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Research Report

DNA Damage and Oxidative Stress Induced by Proton Beam in *Cymbidium* Hybrid

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Abstract. We investigated the radiation damages caused by two types of proton beam and gamma ray in the *Cymbidium* hybrid RB001 [(*C. sinensis* × *C. goeringii*) × *Cymbidium* spp.] to characterize proton beam as a new mutagen for *Cymbidium* mutation breeding. The protocorm-like bodies (PLBs) of *Cymbidium* hybrid were irradiated with a 45 MeV proton beam [mean linear energy transfer (LET) = 1.461 keV·μm^{-1}], a 100 MeV proton beam (LET = 0.7306 keV·μm^{-1}), and gamma ray (LET = 0.2 keV·μm^{-1}). The PLBs treated with radiation doses of 0-100 Gy were analyzed using the comet assay and their physiological responses as indices of radiation damage. In the comet assay, the 45 MeV proton beam caused significant damage to the DNA integrity, but the 100 MeV proton beam and gamma ray showed relatively little radiation damage between the untreated control and treated PLBs. Malondialdehyde (MDA) content, an index of the indirect effects of ionizing radiation, was increased slightly by the 45 MeV proton beam with higher-LET, but highly increased by the 100 MeV proton beam with lower-LET. These results suggested that the 100 MeV proton beam caused extreme oxidative stress. Therefore, proton beam have unique characteristics in DNA mutagenesis pattern according to its LETs. Based on these results, proton beam is expected to be a useful tool for developing new mutant varieties of *Cymbidium*.

***Additional key words*:** comet assay, gamma ray, linear energy transfer, physiological responses

Introduction

Cymbidium species are the most popular and important orchids and have been cultivated in many areas including China, Korea, Japan, Southeast Asia, and India for thousands of years (Du Puy et al., 2007). However, the number of farmers cultivating these oriental orchids is decreasing and the amount of imports is increasing in Korea (Ministry of Agriculture, Food and Rural Affairs, 2012). Therefore, it is necessary to develop new *Cymbidium* cultivars with novelty in traits such as flower color, shape, and plant height to increase cultivation in the country.

Radiation technology is one of the most common techniques used to create novel variations by artificial mutation in ornamental plant breeding. According to a report by the FAO/IAEA organization, a total of 3,100 mutant varieties from 170 different plant species including cereals, beans, and flowers have been officially registered so far and most of which were developed by treatment with ionizing radiations, chiefly gamma ray (Joint FAO/IAEA Programme, 2014; Tanaka et al., 2010).

The type of mutagenic treatment is an important factor for successful results in mutation breeding. Gamma-ray irradiation has advantages over treatment with chemical mutagens such as ethyl methane sulfonate (EMS) in that it induces DNA damage relatively randomly, resulting in various types of mutations including point mutations, indels, and chromosome aberrations (Tanaka et al., 2010). Therefore, gamma-ray irradiation has been the main treatment used in various plants, particularly ornamental plants such as orchids (Kikuchi, 2000), *Anthurium* (Puchooa, 2005), chrysanthemum (Kumar et al., 2012; Yamaguchi et al., 2008), and *Paphiopedilum* (Luan et al., 2012), for over 80 years. Recently, irradiation with ion beams, which are different from gamma-ray in that they are generated by accelerators and has specific mass and charge according to types of ions, has been proven to be very effective in mutation breeding in terms of biological effectiveness, mutation frequency, and range of mutation spectrum compared with gamma ray (Hase et al., 2012). New varieties in diverse crops including rice, verbena, carnation, and chrysanthemum have been developed using heavy-ion beams (Kanaya et al., 2008; Nagatomi, 2003; Okamura et al., 2003; Tanaka et al., 2010).

Mutagenic effect of radiation is greatly different according to linear energy transfer (LET) which describes how much energy an ionizing particle transfers to the material transversed per unit distance. LET of ion beams is much higher than that of gamma-ray and widely vary according to ion type (Shu et al., 2012). Despite strong potential of ion beams in mutation breeding, the effects of ion beam irradiation have been characterized only for very limited ion types with specific energy and LET (Kazama et al., 2008; Tanaka et al., 2010). The frequency and type of mutations have been mainly characterized in heavy-ions such as C, N, and Ar ions which show high LET, usually between 20-500 keV·μm^{-1} (Hirono et al., 1970; Mehnati et al., 2005; Tanaka et al., 1997; Yokota et al., 2003). However, in proton beam, which has much lower LET (usually lower than 2 keV·μm^{-1}) than heavy-ion beams, few researches have been performed for application in mutation breeding although it has been widely studied for proton therapy on medical purpose (Schulz-Ertner and Tsujii, 2007). In agronomic field, changes of physiological and metabolic properties such as starch composition and gibberellin content have been investigated recently in yam (Kim et al., 2011a, 2011b), barley (Kim et al., 2013), and rice (Kim and Kim, 2013).

Treatment with ionizing radiations has various direct and indirect effects on plant cells. Single-strand breaks (SSB) or double-strand breaks (DSB) can be induced by ionizing radiation with enough energy to directly ionize DNA (Boudaïffa et al., 2000). In addition, reactive oxygen species (ROS) radicals can be generated by energy deposited in water and other bio-molecules. Highly reactive oxygen species around DNA can attack DNA to induce breaks or non-DSB DNA legions as well as they can cause structural changes in proteins resulting in loss-of-function (Shu et al., 2012). DNA damage can be directly investigated using comet assay analysis. The comet assay (single cell gel electrophoresis, SCGE) is a technically simple, highly sensitive, fast and economical test for detecting DNA damage in plant cells (Gichner et al., 2004, 2006, 2008; Liman et al., 2011; Menke et al., 2000). Meanwhile, the effects of ionizing radiation on the generation of ROS can be monitored indirectly by measuring the quantity of biological indicator metabolites such as malonaldehyde (MDA) which is produced by lipid peroxidation under oxidative stress or the activity of proteins involved in the integrated endogenous enzymatic defense system against oxidative stress, which includes peroxidase (POD), ascorbate peroxidase (APX), and catalase (CAT) (Del Rio et al., 2005; Wang et al., 2010; Zaka et al., 2002).

In this study, we investigated the biological effects caused by irradiation with proton beam in regard to mutation breeding. Comparison between the effects of gamma radiation and proton beam with different LETs on the PLBs of *Cymbidium* hybrid was performed by the comet assay and the examination of physiological responses.

Materials and Methods

Plant Materials and Mutation Induction

The PLBs of *Cymbidium* hybrid RB001 [(*C. sinensis* × *C. goeringii*) × *Cymbidium* spp.] were used in this study. In vitro PLBs were maintained on Hyponex medium (N:P:K = 6.5:6:19, Hyponex Japan Co., Osaka, Japan), pH 5.35 with 3% sucrose and 0.4% plant agar (Duchefa B.V., Haarlem, the Netherlands). The PLBs were irradiated at room temperature with 45 MeV (LET = 1.461 keV·μm^{-1}) and 100 MeV (LET = 0.7306 keV·μm^{-1}) proton beam at a dose of 0, 20, 40, 80 and 100 gray (Gy). The LET values of the beam were calculated at the surface of the water. For comparison with radiation mutagens, explants cultured in a plastic dish were irradiated at five different doses of gamma ray (0, 20, 40, 80, and 100 Gy; LET = 0.2 keV·μm^{-1}) emitted from a ^{60}Co source at the Korea Atomic Energy Research Institute. After irradiation, the PLBs were immediately transferred onto the same medium. Cultures were incubated at 25 ± 1°C under a 16/8 h (day/night) photoperiod, provided with white fluorescent tubes at an intensity of 50 μmol·m^{-2}·s^{-1}.

Comet Assay

For the comet assay, the same procedure was used as in Dhawan et al. (2009), with some modifications. The comet assay was conducted one week after proton beam and gamma ray irradiation. Briefly, the PLBs at 1 week after radiation treatments were placed in a petri dish kept on ice, and spread with 2 mL phosphate-buffered saline (11.9 mM phosphates, 137 mM sodium chloride, and 2.7 mM potassium chloride, pH 7.5). The PLBs were immediately chopped with a razor blade and isolated PLB nuclei were collected in the buffer. The first layer of a microscope slide was precoated with 1% normal melting point agarose (NMPA) and thoroughly dried at room temperature. Next, 180 μL of 0.5% low melting point agarose (LMPA) at 40°C was mixed with 20 μL of the nuclear suspension, and then dropped on top of the first layer, which was additionally precoated with a dried layer of 0.5% LMPA. Each drop was covered with a coverslip and solidified on ice. After removal of the coverslips, the nuclei were immersed in ice-cold lysing solution (2.5 M NaCl, 100 mM EDTA, and 10 mM Trizma base, pH 10.0) for 2 hours. After lysing, the nuclei were allowed to unwind for 10 min in electrophoretic buffer containing 300 mM NaOH and 1 mM EDTA at pH 13. Electrophoresis was then conducted for 10 min at 25 V in a chamber cooled on ice. Next, the slides were washed twice with 0.4 M Tris buffer and kept in cold 100% ethanol for 20 min for dehydration, and then dried at room temperature overnight. Finally, the dried microscope slides were stained with 80 μL propidium iodide solution (25 μL·mL^{-1}) for 10 min, covered with a

coverslip, and analyzed using a on a fluorescent microscope (40× objective). Three slides were evaluated per treatment, and each treatment was repeated at least twice. The data were expressed as mean ± standard error (SE).

Measurement of Malondialdehyde Content

Lipid peroxidation was determined by measuring the MDA content of PLBs at 1 week after irradiation. The MDA content was measured using the trichloroacetic acid (TCA) method described by Wang et al. (2010), with a few modifications. Briefly, PLBs (0.1 g) were mixed with 1 mL of 10% TCA. The homogenate was centrifuged at 13,800 xg for 10 min, and 600 μL of the supernatant was mixed with 600 μL of 0.67% 2-thiobarbituric acid (TBA). The mixture was heated at 100°C for 30 min, and immediately cooled on ice. The MDA content was measured spectrophotometrically at 450, 532, and 600 nm and calculated as follows:

MDA content = $6.453 \times (A_{532} - A_{600}) - 0.563 \times A_{450}$.

For each treatment, three replicates were used, and the data were expressed as mean ± standard error (SE).

Results

DNA Damage by Radiation in *Cymbidium* Hybrid

There was a significant correlation between the type of mutagenic treatment and DNA damage in *Cymbidium* hybrid (Fig. 1). Various changes in the overall distribution of DNA were detected in individual *Cymbidium* cells after exposure to gamma ray and proton beam (45 and 100 MeV) at doses from 0 to 100 Gy.

To clarify the different effects among the mutagenic treatments, we compared the percentage of head and tail DNA, and tail length (Fig. 2). The percentage of head DNA after 45 MeV proton beam irradiation decreased significantly with increasing dose up 40 Gy from 85.43 ± 1.20 (untreated control) to 66.08 ± 1.97% (40 Gy treatment), and maintained

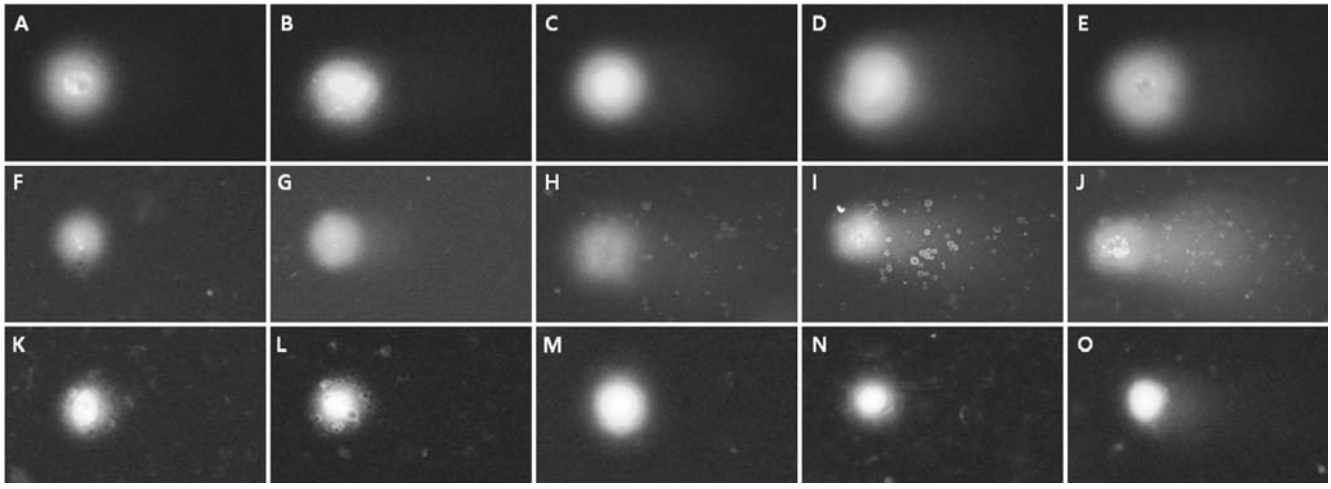

Fig. 1. Comet assay images of nuclei of *Cymbidium* hybrid RB001 [(*C. sinensis* x *C. goeringii*) x *Cymbidium* spp.] PLBs after radiation: A, control, gamma-ray; B, 20 Gy gamma-ray; C, 40 Gy gamma-ray; D, 80 Gy gamma-ray; E, 100 Gy gamma-ray; F, control, 45 MeV proton beam; ,G, 20 Gy, 45 MeV proton beam; H, 40 Gy, 45 MeV proton beam; I, 80 Gy, 45 MeV proton beam; J, 100 Gy, 45 MeV proton beam; K, control, 100 MeV proton beam; L, 20 Gy, 100 MeV proton beam; M, 40 Gy, 100 MeV proton beam; N, 80 Gy, 100 MeV proton beam; and O, 100 Gy, 100 MeV proton beam.

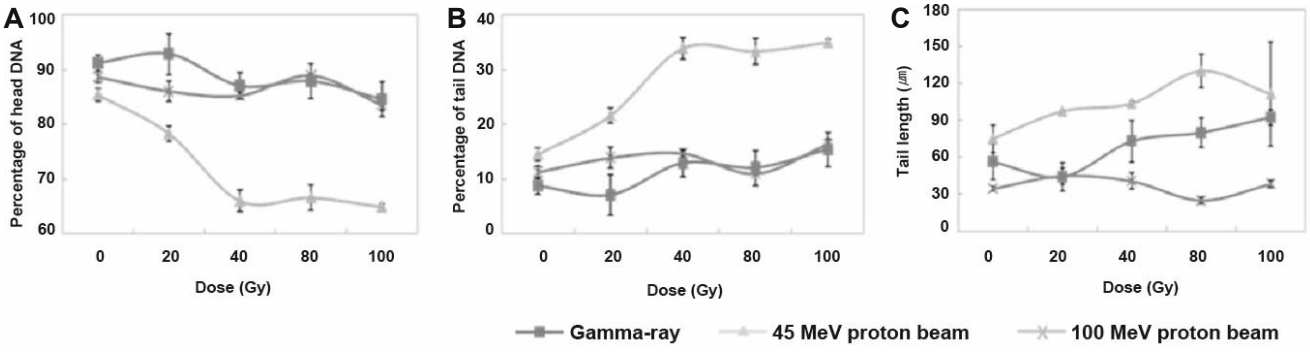

Fig. 2. Results of the comet assay analysis on *Cymbidium* hybrid RB001 [(*C. sinensis* x *C. goeringii*) x *Cymbidium* spp.] cells at 1 week after radiation treatments: A, proportion of head DNA; B, proportion of tail DNA; and C, tail length. Error bars indicated ± S.E., n = 3.

similar levels after 40 Gy (Fig. 2A). For 100 MeV proton beam, the percentage of head DNA of PLBs decreased with increasing irradiation dose from 88.69 ± 1.05 (untreated control) to 83.60 ± 0.93% (100 Gy treatment). Gamma ray, in which the percentage of head DNA of PLBs decreased gradually with increasing irradiation dose from 91.23 ± 1.44 (untreated control) to 84.59 ± 3.15% (100 Gy treatment), showed degree of DNA damage similar with that of 100 MeV proton beam, (Fig. 2A). Conversely, the percentage of tail DNA increased with increasing dose for the 45 MeV proton beam and gamma ray (Fig. 2B). With increasing dose rate, in the range of 0-100 Gy, the tail length after gamma-ray irradiation increased from 56.28 ± 14.52 (untreated control) to 92.56 ± 6.21 μm (100 Gy treatment) (Fig. 2C). The tail length after 45 MeV proton beam irradiation also increased with an increasing dose up to 80 Gy, but decreased slightly at 100 Gy. However, the tail length after 100 MeV proton beam irradiation showed almost no difference under the radiation treatment, ranging from 34.58 ± 1.93 (untreated control) to 38.44 ± 2.90 μm (100 Gy treatment).

Malondialdehyde Content to Proton Beam and Gamma Ray Irradiation in *Cymbidium* hybrid

To compare the oxidative damage among the proton beam and gamma ray, the MDA contents were measured at five different radiation dosages (0, 20, 40, 80, and 100 Gy). The MDA contents of gamma-ray irradiated PLBs increased gradually with increasing dose (Fig. 3A). On the other hand, the MDA contents of 45 MeV proton beam irradiated PLBs gradually increased with increasing dose up to 40 Gy, but decreased after 80 Gy (Fig. 3B). The MDA content after 80 Gy (average 5.45 ± 0.15 nmol·g^{-1} F.W.) was lower than that in the control PLBs (average 5.69 ± 0.01 nmol·g^{-1} F.W.). The MDA contents of 100 MeV proton beam irradiated PLBs increased significantly with increasing dose up to 80 Gy, but decreased at 100 Gy (Fig. 3C). However, the MDA contents were all higher than those in the control PLBs. These results showed that the MDA content in the 100 MeV proton beam treatment had the highest increase rate among the two types of proton beam and gamma ray.

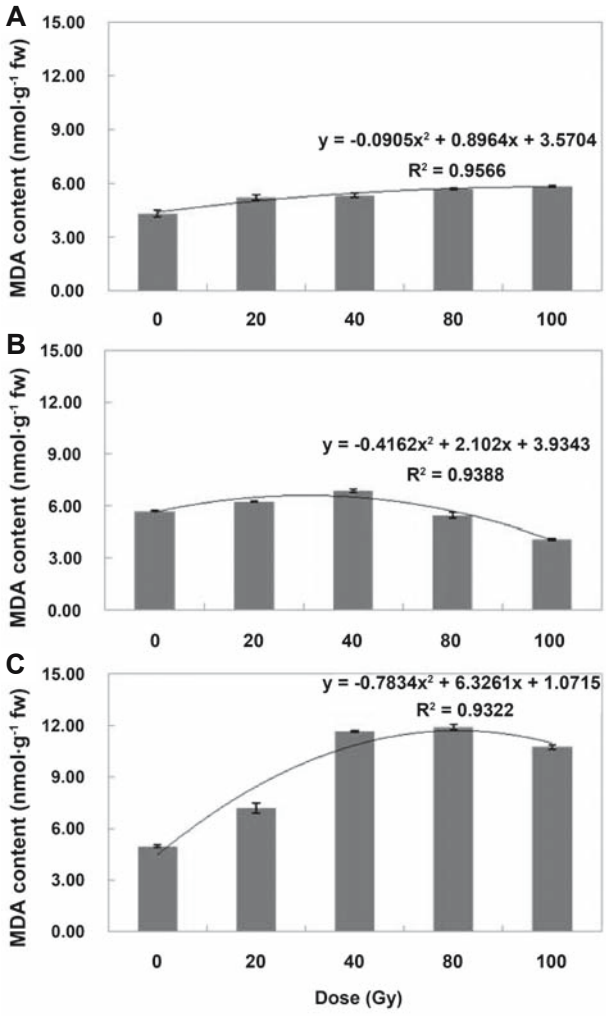

Fig. 3. Malondialdehyde (MDA) contents of 20, 40, 80, and 100 Gy-irradiated PLBs after gamma-ray and proton beam irradiation in *Cymbidium* hybrid RB001 [(*C. sinensis* x *C. goeringii*) x *Cymbidium* spp.]: A, Gamma-ray; B, 45 MeV proton beam; and C, 100 MeV proton beam. Error bars indicated ± S.E., n = 3.

Discussion

Ion beam are expected to be utilized widely as new mutagens because high energy levels can be directed on a target in a densely focused manner as compared with low LET radiations, such as gamma ray and X-ray (Kahl and Meksem, 2010). In addition, the mutation frequencies from ion beam are much higher than those of gamma ray (Okamura et al., 2003). Characterization and utilization of ion beam in plant breeding have been focused on carbon ions in previous researches (Hase et al., 2002; Shikazono et al., 1998, 2005; Yokota et al., 2003), although ion beam may show different characteristics and efficiency in mutation induction according to ion type.

Proton beam is distinct from both gamma ray and heavy ion beam in that the proton beam is consisted of particles having mass and electrical charge, unlike gamma ray, and has a LET that is much lower than that of heavy ion beam and closer to that of gamma ray (Kazama et al., 2008; Shu et al., 2012). Therefore, the biological effectiveness and type of damage from proton beam may be unique, suggesting that proton beam has potential as a new mutagen that may cause different patterns of mutation. The effects of proton beam on plant cells have been investigated using crops such as yam (Kim et al., 2011a, 2011b), rice (Kim and Kim, 2013) and barley (Kim et al., 2013). However, all these studies

have focused on changes in metabolite properties, which are not related to the mutation of DNA.

To characterize the effects of proton beam on DNA, comet analysis was performed using *Cymbidium* hybrid PLB samples treated with proton beam at two different LETs and gamma ray. The comet assay is a well-established, simple, versatile, rapid, visual, sensitive, and extensively used tool to assess DNA damage and repair quantitatively as well as qualitatively in individual cell populations (Olive and Banáth, 2006). The advantages of the comet assay include its demonstrated sensitivity for detecting low levels of DNA damage, low cell number requirement (~10,000) per sample, flexibility to use proliferating as well as non-proliferating cells, low cost, ease of application, and the short time needed to complete a study (Gedik et al., 1992). Here, irradiation of the higher-LET proton beam (1.461 keV·μm^{-1}) resulted in a much higher frequency of DNA breakage than the lower-LET proton beam (0.7306 keV·μm^{-1}) and gamma ray (0.2 keV·μm^{-1}). This result is consistent with a previous report in that an increased DNA breakage yield in the higher-LET radiation treatment was detected, in comparison with helium ion beam (9.4 and 17.7 keV·μm^{-1}) and lower-LET carbon ion beam (94.8 and 124 keV·μm^{-1}) (Yokota et al., 2007). Comparing the lower-LET proton beam and gamma ray, treatment with the lower-LET proton beam resulted in a similar proportion of damaged and undamaged DNA to gamma ray, but generated a smaller portion of short DNA fragments which was represented by smaller tails in the comet-shaped DNA spots. Yokota et al. (2007) reported that the proportion of short DNA fragments was larger in treatments with high-LET heavy ion beam such as carbon and neon beam than with gamma ray, implying that higher-LET radiation may result in more closely spaced double-strand breaks in the DNA, and thus might result in severe chromosomal aberrations. In their research, helium ion beam, which show comparatively low-LET, caused similar DNA fragmentation patterns to gamma ray. Therefore, lower-LET proton beam may induce DNA breaks more randomly in terms of localization of breaks than gamma ray and other ion beam that have been studied. The reason for this is mostly unknown, although DNA fragmentation patterns have been shown to be dependent on the ion type of the radiation even when the LET is similar (Yokota et al., 2007).

We also examined the indirect effects of the ionizing radiations by quantification of malonaldehyde (MDA). The MDA is an end product of lipid peroxidation in biomembranes, and usually reflects the level of lipid peroxidation (Wang et al., 2010). As lipid peroxidation is the symptom mostly ascribed to oxidative damage, it is often used as an indicator of stress-induced damage at the cellular level (Khan and Panda, 2008). Ionizing radiation was shown to induce oxidative stress with overproduction of ROS such as superoxide radicals (O_2^-), H_2O_2 and hydroxyl radicals (HO) in plant cells (Larson, 1988). More than half of the biological damage caused by low LET radiation (e.g. X-ray, gamma ray) is the result of indirect radiation effects caused by ROS reactions, while higher-LET radiation (e.g. ion beam) is more likely to affect DNA by direct ionization. Our higher-LET proton beam (1.461 keV·μm^{-1}) caused a small increase of MDA (0-40 Gy), but the lower-LET proton beam unexpectedly caused a much higher increase of MDA as compared with the higher-LET proton beam and gamma ray, and thus had a greater effect on oxidative stress induction. This result implies that the lower-LET proton beam treatments may be expected to induce a higher proportion of DNA damage caused by indirect radiation effects compared with higher-LET proton beam. When the results of comet assay are considered together, large portion of DNA lesions caused by the lower-LET proton beam may be non-DSB oxidative DNA lesions. The intermediate LET value (0.7306 keV·μm^{-1}), which was between those of gamma ray (0.20 keV·μm^{-1}) and higher-LET proton beam (1.461 keV·μm^{-1}), is thought to be responsible for maximizing oxidation stress induction, but the detailed mechanism is unknown. It may also be possible that the detrimental effects of severe radiation treatments affected survival of cells or normal lipid metabolism, hampering the correlation between MDA quantity and degree of irradiation. Although MDA quantity and degree of oxidative stress show close relationship within a given range of oxidative stress (Wang et al., 2010), excessive treatments of oxidative stress-inducing agent can result in inhibition of mitotic cell division and break of proportional relationship between MDA content and oxidative stress (Unyayar et al., 2006). Irradiation of lower-LET proton beam (0.7306 keV·μm^{-1}) which exceed to 80 Gy or higher-LET proton beam (1.461 keV·μm^{-1}) resulted in severer DNA damage than in the treatments of lower-LET proton beam bellow 60 Gy or gamma-ray, in which co-relationship between MDA content and dose of radiation was maintained. This may be the explanation why irradiation of lower-LET proton beam resulted in higher production of MDA than that of higher-LET proton beam. Further analysis using various indicators of oxidative stress is required to confirm the relationship between oxidative stress and the type of radiation observed in this study.

In conclusion, we report the biological effects of proton beam irradiation on DNA damage in *Cymbidium* hybrid. Comparison between the effects of gamma radiation and proton beam with different LETs on the PLBs of *Cymbidium* hybrid showed that higher-LET proton beam may induce more DNA breaks than the lower-LET proton beam and gamma ray, whereas lower LET proton beam are expected to cause indirect radiation effects that may induce smaller DNA alterations. These results are expected to be useful in the design of mutation breeding programs using proton

radiation as a new mutagen.

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