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Ion Beam as a Noble Tool to Induce Apoptosis-Like Cell Death in Roots of Maize (*Zea mays* L.)

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Abstract

Maize seedlings were irradiated with $^{20}Ne^{8+}$ generated by the AVF cyclotron. The growth suppression at the different dosages of ion beam was observed in both root and coleoptile. DNA laddering was also observed at the dosage over 100 Gy. In addition, abnormal morphological changes including chromatin condensation, typical of apoptosis in animal cells, were noted by light and electron microscopy. Our results indicate that the ion beam can serve as an effective tool to induce apoptosis-like cell death in plant cells.

Abbreviations

DAPI; 4', 6'-diamidino-2-phenylidole, LET; linear energy transfer

High linear energy transfer (LET) ionizing radiations such as ion beams cause more localized dense ionizations within the cells compared to low LET radiations such as X- and γ-rays (Kraft *et al.*, 1992). It is thought that the ion beam mainly produces double strand breaks of DNA with damaged end groups, where repair abilities would be low (Hagen, 1994; Goodhead, 1995). Therefore, it is likely that high LET radiations, rather than low LET radiations (e.g., X- and γ-rays) might be useful for the generation of DNA deletions. The ion beam appears to have specific effects on various biological functions. For instance, Fujii *et al.* (1966; 1967) reported that heavy ions were more effective than low LET radiations on the inhibition of germination, survival and induction of mutations in *Arabidopsis*. Furthermore, it has been demonstrated that the relationship between dosage and pollen germination rate was different in ion beam and γ-ray treated tobacco; ion beams were more effective in reducing germination and survival rates than γ-rays (Inoue *et al.*, 1993).

In mammalian cells, apoptosis can be induced by radioactive rays such as X-or γ-rays (Potten, 1992; Blankenberg *et al.*, 1999). Such experimental models have contributed to our understanding of the molecular and cellular processes of programmed cell death (PCD). In plants, Nagata *et al.* (1999) reported that 1 k-3 kGy of γ-ray irradiation induces additional formation of trichomes in the mature leaves of *Arabidopsis*. However, low LET ionizing radiation, such as X- or γ-rays, failed to successfully induce apoptosis in plants (Frylink *et al.*, 1987; Nagata *et al.*, 1999).

Here we report for the first time that a treatment of maize seedlings with ion beam ($^{20}Ne^{8+}$) causes apoptosis-like cell death, suggesting that this tool can serve as an effective mean of inducing PCD in plant cells.

Ion beam irradiation

Maize (*Zea mays* L.) kernels treated with 10% sodium hypochlorite for 5 min were placed on 1% agar, followed by incubation at 23 ℃ under continuous illumination. Two-day-old seedlings on agar plates were irradiated with $^{20}Ne^{8+}$ (350 MeV) generated by the AVF cyclotron (JAERI, Takasaki, Japan) in the atmosphere. LET values and projectile ranges were calculated with ELOSS code developed by Japan Atomic Energy Research Institute (JAERI). The particle fluence was determined by microscopic counting of etched particle tracks on diethyleneglycol-bis-allylcarbonate (CR-39) film track detector. To convert particle fluence to dose in Gy, the following relationship was used:

$$\text{Dose [Gy]} = 1.6 \times 10^{-9} \times \text{LET [keV/}\mu\text{m]} \times \text{Fluence [particles/cm}^2\text{]}$$

All irradiations were carried out within 2 min at room temperature (25 ℃). After irradiation, seedlings were maintained at 25 ℃ under continuous light and seminal roots were used for further analysis.

Ion beam induced apoptosis-like cell death

To determine the biological effects of ion beam irradiation on two-day-old maize plants, we analyzed the root growth 48 h after treatment. Irradiation resulted in a marked shortening of the relative root length as well as coleoptile, and such shortening was $^{20}Ne^{8+}$-dose dependent (**Fig. 1A** and **B**). Plant cells are well known to be highly resistant to ionized radiation compared to animal cells (Bark *et al.*, 1989; Kranz *et al.*, 1996). Our results are consistent with these early findings, i.e., root elongation was not completely inhibited at 10 kGy.

During the early stages of apoptosis in animals, specific endonucleases attack nuclear DNA at the internucleosomal linker regions between nucleosomal cores, resulting in the production of low molecular weight oligonucleosomal DNA fragments, about 180 bp in size (Wyllie, 1980; Wyllie *et al.*, 1984; Cohen, 1993). To confirm whether or not radiation induced-growth defect of maize seedlings is associated with similar nuclear changes, we analyzed the irradiated plants for the appearance of DNA laddering. Roots of maize seedlings treated with different doses of Ne ions were used for DNA analysis. As shown in **Fig. 2**, DNA fragmentation was observed at a relatively high dosage. Low molecular weight DNA fragments were noted at 100 Gy, and DNA laddering increased significantly with higher dosages.

To analyze the cytological changes induced by the irradiation, the seminal roots (5-mm long from the tip) of the seedlings (48 h after irradiation) were fixed and examined by DAPI staining. As seen in **Fig. 3B**, chromatin condensation and fragmentation were identified in cells irradiated with 10 kGy.

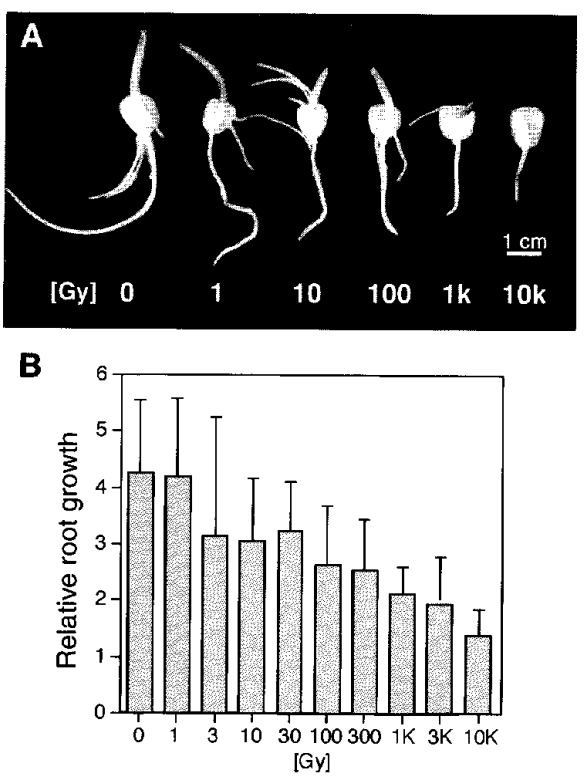

Fig. 1 Growth inhibition of maize seedlings by Ne ions. Maize seedlings (2 day-old) were irradiated with different dosages (1 Gy-10 kGy) of $^{20}Ne^{8+}$. The photograph was taken at 48 h after irradiation (A) and the length of seminal roots was measured and expressed relative to the length of time O (B). Scale bar = 1 cm. Data represent the mean ± SD of 10-15 samples.

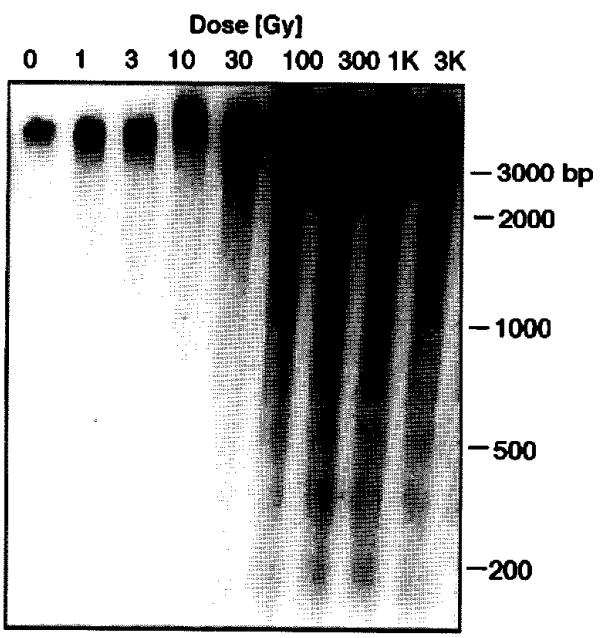

Fig. 2 Detection of DNA laddering in irradiated maize roots. Roots irradiated with different dosages (0 Gy-3 kGy) were collected at 48 h after irradiation, and were ground in liquid N_2 with a mortar and pestle to a fine powder. The extraction buffer (5 mM Tris HCl, pH 8.0, 20 mM EDTA and 0.5 % Triton X-100) was then added to the sample, followed by treatment with an equal volume of phenol-chloroform (1:1, v/v). Following centrifugation (8,000 ×*g* for 15 min), the supernatant was used to precipitate total nucleic acids by the addition of an equal volume of isopropanol. In the next step, 10 μg of the extracted total DNA was treated with RNase (0.01 mg/mL) then electrophoresed on a 1.5% agarose gel, followed by staining with ethidium bromide. Negative photo images were examined under UV transilluminator (TOYOBO FAS1000, Japan). bp, base pairs.

Further analysis showed that these morphological changes in nuclei appeared 24 h after irradiation (data not shown).

Electron microscopic examination confirmed the above morphological changes in irradiated cells. Condensed and dispensed chromatins were found in the concave nuclei (**Fig. 3D**) although significant morphological changes were not observed in the mitochondria (data not shown). The cellular structural changes observed in this study mimicked those of apoptosis in animal cells. Since necrosis is characterized by cell swelling and lysis associated with leakage of cellular contents (Cohen, 1993), the observed ion beam-induced changes noted in our study does not represent necrosis but rather a cell death process that resembles apoptosis of animal cells. Recently, Danon and Gallois (1998) reported that a high dose (40 kJ/m^2) of UV-C induced oligonucleosomal DNA fragmentation in *Arabidopsis* seedlings, although nuclei were not examined by electron microscopy.

In general, the irradiation dosage required to induce apoptosis of animal cells is < 10 Gy (Yamada and Ohyama, 1980; Ohyama *et al.*, 1985). Significantly, about 10-100 times higher dose was required for the induction of apoptotic changes in plant cells. The high resistance of these cells to radioactivity can be explained by the presence of radioprotective chemicals such as flavonoids and sinapic esters in plant cells (Jansen *et al.*, 1998), different base ratio (Kaplan *et al.*, 1962), DNA content and nuclear volume (Baetcke *et al.*, 1967), as well as other yet unknown mechanisms.

Several genes are known to be involved in the process of apoptosis, but no such genes have yet been identified in plants. In this respect, H_2O_2 can be considered as an inducer of plant PCD. Specifically, acting as an oxidative burst, H_2O_2 has been shown to induce plant cell death (Levine *et al.*, 1994; Tenhanken *et al.*, 1995). In this regard, radiation is known to exert deleterious effects through the intermediacy of reactive oxygen species (Imlay and Linn, 1988).

Since ion beam constitutes one form of higher LET ionizing radiation; it can deposit high energy on the target as opposed to low LET radiations such as electron beam, X- and γ-rays. Shikazono (1998) reported that carbon ion-induced mutations in *Arabidopsis* were caused by various types of DNA alterations including deletion, rearrangement, and point mutations in higher plants. Furthermore, it has been suggested that the ion beams cause clustered damage of DNA which is difficult to repair rapidly (Goodhead, 1995). Thus, the ion beam may be a useful alternative to low LET radiation such as X-

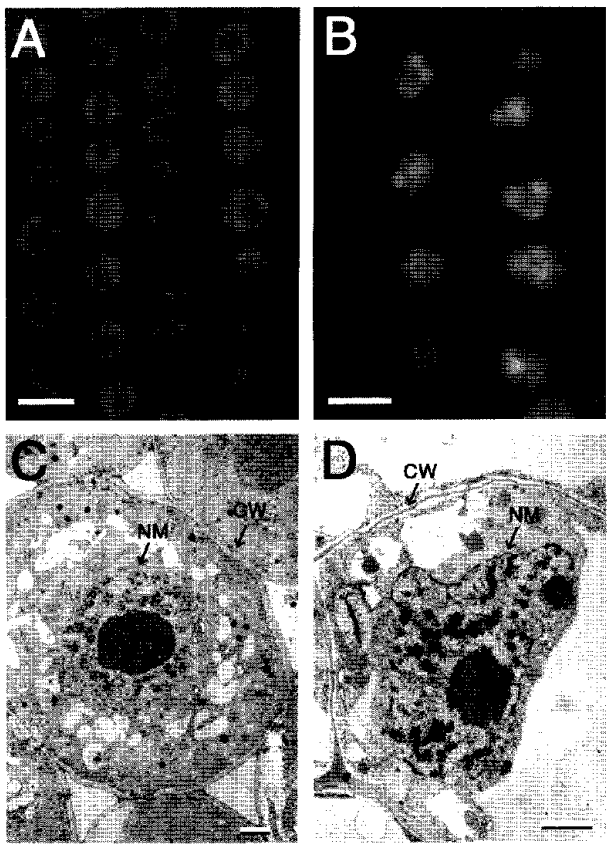

Fig. 3 Nuclear morphology of non-irradiated (A, C) and irradiated (B, D) root meristem cells. Maize roots treated with 3 kGy of Ne ions were collected at 48 h after irradiation and fixed overnight in 4% paraformaldehyde - 20 mM cacodylate buffer (pH 7.0) at 4 ℃ for 18 h. Tissues were then dehydrated in a series of concentration-graded ethanol solutions (50 to 100%) and embedded in Technovit 8100 (Kulzer, Wehrheim, Germany). Longitudinal 10-μm thick sections were cut with a microtome (Yamato Kohki, Japan) and stained with DAPI (A and B; 4', 6'-diamidino-2-phenylidole) for examination of nuclei, followed by observation under a fluorescence photomicroscope (LEITZ DM RD, Leica, Wetzlar, Germany). Scale bar = 0.01 mm. Root samples of non-irradiated (C) and irradiated seedlings with 3 kGy of Ne ions (D) were examined by an electron microscope. Corrected root tips were fixed in 2.5% glutaraldehyde in cacodylate buffer, pH = 7.2, and treated with OsO_4, as described in detail by Ueda *et al.* (1996), with a minor modification. The substituted samples were embedded in Spurr's resin and thin serial sections were prepared with an Ultracut N or Ultracut S microtome (Reichert, Vienna, Austria). Sections were stained with uranyl acetate and lead citrate (UA/Pb) and observed under an electron microscope (Zmodel 2010; JEOL, Akishima-shi, Japan). Scale bar = 2 μm. Each root sample was collected at 48 h after irradiation. CW, cell wall; NM, nuclear membrane.

and γ-rays as a powerful inducer of apoptosis-like cell death in plants.

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