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Physiological and molecular characterization of the enhanced salt tolerance induced by low-dose gamma irradiation in *Arabidopsis* seedlings

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ABSTRACT

It has been established that gamma rays at low doses stimulate the tolerance to salt stress in plants. However, our knowledge regarding the molecular mechanism underlying the enhanced salt tolerance remains limited. In this study, we found that 50-Gy gamma irradiation presented maximal beneficial effects on germination index and root length in response to salt stress in *Arabidopsis* seedlings. The contents of H_2O_2 and MDA in irradiated seedlings under salt stress were significantly lower than those of controls. The activities of antioxidant enzymes and proline levels in the irradiated seedlings were markedly increased compared with the controls. Furthermore, transcriptional expression analysis of selected genes revealed that some components of salt stress signaling pathways were stimulated by low-dose gamma irradiation under salt stress. Our results suggest that gamma irradiation at low doses alleviates the salt stress probably by modulating the physiological responses as well as stimulating the stress signal transduction in *Arabidopsis* seedlings.

1. Introduction

Salinity is a major abiotic stress that adversely affects plant growth and development. It can cause an elevation of Na^+ and Cl^- concentration in the cytoplasm, which decreases the ability of a plant to take up water and leads to dehydration at cellular level and ultimately the osmotic stress [1]. The excessive amounts of Na^+ and Cl^- ions also result in an ionic imbalance by reducing the uptake of beneficial ions such as K^+, Ca^{2+}, and Mn^{2+} [2]. Furthermore, the increased salt concentrations negatively affect plants growth by impairing metabolic processes and decreasing cytosolic enzyme activities and photosynthetic efficiency [3]. Recently, increasing the salt tolerance of plants has received considerable attention.

Plant salt tolerance involves a complex network of different mechanisms through which plants can sense the stresses and trigger proper responses and signal transduction. Based on the decades of research into the effects of salinity on plant physiology and development, a number of signaling pathways in response to salt stress have been established. The excess cytoplasmic Na^+ concentrations under salt stress can activate the SOS (Salt Overly Sensitive) signaling pathway to maintain low Na^+ [4]. Plants have various members of mitogen-activated protein kinase (MAPK) family that are involved in the regulation of responses to salt and hyperosmosis stress [5]. Furthermore, phospholipid signaling system, such as phosphatidylinositol 4,5-bisphosphate (PIP_2) and the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), also plays an important regulatory role in response to salt stress [6,7]. Therefore, the identification of molecular components in salt-stress-associated signaling pathways is essential for the engineering of salt-tolerant plants.

Gamma rays characterized with the penetration power facilitates its wide application in techniques for plant improvement. The effects of gamma irradiation on the morphology changes and biological responses of plants are dependent on radiation doses [8]. It has been proved that the use of gamma rays at relative low doses has positive effects on a range of plants. The exposure to low-dose gamma irradiation not only stimulates the vegetative growth of plants, such as seed germination index, root and shoot length in lettuce [9], but also improves growth traits related to production, such as panicle number and length, and number of seeds per panicle in rice [10]. Furthermore, the rice seedlings germinated from seeds irradiated with gamma irradiation at low doses showed enhanced biomass production and better growth under saline

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condition compared with control [11]. However, the knowledge about the molecular mechanism underlying the salt tolerance induced by low-dose gamma irradiation still remains limited.

The present study aims to investigate the physiological and molecular responses to salt stress with gamma irradiated *Arabidopsis* seeds and explore the mechanism underlying the salt tolerance induced by low-dose gamma irradiation in *Arabidopsis* seedlings.

2. Materials and methods

2.1. Plant materials, growth conditions and stress treatments

Seeds of *Arabidopsis* Columbia-0 were used for gamma irradiation. For growing seedlings on agar-containing plates, *Arabidopsis* seeds were pretreated with 70% ethanol for 1 min, surface-sterilized in 2.5% bleach for 10 min, and washed with distilled water. The seeds were planted on 1/2 MS medium (Sigma) supplemented with 1% (w/v) sucrose, 1% (w/v) agar (pH 5.8), and placed at 4 °C in the dark for 48 h before germination. Growth conditions were at 23 °C with a 16-h-light/8-h-dark cycle. For salt treatment, the seeds were grown for 7 days after germination in agar-containing plates supplemented with 50 mM or 100 mM NaCl.

2.2. Gamma irradiation

Uniform seeds were randomly divided into two groups: (1) non-irradiated seeds for the controls and (2) seeds exposed to gamma irradiation. Gamma irradiation was conducted using a ^{60}Co [Cobalt-60] gamma source at a dose rate of 8.5 Gy/min. The doses of exposure used in this study were 25, 50, 75, 100, and 150 Gy.

2.3. Morphological observations

Three replicates of 100 seeds each were used for the seed germination test. The seeds were considered germinated when they exhibited a radical extension of 0.2 cm. The germinated seeds were counted daily for a period of five days to determine the germination index.

The germination index is a quantitative expression of germination that relates the daily germination rate to the maximum germination value. The germination index (GI) was calculated using the following formula:

$$GI = \frac{N_1}{1} + \frac{N_2}{2} + \frac{N_3}{3} + \cdots \frac{N_n}{n}$$

where N_1, N_2, N_3, ..., N_n represents the number of seeds that germinated on day 1, 2, 3, ..., n. Each result represents the mean of three biologic replicates.

The root length of irradiated samples treated with salt was measured on the seventh day after vernalization for two days. 30 individual seedlings were measured for each treatment. All of the data represent the mean of three biologic replicates.

2.4. Measurement of ROS content

Seven-day old seedlings (0.50 g of FW) were homogenized in 5 mL of cold acetone, and the homogenate was centrifuged at 10,000*g* and 4 °C for 15 min. The supernatant was collected and added into concentrated hydrochloric acid solution of 0.1 mL 20% $TiCl_4$ and 0.2 mL concentrated ammonia. After a 5-min reaction at 25 °C, the reaction mixture was centrifuged at 8000*g* and 4 °C for 10 min. The pellets were washed with cold acetone twice and added into 3 mL 1 M H_2SO_4. The absorption at 410 nm was measured, and the concentration of H_2O_2 was determined using a standard curve plotted with known concentrations of H_2O_2.

2.5. Determination of antioxidant enzyme activity

Seven-day-old seedlings (0.20 g of FW) were homogenized in a mortar and pestle with 2 mL of 50 mM ice-cold phosphate buffer (pH 7.8) containing 4% PVP and 1 mM EDTA. The homogenate was centrifuged at 10,000 rpm/min and 4 °C for 15 min. The supernatant was used for assaying the activities of SOD, POD and CAT. All of the procedures were conducted at 4 °C.

The superoxide dismutase (SOD, EC1.15.1.1) activity was determined by monitoring the inhibition of the photochemical reduction of NBT. The reaction mixture (3 mL) contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 130 mM methionine, 0.75 mM NBT, 0.02 mM riboflavin, and 0.1 mL enzyme extract. Riboflavin was added as the last component, and the reaction mixtures were illuminated for 15 min. Non-illuminated and illuminated reactions without the supernatant served as calibration standards. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

The peroxidase (POD, EC 1.11.1.7) capacity was measured using guaiacol. The enzyme extract (0.02 mL) was added to the reaction mixture containing 0.02 mL guaiacol solution and 0.01 mL hydrogen peroxide solution in 3 mL of phosphate buffer solution (pH 7.0). The addition of enzyme extract started the reaction and the increase in absorbance was recorded at 470 nm for 5 min.

The catalase (CAT, EC 1.11.1.6) activity was determined by measuring the initial rate of disappearance of H_2O_2. The reaction solution (3 mL) consisted of 50 mM phosphate buffer (pH 7.0), 20 mM H_2O_2, and 0.1 mL enzyme extract. The reaction was initiated by adding the enzyme extract. The decrease of H_2O_2 was monitored at 240 nm for at least 3 min.

2.6. Lipid peroxidation assay

Malondialdehyde (MDA) concentration was determined by the trichloroacetic acid (TCA) reaction. Samples of seedlings (0.30 g fresh weight, FW) were collected and ground sufficiently with 5 mL 10% TCA and a little of SiO_2. After centrifugation with 4000 rpm/min for 10 min, two milliliter supernatant sample was combined with 2 mL 0.6 (w/v) thiobarbituric acid and incubated in boiling water for 15 min. The reaction was stopped by placing the tubes in an ice bath. The mixture was centrifuged at 4000 rpm/min for 15 min and the supernatant was assayed at 532 and 450 nm.

2.7. Measurement of proline content

Seedlings (0.3 g FW) were homogenized with liquid nitrogen. Tissue powders were suspended in 5 mL of 3% sulfosalicylic acid and incubated in boiling water for 10 min. After centrifugation with 3000 rpm/min for 10 min, 2 mL supernatant was mixed with 4 mL acid ninhydrin, 2 mL acetic acid and 2 mL distilled water. The mixtures were incubated at 100 °C for 1 h, and the reaction terminated in an ice bath. The reaction mixtures were extracted with 4 mL toluene and upper phases were collected. The absorbencies were read at 520 nm. The proline concentration was determined from a standard curve and calculated on a fresh weight basis.

2.8. RNA isolation and real-time quantitative RT-PCR

The total RNA from seven-day-old seedlings germinated from irradiated seeds treated with 50 mM or 100 mM NaCl was extracted using the SV Total RNA Isolation Kit (Promega), and the cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen). Polymerase chain reactions were performed in triplicate with a Mastercycler EP Realplex thermal cycler (Eppendorf,

Hamburg, Germany). The reaction system was as followed: 10 μL 2×SYBR® Premix *Ex Taq™* (Takara Bio, Japan), 1 μL of the reverse transcription reaction (1:5 diluted), and 200 nM of each gene-specific primer in a final volume of 20 μL. The following standard thermal profile was used for all PCRs: pre-denaturation at 95 °C for 2 min; denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and prolongation at 72 °C for 20 s, 40 cycles. Data were analyzed using the Realplex 2.2 software (Eppendorf) to calculate cycle threshold (CT) values. The data were analyzed using the comparative C_t ($2^{-\triangle\triangle Ct}$) method. To compare the data from different PCR runs or cDNA samples, the C_t values for the genes were normalized to the C_t value of 18 sRNA, which was a housekeeping gene included in each PCR run. Primers of the genes used are listed in Table S1.

2.9. Statistical analysis

The experiments were completely random designs with at least three replications. For each experiment described above, the results are expressed as the means ± standard errors. One-way analysis of variance (ANOVA) with LSD test was applied to determine the significance of the results between different treatments.

3. Results

3.1. Effects of gamma irradiation on seed germination and plant growth under salt stress

The germination potential of the irradiated seeds under salt stress, which is expressed as the germination index (GI), was higher than that of control seeds with increasing gamma-ray doses of up to 75 Gy. In response to salinity stress with 50 mM NaCl, the exposure to doses of 50 Gy resulted in highest increase of the germination process, compared with the control. The GI obtained with a dose of 100 Gy was slightly decreased, whereas that obtained with 150 Gy was significantly decreased (Fig. 1A). Similar results were obtained under stress of 100 mM NaCl (Fig. 1B).

The biometric measurements of the roots that emerged from the irradiated seeds (25–75 Gy) showed an increase in the root length under salt stress compared with controls. In the treatment with 50 mM NaCl, the maximum increase in root length was recorded with the dose of 50 Gy. An absorbed dose of 100 Gy had no effects on the root length, while with the higher dose of 150 Gy, the radicular system severely decreased compared with the control (Fig. 1C). Similar results were obtained in response to 100 mM NaCl (Fig. 1D). Based on the analysis above, it can be concluded that gamma-ray treatment with a dose of 50 Gy exhibits maximal salt tolerance in terms of the overall growth parameters in comparison with other doses. Thus, we used seeds that were irradiated with the dose of 50 Gy for the subsequent studies.

3.2. Effects of low-dose gamma irradiation on ROS level and lipid peroxidation in response to salt stress

The results in Fig. 2A showed that the concentration of H_2O_2 in control seedlings treated with 50 mM NaCl was 0.712 ± 0.007 μmol g^{-1} FW; while, pretreatment of 50-Gy gamma irradiation decreased the H_2O_2 concentration in seedlings significantly compared with the control under the same stress. Although the treatment with 100 mM NaCl induced higher ROS level, a marked decline of 21% in the H_2O_2 concentration was observed in the irradiated plantlets compared with the non-irradiated samples.

Malondialdehyde (MDA) level, often used as an index for lipid peroxidation, was also measured (Fig. 2B). In response to 50 mM salt stress, MDA content in control seedlings was 0.486 ± 0.004 μmol g^{-1} FW; while, the MDA content was decreased significantly in 50 Gy-gamma ray-irradiated samples compared

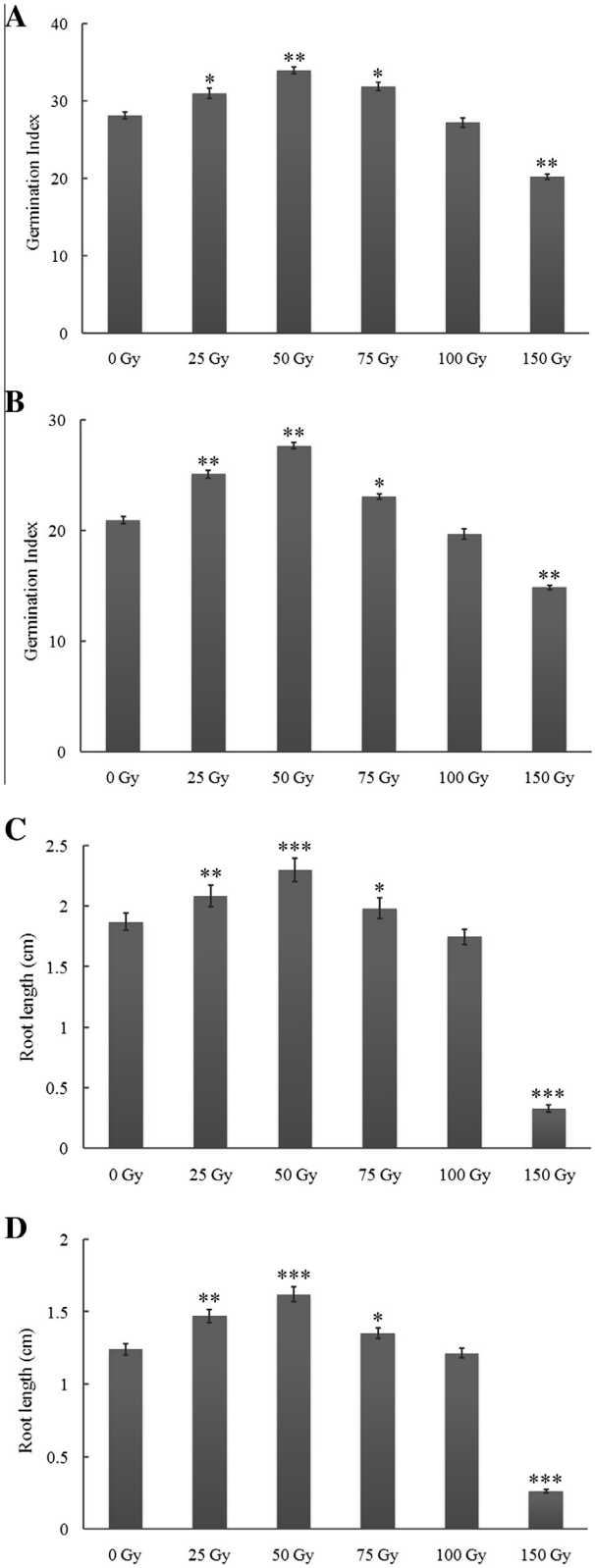

Fig. 1. Analysis of the germination index (A, B) and root length (C, D) in *Arabidopsis* seedlings germinated from the irradiated seeds with a range of gamma rays in response to 50 mM (A, C) or 100 mM NaCl (B, D). 0 Gy was used as the control. The difference in the biological effects was determined through comparisons with the control. *Significant difference ($P < 0.05$). **Highly significant difference ($P < 0.01$). ***Extremely significant difference ($P < 0.001$).

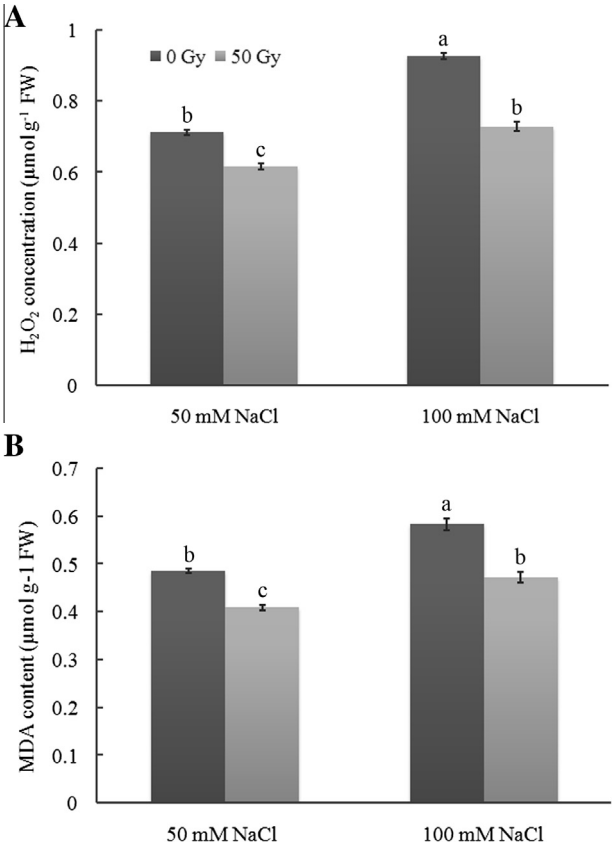

Fig. 2. Analysis of the H_2O_2 concentration (A) and MDA content (B) in *Arabidopsis* seedlings germinated from the 50-Gy gamma-irradiated and control seeds (0 Gy) in response to 50 mM or 100 mM NaCl. Means with different letters above bars were significantly different at $P < 0.05$.

with the control. High MDA content was accumulated with the increase of NaCl concentration. However, pretreatment with low-dose gamma irradiation caused a significant reduction of 19% in the MDA content compared the control under stress with 100 mM NaCl.

3.3. Effects of low-dose gamma irradiation on antioxidant enzyme activities in response to salt stress

Activities of several representative antioxidant enzymes, including SOD, POD, and CAT in *Arabidopsis* seedlings were determined to assess the physiological mechanism of low-dose gamma irradiation in regulation of these antioxidant enzymes upon salt stress. As shown in Fig. 3A, the POD activity under 50 mM NaCl treatment was measured as 1.64 ± 0.1 U min^{-1} g^{-1} FW in control seedlings, while the POD activity in seedlings subjected to 50-Gy gamma irradiation was increased to 2.24 ± 0.12 U min^{-1} g^{-1} FW under the same growth condition; this increase was statistically significant ($P < 0.05$). The analysis of POD activity in 100 mM-NaCl-treated seedlings germinated from the irradiated seeds revealed a marked increase of 23% compared with the level observed in the control. In the case of SOD, pretreatment with 50-Gy gamma irradiation increased the SOD activities significantly in seedlings under stress with 50 or 100 mM NaCl compared with the levels observed in the corresponding control (Fig. 3B). The CAT activities were also found to be greatly increased in the treatment with low-dose gamma irradiation upon salt stress. In response to 50 mM NaCl, the CAT activities increased by 21% in

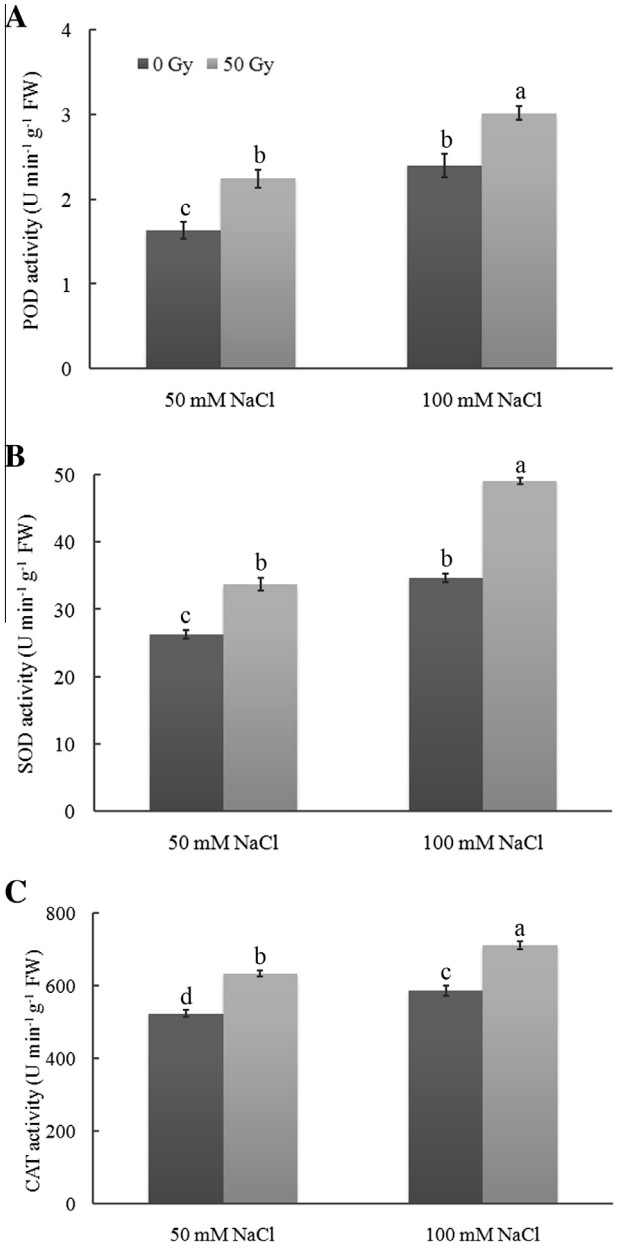

Fig. 3. Effects of 50-Gy gamma irradiation on the activities of antioxidant enzymes under salt stress. See notes to Fig. 2.

gamma-irradiated plantlets compared with the non-irradiated control. Similarly, a marked increase in the CAT activities was obtained in seedlings pretreated with 50-Gy gamma irradiation in response to 100 mM NaCl compared with the activities observed in control seedlings (Fig. 3C).

3.4. Effects of low-dose gamma irradiation on proline accumulation in response to salt stress

To explore whether increased salt tolerance under low-dose gamma irradiation is related to proline accumulation during NaCl stress, seedlings were analyzed for proline levels (Fig. 4). In the treatment with 50 mM NaCl, the proline was significantly accumulated in plantlets irradiated with low gamma rays compared with the proline content in control seedlings. The analysis of proline

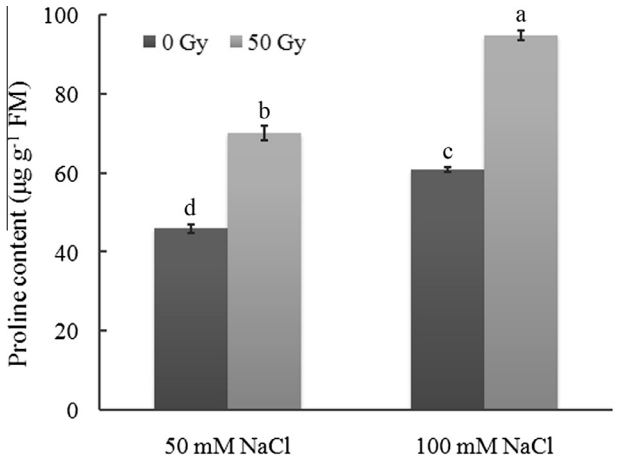

Fig. 4. Effects of 50-Gy gamma irradiation on the proline content under salt stress. See notes to Fig. 2.

accumulation in response to high concentration NaCl revealed a marked increase of 55% ($P < 0.05$) in the irradiated plantlets compared with the non-irradiated samples.

3.5. Expression analysis of genes related to salt stress signaling pathways under low-dose gamma irradiation

To gain more insights into the mechanisms underlying the salt tolerance induced by low-dose gamma irradiation, we characterized the expression of several selected genes associated with salt stress signaling (Table 1). The SOS signaling component SOS3 was transcriptionally up-regulated in gamma irradiated seedlings in response to low or high salt stress compared with the control seedlings. The expression levels of *SOS1* and *SOS2* were up-regulated only during 100 mM NaCl stress. An opposite trend was observed for *MPK4* and *MPK6* gene transcripts in seedlings exposed to 50 or 100 mM salt stress under low-dose gamma irradiation compared with the non-irradiated control seedlings. Moreover, four salt-responsive genes were strongly induced in response to salt stress under 50-Gy gamma irradiation. The transcript levels of *AREB1* and *DREB2B* were significantly enhanced by over 1.5-fold in seedlings irradiated with 50-Gy gamma rays under low or high salt stress compared to the levels in the control seedlings. Similar expression levels of *RD29A* and *RD29B* were recorded in the irradiated seedlings compared with the control, except that the expression of *RD29A* was reduced in response to 100 mM NaCl.

Table 1
Expression levels of the selected genes associated with salt stress signaling in control and irradiated *Arabidopsis* seedlings exposed to 50 or 100 mM NaCl. Data are mean ± standard error of at least 3 replicates. Means with different letters were significantly different at $P < 0.05$.

Gene	Relative gene expression level			
	50 mM NaCl		100 mM NaCl	
	0 Gy	50 Gy	0 Gy	50 Gy
SOS1	0.973 ± 0.038b	0.871 ± 0.009c	1.011 ± 0.065b	1.334 ± 0.046a
SOS2	0.963 ± 0.052a	0.567 ± 0.044c	0.520 ± 0.033c	0.753 ± 0.029b
SOS3	1.043 ± 0.061c	2.567 ± 0.025a	1.892 ± 0.019b	2.819 ± 0.041a
MPK4	0.657 ± 0.064b	0.481 ± 0.042c	1.051 ± 0.072a	0.978 ± 0.028a
MPK6	0.821 ± 0.036b	0.492 ± 0.072c	1.04 ± 0.056a	0.513 ± 0.07c
AREB1	1.047 ± 0.067d	2.567 ± 0.05c	3.399 ± 0.017b	5.224 ± 0.077a
DREB2B	1.036 ± 0.051d	1.586 ± 0.023c	1.873 ± 0.046b	3.352 ± 0.082a
RD29A	1.043 ± 0.061d	2.484 ± 0.219c	16.855 ± 0.248a	11.158 ± 0.109b
RD29B	1.003 ± 0.022d	1.754 ± 0.069c	2.723 ± 0.04b	3.878 ± 0.095a

4. Discussion

Radiation hormetic effects have been defined as stimulation by small doses of ionizing radiation and inhibition at large doses, and this definition has been validated in a variety of organisms. An increasing body of evidence shows that gamma irradiation at relative low doses improves the plant growth in response to salt stress. In the present study, we investigated the physiological and molecular responses of *Arabidopsis* seedlings germinated from irradiated seeds to salt stress. The results showed that gamma irradiation (less than 75 Gy) notably stimulated the growth of seedlings, including the germination index and primary root length under salt stress compared with the non-irradiated samples. In particular, the 50-Gy gamma irradiation displayed the maximal positive effects on all of the growth parameters analyzed, and this finding is consistent with other reports. Pre-exposure to gamma irradiation at a dose of 50 Gy alleviates the adverse effects of salinity on the growth parameters of cowpea plants, such as root and shoot length, dry weight of root and shoot, area of leaves and photosynthetic pigments [12,13]. The biopositive effects of low-dose gamma irradiation on plant growth under salt stress may be attributed to synthesis activation of nucleic acids and total soluble protein during seedling growth after seed irradiation [12,14]. In our study, we found that low-dose gamma irradiation modulates the anti-oxidative defense and gene expression related to salt stress pathways in *Arabidopsis* seedlings in response to salt treatment (see discussion below).

Various environmental stresses trigger the generation of reactive oxygen species (ROS) including short-lived free radicals and the predominant secondary ROS, H_2O_2. The excess ROS cause lipid peroxidation and disturb normal cellular metabolism. In this study, we found that the contents of H_2O_2 and MDA, an indicator of lipid peroxidation, were significantly reduced in the irradiated seedlings compared with controls under salt stress. The enhanced defense to ROS damage was probably due to the increased activation of antioxidant enzymes which can improve salt tolerance [15]. Our results indicated that the enzymatic activities of POD, SOD and CAT were all significantly increased to different extents in response to salt stress under low-dose gamma irradiation compared with the controls. Moreover, CAT showed much higher activities than SOD and POD in both the irradiated and non-irradiated samples, suggesting that H_2O_2 is the main free radical of the ROS induced by salt stress. Our results are supported by previous reports. The increased activities of SOD, POD, APX and CAT and reduced lipid peroxidation and H_2O_2 values were recorded in gamma irradiated lemon shoot under salinized condition [16]. Recently, Macovei et al. (2014) reported that pretreatment with low-dose gamma irradiation increased the enzymatic activities of APX, CAT, and GR and corresponding gene expression profiles in rice seedlings subjected to salt stress [17], indicating that the stimulated transcription of genes for antioxidant enzymes contributes to the salt tolerance enhanced by low-dose gamma irradiation. Taken together, our results suggest that increased activities of POD, SOD, and CAT induced by low-dose gamma irradiation help scavenge the excess ROS produced under salt stress and contribute to the maintenance of the relative nontoxic level of ROS in cells.

Proline, which contributes to the balance in osmotic potential, commonly accumulates in stressed seedlings. Soybean plants irradiated with low gamma rays promoted the accumulation of proline under salt or drought stress [13,18]. Our results also indicated that proline level was significantly increased in 50-Gy gamma irradiated seedlings under salt stress compared with the non-irradiated samples. Given that proline can confer enzyme protection and stabilize the structure of membrane and macro molecules upon stress, our results suggest that the enhanced salt tolerance in irradiated

Arabidopsis seedlings was partly due to the induction of proline. However, the molecular mechanism of proline accumulation by gamma irradiation needs further investigation.

The genome-scale profiling of transcripts has been shown that the gamma-radiation-responsive genes in *Arabidopsis* are distinctively regulated and most of the altered genes are associated with general metabolism, ROS scavenging and signal transduction [19,20]. However, it is unclear how low-dose gamma irradiation regulates the expression of genes responsible for the signaling pathways under salt stress. In our study, the increased mRNA levels of SOS-signaling-related genes were observed in the irradiated samples under salt stress compared with the controls. These results maybe explain the less Na uptake in gamma-irradiated seedlings [11], since SOS2 and SOS3 together activate and regulate the transport activity of SOS1, a plasma membrane Na^+/H^+ antiporter, which is required for the export of Na^+ out of the cell [1]. However, the genes selected from MAPK signaling were down-regulated, indicating that MAPK-related genes were insensitive to low-dose gamma irradiation in NaCl-treated seedlings. Interestingly, both the early-response genes *AREB1* and *DREB2B* and the delayed-response genes *RD29A* and *RD29B* [1] were markedly up-regulated in the irradiated seedlings exposed to salt stress, suggesting that the stress-responsive genes were stimulated directly or indirectly by low gamma rays, which contributes to the tolerance to salt stress. Thus, the vastly different transcriptional profiles support the enhanced tolerance to salt treatment as a result of low-dose gamma irradiation in *Arabidopsis* seedlings.

In conclusion, applying low-dose gamma irradiation to *Arabidopsis* seeds prior to NaCl treatment could obviously alleviate the detrimental effect of salt stress on growth through improving antioxidant enzyme system, reducing oxidative stress, promoting proline accumulation and stimulating salt stress signal transduction. Our results extend the available knowledge of the mechanisms underlying the salt tolerance induced by low-dose gamma irradiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.086.

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