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Oxidative Metabolism Involved in Non-targeted Effects Induced by Proton Radiation in Intact *Arabidopsis* Seeds

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Partial irradiation/Non-targeted effects/Oxidative metabolism/*Arabidopsis thaliana*.

Non-targeted effects induced by ionizing radiation have been demonstrated both *in vitro* and *in vivo*. Previously, we have also demonstrated the existence of non-targeted effects in intact *Arabidopsis* seeds following low-energy heavy-ion radiation. In the present study, 6.5 MeV protons with 8×10^{11} ions/cm^2 and 2×10^{11} ions/cm^2 fluence respectively were used to irradiate non-shielded or partial-shielded *Arabidopsis* seeds to further explore the mechanisms which regulate *in vivo* non-targeted effects and to investigate the difference between damage caused by non-targeted effects and direct irradiation. Results showed that excess reactive oxygen species (ROS) are present in the non-irradiated part of the partially irradiated samples, indicating that *in vivo* non-targeted effects can promote the generation of excess metabolic ROS in the non-irradiated shoot apical meristem/root apical meristem cells. Furthermore, pretreatment with 0.5% ROS scavenger dimethyl sulfoxide (DMSO) or 0.02 mM reactive nitrogen species (RNS) scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) significantly suppresses the non-targeted effects in the partially irradiated samples, while in the whole-body irradiated samples, the cPTIO pretreatment has no effect. On the other hand using antioxidant enzyme assays, superoxide dismutase activity was found to increase for partial irradiated samples and decrease for the whole-body exposed seeds. Taken together, these results implicate that damage caused by non-targeted effects is different from that induced by direct irradiation *in vivo*. Metabolic products such as ROS and RNS are involved in the *in vivo* non-targeted effects.

INTRODUCTION

Formerly, risk assessment of ionizing radiation is based on the detrimental effects occurring in irradiated cells. In recent decades, this classic radiobiological model has been expanded to include non-targeted effects. These effects include genomic instability, clastogenic factors, abscopal and bystander effects.[1)] Radiation-induced genomic instability is characterized by a number of delayed adverse responses including chromosomal abnormalities, gene mutations, and cell death.[2)] Clastogenic factors are found in blood plasma from some irradiated individuals and can cause chromosome breakage in non-irradiated peripheral blood lymphocytes.[3)] Abscopal effects are defined as significant responses occurring in tissues clearly separated from the irradiated volume.[4)] Bystander effects are those effects occurring in cells or individuals which are not directly traversed by radiation but are caused by signals from irradiated cells or individuals.[1)] Many *in vitro* studies have suggested possible mechanisms of non-targeted bystander effects. Bisayee *et al.* used reactive oxygen species (ROS) scavenger dimethyl sulfoxide (DMSO) and gap junction inhibitor lindane to treat co-cultured irradiated and bystander cells, demonstrating that at least a part of non-targeted effects are initiated by free radicals and are likely to be mediated by gap junction intercellular communication.[5)] Yang *et al.* reported that adding Cu-Zn superoxide dismutase (SOD) or catalase (CAT) to the medium decreases the formation of micronuclei and induction of $p21^{Waf1}$ and γ-H2AX foci in bystander human fibroblasts cells. This suggests oxidative metabolism also plays a

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role in the signaling pathways *in vitro* bystander cells.[6] Shao *et al*. reported that downstream of radiation-induced nitric oxide (NO), transforming growth factor β1 (TGF-β1), can be released from targeted T98G cells and plays a key role as a signaling factor in the non-targeted effects by further inducing free radicals and then DNA damage in non-targeted bystander cells.[7] According to these reports, several mechanisms, including secreted soluble factors, oxidative metabolism and gap junction intercellular communication, have been proposed to explain radiation-induced non-targeted effects. There are also evidences that non-targeted effects occur in complex artificial three-dimension model systems, plants and animals.[3,8,9] Belyakov *et al*. proposed that the target for radiation damage is larger than the initial irradiated tissue volume in a three-dimensional human tissue model system.[8] Koturbash *et al.* reported radiation induces DNA damage in bystander tissue more than 1 cm away from the directly irradiated site in mice.[10] Previously, we used 30 KeV ^{40}Ar particles with 1.5×10^{17} ions/cm^2 fluence, whose penetration range in water is just 0.07 μm, to irradiate the intact *Arabidopsis* seeds and demonstrated the existence of low-energy heavy-ion radiation-induced non-targeted effects in intact *Arabidopsis* seeds.[11] However, the mechanisms regulating the signaling responsible for *in vivo* non-targeted effects are not fully understood.

Ionizing radiation is usually used in plant breeding to find new mutagenic source for genetic modification.[12] The postembryonic development and the heredity of a plant are entirely decided by the shoot apical meristem (SAM) and root apical meristem (RAM) cell groups in seeds.[13,14] Therefore, the SAM and RAM cell groups are considered to be the most important irradiation targets of seeds. Traditionally, only high-energy ions are used to find new mutagenic. In the past decades, low-energy ion irradiation also has been shown to have a wide range of biological effects, and is used as a new mutagenic source for genetic modification.[15–17] However, the SAM in seed can not be damaged directly by low energy ions due to their limited penetration. The mechanisms of indirect damage in plant are still not fully understood. Previously, we have used low-energy ions to irradiate *Arabidopsis* seeds and followed the SAM and RAM development.[18] Results showed that despite their SAM/RAM not been directly traversed by low energy ions, these cells also exhibit radiation damage. In the present study, in order to further explore the mechanisms of *in vivo* non-targeted effects and the difference between damage induced by non-targeted and direct irradiation, we used 6.5 MeV protons, which can penetrate through the whole *Arabidopsis* seeds, to irradiate partial shielded or non-shielded samples. After the irradiation, we compared the postembryonic development of the irradiated samples with the controls, and measured the ROS distribution in all samples. Furthermore, we used ROS scavenger DMSO or RNS scavenger cPTIO to block possible signaling pathways. Whole-body irradiated samples were also pretreated with the same scavengers. Antioxidant activities in both the partially and whole-body irradiated samples were assayed to investigate variation of oxidative metabolism in non-targeted and direct irradiation effects.

MATERIAL AND METHODS

Irradiation protocols

Wild type *Arabidopsis thaliana* (ecotype Columbia) were used in this study. The average length of the seeds is about 400–500 μm and the width is about 200–300 μm.[19] Proton irradiation was carried out with a 2 × 6 MV tandem accelerator (Tandem, Peking University, Beijing, China). Ions were extracted in air through a vacuum window made of a 5.8 μm thick titanium foil. The penetration range of the 6.5 MeV protons in water is about 570 μm, linear energy transfer (LET) in seed is about 7 KeV/μm. All the values of proton character are from the SRIM 2006 code (the Stopping and Range of Ions in Matter).[20,21] And according to the reported paper, the ROS generated around the track can only diffusion 4 nm.[22] Therefore, the diameter of direct damaged area around the incident particles is about 4 nm. Considering the dimension of seeds and SRIM calculations, 6.5 MeV protons can penetrate through the entire *Arabidopsis* seeds. All samples were incubated on a moistened filter paper at 4°C for three days before the irradiation (jarovization).

To investigate the direct and non-targeted effects in *Arabidopsis* seeds, samples were irradiated with two different protocols, whole-body irradiation or partial irradiation (Fig. 1 A and B). For the whole-body irradiation, seeds were placed on the water-containing filter papers, and were irradiated by 6.5 MeV protons from one side of the seed surface without shielding. For the partial irradiation, the seeds were shielded with a mask fabricated from two silicon wafers with ~100 μm width slit. To keep the same water content, water-containing filter papers are shielded on another side of the samples. We confirmed that the 6.5 MeV protons can not

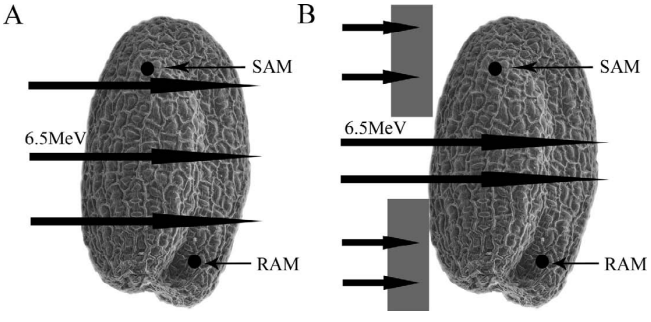

Fig. 1. Diagram of irradiation protocols. (A), 6.5 MeV protons irradiate *Arabidopsis* seed without silicon mask shielding (Whole-body irradiation); (B), 6.5 MeV protons irradiate *Arabidopsis* seed with silicon mask shielding (Partial irradiation). Black circles are the RAM and SAM cell groups respectively.

penetrate through the 500 μm thick silicon wafer. The fluence of ions in the whole-body irradiation is about 2×10^{11} ions/cm^2. To ensure that the same doses are delivered, the fluence of ions in the partially irradiated seeds is about 8×10^{11} ions/cm^2. The control samples were treated with the same process but without radiation exposure.

Growth of Arabidopsis thaliana

Samples were cultured in a growth chamber at 20 ± 0.5°C, 80% relative humidity, and illuminated with a 16 h light/8 h dark cycle (40 μmol photons $m^{-2}s^{-1}$, Philips). To observe the germination rate and the primary root length, seeds/seedlings were grown vertically in the 90 mm Petri dishes just post irradiation. In order to measure the survival ratio and malformation rate, seeds were grown in MS-culture dishes just post irradiation, and then the seedlings were moved to soil from the MS-culture dishes on day 7 post irradiation.

Phenotypic analysis

The germination rates were recorded every 12 hours post irradiation, and the root development differentiations on day 4 after transplanting to MS culture medium were recorded. In brief, pictures were captured with a microscope (Olympus, SZ61TRC) and digital camera (Sony, T400), and then analyzed with Adobe Photoshop 7.0 TM software. The survival ratio was recorded on day 24 after sowing. Seedlings with more than three rosette leaves were counted as surviving. At least 40 seeds/seedlings were used for the germination rate and the root length measurement and at least 100 seeds/seedlings were used for the survival ratio. Data from three independent experiments were pooled together.

Morphologies of mature *Arabidopsis* seedling are observed in this work. The multi-SAM malformation is characterized by the plant having two or more SAM. The dwarf seedling is characterized by the height of mature seedling less than 5 cm. And the infertility malformation is characterized by the mature plant with no seeds. The malformation rates were calculated from the ratio of the malformation number to the total number of irradiated seeds. At least 300 seeds were used for the malformation rate. Data from three independence experiments were pooled together.

Hydrogen peroxides (H_2O_2) assay

In this experiment, DAB was used to measure the amount of H_2O_2 in seeds. After irradiation, embryos without seed coat were immersed in 1 mg/ml DAB solution for 8 hrs in darkness at 25°C. The reaction was then terminated in boiling ethanol (96%) for 10 minutes. Once images of embryo were captured using a microscope (Olympus SZ61TRC), the intensity values were recorded using the Adobe Photoshop 7.0 TM [23,24] with the color been removed by the software. The intensity value of white was record as 0, while that of black was 100.

Treatment with DMSO or cPTIO

In the present experiment, to further investigate the mechanisms of *in vivo* non-targeted effects, the ROS scavenger DMSO (Sigma USA) or the NO scavenger cPTIO (sigma USA) were used to treat the whole-body and partially irradiated samples. The concentrations of the DMSO and cPTIO were 0.5% and 0.02 mM respectively. Pretreatment of non-irradiated samples with 0.5% DMSO or 0.02 mM cPTIO has no effects on the postembryonic development. These concentrations of DMSO and cPTIO were also reported as being non-cytotoxic in the other experiments.[9,25–27] The exogenous scavengers were added during the jarovization period.

SOD activity assayed

We separately investigated the enzyme activities of the whole-body and partially irradiated groups. Enzyme activities of the seedlings were assayed on day 10 post transplanting to MS culture medium. 60 mg leaves of each group were used. SOD activity was assayed with the nitroblue tetrazolium (NBT) method.[28] NBT was reduced to blue black formazan deposits by O_2^-, which has a strong absorbance at 560 nm. However, the presence of SOD inhibits this reaction. To determine the SOD activity, the leaves were ground in liquid nitrogen. The final volume was adjusted to 1.5 ml using 50 mM phosphate buffer (pH 7.8). The mixture was then centrifuged at 1000 rpm for 20 min and the supernatant crude liquid enzyme extracted. The reaction mixture was consisted of 1.5 ml of crude extract liquid enzyme, 0.3 ml of 130 mM methionine (Met), 0.3 ml of 750 μM NBT, 0.3 ml of 100 μM ethylene diamine tetra acetic acid (EDTA), 0.3 ml of 20 μM riboflavin and 0.3 ml of water. The mixtures were under 4000 lx light for 20 min, and then the absorbance of formazan in the supernatants was measured at 560 nm.

CAT activity assayed

The steps of CAT activity measurement are similar to the assay of SOD activity but with different reaction mixture. The reaction mixture contained 0.3 ml of 0.1 M hydrogen peroxide (H_2O_2) and 200 μl of enzyme extracted liquid in 50 mM phosphate buffer (PH 7.8), and was adjust to a final volume of 1.5 ml with water. Reaction mixture was incubated for 2 min at 37°C and the absorbance of each sample was detected for 5 min at 240 nm. The changes in absorbance are proportional to the breakdown rate of hydrogen peroxide.[29]

Statistic analysis

In all cases, the average and standard deviation (SD) were calculated. The statistical significance of the experiments was confirmed by performing Student's *t*-test. A p value of 0.05 or less between groups was considered to be significant.

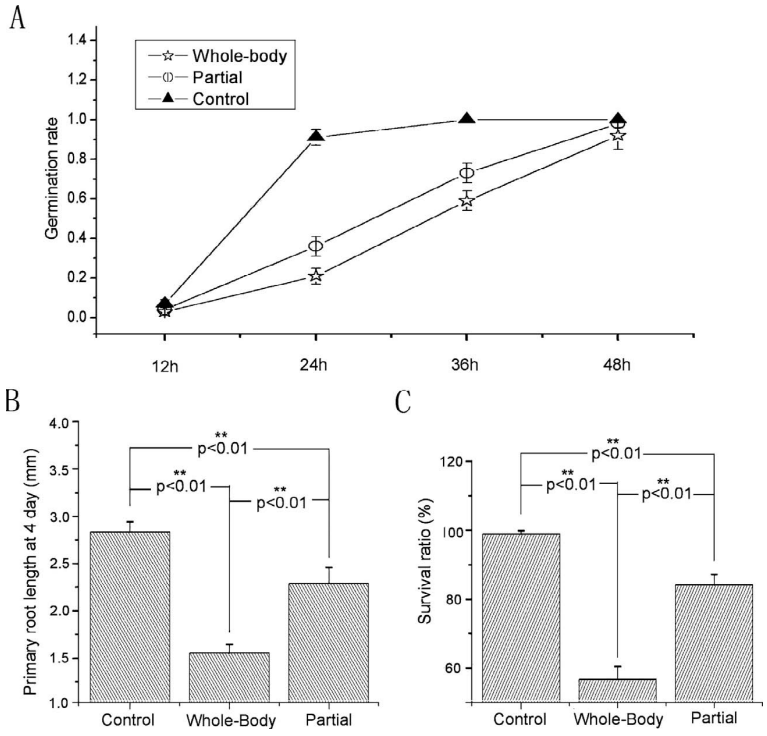

Fig. 2. Both the partial and whole-body irradiation inhibit postembryonic development of samples. (A), Germination rate in 48hrs post transplanting, ☆ means the results of whole-body irradiated group, ○ means the results of partial irradiated group, ▲ means that of the controls; (B), primary root length on day 4 post transplanting; left is the data of whole-body irradiated group, middle is that of the partial irradiated group, while the right is the controls; (C), survival ratio on day 24 post sowing, right is the data of the partial irradiated group, middle is that of whole-body irradiated group, while the left is the controls; At least 40 seeds/seedlings were used for the germination rate and the root length measurement. And at least 100 seeds/seedlings were used for the survival ratio. Data from three independent experiments were pooled. ** means $p < 0.01$, * means $p < 0.05$.

RESULTS

Postembryonic development

End-points of postembryonic development including germination rate, root development and survival ratio are useful for studying plant development and environment induced damage.[30] The germination rate of the whole-body and partially irradiated samples were significantly lower than that in the control seeds at 24 and 36 hrs post irradiation. The germination process was delayed to about 48 hrs after inoculation in the whole-body and partially irradiated groups, while the control seeds had almost completed germination within 36 hrs (Fig. 2 A). The average primary root length of the part irradiated samples on day 4 was significantly shorter than that of the controls (Fig. 2 B). And as shown in Fig. 2 C, the survival ratio of the part irradiated group was also significantly lower than that of the controls. In conclusion, despite the partial irradiated and whole-body irradiated groups were treated with the same dose irradiation, significant differences were found between the two groups in the survival ratio and the primary root length (Fig. 2 B and C).

Table 1. Malformation rates of irradiated *Arabidopsis* seedlings.

	Control (%)	Whole-body (%)	Partial (%)
Multi-SAM	0	1.8 ± 0.9	0.6 ± 0.4
Dwarf	0	4.9 ± 2.4	3.3 ± 1.9
Infertility	0	1.4 ± 1.1	1.2 ± 0.8

Malformation ratio

Some malformations in morphology were observed both in whole-body and partial irradiated samples. Among them, the multi-SAM, dwarf and infertility malformations occurred with relatively high frequency. Multi-SAM malformation has been reported in our previous paper,[31] which is characterized by the plant having two or more SAM. Dwarf seedlings are those mature seedlings which is much lower than normal mature seedlings. The average length of normal seedling is more than 15 cm, while the dwarf seedling is no

more than 5 cm. The malformation rates are listed in Table 1.

As shown in Table 1, whole-body irradiation induced malformation rates are a little higher than partial irradiated samples. In addition, the data suggested that partial irradiation also significantly increased the malformation rates, which suggests that the partial irradiation might affect the postembryonic development of seeds.

ROS distribution

Hydrogen peroxide is the most important long lifetime

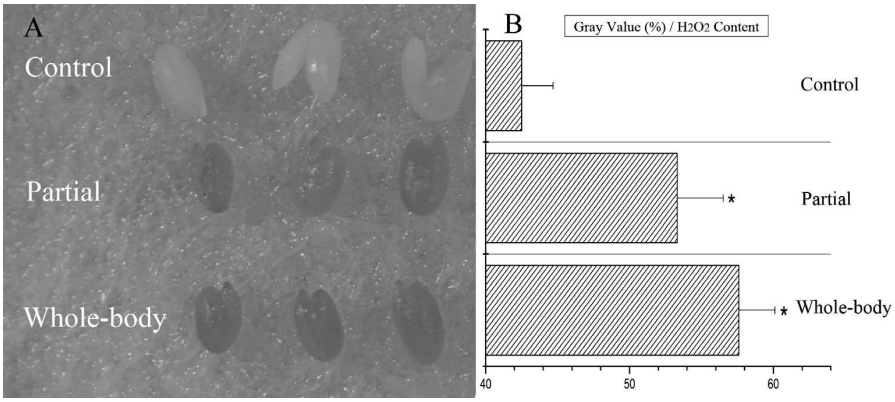

Fig. 3. H_2O_2 assayed with DAB histochemical staining. (A), image of DAB stained seeds, from the top group to the base one respectively are the control, partial irradiated group and whole-body irradiated one; (B), gray value was recorded from the image of staining samples, and it shows the H_2O_2 content in each group. At least 10 embryos from 3 independent experiments were randomly selected to assay the gray values.

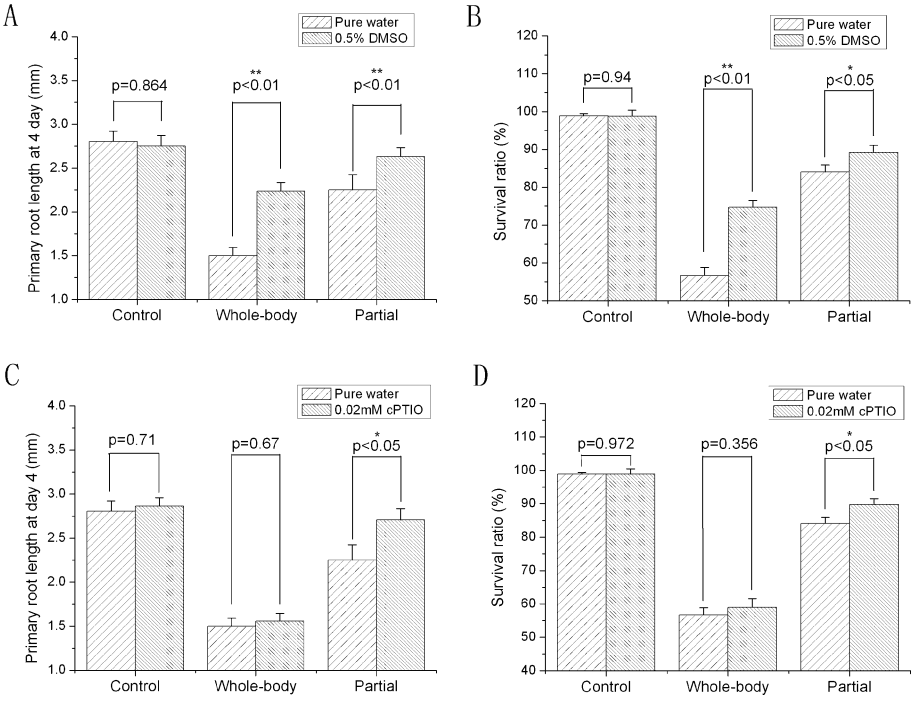

Fig. 4. Both the ROS scavenger DMSO and NO scavenger cPTIO suppress the postembryonic alteration of partial irradiated groups. (A), the average primary root length of 0.5% ROS scavenger DMSO or water pretreated whole-body irradiated, partial irradiated and control seeds/seedlings respectively (B), survival ratios of 0.5% ROS scavenger DMSO or water pretreated whole-body irradiated, partial irradiated and control seeds/seedlings respectively (C), the average primary root length of 0.02 mM NO scavenger cPTIO or water treated whole-body irradiated, partial irradiated and control seeds/seedlings respectively (D), survival ratios of 0.02 mM NO scavenger cPTIO or water treated whole-body irradiated, partial irradiated and control seeds/seedlings respectively. At least 100 seeds/seedlings were used for the survival ratio. At least 40 seeds/seedlings were used to calculate the root length. Data from three independent experiments were pooled. ** means $p < 0.01$, * means $p < 0.05$.

ROS molecule. DAB reacts rapidly with hydrogen peroxides when the presence of peroxidase, forming a brown polymerization product.[32] After immersed in 1 mg/ml DAB solution for 8 hrs, both the whole-body and partially irradiated embryos turned much browner than the controls (Fig. 3 A). Figure 3 B shows the mean gray values in the sample images. The results suggest that the amounts of ROS for both the partially and the whole-body irradiated seeds were much higher than that of the controls. In addition, the non-irradiated cells in part irradiated embryos also turned brown, which suggests that the ROS was detected in the non-irradiated cells of embryos.

Treatment with ROS scavenger DMSO

The exogenous antioxidant DMSO can effectively scavenge free radicals, and is widely used as the ROS scavenger in experiments *in vitro* and *in vivo*.[25,33] As Fig. 4 shows, the postembryonic development including primary root length and survival ratio was significantly rescued in partially/whole-body irradiated groups when pretreated with DMSO. These results indicate that ROS is important for both direct irradiation and non-targeted effects of irradiation.

Treatment with NO scavenger cPTIO

Figure 4 C and D show the average primary root length and survival ratio of the samples pretreated with 0.02 mM cPTIO before irradiation. In the whole-body irradiated groups, both the average primary root length and survive ratios of cPTIO pretreated samples have no rescue effects. However, in the partially irradiated groups, postembryonic developments of the cPTIO pretreated samples have been significantly rescued comparing to the controls. The results suggest that NO plays an important role in the *in vivo* non-targeted effects but might have no effect on the directly irradiation induced damage.

Antioxidant enzyme activity

SOD and CAT are important metabolic superoxide scavengers in cells. The SOD reduces superoxide to hydrogen peroxide, and then the CAT resolves hydrogen peroxide to water. Figure 5 shows that the enzyme activities of metabolic SOD and CAT were enhanced in the part irradiated group. Addition of DMSO or cPTIO showed that SOD and CAT activities were significantly decreased for the partially irradiated samples (Fig. 5 A and B). However, SOD activity was inhibited in the whole-body irradiated group and had no response to the pretreatment of exogenous DMSO or cPTIO. In addition, the CAT activity of the whole-body irradiated group had no significant changes comparing to the controls (Fig. 5 C and D).

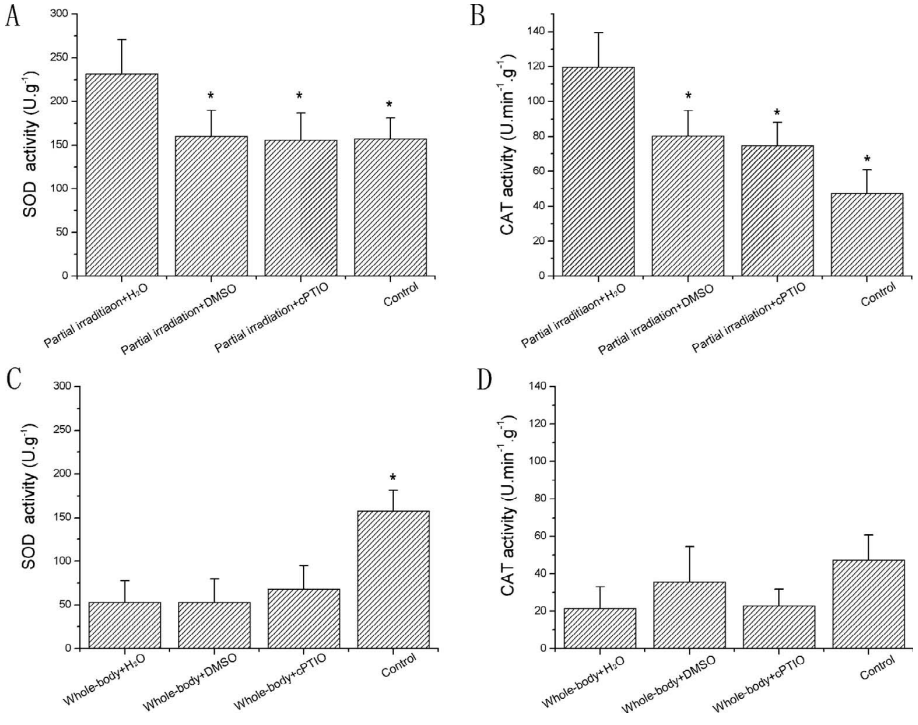

Fig. 5. SOD and CAT activities in water, 0.5% DMSO and 0.02 mM cPTIO pretreated samples. (A), SOD activity in partial irradiated samples (B), CAT activity in partial irradiated samples (C), SOD activity in whole-body irradiated samples (D), CAT activity in whole-body irradiated samples. And at least 40 seedlings were randomly selected to assay the enzyme activity. Data from 3 independent experiments were pooled. * means this group has significant differences with water pretreated partial irradiated samples (A, B) or whole-body irradiated samples (C, D).

DISCUSSION

Verification of radiation induced non-targeted effects and understanding the mechanisms in multicellular systems are important. Previously we have demonstrated the existence of non-targeted effects *in vivo* and suggested that ROS as well as auxin play important roles in the radiation induced non-targeted effects.[18] In the present study, in order to further explore the mechanism of *in vivo* non-targeted effects and to investigate the difference between non-targeted and direct irradiation damage *in vivo*, 6.5 MeV protons were used to irradiate completely or partially shielded *Arabidopsis* seeds.

Germination commences with the uptake of water by seed (imbibition), and is completed when the radicle extends to the testa.[34] After the germination, all of the aerial structures, the stems, leaves and flowers, are derived from the SAM of embryo. In this experiment, there is no significant difference between the germination percentage of whole-body/partially irradiated seeds and the controls at 48 hrs post irradiation. However, the survival ratio of the irradiated seeds significantly decreased. These deaths of seedlings after germination suggest that the SAM cells must be damaged. The slow growth of primary roots in the irradiated samples also demonstrates the RAM cells were damaged.[35] In addition, the malformation rates also clearly proved that the partial irradiation can affect the postembryonic development of SAM cells as whole-body irradiation, though the SAM cells in the partial irradiated samples are not direct damaged by the incident particles. In the whole-body irradiated samples, the SAM and RAM cells can be direct irradiated. The mechanisms of cell damage induced by direct irradiation have been clearly investigated. The critical molecules in cells such as DNA can be directly damaged by the incident ions and the free radicals which generated from the radiolysis around the incident ions.[36] However, in the partially irradiated samples, both the SAM and RAM were protected from direct ions irradiation. The alterations of postembryonic development of the part irradiated seeds definitely proved the existence of non-targeted effects *in vivo*.

In live systems, excess ROS is generated from the radiolysis of water surrounding the incident ion tracks. The ROS is found to persist for milliseconds, and results in oxidative damage on biomolecules such as DNA, proteins, and lipids. The radiolysis generated ROS is believed to be the main damage factor in the direct irradiation.[36] These known theories can easily explain our findings that when pretreated with DMSO, the postembryonic development of whole-body irradiated samples was effectively rescued. However, the action range of radiolysis generated ROS is just several nanometers,[36] which means it is impossible to spread to the SAM/RAM cells. It is well known that there are two different sources of excess ROS in the irradiated cells. Part is from the radiolysis of incident ions with the water in cells and the other part is generated from the metabolism under biotic and/or abiotic stimulation.[37] H_2O_2 is considered to be one of the most important long lifetime ROS. The detected H_2O_2 in the non-irradiated SAM/RAM cell groups most likely is the metabolic H_2O_2. It is probable that signals from irradiated cells stimulate the non-irradiated cells to generate excess metabolic ROS. Feinendegen *et al*. have reported that the excess metabolic ROS can result in cell damage and cell apoptosis.[38] In the present study, it is probable that cells with unirradiated SAM/RAM are damaged by the excess metabolic ROS and result in development alterations. So the pretreatment of partially irradiated samples with DMSO has significantly effects to suppress the damage on SAM/RAM cell groups. These results indicate that the metabolic ROS is one of the most important factors in the *in vivo* non-targeted effects.

In biological systems, normal metabolic NO emerges as a key signaling molecule due to its diffusion potentials and high level of metabolic activity.[39] The diffusible nature of these species coupled with their reactivity make them logical candidates to initiate redox regulatory cascades allowing for communication between different cellular compartments.[40] In the present study, we detected that the NO scavenger cPTIO has effective rescue effect in the partially irradiated samples, but has no effect in the whole-body irradiated samples. This supports the hypothesis that NO is playing a key role in cell signaling between irradiated and non-irradiated cells. Similar results have been observed in the *in vitro* non-targeted bystander effects. Shao *et al*. reported that downstream of radiation-induced NO, TGF-β1 can be released from targeted T98G cells and plays a key role as a signaling factor in the non-targeted effects.[7] In addition, many studies reported that metabolic ROS and NO molecules can act cooperatively to transmit signals in cells.[41,42] The NO molecules stimulate the mitochondria to generate metabolic ROS and in turn are up-regulated by the excess ROS. Both the DMSO and cPTIO can decrease the non-targeted effects induced damage on SAM/RAM cells, implicating that the metabolic ROS and NO may jointly act to transmit the signals in the *in vivo* non-targeted effects.

SOD activity is decreased in the whole-body irradiated group while increased in the part irradiated group. The different alterations of SOD activity implicate there are distinctions between the damage mechanisms of direct irradiation effects and that of non-targeted effects. In the whole-body exposed seeds, the SAM/RAM were damaged through the incident ions and radiolysis generated ROS, while in the partially irradiated seeds, cells were only affected by the excess metabolic ROS. Oxidative metabolism in cells is a balance between oxidants and antioxidants.[43] Such balance might be the reason for the SOD activity increase. The non-irradiated SAM cells enhance the antioxidant activities to scavenger the excess metabolic ROS. Therefore, when the excess metabolic ROS is suppressed by the exogenous scavenger, the

antioxidant activities are also suppressed. In addition, the antioxidant activities also can be suppressed by the exogenous scavenger cPTIO (Fig. 5 A and B). As mentioned above, NO plays a key role in cell signaling between irradiated and non-irradiated cells. Therefore, scavenging the NO signals could reduce the SAM cells damage, and then suppress the enhanced antioxidant activities in partial irradiated samples. However, damage induced in direct irradiated SAM cells cannot be rescued by the SOD or the exogenous ROS scavenger, therefore, pretreatment with DMSO or cPTIO have no significant effect on the antioxidant activities.

In conclusion, results show that the mechanisms of non-targeted effects are different from those regulating the direct effects. Direct radiation damage is induced by ion traversals and radiolysis generated ROS, while non-irradiated cells may be damaged by the excess metabolic ROS. The long-distance signal regulating non-targeted effects is probably cooperatively transmitted by the metabolic ROS and RNS.

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