Biochemical Responses of Soybean (*Glycine max* L. Merr.) to Proton Beam Irradiation

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ABSTRACT The present study evaluated the biochemical effects of proton beam irradiation in soybean. Seeds of two Korean elite cultivars (Kwangan and Pungsannamul) were irradiated by a 57-MeV proton beam in the range of 50-400 Gy. We measured the contents of malondialdehyde (MDA), antioxidant enzymes, and chlorophyll. MDA contents in proton beam-irradiated plants were higher than those in control plants. The activity of antioxidant enzymes differed between the two cultivars. In Kwangan, ascorbate peroxidase (APX) activity increased by 29% relative to the control at 55 Gy and decreased by 58% at 308 Gy. Superoxide dismutase (SOD) and peroxidase (POD) activities decreased by 47 and 25% relative to the control at 55 Gy and recovered to 87 and 56% of that at 55 Gy with 172 Gy and 117 Gy, respectively. In Pungsannamul, APX and SOD decreased by 32 and 35% relative to the control at 62 Gy, with the highest value observed at 243 Gy. In terms of the chlorophyll content, the two varieties responded similarly to proton beam irradiation, whereas in Kwangan, no significant reduction was observed above 100 Gy when compared with the control. Proton beam irradiation affected chlorophyll b more than chlorophyll a. These results show that the activity of antioxidant enzymes decreased in response to irradiation with approximately 50 Gy proton beams, then increased gradually with increasing doses, followed by a moderate decrease at higher doses. According to correlation with MDA contents and antioxidant enzyme activities, defense system of Pungsannamul was less activated by proton-beam irradiation than that of Kwangan.

Keywords Soybean, Mutant, Proton beam, Biochemical response

INTRODUCTION

New varieties have been developed through cross-breeding techniques; however, hybridization with the same or very closely related species is limited in its ability to create new and diverse cultivar traits (Lee *et al.* 2015a). Mutation breeding is an alternative method used to generate variation and is used to improve agronomic traits, such as resistance to stress, physical parameters of grain, and nutritional value (Ling *et al.* 2013; Lee *et al.* 2015a). Mutations are induced by chemical or physical mutagens. Chemical mutagens such as ethylmethanesulfonate (EMS), N-nitroso-N-methylurea (NMU) and colchicine have long been used to develop new traits in crops, but these che-

micals are carcinogenic and difficult to use (Sung *et al.* 2013). Ionizing radiation can overcome the limitations of chemical treatment and generate new mutations over a short period of time (Lee *et al.* 2015b).

For 80 years, gamma-rays have been used to induce mutations and generate new agronomic traits, mainly in crop mutation breeding programs. Gamma-rays emitted from a ^{60}Co source are a type of low linear energy transfer ($\text{LET} = 0.2 \text{ keV}/\mu\text{m}$) radiation, and induce mutations ranging from point mutations to chromosomal aberrations (Du *et al.* 2014). Recently, high energy ion beams, including proton, helium, carbon, and argon ions, have been considered as new effective mutagens. Ion beams were shown to exhibit greater biological effects on growth

Received March 14, 2017; Revised April 20, 2017; Accepted April 24, 2017; Published June 1, 2017

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inhibition and lethality than gamma-rays (Tanaka *et al.* 2010). In addition, ion beams induced a high frequency and a broad spectrum of mutant phenotypes (Tanaka *et al.* 2010).

The biological effects of ionizing radiation are induced in multiple steps, from the initial absorption of energy to the final biological injury (Esnault *et al.* 2010). The initial reactions include the formation of an H atom and an OH radical following water radiolysis. OH radicals are the most highly reactive oxygen species (ROS) (Gill and Tuteja 2010). OH radical rapidly attacks macromolecules and leads to irreparable metabolic dysfunction and cell death (Scandalios 1993). In addition, water radiolysis produces secondary ROS such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radicals ($^{\bullet}OH$), and singlet oxygen (Wi *et al.* 2007). ROS is also generated by various metabolic pathways, such as photorespiration and the oxidation of fatty acids in mitochondria and chloroplasts (Garg and Manchanda 2009; Weisany *et al.* 2012), as well as biotic and abiotic factors, such as drought, salinity, chilling, metal toxicity, UV-B radiation, and pathogen attack (Sharma *et al.* 2012; Weisany *et al.* 2012). The high accumulation of ROS by ionizing radiation can cause oxidative damage to lipids, proteins, and DNA, which result in increased peroxidation (degradation) of lipids, site-specific amino acid modification, fragmentation of peptide chains, DNA-strand breakage, and various nucleotide modifications (Sharma *et al.* 2012).

Conversely, excess accumulation of ROS in plants triggers the activity of antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), and catalase (CAT) (Lee *et al.* 2016). SOD acts as the first enzyme in the scavenging process, converting superoxide radicals to H_2O_2 (Kim *et al.* 2012). APX and POD play important roles in H_2O_2 detoxification in cells (Foyer and Noctor 2005; Wi *et al.* 2007). APX uses ascorbate as the reducing agent of H_2O_2 (Foyer and Noctor 2005). CAT also dismutates H_2O_2 into H_2O and O_2 , mostly in peroxisomes (Gill and Tuteja 2010). Evaluating the contents of antioxidant enzymes can be used to determine the level of oxidative stress caused by ROS.

Proton beams are composed of particles with a mass and electrical charge, and a lower LET than heavy ion beams

(Lee *et al.* 2015b). In this study, we irradiated soybean seeds of two elite Korean varieties with different doses of proton beams. We measured physiological parameters such as antioxidant enzymes, chlorophyll, and malondialdehyde (MDA) to study the effects proton-beam irradiation.

MATERIALS AND METHODS

Plant material

Two Korean soybean cultivars including ‘Pungsannamul’ and ‘Kwangan’ were used in this study. Pungsannamul has a small seed size (10.6 g per 100 seeds) and is suitable for soybean sprouts (Suh *et al.* 1997). Kwangan also has a small seed size (11.6 g per 100 seeds) and is suitable for soybean sprouts (Lee 2012).

Proton-beam irradiation

The seeds of all cultivars were irradiated using a proton beam at 57 MeV with 100 MeV proton linear accelerator of the Korea Multi-Purpose Accelerator Complex (KOMAC) in Gyeongju, Korea. Pungsannamul was irradiated at doses of 62, 110, 168, 243, and 316 Gy. Kwangan was irradiated at doses of 55, 117, 172, 246, and 308 Gy. Irradiated and non-irradiated seeds were planted in 50-cell plastic trays ($27 \times 53 \times 11.2$ cm) filled with potting mix (coco peat, peat moss, zeolite, perlite, caldolomite, wetting agent, and fertilizer) and cultured in a greenhouse. Each seed was planted in an individual cell. Three replicates were performed for each treatment, and each replicate comprised of one tray of 50 plants. Leaves from irradiated and non-irradiated plants were harvested 4 weeks after planting and stored at $-80^{\circ}C$ for biochemical analyses.

Measurement of MDA content

The extent of lipid peroxidation was determined by measuring MDA content using the trichloroacetic acid (TCA) method as determined by Zhang *et al.* (2011). Leaf samples (0.2 g) were homogenized with 1.5 mL 10% TCA for 30 minutes in a refrigerator. Samples were vigorously vortexed before centrifugation at 10,000 g for 20 minutes at $4^{\circ}C$ and supernatants were mixed with an equal volume of

0.67% 2-thiobarbituric acid. The mixtures were heated at 95°C for 30 minutes followed by cooling on ice for 5 minutes and centrifugation at 10,000 g for 5 minutes at 4°C. The MDA concentrations were determined by measuring the absorbance at 450, 532, and 600 nm using an Epoch microplate spectrophotometer (Bio Tek Instruments Inc., USA) and were calculated as follows:

$$\text{- MDA } (\mu\text{mol L}^{-1}) = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450} = C1$$

$$\text{- MDA (nmol [g FW]}^{-1}) = C1 \times V/W$$

Measurement of antioxidant enzymes

The total soluble protein content in irradiated and non-irradiated plants was determined using the Bradford method (Bradford 1976). Powdered samples (0.2 g each) in a 2.0-mL tube were mixed with 1.2 mL 0.2 M potassium phosphate buffer (pH 7.8 + 0.1 mM EDTA) and homogenized thoroughly, followed by centrifugation at 10,000 rpm for 20 minutes at 4°C. To determine the total soluble protein content, 2 μL of supernatant was added to 8 μL ddH₂O and 190 μL 1X dye reagent. The absorbance at 595 nm was determined using a spectrophotometer. The absorbance was compared to a standard curve plotted using bovine serum albumin (BSA) (Bio-Rad Laboratories, Inc., USA) with standard concentrations of 0, 125, 250, 500, 750, 1,000, 1,500, and 2,000 $\mu\text{g/mL}$. The total soluble protein content of the samples is expressed in mg/g fresh weight (FW) of plant material.

APX activity was measured using the method described by Nakano and Asada (1981). The reaction solution contained potassium phosphate buffer (pH 7.0), 5 mM ascorbate, 1 mM EDTA, and 50 mM H₂O₂. Enzyme activity was determined from the initial rate of the reaction using the extinction coefficient of ascorbate, and the absorbance was monitored at 290 nm using a UV spectrophotometer for 5 minutes.

SOD activity was measured by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture consisted of 50 mM potassium phosphate (pH 7.8), 100 mM EDTA, 1 mM NBT, 100 mM methionine, 0.2 mM riboflavin, and 1% Triton-X100. The reaction mixture was placed under strong light conditions for 10 minutes. SOD activity was

measured at a wavelength of 560 nm by UV spectrophotometry (Beyer and Fridovich 1987).

POD activity was measured using the method described by Kim *et al.* (2012). The reaction mixture contained 100 mM potassium phosphate (pH 6.0), 100 mM pyrogallol, and 100 mM H₂O₂. POD activity was measured by monitoring changes in the absorbance at 420 nm for 1 minute, which resulted from the oxidation of pyrogallol.

Measurement of chlorophyll content

Fresh leaf samples (0.2 g) were ground in liquid nitrogen using a mortar and pestle, and homogenized in 1.5 mL 80% acetone. The homogenized samples were then kept overnight in the dark at 4°C. The samples were centrifuged at 3,000 rpm at 4°C for 15 minutes. The supernatant was transferred to a new tube. The absorbance of each supernatant at 645 and 663 nm, and concentrations, were calculated as previously described (Ni *et al.* 2009).

Statistical analysis

In this study, three replicates were used to assess biochemical responses. The results were subjected to analysis of variance using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Separation of means was performed using the DMRT test at $P < 0.05$.

RESULTS

Effect of proton beam irradiation on MDA content

In this study, the seeds of two soybean cultivars were irradiated with five different doses of proton beam, and the MDA content was determined using the leaves of 4-week-old soybean plants (Fig. 1). In Kwangan, there was no significant difference among the control and proton beam-irradiated plants. However, all proton beam-irradiated plants showed higher MDA contents than the control plants. In particular, the MDA content of Kwangan peaked at a dose of 55 Gy, and then decreased with increasing doses. In Pungsannamul, the MDA contents of treated plants tended to increase with increasing dose. A dose of 316 Gy resulted in the highest MDA value, which was increased by 46% in comparison to the control.

Fig. 1. Effect of proton beam irradiation on the total MDA content of leaves from soybean cultivars. Mean separation within columns by LSD test at $P = 0.05$. (A) Kwangan (B) Pungsannamul.

Fig. 2. Effect of proton beam irradiation on the total SOD activity of leaves from soybean cultivars. Mean values were separated using the DMRT test at $P < 0.05$. (A) Kwangan (B) Pungsannamul.

Effect of proton beam-irradiation on antioxidant enzymes

To evaluate the effect of proton beam treatments on the activities of antioxidant enzymes, SOD, POD, and APX activities were measured in two soybean cultivars. In both cultivars, the highest SOD activity was found in control plants and the lowest SOD activity was observed in plants irradiated with proton beams below 100 Gy (Fig. 2). In

Kwangan, SOD activity decreased to 48% that of the control at 55 Gy and recovered to 87% that of 55 Gy at 172 Gy. In Pungsannamul, SOD activity decreased by 35% that of the control at 62 Gy, and recovered to 38% that of 62 Gy at 243 Gy. The recovered SOD activity in both Kwangan and Pungsannamul remained lower than that of the control. Furthermore, the SOD activities of both cultivars decreased again in response to proton beam-irradiation over 300 Gy.

In Kwangan, POD activity followed a similar pattern to

Fig. 3. Effect of proton beam irradiation on the total POD activity of leaves from soybean cultivars. Mean values were separated using the DMRT test at $P < 0.05$. (A) Kwangan (B) Pungsannamul.

Fig. 4. Effect of proton beam irradiation on the total APX activity of leaves from soybean cultivars. Mean values were separated using the DMRT test at $P < 0.05$. (A) Kwangan (B) Pungsannamul.

SOD activity. POD activity decreased to 25% that of the control at 55 Gy and recovered to 56% that of 55 Gy at the dose of 117 Gy. Then, POD activity decreased again at doses exceeding 200 Gy. In Pungsannamul, the highest value observed was 114% that of the control at 243 Gy, and the lowest value was 92% that of the control at 168 Gy (Fig. 3).

APX isoforms have a higher affinity for H_2O_2 than CAT or POD (mM range), which may play a more crucial role in the management of ROS during stress (Gill and Tuteja 2010; Sharma *et al.* 2012). The APX activities of the two

cultivars showed a reverse tendency. In Kwangan, a dose of 55 Gy resulted in the highest activity, which was increased by 29% compared with the control. Conversely, a dose of 308 Gy resulted in the lowest value, which decreased by 58% compared with the control. In Pungsannamul, APX activity seemed to follow a similar trend to SOD activity. A dose of 62 Gy resulted in the lowest value, decreasing to 32% of that observed in the control plant. APX activity increased with increasing proton beam dose, up to 243 Gy, after which activity decreased again (Fig. 4).

Effect of proton beam irradiation on chlorophyll contents

The chlorophyll content of two varieties, Kwangan and Pungsannamul, followed a similar tendency, such that the content of chlorophyll b and total chlorophyll increased in response to low-dose proton beams and decreased above 100 Gy (Fig. 5). Borzouei *et al.* (2010) reported an increase in chlorophyll content in wheat seedlings irradiated by low-dose gamma-rays, which might be due to growth stimulated by gamma-ray irradiation. In the present study, proton beams around 50 Gy increased plant height compared with the control (data not shown). Therefore, low-dose proton beams could also stimulate plant growth resulting in some increase in chlorophyll content.

In response to proton beams above 100 Gy, chlorophyll b and total chlorophyll contents in Kwangan were not significant different compared with those of the control, while Pungsannamul showed a significant reduction of chlorophyll b and total chlorophyll contents compared with the control.

DISCUSSION

In the present study, biochemical responses of two proton beam-irradiated soybean cultivars are reported. In Kwangan, the MDA contents of all treatments were slightly higher than that of control, but these increases did not show significant different. In Pungsannamul, the MDA content increased significantly in response to increasing proton beam dose and the increase at a dose of 316 Gy was 46% compared to the control. Lipid peroxidation is a sign of oxidative damage, and the level of lipid peroxidation can be determined by measuring the MDA content, which is an end-product of lipid peroxidation, and has been used as indicator of plant membrane stress (Kim *et al.* 2015; Lee *et al.* 2015b). Lee *et al.* (2015b) reported that MDA contents increased in response to increasing gamma-ray and proton beam doses, and that *Cymbidium* irradiated with proton beams at 100 MeV showed higher MDA contents than *Cymbidium* irradiated with gamma-rays. In addition, Alikamanoglu *et al.* (2011) reported MDA content of irradiated soybean had similar tendency with that of Pungsannamul. However, the increase at 300 Gy gamma-ray exposures was 12% compared to non-irradiated controls. These results indicate that proton beams lead to greater

Fig. 5. Effect of proton beam irradiation on the chlorophyll content of the leaves of soybean cultivars. Mean values were separated using the DMRT test at $P < 0.05$. (A) Kwangan (B) Pungsannamul.

oxidative damage of plant membranes than gamma-ray.

Antioxidant enzymes play key roles in cellular defense mechanisms against oxidative damage induced by ROS. SOD enzymes represent the first-line defense against oxidative stress. The SOD activity of the two cultivars had followed a similar ‘falling-rising-falling’ pattern with increasing doses of proton beams. This ‘falling-rising-falling’ of SOD activity was also found in rice in response to N^+ beams (Zhao *et al.* 2013). In addition, SOD and POD activities in Kwangan responded in a similar way to proton beam irradiation, while SOD and APX activities in Pungsannamul responded similarly. Furthermore, we found that SOD activity had the opposite effect on MDA content. The results of a previous study revealed similar results, such that SOD activity had a negative relationship with MDA content or lipid peroxidation because SOD scavenged superoxide, which interrupts vital biomolecules and inactivates CAT and POD (Esfandiari *et al.* 2007).

In the present study, the activity of antioxidant enzymes differed between cultivars. The sum of APX and POD activity below 200 Gy seemed to be similar trend in the two cultivars. Above 200 Gy, the total activity reported for Pungsannamul was higher than that for Kwangan. According to Kim *et al.* (2012), diverse antioxidant response systems in higher plants have evolved, and different mechanisms exist for scavenging ROS generated by water radiolysis when exposed to different ionization treatments. This is because radio-sensitivity depends on chromosome density, DNA contents, the ability to restore damaged DNA, the activity of antioxidant enzymes, and the moisture content of a species or cultivar (Oh *et al.* 2003).

Reduced chlorophyll contents are a useful indicator of leaf senescence and an important parameter of physiological reactions to stresses (Kim *et al.* 2012). The highest value of total chlorophyll was observed following irradiation below 100 Gy and was gradually decreased in the two cultivars above 100 Gy. However, a significant decrease was only observed in Pungsannamul. Consistent with the present results, Ling *et al.* (2013) reported that the highest chlorophyll content appeared in response to 40 Gy and a reduction in chlorophyll content was observed above 100 Gy following irradiation with carbon ion beams in rice. Kalimullah *et al.* (2003) suggested that 1H heavy-ion

radiation inhibited chlorophyll biosynthesis. In addition, a reduction in the chlorophyll concentration was observed following irradiation due to the breakdown of the porphyrin ring of the chlorophyll molecule. Interestingly, high-dose irradiation induced noticeable structural changes in chloroplasts, which exhibited swollen and destructed thylakoids (Wi *et al.* 2007). In the same way as heavy-ion beam and gamma-ray irradiation, proton beams might destroy photosynthetic pigments and adversely affect the photosynthetic capacity of plants.

In this study, proton beam irradiation was also found to result in a greater reduction in the contents of chlorophyll b compared with chlorophyll a in both cultivars. Similar results have been reported in previous studies on *Orthosiphon stamineus* (Kiong *et al.* 2008) and *Zea mays* (Marcu *et al.* 2013) exposed to gamma irradiation, and *Oryza sativa* irradiated by carbon ion beams (Ling *et al.* 2013). This severe reduction in chlorophyll b following ionizing radiation is due to the destruction of chlorophyll b biosynthesis or degradation of its precursors (Ling *et al.* 2013). During photosynthesis, chlorophyll b is present only in the light harvesting antenna complex I (LHC I) and light harvesting antenna complex II (LHC II), while chlorophyll a is present in the photosystem cores and light harvesting antenna complexes (Voitsekhovskaja and Tyutereva 2015). Therefore, the reduced levels of chlorophyll b following ionizing radiation suggest that the LHC I and LHC II of photosystems are damaged.

The results of this study showed that proton beams affect soybean plants by inducing plant membrane damage and oxidative stress and reducing chlorophyll contents. The changes in antioxidant enzyme activities against oxidative damage were shown to be ‘falling-rising-falling’ pattern with increasing doses of proton beams in a cultivar-dependent manner.

ACKNOWLEDGEMENTS

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP: Ministry of Science, ICT and Future Planning; No. NRF-2016M2B2A4908203) and the Korea

Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries (IPET) through the Agri-Bio Industry Technology Development Program, as well as by the Ministry of Agriculture, Food and Rural Affairs (MAFRA), Republic of Korea (No. 315025-03-1-HD060).

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