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Assessment of biological changes in wheat seedlings induced by $^{12}C^{6+}$-ion irradiation

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Abstract The present study was designed to evaluate the effect of $^{12}C^{6+}$ ion beam (10～80 Gy) on biological changes of wheat seedlings. Reactive oxygen species (ROS)-related biomarkers and the quantification of plant survival and growth were examined at 10 day after carbon ions irradiation (LET: 30.8 keV/μm). The results showed that heavy ions obviously enhanced ROSs reflected by the production of O_2^- and H_2O_2 as well as TBARS, and treatment with 20 Gy achieved the peak value, suggesting that higher mutagenic potential may occur at 20 Gy. Simultaneously, increase of SOD activity was induced by heavy ions to counteract ROS accumulation. On the other hand, higher doses at 40 and 80 Gy inhibited wheat growth and survival in comparison with the control, and reversely lower doses at 10 or 20 Gy stimulated wheat growth and survival. In conclusion, the above observations imply that a dose range of 20~40 Gy is likely promised for wheat mutation breeding.

Key words $^{12}C^{6+}$ ion, Wheat, Reactive oxygen species, Plant growth, Plant survival

CLC number Q691.5

1 Introduction

As a new mutation source, heavy ion beam is advantageous over γ-rays, X-rays and other physical mutation treatments in terms of higher mutation rate, wider mutational spectrum and stability [1]. Heavy ion beams are used to treat e.g wheat[2], maize[3], sorghum[4] and flowers[5] for developing new plant varieties. Therefore, it is indispensable to investigate the mechanism of heavy ion induced mutation so as to obtain a plant of good inheritable character with an optimum dose.

Generally, potential mutagenic effects of ionizing radiation exposure result from direct DNA damage or indirectly from the reactive oxygen species (ROSs) produced, such as superoxide radicals (O_2^-), hydroxyl radicals (OH^-) and hydrogen peroxides (H_2O_2)[6]. The ROSs react rapidly with almost all structural and functional organic molecules, including proteins, lipids and nucleic acids, causing disturbance of cellular metabolism[7]. Furthermore, the endogenous ROSs are signaling and effector molecules in plant growth, development, stress responses, biotic interactions[8], pathogen defence, and even gene expression regulation[9]. The close link between ROS generation and photosynthetic metabolism are particularly important. Plants have evolved various detoxifying systems to avoid oxidative damage by ROSs. One of the protective system is enzymatic system, including SOD (superoxide dismutases), CAT (catalases), POD (peroxidases), APX (ascorbate peroxidases), and other enzymes implicated in the Halliwell and Asada cycle (as corbate-glutathione pathway)[10]. However, little has been known about ROS regulations in plant in response to heavy ion treatment. This study was

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designed to evaluate the changes of ROS-associated biomarkers and morphology of plant exposed to different doses of $^{12}C^{6+}$ ions.

2 Materials and method

2.1 Seeds selection and seedling culture conditions

Selected dry wheat seeds (Yong L. No. 4, from Gansu Academy of Agricultural Sciences) were treated with 90.4 MeV/u $^{12}C^{6+}$ ions. The irradiated seeds were germinated in moistened plates at 25°C and sowed in plastic pots filled with perlite. Seedlings were grown at 25°C with 12 h photoperiod in an environmental growth chamber. The lost water was supplemented every day. The plants were irrigated by Hoagland solution. Ten days after the irradiation, leaf samples, in replication of three, were randomly taken from uniform plants. Fresh leaf tissues were used to determine O_2^-, or frozen with liquid N_2 and stored at –80°C for measuring H_2O_2 and lipid peroxidation.

2.2 Irradiation

The irradiation was carried out on HIRFL (Heavy Ion Research Facility in Lanzhou) at Institute of Modern Physics, Chinese Academy of Sciences. From a vertical beam line, 100 MeV/u $^{12}C^{6+}$ ions were extracted from the vacuum system to bombard the seeds in dishes at room temperature. The dishes were placed on a rotating wheel with remote control, and the seeds received 10, 20, 40 or 80 Gy at a dose rate of 10 Gy/min. Mean energy of the ions impinging on the seeds was 90.4 MeV/u, corresponding to a mean LET of 30.8 keV/μm.

2.3 Determination of hydrogen peroxide

H_2O_2 contents were determined by peroxidase-coupled assay according to Veljovic-Jovanovic *et al*[11]. Samples of 0.5g fresh leaf tissue were ground in liquid N_2 and the powder was extracted in 1mol/L $HClO_4$ in the presence of 5% PVP. After centrifugation at 12000 g for 10 min, the supernatant was neutralized to pH 5.6 and incubated for 10 min with 1 U ascorbate oxidase to oxidize ascorbate prior to assay. The reaction mixture contained 0.1 mol/L phosphate buffer (pH 6.5), 3.3 mmol/L DMAB, 0.07 mmol/L MBTH, 0.3 U POX and 200 μL supernatant. The absorbance at 590 nm was monitored.

2.4 Determination of superoxide radical

O_2^- was measured as described in Ref.[12]. Fresh leaf tissue of 1.0 g was homogenized in 3 mL of 65 mmol/L potassium phosphate buffer (pH 7.8) and centrifuged at 12000 g for 20 min. The supernatant was incubated at 25°C for 20 min, with the incubation mixture containing 1 mL of 65 mmol/L potassium phosphate buffer (pH7.8), 1 mL of 10 mM hydroxyl-aminoniumchloride and 1 mL supernatant. After incubation, 17 mmol/L sulphanilic acid and 7 mmol/L α-naphthyl amine were added to the incubation mixture. The reaction lasted for 20 min at 25°C, before ethylether of the same volume was added and centrifuged at 5000g for 5 min. The absorbance in the aqueous solution was read at 530 nm. The production rate of O_2^- was calculated based on a standard curve.

2.5 Analysis of lipid peroxidation

Oxidative damage to lipids was estimated by measuring the amount of thiobarbituric acid reactive substances (TBARS) in sample homogenates, prepared in 4 mL 10% trichloroacetic acid containing 0.25% thiobarbituric acid (TBA) and heated at 95°C for 30 min, as described by Hodges *et al*.[13]. The absorbance of the supernatant was measured at 532 nm.

2.6 Determination of SOD activity

Total SOD activity was assayed by NBT method[14], with 6 mL reaction mixture containing 50 mmol/L K_2PO_4 buffer (pH 7.8), 13 mmol/L methionine, 75 μmol/L NBT, 2 μmol/L riboflavin, 0.1 mmol/L EDTA and 50 μL enzyme extract. It was illuminated at 100 μmol photons·m^{-2}·s^{-1} for 30 min. One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the NBT reduction as monitored at 560 nm. Protein content was determined according to method in Ref.[15] with BSA as standard.

2.7 Growth and survival quantification

All seedlings were randomly selected under each pot and harvested. Plant height was measured and

plant survival was counted on Day 10 after irradiation.

2.8 Statistical analysis

Each experiment was repeated at least three times. Values were expressed as means ± standard errors (S.E.M). The significance of differences between groups was determined by analysis of variance (ANOVA) with the multiple comparison tests. A p-value < 0.05 was considered as a statistically significant difference.

3 Results and discussion

O_2^- and H_2O_2 are the most important ROSs in biological systems, and can be an index of oxidative damage. The changes in O_2^- and H_2O_2 level induced by the $^{12}C^{6+}$ ions are shown in Fig. 1. Compared with respective control, O_2^-production at 20 Gy (p<0.0001), and H_2O_2 content at 10 (p<0.05) and 20 Gy (p<0.01) are significantly different in wheat seedlings. The seeds irradiated to 20 Gy by the $^{12}C^{6+}$ ions result in ROS accumulation and the maximal production of ROSs which are responsible for initiating biological damage, and further lead to mutagenesis by attacking proteins, lipids and nucleic acids[16]. Moreover, Pickert *et al*[17] found that early damages were characteristic for plant mutation. Therefore, we speculate that 20 Gy possibly induce higher mutagenic potential.

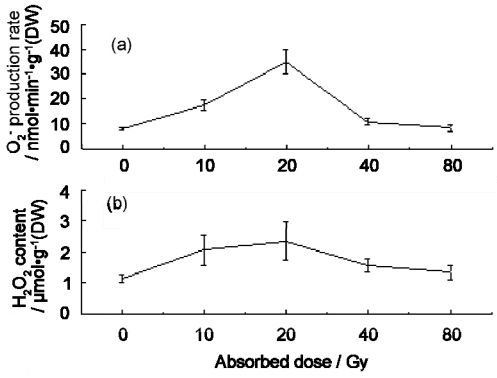

Fig.1 O_2^-production (a) and H_2O_2 content (b) in wheat seedlings 10 d after $^{12}C^{6+}$ irradiation of the seeds. DW: dry weight.

Under normal conditions, cells have enzymatic and non-enzymatic mechanisms to scavenge ROSs. Among these, SOD, a primary ROS scavenger, converts O_2^- to H_2O_2 and oxygen. In the study, no dose-related phenomena could be seen with the $^{12}C^{6+}$ ion treatment, which enhanced SOD activities to 40% and 30% at 10 Gy (p<0.05) and 80 Gy (p<0.05) compared with control in wheat seedlings (Fig.2). But 20 Gy did not induce SOD activity significantly (Fig.2), suggesting that SOD gradually showed insufficient antioxidant potential with increasing O_2^- production. A possible explanation is deceased synthesis of enzyme or oxidative inactivation of enzyme protein[18].

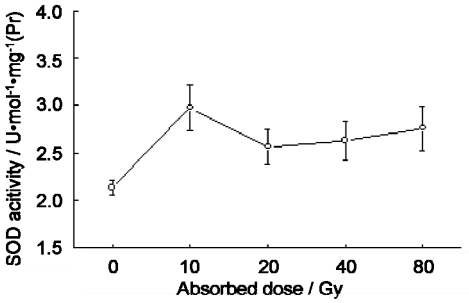

Fig.2 SOD activities in wheat seedlings 10 d after $^{12}C^{6+}$ irradiation of the seeds. Pr: protein.

If ROSs are not effectively eliminated, they could cause cell oxidative injury, for example, peroxidation of membrane, proteins (receptors and enzymes) and DNA. The ability of cell defense against free radicals produced by irradiation is referred to as oxidative stress[19], which can be measured by the TBARS. As shown in Fig.3, TBARS increased 2.3-, 3.0-, 1.3- and 1.1-fold at 10 Gy (p<0.0001), 20 Gy (p<0.0001), 40 and 80 Gy in comparison with the control, respectively. The data suggest that the $^{12}C^{6+}$ treatment might lead to oxidative stress in wheat seedling at different degrees.

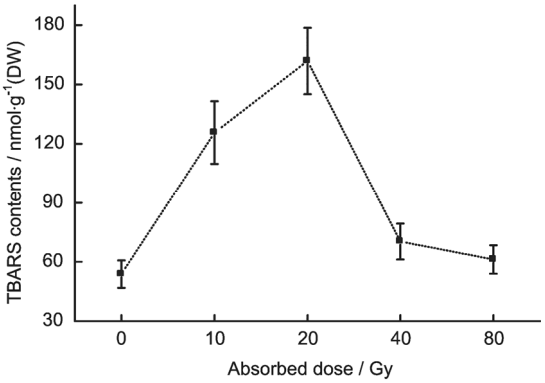

Fig.3 TBARS contents in wheat seedlings 10 d after $^{12}C^{6+}$ irradiation of the seeds. DW: dry weight.

Compared with the control, wheat heights increased by 6% and 14% at 10 and 20 Gy (Table 1), respectively, indicating that the low doses stimulated the plant growth. But at 40 and 80 Gy, plant heights decreased by 5% and 9%, indicating that high doses

inhibited the plant growth. Similarly, the survival rate was higher at 20 Gy (Table 1, $p<0.01$), but decreased significantly at 80 Gy ($p<0.001$). These demonstrate that high dose $^{12}C^{6+}$ irradiation did not benefit plant mutation breeding. Yamaguchi *et al*[20] observed that high dose irradiation resulted in a lower survival rate, and did not increase the levels of mutation frequency in rice. Thereby, an optimum dose may exist in 20~40Gy for best effect on wheat mutation by $^{12}C^{6+}$ ion beam treatment, but further investigation is needed.

Table 1 Plant survival and plant height of wheat seedlings 10 d after $^{12}C^{6+}$ irradiation of the seeds

Index	Absorbed dose / Gy				
	0	10	20	40	80
Plant survival / %	80.0±0.029	85.0±0.035	92.5±0.038	75.0±0.033	67.5±0.041
Plant height / cm	13.8±2.639	14.6±2.471	15.8±2.092	13.1±3.067	12.6±4.024

4 Conclusion

In conclusion, the study described here demonstrates that $^{12}C^{6+}$ ion beam irradiation induces the production of O_2^- and H_2O_2 as well as TBARS in wheat seedling with different doses, and 20 Gy achieved the peak value. Correspondingly, increased SOD activity neutralizes ROS production. In the index of plant morphology, lower dosages stimulated the plant growth and survival rate and higher dosages restrained them. In conjunction with all data, we thought the range of 20~40 Gy for $^{12}C^{6+}$ ion beam treatment is suitable for plant mutation.

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