typical image of DNA separation is shown in Fig. 1. Genomic DNA of sham-irradiated tobacco protoplasts was rarely migrated into the agarose gel. In contrast, a major fraction of DNA was migrated at immediately after irradiation and then the fraction was decreased with increasing post-irradiation cultivation time at 27°C.

In Fig. 2, the remaining DSBs are shown as a function of post-irradiation cultivation time. The doses were chosen to compare the DSB rejoining efficiencies between different types of cell lines: irradiation at 300 and 200 Gy yields an equal number of DSBs in tobacco protoplasts and CHO-K1 cells.
of remaining DSBs between tobacco protoplasts and CHO-K1 cells at 1, 2, 4 and 24 h post-irradiation. The rejoining kinetics of tobacco protoplasts were well represented by a biphasic-exponential curve including fast- and slow-phase components: 

\[ F(t) = F_{\text{fast}} \times \exp\left(-t/\tau_{\text{fast}}\right) + F_{\text{slow}} \times \exp\left(-t/\tau_{\text{slow}}\right), \]

in which \( F(t) \) represents the fraction of remaining DSBs at time \( t \), \( \tau_{\text{fast}} \) and \( \tau_{\text{slow}} \) are the time constants, and \( F_{\text{fast}} \) and \( F_{\text{slow}} \) are the fraction of DSBs that were rejoined at fast and slow phases, respectively. In tobacco protoplasts, \( F_{\text{fast}} \) and \( F_{\text{slow}} \) were 0.43 ± 0.07 and 0.57 ± 0.06, respectively and the half times of DSBs at fast and slow phases were 5.4 ± 2.1 min.

Fig. 1. A typical image of the migration pattern of tobacco genome DNA. Each lane represents sham-irradiated (lane 1), irradiated with 300 Gy and post-irradiation cultured for 0, 4 and 24 h (lanes 2–4), and a size standard (lane M). Agarose plugs are seen at the top of the image.

Fig. 2. DSB rejoining kinetics. Tobacco protoplasts (●) and CHO-K1 cells (○) were irradiated with 300 and 200 Gy, respectively and were post-irradiation cultured at 27 and 37°C, respectively. The percentage of remaining DSBs is shown as a function of post-irradiation cultivation time. Each data represents a mean ± SE of 2 to 6 independent experiments. Biphasic exponential curve was fitted to the data of tobacco protoplasts using a least-squares method. NS.: not significant.

Fig. 3. DNA fragment-size distribution. Tobacco protoplasts (top panel) and CHO-K1 cells (bottom panel) were irradiated with 300 and 200 Gy, respectively and were post-irradiation cultured at 27 and 37°C, respectively. The frequency of remaining DNA fragments at each post-irradiation cultivation time is shown as a function of fragment size. Each data represents a mean ± SE of 2 to 6 independent experiments. Experimentally-determined frequencies were fitted to the random fragmentation model using a least-squares method.
and 64.9 ± 10.9 min, respectively. They were similar to those reported in normal human fibroblasts. These led the unexpected conclusion that the DSB rejoining efficiency was comparable between tobacco protoplasts and mammalian cells regardless of their different radiosensitivities. Why were their DSB rejoining efficiencies close together? A possible interpretation is that most of DSBs induced by γ-rays are originated in simple type damage such as nicks and heat-labile sites. These damages might be immediately rejoined without using a complex repair system, which may result in the comparative rejoining efficiencies among different organisms. It has been reported that distinct radiosensitivities among mammalian cell lines are often independent of their ability of rejoining DSBs. The results of this study may support the possibility that the DSB rejoining efficiency is of little importance in the radiation tolerance of tobacco protoplasts. If this is true, which factors do contribute their radiation tolerance? The importance of unrejoined and misrejoined DSBs remained to be unclear because the PFGE assay could neither detect a few DSBs on genome DNA nor analyze the accuracy of DSB rejoining.

Further investigation of the rejoining efficiency was performed with tobacco protoplasts subjected to different levels of initial DSB induction: 200 Gy (irradiated dose is equal to CHO-K1 cells) and 600 Gy (initial DSB yield per bp DNA is equal to CHO-K1) along with 300 Gy (initial DSB yield per cell is equivalent to CHO-K1). No obvious tendency was found in the rejoining efficiency among them (Table 1), indicating that the rejoining efficiency of tobacco protoplasts is almost identical to that of CHO-K1 cells regardless of the initial levels of DSB induction within the doses employed.

In summary, we investigated the DSB rejoining kinetics in tobacco protoplasts and CHO-K1 cells following γ-irradiation. The present study is the first report that quantified the DSB rejoining of higher plant cells and revealed that there was no significant difference in the rejoining efficiency between tobacco protoplasts and CHO-K1 cells under their optimal cultivation conditions.

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**Table 1.** DSB rejoining efficiency at different doses.

| Cell  | Dose (Gy) | % remaining DSBs (mean ± SE of 3–6 independent experiments) | Post-irradiation time (h) |
|-------|-----------|-------------------------------------------------------------|---------------------------|
| Tobacco | 200       | 24.1 ± 14.5                                                 | 11.1 ± 11.1               |
|        | 300       | 5.2 ± 1.7                                                   | 2.9 ± 2.2                 |
|        | 600       | 16.8 ± 3.5                                                 | 0.2 ± 0.1                 |
| CHO    | 200       | 10.0 ± 5.2                                                  | 0.8 ± 0.5                 |

*There was no significance in the percentages of remaining DSBs between tobacco protoplasts and CHO-K1 cells.*
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