

Silicon-mediated Mitigation of Wounding Stress Acts by Up-regulating the Rice Antioxidant System

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Silicon (Si) is essential for normal growth and development in plants and is also beneficial for their responses to wounding. However, the mechanisms by which Si acts to mitigate the effects of wounding is not fully understood. This effect possibly occurs through a reduction in the oxidative stresses associated with wounding. Here, we tested this possibility by investigating the effects of applying different concentrations of Si (0.5 and 1.0 mM) to rice plants under wounding stress for a period of 6 and 12 h. We found that a higher uptake of Si was significantly associated with an increase in leaf chlorophyll content. In response to wounding induced oxidative stress, the extent of lipid bilayer peroxidation was reduced in a dose-dependent manner by Si application for 6 or 12 h. Activity of the catalase enzyme was initially lowered by Si treatment; however, at 1.0 mM Si, catalase activity increased significantly after 12h of wounding stress. A similar response was also observed for a peroxidase enzyme. Polyphenol oxidase showed a significant reduction in activity. We conclude that Si application does not only improve leaf chlorophyll content but can also overcome the oxidative stress due to wounds or physical injuries.

Keywords: silicon, antioxidant enzymes, wounding stress, lipid peroxidation, *Oryza sativa japonica*, time and dose dependent effect

Instructions

Mechanical or physical injuries and wounds to crop plants can drastically hinder both plant growth and yield, especially of rice plants. Such wounds can develop by lodging due to the effects of strong winds or by herbivory attacks. The maintenance of physical strength and integrity in rice plants is essential to achieving sustainable yields. In this regard, the application of silicon (Si) offers a possible means of avoiding negative effects (Ma and Takahashi 20002; Massey and Hartley, 2009). Si is one of the most abundant minerals in the soil. It has strong affinity with oxygen in the soil and usually exists as

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silica, silicic acid, and silicates, depending upon soil pH (Ma and Yamaji 2006). In natural conditions, plants uptake Si in its silicic acid form [$n\text{Si}(\text{OH})_4$] via the roots (Ma and Takahashi 2002). From the roots, the Si is transferred to the shoot through vascular bundles and forms silica cells and silica bodies (Ma and Yamaji 2008; Isa et al. 2010). The accumulated silicon in rice leaves improves photomorphogenesis, which in turn, increases the photosynthetic rate and also strengthens the cuticle membranes. The provision of additional Si to the rice plant promotes growth parameters such as length, weight, and chlorophyll contents (Kim et al. 2014a). Similar effects have also been reported in soybeans, cotton, poinsettia, wheat, maize, rice, sorghum cucumber, and tomatoes (Li et al. 1989; McAvoy and Bible 1996; Gong et al. 2005; Hattori et al. 2005; Liang et al. 2005; Feng et al. 2009; Hamayun et al. 2010; Kim et al. 2011; Kim et al. 2014b, c).

Wounds expose plants to microbial pathogens that further increase the intensity of stress. As a consequence, reactive oxygen species (ROS) are produced (Leon et al. 2001). Various ROS such as superoxide (O_2^-), hydroxyl radical (OH^-), oxygen (O_2) and hydrogen peroxide (H_2O_2) are rapidly formed in plant cells, where they can potentially interact with many cellular components (Zhu et al. 2004). The induction of ROS triggers peroxidative reactions and damages cellular membranes and essential macromolecules, such as photosynthetic pigments, proteins, nucleic acids, and lipids (Lin and Kao 2000; Zhu et al. 2004).

Stressful conditions result in the breakdown of functional membranes and the formation of malondialdehyde (MDA). This α -linoleic acid is a major component of plant membranes and is a precursor of the phytohormone jasmonic acid (Weber et al. 2004; Wastermack et al. 2006; Kim et al. 2014a). MDA activity is often used as an indicator of the stress perceived by plant cell and the level of damage due to abiotic stress. In response to stress, other antioxidation enzymes, such as catalases, play an important role in the conversion of excess H_2O_2 generated through the reactions that produce ROS. Catalase converts H_2O_2 to water and oxygen, thus protecting the plant cell from death due to ROS toxicity (Vranová et al. 2002; Afiyanti and Chen. 2014). In a similar fashion, peroxidases act as oxidative reductive enzymes. When a plant suffers an attack by a pathogenic agent, its cells induce defense mechanisms, such as oxidation of phenols, suberization, and lignification, as well as produce peroxidases that affect plant growth and development, such as cell growth and expansion, differentiation, and development and auxin metabolism (Fang and Kao 2000; Lagrimini et al. 1997; Mohammadi and Kazemi 2002). Polyphenol oxidase, another oxidative stress enzyme, is a copper-containing enzyme that catalyzes the *o*-hydroxylation of monophenols to *o*-diphenols (Araji et al. 2014). Polyphenol oxidase is involved in the lignification of plant cells under biotic stress conditions. The enzyme has an important role in post-harvest browning, which is induced by cuts or damage to plant tissues, through the polymerization of polyphenol oxidase-generated quinones that generate phytomelanins (Araji et al. 2014; Mayer 2006; Mesquita and Queiroz, 2013).

Reducing the level of ROS generation and regulating ROS-induced cellular toxicity would be an ideal scenario for maintaining sustainable plant growth and development (Becana 2000; Zhu et al. 2004; Shen et al. 2010; Kim et al. 2014a and c). Recently, the application of Si was reported to improve crop growth and to aid regulation of stress

responses in plants (Liang et al. 2007; Sivanesan and Park 2014). However, little is known regarding plant responses to Si in relation to the regulation of oxidative stress after wounding or physical injury. The present study was initiated to investigate the effects of the application of Si on oxidative stress in rice plants subjected to wounding. Furthermore, for first time such study was conducted in time and dose dependent manner (Claeys et al. 2014) to assess the sensitivity of rice towards wounding stress and then enhancing resistance using Si mineral nutrition supplementation by fine regulation of underlying antioxidants mechanism.

Materials and Methods

Plant growth and Si application

Seeds of *Oryza sativa* L. cv. Dongjin were obtained from the National Institute of Crop Science, Rural Development Administration, South Korea. The seeds were thoroughly washed and soaked in double-distilled water (DDW). After 3 days soaking in DDW, germinated rice seedlings were transplanted to autoclaved sand medium (moisture content 18–23%, pH 4.5–5.5, EC 2.0 ds/m, bulk density 0.7–1.0 mg/m³, grain size 125–250 μm, nitrogen 800–2500 mg/kg, phosphorus 150–850 mg/kg; other components included zeolite, diatomite, and vermiculite) and placed in a growth chamber. During plant growth, the growth chamber (KGC-175 VH, KOENCON) conditions were adjusted to 12-h light (08:00~20:00 h; 30 °C; relative humidity 70%) and 12-h dark (20:00–08:00 h, 25 °C; relative humidity 70%). Yoshida solution was applied as a nutrient source for 2 weeks to rice seedlings (Yoshida et al. 1959). The pH of the Yoshida solution was maintained at 5.0–5.3 during the growth period. After 2 weeks, the rice plants were transplanted to pots (25 cm × 20 cm × 20 cm) containing only DDW to wash out nutrients and assess the role of Si alone for 1 day. Thereafter, fresh DDW and 0.5 or 1.0 mM Si (Na₂SiO₃) was applied for 6 or 12 h under growth chamber conditions as described earlier. After each Si treatment, wounding stress was applied by a 1 mm incision in the plant shoots at three different positions: near the first, second, and third leaves (Fig. S1*). Before harvesting, data were recorded for leaf chlorophyll content (Soil-plant analysis development unit for measuring leaf chlorophyll content with SPAD-502 Minolta, Japan), plant height and fresh weight. Thirty minutes after injury, rice plant tissues were frozen in liquid nitrogen and kept at –70 °C until used for analyses of antioxidant activities.

Determination of Si accumulation

Rice shoots were soaked in 0.5 M HCl for 20 s, rinsed with DDW, and dried for 72 h in an oven at 80 °C. The samples were weighed, ground to a fine powder, and digested in 5 mL of a tertiary mixture of HNO₃:H₂SO₄:HClO₄ (10:1:4 v/v/v). The Si content was determined using inductively coupled plasma mass spectrometry (Optima 7900DV, Perkin-Elmer, Waltham, MA, USA).

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

Determination of antioxidant enzyme activities and lipid peroxidation

Fresh leaves (100 mg) were homogenized in 50 mM Tris-HCl buffer (pH 7.0) containing 3 mM MgCl₂, 1 mM EDTA, and 1.0% polyvinylpyrrolidone and then centrifuged at 5590 × g for 15 min at 4 °C; the supernatant was used for antioxidant enzyme analysis. All parameters are expressed as activity per mg protein. Catalase (CAT; EC 1.11.1.6) activity was assayed by the method of Aebi (1984). A crude enzyme extract was reacted with 0.5 mL of 0.2 M H₂O₂ in 10 mM phosphate buffer (pH 7.0). CAT activity was estimated by the decrease in absorbance of H₂O₂ at 240 nm, and 1 unit of CAT was defined as micrograms of H₂O₂ released per milligram of protein per minute.

Peroxidase (PO; EC 1.11.1.7) and polyphenol oxidase (PPO; EC 1.10.3.2) activities were measured as described by Kar and Mishra (1976) with some modification. Leaf samples were homogenized with 0.1 M phosphate buffer at pH 6.8 and centrifuged at 2 °C for 15 min at 15,300 × g in a refrigerated centrifuge. The clear supernatant was analyzed for enzyme activity. The assay mixture for PO activity consisted of 0.1 M phosphate buffer (pH 6.8), 50 µL of pyrogallol, 50 µL of H₂O₂, and 0.1 mL of enzyme extract. The mixture was incubated for 5 min at 25°C, after which the reaction was stopped by adding 0.5 mL of 5% (v/v) H₂SO₄. The amount of purpurogallin formed was determined by the absorbance at 420 nm. The same assay mixture as that used for PO but without H₂O₂ was used to assay the activity of the PPO. The amount of purpurogallin was measured by absorbance at 420 nm. One unit of the PO and PPO was defined as an increase of 0.1 unit of absorbance.

Lipid peroxidation was determined by the method of Ohkawa et al. (1979). For this assay, 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid (pH 3.5), and 1.5 mL of 0.81% thiobarbituric acid aqueous solution were added in succession in a reaction tube. Then, 0.2 mL of tissue homogenate, extracted with 10 mM phosphate buffer (pH 7.0), was added. The mixture was then heated in boiling water for 60 min. After cooling to room temperature, 5 mL butanol:pyridine (15:1 v/v) solution was added. The upper organic layer was separated, and the optical density of the resulting pink solution was recorded at 532 nm using a spectrophotometer. Tetramethoxypropane was used as an external standard, and experiments were repeated three times.

Statistical analysis

The experiment was independently repeated three times in completely randomized design under same conditions. The data from each repetition of the same experiment were pooled together for statistical analysis. To identify significant differences between treatments, analyses of variance, multiple mean comparisons, and Duncan's Multiple Range Test (DMRT) were carried out using SAS 9.1.

Results

Effects of Si on plant growth after a wounding stress

Exogenous application of 0.5 or 1.0 mM Si to rice seedlings did not significantly alter plant growth attributes such as plant height and weight compared to plants that received only DDW (Table 1). However, the content of the photosynthetic pigment chlorophyll increased in plants treated with 0.5 or 1.0 mM Si (Table 1). Although the changes in chlorophyll contents showed evidence of a dose effect with regard to Si, the duration of the treatment did not have a significant effect. Therefore, the growth characteristics of rice seedlings were not affected by short-term application of Si; however, chlorophyll, a component of plastids, was affected by the Si treatments.

Table 1. Effect of silicon (Si) application on the growth attributes of rice plants

Treatments		Length (cm/plant)	Fresh Weight (g/plant)	Chlorophyll Content (SPAD)
6 h*	Control	36.2±0.5 ^a	0.596±0.3 ^a	35.5±0.2 ^b
	0.5 mM	36.1±0.1 ^a	0.611±0.2 ^a	36.3±0.5 ^a
	1.0 mM	36.3±0.5 ^a	0.604±0.3 ^a	36.5±0.6 ^a
12 h*	Control	36.3±0.2 ^a	0.607±0.2 ^a	35.7±0.3 ^b
	0.5 mM	36.2±0.1 ^a	0.621±0.3 ^a	36.5±0.7 ^a
	1.0 mM	36.4±0.4 ^a	0.615±0.4 ^a	36.6±0.4 ^a

*Time in silicon solution in hydroponic culture without any other nutrients. In each column, different letter(s) indicate significant differences at $P < 0.05$ by Duncan's Multiple Range Test (DMRT). The data show the mean of three replicate experiments at each treatment ($n = 10$) with standard error (SE). Control plants were treated with DDW.

Silicon accumulation in rice seedlings

After application of Si to rice seedlings for 6 h or 12 h, the levels of Si in the leaves were examined to confirm Si uptake. We found an approximately three-fold increase in Si concentrations in plants from both treatment groups. Therefore, the rice plant absorbed Si during the 6 h or 12 h treatments (Fig. S2).

Lipid peroxidation levels during wounding stress

Next, we examined lipid peroxidation levels in rice seedlings treated with Si as described earlier. Our results showed that MDA activities in plants from the 0.5 mM and 1.0 mM Si treatments were slightly increased compared to control plants during the Si application period; however, after the wounding stress, MDA activities fell in the Si treated plants compared to controls (Fig. S3).

Antioxidant enzymes activity during wounding stress

We evaluated wound-induced oxidative stress modulation by analyzing the activities of three different antioxidant enzymes. CAT activity in plants that received a 6 h Si application and were subjected to wounding stress was significantly reduced compared to those without the wounding stress. In the 12 h Si treatment, CAT activities in control and Si treated plants were significantly increased after wounding stress compared to those without wounding stress (Fig. S4). The second antioxidant, PO, showed slightly reduced activities in control plants after wounding; however, in the 6 h treatment group, PO activity rose significantly after wounding in both 0.5 mM and 1.0 mM Si applications (Fig. S5). A similar pattern of responses was seen in the 12 h treatments, i.e., PO activity decreased in control plants after wounding but increased by approximately 1.8 to 2.0 fold in the 0.5 mM and 1.0 mM Si groups (Fig. S5). The third antioxidant studied, PPO, showed similar responses as PO. In control plants, PPO activity fell after wounding whereas it rose significantly after wounding in plants from the 6 h and 12 h Si application groups (Fig. S6).

Discussion

Growth of the human population has placed enormous pressure on the limited agricultural resources available for sustainable food supply. The yields of many agricultural crops, such as rice, wheat, and barley, do not meet current global food demands as stresses induced by changing environmental conditions results in reduced productivity (Kim et al. 2014 a, b, c). Treatment with silicon (Si) has been reported to alleviate many abiotic and biotic stresses in agricultural crops, thus leading to the incorporation of silicates into many fertilizers. How Si exerts such a protective effect has yet to be fully elucidated; it has been speculated that Si acts through physical and/or biochemical defense systems (Ma and Takahashi 2002; Richmond and Sussman, 2003; Fauteux et al. 2005). Si is a major constituent of many plants, but is not generally classified as 'essential'; although Si is regarded as an essential element in a number of species of the Poaceae and Cyperaceae, it has not been possible to demonstrate that it is essential to all higher plants because direct evidence is still lacking (Epstein 1994; 1999; Liang et al. 2007). Previous research has reported that exogenous Si can increase plant tolerance to high manganese concentrations (Horst and Marschner 1978), drought (Lux et al. 2002), heavy-metal contamination (Neumann and zur Nieden 2001; Kim et al. 2014a), and resistance to pests and pathogens (Belanger et al. 2003; Liang et al. 2007; Richmond and Sussman, 2003). Furthermore, Si contributes to stress resistance in other crops such as the cucumber (Zhu et al. 2004), soybean (Hamayun et al. 2010), tomato (Heine et al. 2007), and wheat (Gong et al. 2005). For these reasons, the role of Si in plant metabolism has recently received increasing attention (Liang et al. 2007).

In the present study, Si treatment improved various aspects of plant growth. Previous studies reported that Si applications below 2.0 mM significantly increased shoot length, plant biomass, and chlorophyll content of rice plants compared to untreated controls. Chemically, silicic acid polymerizes into silica gel ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$) when the concentration

of silicic acid exceeds 2.0 mM (Ma and Takahashi 2002; Mittler et al. 2011). Therefore, a previous investigation used a concentration gradient up to 2.0 mM to investigate the physiological impacts of Si (Kim et al. 2012). Si-transporter genes were found to be exponentially regulated within a short time span, ranging from a few minutes to several hours, leading to the accumulation of Si inside plant tissues (Ma and Yamaji 2008). These Si deposits protect rice cells and tissues from multiple abiotic and biotic stresses, including physical injury or herbivory (Cai et al. 2009; Bockhaven et al. 2012).

Plants possess efficient systems for scavenging active oxygen species in order to protect themselves from destructive oxidative reactions (Foyer et al. 1994). Antioxidant enzymes are key elements in these defense mechanisms. Oxidative stresses induced by wounding results in the accumulation of reactive oxygen species (ROS). Major antioxidant enzymes, such as CAT, PO and PPO, have important roles in the scavenging of ROS (Esterbauer et al. 1991). In our study, we found enhanced activities of CAT, PO, and PPO in Si-treated wounded and non-wounded rice plants as compared to their respective control plants. CAT converts hydrogen peroxide to water and molecular oxygen. To counteract wound induced ROS, Si-treated rice plants increased their levels of CAT, PO and PPO. A similar trend of higher antioxidant enzyme activities was also reported by Reyes and Luis (2003), Gong et al. (2005), and Liang et al. (2005). The effectiveness of these enzymes even at their maximum catalytic rates is reduced by low substrate affinities due to the need for the simultaneous access of two H_2O_2 molecules to active sites in the catalytic reaction. An alternative mode of H_2O_2 destruction is via PO, which is found throughout cells and has a greater affinity for H_2O_2 than CAT. PO, however, requires a reductant, since it reduces H_2O_2 to H_2O during wounding stress. PPO is another important frontline oxidative enzyme mainly responsible for oxidation of phenolic compounds into quinines (Araji et al. 2014). The quinones change plant materials into a nutritionally unavailable form for pests (Constabel and Ryan 1998). Thus, accumulation of anti-oxidative enzymes in Si-treated plants provides a defensive strategy to cope with wounding stress. Wounding stress induced by herbivory or lodging can increase the level of hydrogen peroxide inside plant tissues (Leon et al. 2001).

The levels of lipid peroxidation indicate the amount of oxidative stress damage to lipid membranes due to wounding stress (Davey et al. 2005; Esterbauer et al. 1991). Here, we found that lipid peroxidation was significantly higher in non-Si-treated plants compared to Si-treated plants under wounding stress. Higher MDA formation indicates a higher degree of lipid peroxidation (Dionisio-Sese and Tobita 1998). Si accumulation in rice provides a physical barrier by forming a 2.5 to 1 μ m layer beneath the cuticle layer (0.1 mm) of leaf blades (Currie and Perry 2007). Silicified cells resulting from Si accumulation are also observed in the epidermis and vascular bundles of the stem as silica cells and silica bodies (phytoliths) (Ma and Takahashi 2002). The accumulation of Si results in a considerable increase in rigidity of the lipid membrane in Si-treated compared to non-Si treated plants under wounding stress. This layer can mechanically impede penetration of wounds and thereby disrupt the infection process (Ma and Yamaji 2008). Thus, Si seems to act as a physical barrier against wounding stress. Liang et al. (2005) suggested that Si

might be involved in the metabolic or physiological and/or structural activities in higher plants exposed to abiotic and biotic stresses.

According to our results, MDA activity was significantly increased in non-Si treated plants after wounding but MDA activity was decreased following Si application. Generally, plants are faced with biotic stress condition such as attacks by herbivores or cell membrane damage due to lipid peroxidation (Kim et al. 2014b). Si is taken up in the silicic acid form by the rice root and is transported to other parts such as the grain, straw and hull in the same form. Although absorbed Si in rice is easily transferred between different structures owing to the activation of silicon transport genes, it nevertheless shows differences in concentration in the rice plant with higher levels in rice straw and rice hull (Van Hoest 2006; Currie and Perry 2007).

In conclusion, our results indicate that application of exogenous Si to rice plants can improve crop growth and increase the mechanical strength of the plants to overcome losses from wounding stress as compared to non-Si-treated plants. Additionally, we observed that Si can regulate wound-related stress to plants by increasing the activities of antioxidant enzymes and decreasing the level of lipid peroxidation compared to non-Si-treated plants. These results further strengthen the role of Si as an efficient treatment for the improvement of plant resistance to abiotic stresses, as noted in earlier studies (Eraslan et al. 2008; Li et al. 2005). To improve crop yields, our results support the application of Si to control the negative effects of wounding stress to crop plants. However, additional studies are still needed at the transcriptomics level to further elucidate the role of Si for wider applications in crop plants.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at <http://www.akademai.com/content/120427/>

Electronic Supplementary *Figure S1*. Wounding stress application to a rice plant grown in Yoshida solution for two weeks. The physical injuries were made on the rice plants using a blade. The wounds were made over a 30 min period

Electronic Supplementary *Figure S2*. Si accumulation in rice plants treated with silicon. Error bars indicate standard errors (n = 3) and different letters show significant differences at $P < 0.05$

Electronic Supplementary *Figure S3*. Effect of silicon (Si) application for 6 h and 12 h on lipid peroxidation levels in control and wounded rice plants. Error bars indicate standard errors (n = 3) and different letters show significant differences at $P < 0.05$

Electronic Supplementary *Figure S4*. Effect of silicon (Si) application on catalase (CAT) activity in control and wounded rice plants. Error bars indicate standard errors (n = 3) and different letters show significant differences at $P < 0.05$

Electronic Supplementary *Figure S5*. Effect of silicon (Si) application on peroxidase (PO) activities in control and wounded rice plants. Error bars indicate standard errors (n = 3) and different letters show significant differences at $P < 0.05$

Electronic Supplementary *Figure S6*. Effect of silicon (Si) application on polyphenol oxidase (PPO) activities in control and wounded rice plants. Error bars indicate standard errors (n = 3) and different letters show significant differences at $P < 0.05$