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Silicon Application to Rice Root Zone Influenced the Phytohormonal and Antioxidant Responses Under Salinity Stress

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Abstract Silicon (Si) application shows beneficial effects on plant growth; however, its effects on the phytohormone and enzymatic antioxidant regulation have not been fully understood. We studied the effects of short-term (6, 12, and 24 h) silicon (0.5, 1.0, and 2.0 mM) application on salinity (NaCl)-induced phytohormonal [abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA)] and antioxidant regulation in *Oryza sativa*. The results showed that Si treatments significantly increased rice plant growth compared to controls under salinity stress. Si treatments reduced the sodium accumulation resulting in low electrolytic leakage and lipid peroxidation compared to control plants under salinity stress. Enzymatic antioxidant (catalase, peroxidase and polyphenol oxidase) responses were more pronounced in control plants than in Si-treated plants under salinity stress. Stress- and defense-related phytohormones like JA were significantly downregulated and SA was irregular after short-term Si applications under salinity stress compared to control. Conversely, ABA was significantly higher after 6 and 12 h but insignificant after 24 h in Si-treated plants under salinity stress. After 6 and 12 h, Si and salinity stress resulted in upregulation of zeaxanthin epoxidase and 9-*cis*-epoxycarotenoid dioxygenase 1 and 4 (*NCED1* and *4*), whereas 24-h treatments significantly downregulated the expressions of these genes compared to those in the control. *NCED3* expression increased after 6 and 24 h but it was insignificant after 12 h of Si application compared to control. The current findings indicate that increasing the Si concentrations for longer periods of time can regulate the salinity-induced stress by modulating phytohormonal and enzymatic antioxidants' responses.

Keywords Abscisic acid biosynthesis · Enzymatic antioxidant · Phytohormone · Silicon · Salinity stress

Introduction

Crop plants are frequently exposed to a wide array of environmental stresses, including salinity, which adversely affect their growth and yield (Munns and Tester 2008). Globally, about 800 million hectares of land are affected by salinity, which is almost 6 % of world's total area. With the changing global climate this rate will further increase resulting in 50 % losses in crop production by the year 2050 (Munns and Tester 2008). Rice is a major food staple for more than 70 % of the human population. Rice grown in paddy fields is often exposed to salinity stress because of the higher rate of evaporation in summer. Salinity stress hampers plant growth by (1) reducing osmosis, (2) increasing the toxicity of ions (Na^+, Cl^-, and SO^{2-}) inside the plant cell, and (3) influencing nutritional imbalance (Hasegawa and others 2000). Though the sodium (Na^+) ion acts as a micronutrient in plant growth, its excess can inhibit the plant's health. High salt concentrations in turn increase the cytosolic Ca^{2+} concentration and subsequently activate plasma membrane-localized anion channels (Lee

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and Luan 2012), guard cell depolarization, potassium efflux, loss of guard cell turgor and volume, and finally result in stomatal closure (Hasegawa and others 2000).

The accumulation of toxic sodium ions results in unprecedented production of reactive oxygen species (ROS) which can severely damage the cellular apparatus of the plant (Munns and Tester 2008). ROS damage the lipid membrane, proteins, and nucleic acids through the alteration of normal cellular metabolism (Imlay 2003). To alleviate and repair the damage by ROS, plants have developed a complex antioxidant enzyme system (Choudhury and others 2013). Enzymatic antioxidants such as catalase, peroxidase, and polyphenol oxidase are recruited to detoxify the ROS attack. Salinity stress, on the other hand, reduces the ability of plants to scavenge ROS. Lipid peroxidation induces oxidative stress and stimulates root H^+-ATPase in the membranes (Liang and others 2003). The desaturation of membrane-based fatty acids can adversely affect phytohormonal biosynthesis of mainly jasmonic acid (JA) and salicylic acid (SA), thus weakening plant defense responses against herbivory and pathogenic attack.

JA and SA are stress-signaling phytohormones. Both hormones respond against biotic and abiotic stresses (Wasternack and others 2006; Zarate and others 2007). SA is known to induce systemic acquired resistance against pathogens, but recently its role has also been emphasized in abiotic stress tolerance like drought and salinity (Hara and others 2012). JA also stimulates defense-related signaling in response to herbivory and it is also important in accumulation of abscisic acid (ABA) during osmotic stress (Han and others 2013; Ollas and others 2013). ABA is accumulated in plant tissues to avoid water loss by functioning in stomatal closure during salinity stress (Munns and Tester 2008; Ollas and others 2013). ABA is synthesized from carotenoid through an indirect pathway and makes zeaxanthin. Zeaxanthin is converted to all-*trans*-violaxanthin by two-step epoxidation catalyzed by zeaxanthin epoxidase (ZEP) in plastids (Agrawal and others 2001). 9-*cis*-Epoxycarotenoid dioxygenase (NCED) catalyzes oxidative cleavage of the 9-*cis* isomer of violaxanthin or neoxanthin and produces a C_{15} product xanthoxin and a C_{25} metabolite (Qin and Zeevaart 1999). Xanthoxin is converted to abscisic aldehyde and then abscisic aldehyde is oxidized to ABA by abscisic aldehyde oxidase (Seo and others 2000). The two enzymes (*ZEP* and *NCED*) have a key role in ABA biosynthesis as mediator. Though the function of these phytohormones under salinity stress has been widely elaborated, their role in silicon (Si) treatments is not fully understood.

To mitigate such stresses, crop plants adapt a defensive strategy at multiple levels, including the physiological and molecular levels. Si has been regarded as a beneficial element in plant growth and development (Epstein 1994). It is the second most abundant element in soil. Si concentration ranges from 1 to 10 % or even higher in plants growing in the terrestrial environment (Epstein 1994). Generally, rice is known as a Si-accumulating plant and it is deemed important for sustainable production of rice (Yamaji and Ma 2011). Its role and function in plant physiology is still poorly understood, whereas direct evidence mimics the role of Si for being part of plant constituents (Epstein 1994, 1999; Ma and others 2001; Liang and others 2003; Ma and Yamaji 2008). On the other hand, studies have revealed that Si is beneficial to higher plants, particularly for grasses and various cultivated crops like rice, wheat, tomato, and cucumber (Epstein 1994, 1999; Liang and Ding 2002; Liang and others 2007; Zuccarini 2008; Hamayun and others 2010; Parveen and Ashraf 2010; Kim and others 2011; Vaculik and others 2012; Saleh and others 2013). In the last decade, studies have revealed the mitigating role of Si under various biotic (plant diseases and pests) and abiotic stresses (heavy metals, drought, and salinity) to crop plants (Ma and others 2011; Lee and Luan 2012). However, the role of Si in phytohormonal regulation at the transcriptomic level during salinity stress has yet to be elucidated.

In the present study, we aimed to assess the effects of exogenous Si application on rice plant growth, oxidative stress, and phytohormonal regulation under salinity stress. The time- and dose-dependent response of Si applied to the root zone of rice plants was also elucidated to understand the stress perception and tolerance. Although the responses of various antioxidants (Liang and others 2007; Parveen and Ashraf 2010) and some phytohormones, mainly JA and SA (Kim and others 2011), have been previously reported, ABA and its biosynthesis are poorly known. In this regard, the mRNA expressions of ABA biosynthesis-related genes (*ZEP*, *NCED1*, *NCED3*, and *NCED4*) of rice plants were assessed in Si-treated plants under salinity stress.

Materials and Methods

Plant Material, Cultivation Method, Concentration of Si, and Salinity Treatment

Rice seeds (*O. sativa* L. cv 'Dongjin') were procured from the National Institute of Crop Science, Rural Development Administration, the Republic of Korea. Seeds were surface sterilized with 5 % sodium hypochlorite for 15 min, thoroughly washed with autoclaved double-distilled water, and incubated at 28 °C for uniform germination in the dark. After 3 days, germinated rice seedlings were transplanted into sand medium and transferred to growth chambers. The growth chamber (KGC-175 VH, Koencon) conditions were

adjusted to 12-h light (08:00–20:00, 30 °C, relative humidity = 70 %) and 12-h dark (20:00–08:00, 25 °C, relative humidity = 70 %). Rice seedling was supplied with Yoshida solution (Yoshida and others 1959) as a nutrient source for 2 weeks. During this growth period (14 days), the pH of the Yoshida solution was maintained between 5.0 and 5.3 by the addition of HCl to the medium to reduce polymerization of silicates (Brady and others 1953). The rice plants were then transplanted to plastic pots (25 × 20 × 20 cm) containing 4 l of double-distilled water and fitted with Styrofoam sheets. Holes were made in the Styrofoam sheets and foam was used to fill the gap and support the rice seedlings. The experiment was designed with the following treatments: (1) water control in which plants were grown in distilled water for 6, 12, and 24 h; (2) salinity treatment (100 mM NaCl solution) for 6, 12 and 24 h; and (3) silicon application (0.5, 1.0, or 2.0 mM sodium metasilicate, $Na_2SiO_3{\cdot}5H_2O$) for 6, 12, and 24 h with or without salinity conditions. The salinity stress and Si were applied simultaneously to the rice plant's root zone and grown hydroponically in double-distilled water for a short period (6, 12, and 24 h). The growth chamber was set with the following conditions during the course of the experiment (1000–1600, 1000–2200, and 1000–1000; 30 °C; relative humidity = 70 %; light intensity = 1,000 µmol m^{-2} s^{-1}). To maintain an equal population of plants in each pot, only 24 rice plants were kept. Rice plants were harvested after stress treatment and immediately frozen in liquid nitrogen and shifted to −80 °C. Prior to biochemical and hormonal analysis, plant samples were freeze-dried using a Virtis Freeze Dryer (Gardiner, NY, USA) for 4–7 days, whereas for other experiments such as antioxidant activity and mRNA expression fresh samples were used.

Determination of Root Sodium Contents and Root Electrical Conductivity

Rice roots were harvested at 6, 12, and 24 h after salinity treatment with and without Si treatment. Roots were thoroughly rinsed with double-distilled water and dried at 70 °C for 2 days. Dried samples were ground very fine with a grinder. Ground root samples were treated with 3 ml of H_2O_2 and 5 ml of HNO_3, decomposed by microwaving, and digested with 2–3 % of HNO_3. Na was measured by inductively coupled plasma mass spectrometry (ICP-MS) as described by Jin and Zhu (2000). Plant dried samples were extracted with double-distilled water (plant samples:double-distilled water ratio of 1:10 w/v) and boiled at 100 °C for 1 h. The suspensions were centrifuged at 12,000 rpm for 20 min and filtered through 0.45-µm membrane filters. The filtrates were used to measure electrical conductivity (EC) by a Mettler Toledo (SG78—SevenGo Duo, Seoul, South Korea) conductivity meter.

Abscisic Acid Extraction and Quantification

The endogenous ABA content was quantified from the freeze-dried samples by following the protocols of Qi and others (1998) and Kamboj and others (1999). Plant samples were extracted with 30 ml of extraction solution containing 95 % isopropanol, 5 % glacial acetic acid, and 100 ng of [(±)-3,5,5,7,7,7-d^6]-ABA. The filtrate was concentrated by a rotary evaporator. The residue was dissolved in 4 ml of 1 N sodium hydroxide solution and then washed three times with 3 ml of methylene chloride to remove lipophilic materials. The aqueous phase was brought to approximately pH 3.5 with 6 N hydrochloric acid and partitioned three times into ethyl acetate (EtOAc). EtOAc extracts were then combined and evaporated. The dried residue was dissolved in phosphate buffer (pH 8.0) and then run through a polyvinylpolypyrrolidone (PVPP) column. The phosphate buffer was adjusted to pH 3.5 with 6 N HCl and partitioned three times into EtOAc. EtOAc extracts were combined again and evaporated. The residue was dissolved in dichloromethane (CH_2Cl_2), and passed through a silica cartridge (Sep-Pak; Waters, Milford, MA, USA) which was prewashed with 10 ml of diethyl ether:methanol (3:2 v/v) and 10 ml of dichloromethane. ABA was recovered from the cartridge by elution with 10 ml of diethyl ether (CH_3–$CH_2)_2O$:methanol (MeOH) (3:2 v/v). The extracts were dried and methylated by adding diazomethane for GC/MS-SIM analysis (6,890 N network GC system, and 5,973 network mass selective detector; Agilent Technologies, Palo Alto, CA, USA). For quantification, the Lab-Base (ThermoQuest, Manchester, UK) data system software was used to monitor responses to ions of m/e 162 and 190 for Me-ABA and 166 and 194 for Me-[$^2H_6$]-ABA.

Jasmonic Acid Extraction and Quantification

The endogenous JA level was quantified according to the protocol of McCloud and Baldwin (1997). The freeze-dried samples were ground to a fine powder with a mortar and pestle. The powder (0.1 g) was suspended in a solution of acetone and 50 mM citric acid (70:30 v/v) and [9,10-$^2H_2$]-9,10-dihydro-JA (20 ng) was added as an internal standard. The extracts were allowed to evaporate overnight at room temperature to avoid losses of volatile fatty acids. The resulting aqueous solution was then filtered and extracted three times, each time with 10 ml of diethyl ether. The pooled extracts were then loaded on a solid-phase extraction cartridge (500 mg of sorbent, aminopropyl). After loading, the cartridges were washed with 7.0 ml of trichloromethane and 2-propanol (2:1 v/v). The bound JA and the pertinent standard were eluted with 10 ml of diethyl ether and acetic acid (98:2 v/v). After evaporation of solvents and esterification of the residue with excess

diazomethane, the sample was adjusted to 50 µl with dichloromethane. The extracts were then analyzed by GC–MS (6,890 N network GC system and 5,973 network mass selective detector; Agilent Technologies). To enhance the sensitivity of the method, spectra were recorded in the selected ion mode, that is, in case of JA determination, we monitored the fragment ion at $m/z = 83$ amu, corresponding to the base peaks of JA and [9,10-$^2H_2$]-9,10-dihydro-JA. The amounts of endogenous JA were calculated from the peak areas of JA compared with the corresponding standards. The experiment was repeated three times.

Free Salicylic Acid Extraction and Quantification

Free SA was extracted and quantified as described by Seskar and others (1998). Freeze-dried samples were ground to powder form and 0.1 g was sequentially extracted with 90 and 100 % methanol by centrifuging at 10,000×*g*. The combined methanol extracts were vacuum-dried. The dry pellets were resuspended in 2.5 ml of 5 % trichloroacetic acid, and the supernatant was partitioned with ethyl acetate:cyclopentane:isopropanol (100:99:1 v/v/v). The top organic layer containing free SA was transferred to a 4-ml vial and dried with nitrogen gas. The dry SA was again suspended in 1 ml of 70 % methanol. High-performance liquid chromatography (HPLC) analyses were carried out on Shimadzu with a fluorescence detector (Shimadzu RF-10AXL, excitation and emission, 305–365 nm) fitted with a C18 reverse-phase HPLC column (HP hypersil ODS; particle size = 5 µm; pore size = 120-Å water). The flow rate was 1 ml min^{-1}. The SA analyses were repeated three times.

Lipid Peroxidation and CAT, POD, and PPO Activity

The extent of lipid peroxidation was determined using the method of Ohkawa and others (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 we added 0.2 ml of tissue homogenate from fresh rice plant samples (100 mg) that were extracted with 10 mM phosphate buffer (pH 7.0). 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 color was recorded at 532 nm using spectrophotometry. Tetramethoxypropane was used as an external standard. The level of lipid peroxides was expressed as micromoles of malondialdehyde (MDA) formed per gram tissue weight. The experiments were repeated three times.

Fresh plant samples (100 mg) were homogenized in 50 mM Tris HCl buffer (pH 7.0) containing 3 mM $MgCl_2$, 1 mM EDTA, and 1.0 % PVP, and then centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant thus obtained was used for biochemical analysis. All parameters were expressed as activity per mg protein. Catalase activity was assayed using the method of Aebi (1984). The crude enzyme extract was added with 0.5 ml of 0.2 M H_2O_2 in 10 mM phosphate buffer (pH 7.0). CAT activity was estimated by the decrease in absorbance of H_2O_2 at 240 nm, and one unit of CAT was defined as µg of H_2O_2 released mg^{-1} protein min^{-1}.

Peroxidase and polyphenol oxidase activity was measured using the method of Kar and Mishra (1976) with some modification. The fresh plant samples (100 mg) were homogenized with phosphate buffer [pH 6.8 (0.1 M)] and centrifuged at 2 °C for 15 min at 17,000×*g* in a refrigerated centrifuge. The clear supernatant was taken as the enzyme source. The assay mixture for the peroxidase activity comprised phosphate buffer (pH 6.8), 50 µM of pyrogallol, 50 µM of H_2O_2, and 0.1 ml of enzyme extract. This was incubated for 5 min at 25 °C, after which the reaction was stopped by adding 0.5 ml of 5 % (v/v) H_2SO_4. The amount of purpurogallin formed was determined by taking the absorbance at 420 nm. The same assay mixture as that of peroxidase without H_2O_2 was used to assay the activity of polyphenol oxidase. The absorbance of the purpurogallin formed was taken at 420 nm. One unit of peroxidase and polyphenol oxidase was defined as an increase of 0.1 unit of absorbance.

RNA Extraction and RT-PCR Analysis

Total RNA was extracted from fresh rice plant samples using an RNeasy plant extraction minikit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 µg of total RNA using an oligo(dT)18 primer and SuperScript first-strand synthesis system for reverse transcriptase polymerase chain reaction (RT-PCR). For the real-time PCR, the LightCycler® 480II and LightCycler® 480 SYBR® Green I master mix (Roche Diagnostics, Mannheim, Germany) were used according to the manufacturer's instructions. The real-time PCR was performed using the following thermocycler program: an initial polymerase activation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 20 s, and 72 °C for 35 s. Following the amplification phase, a melting curve analysis was conducted from 65 to 97 °C, with a cooling step at 40 °C for 10 s (lamp rate = 2.0 °C s^{-1}). The fluorescence was monitored using the Mono Hydrolysis Probe setting (483–533 nm) following the 72 °C extension phase of each cycle. The second-derivative maximum method in the

Table 1 Primers used for quantitative RT-PCR

Gene	GenBank accession no.	Primer sequences
*Os*Actin	AY212324	F: 5′ CAATGTGCCAGCTATGTATGTCGCC 3′
		R: 5′ TTCCCGTTCAGCA GTGGTAGTGAAG 3′
OsZEP1	LOC_Os04g37619.1	F: 5′ TATGTGACTGATAATGGAAGTG 3′
		R: 3′ ATGGCTGACTGAAGTCTCTCGT 5′
OsNCED1	AY838897	F: 5′ CTCACCATGAAGTCCATGAGGCTT 3′
		R: 5′ GTTCTCGTAGTCTTGGTCTTGGCT 3′
OsNCED3	AY838899	F: 5′ CCCCTCCCAAACCATCCAAACCGA 3′
		R: 5′ TGTGAGCATATCCTGGCGTCGTGA 3′
OsNCED4	AY838900	F: 5′ TCCATCTCCTTCTCCCTCCTCCCA 3′
		R: 5′ CCTCGCACCCTGCTTGATCTTGCC 3′

LightCycler® 480 quantification software (Roche Diagnostics) was used to analyze the data. The reproducibility parameters were calculated from these data in Microsoft Excel (Microsoft Corp., Redmond, WA, USA). Each cDNA sample was amplified in triplicate and *OsActin* was used as the internal control. Primer sequences are given in Table 1 (Oliver and others 2007).

Statistical Analysis

The data were analyzed statistically for standard deviation and error by using SigmaPlot software (2004; Systat, San Jose, CA, USA). The mean values were compared using Duncan's multiple-range tests at $P < 0.05$ (ANOVA SAS release 9.1; SAS Institute, Cary, NC, USA).

Results

Influence of Silicon on Plant Growth Characteristics

The control rice plants were either exposed to salinity [sodium chloride (NaCl)] stress or given the same amount of distilled water (DW). Plants grown only in DW did not have significantly increased plant growth attributes. The NaCl-treated rice plants had significantly lower shoot length, chlorophyll content, and shoot fresh weight. Si application, on the other hand, resulted in significantly higher plant biomass compared to that of DW-treated plants under salinity. Moreover, the chlorophyll content of Si-treated plants was not significantly different than that of the non-Si-treated control plants. The results of plant growth attributes indicate that the time-dependent treatment of Si in combination with NaCl for 6, 12, and 24 h significantly increased shoot length and fresh weight compared to DW- and NaCl-treated rice plants (Table 2).

The Na content was assessed in the roots of Si- and non-Si-treated plants. The results showed that Na accumulation was significantly higher in NaCl-treated plants that in DW- and Si-treated plants. Na accumulation gradually increased at various Si concentrations ($0.5 < 1.0 < 2.0$) and time (6, 12, and 24 h); however, this increase was significantly lower than the NaCl-treated plants (Table 3). Thus, Si application counteracted the transport of sodium and chloride inside roots.

The results showed that electrolytic leakage (EL) was pronounced with salinity treatment. However, Si treatments greatly minimized the negative influence of salinity on rice plant tissues by counteracting the EL process. Si applications significantly decreased the EL of rice plants under salinity stress. EL increased with Si applications from 0.5 to 2.0 mM, but this increase was not significantly higher than that of NaCl-only-treated rice plants (Table 3).

Si Regulates Salinity-induced Antioxidant Responses

Si application to rice plants mitigated the negative effects of salinity stress by changing the antioxidants' responses. Lipid peroxidation is an indicator of stress and is often measured in terms of malondialdehyde (MDA) content. The application of Si in various concentrations under salinity stress brought a significant decrease in lipid peroxidation (MDA) compared with salinity alone (Fig. 1). The results of lipid peroxidation indicate a time-dependent response against Si concentration. The results showed that the MDA content of the control rice plants grown in DW was not significantly different than that of plants with Si (0.5, 1.0, and 2.0 mM) application for 6 h under salinity stress (Fig. 1). MDA content was reduced with the 6- and 12-h Si treatments compared to the 24-h treatment under salinity stress. After 12 h of Si application and salinity stress, the MDA content was significantly different than that with salinity alone. The result evidenced that Si can alter the response of lipid peroxidation during salinity stress.

Salinity stress to plants can generate toxic ROS that are mitigated by recruiting antioxidant enzymes. The present results showed that Si application (0.5, 1.0, and 2.0 mM) regulated antioxidant enzyme activities in rice plants under

Table 2 Salinity stress modulation after exogenous Si application in a time-dependent manner to rice plants

Treatments	SL (cm plant^{-1})	SFW (g plant^{-1})	CC (SPAD plant^{-1})
6 h			
Control	30.46 ± 0.15 b	0.24 ± 0.04 b	28.94 ± 1.12 a
100 mM NaCl	29.39 ± 0.36 b	0.23 ± 0.02 b	24.87 ± 1.44 b
0.5 mM Si + 100 mM NaCl	31.86 ± 0.46 a	0.28 ± 0.01 a	29.89 ± 1.30 a
1.0 mM Si + 100 mM NaCl	31.79 ± 0.44 a	0.30 ± 0.01 a	30.37 ± 1.41 a
2.0 mM Si + 100 mM NaCl	31.78 ± 0.32 a	0.32 ± 0.02 a	29.39 ± 1.36 a
12 h			
Control	31.96 ± 0.38 ab	0.26 ± 0.01 a	30.26 ± 2.06 a
100 mM NaCl	30.02 ± 0.34 b	0.24 ± 0.03 b	28.34 ± 1.33 b
0.5 mM Si + 100 mM NaCl	32.44 ± 0.43 a	0.31 ± 0.01 a	29.47 ± 2.18 a
1.0 mM Si + 100 mM NaCl	32.61 ± 0.61 a	0.34 ± 0.03 a	30.18 ± 1.62 a
2.0 mM Si + 100 mM NaCl	32.24 ± 0.35 a	0.37 ± 0.05 a	29.29 ± 2.05 a
24 h			
Control	32.89 ± 0.41 a	0.29 ± 0.02 a	30.01 ± 2.06 a
100 mM NaCl	31.33 ± 0.37 b	0.25 ± 0.02 b	29.11 ± 2.41 b
0.5 mM Si + 100 mM NaCl	33.56 ± 0.44 a	0.33 ± 0.03 a	31.51 ± 2.67 a
1.0 mM Si + 100 mM NaCl	33.50 ± 0.39 a	0.37 ± 0.03 a	30.30 ± 2.77 a
2.0 mM Si + 100 mM NaCl	33.37 ± 0.51 a	0.40 ± 0.01 a	30.81 ± 2.69 a

Treatment periods (6, 12, and 24 h) within the same column with different letter(s) are significantly different at $P < 0.05$ by DMRT (Duncan's multiple-range test). Data shown are mean ± standard error of the means ($n = 15$)

SL shoot length per plant, *SFW* shoot flash weight per plant, *CC* chlorophyll content per plant, *SPAD* soil–plant analysis development unit for measuring leaf chlorophyll content

Table 3 Effect of different Si treatments on the electrolytic leakage and sodium ion uptake of the rice plants under salinity stress

Treatments	Na content (mg kg^{-1} DW)	EC (μS cm^{-1} DW)
6 h		
Control	1845.3 ± 5.4 e	727.3 ± 7.6 d
100 mM NaCl	25642.9 ± 5.9 a	1269.7 ± 8.1 a
0.5 mM Si + 100 mM NaCl	10908.0 ± 9.6 d	1092.0 ± 5.2 bc
1.0 mM Si + 100 mM NaCl	16237.6 ± 11.0 c	1189.3 ± 4.6 b
2.0 mM Si + 100 mM NaCl	17664.6 ± 15.0 b	1185.3 ± 1.8 b
12 h		
Control	2021.8 ± 9.2 e	835.7 ± 3.6 d
100 mM NaCl	28071.8 ± 4.4 a	1376.0 ± 2.2 a
0.5 mM Si + 100 mM NaCl	14919.7 ± 24.5 d	1273.0 ± 3.9 b
1.0 mM Si + 100 mM NaCl	20089.5 ± 18.9 c	1226.3 ± 8.5 bc
2.0 mM Si + 100 mM NaCl	23594.2 ± 29.9 b	1245.3 ± 8.2 bc
24 h		
Control	2143.4 ± 16.6 d	819.0 ± 4.0 d
100 mM NaCl	30566.4 ± 28.2 a	1738.3 ± 6.6 a
0.5 mM Si + 100 mM NaCl	16081.0 ± 11.1 c	1336.7 ± 0.3 b
1.0 mM Si + 100 mM NaCl	26884.6 ± 89.0 b	1401.0 ± 4.0 b
2.0 mM Si + 100 mM NaCl	26578.2 ± 96.5 b	1490.3 ± 2.4 b

Treatment periods (6, 12, and 24 h) within the same column with different letter(s) are significantly different at $P < 0.05$ by DMRT (Duncan's multiple-range test). Data are mean ± standard deviation of the means ($n = 5$)

EC electrical conductivity

salinity stress in a time-dependent manner for 6, 12, and 24 h. Among these enzymes, catalase (CAT) activity of Si- and non-Si-treated plants after 6 h significantly increased under salinity stress compared to that of DW-treated controls. However, the presence of Si for 12 and 24 h in combination with salinity caused significantly lower CAT

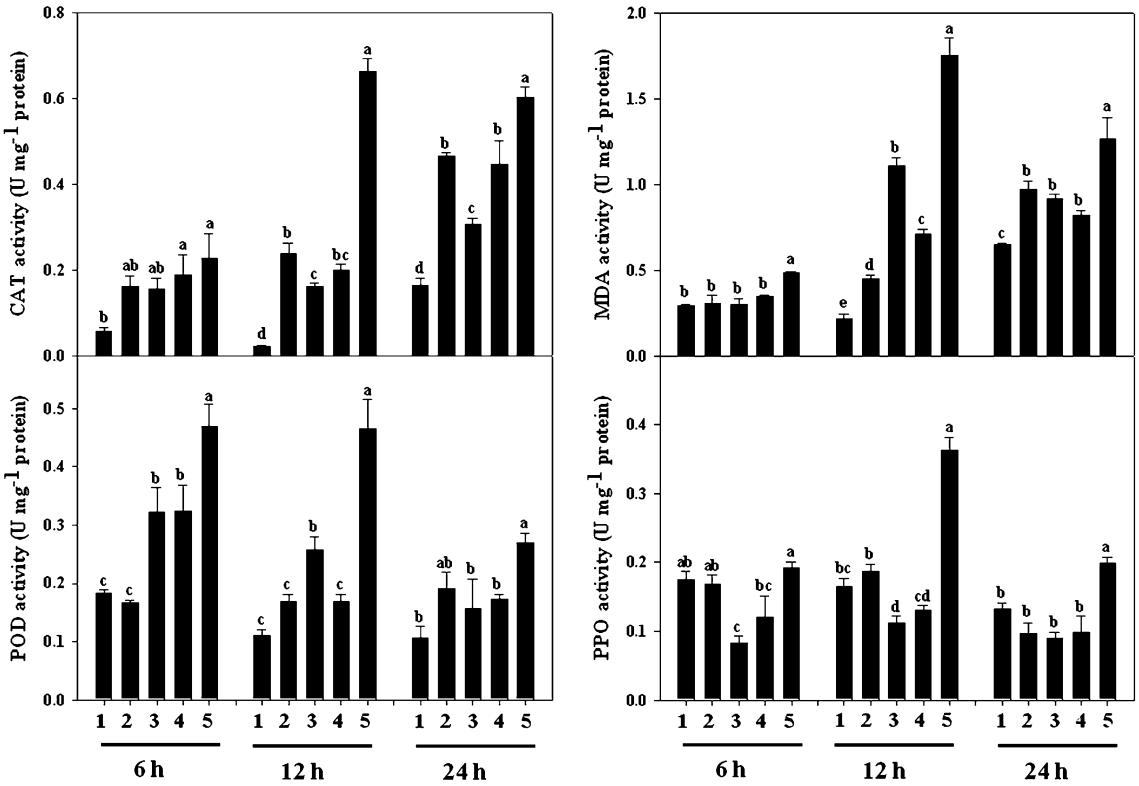

Fig. 1 Effect of Si application and salinity stress on the antioxidant activities of rice plants. Lipid peroxidation (MDA content), peroxidase (POD), polyphenol peroxidase (PPO), and catalase (CAT) were measured in fresh rice plants exposed to salt stress (100 mM) and with short-term Si application (0.5, 1.0, and 2.0 mM). *1*, control (DW-treated); *2*, 0.5 mM Si + NaCl; *3*, 1.0 mM Si + NaCl; *4*, 2.0 mM Si + NaCl; and *5*, NaCl (100 mM). *Error bars* represent the standard error of the means within each treatment. For each duration set (6, 12, and 24 h), the *different letters* indicate significant differences ($P < 0.05$) between treatments as evaluated by Duncan's multiple-range test

activity compared to that of plants treated with salinity alone (Fig. 1). With increasing Si concentration and duration, CAT activity decreased under salinity stress.

Peroxidase (POD) activity was significantly reduced in Si-treated rice plants compared to that in non-Si-treated plants under salinity stress after 6, 12, and 24 h. Salinity stress significantly increased POD activity compared to that in control and Si-treated plants. Polyphenol peroxidase (PPO) activity was significantly different in Si-treated rice plants than that in non-Si-treated plants. Application of NaCl only significantly upregulated PPO activity compared to Si + NaCl-treated plants. However, the PPO activity was almost similar in 0.5-mM Si-treated plants under salinity stress and DW-treated plants after 6 and 12 h (Fig. 1). Application of NaCl only significantly activated the antioxidant apparatus to counteract salinity stress compared to Si-treated plants, indicating a salinity stress-condoning behavior of Si.

Endogenous Abscisic Acid and Its Biosynthesis during Si Application and Stress

The endogenous ABA content often tends to accumulate after a stressful event in the plant's life. The DW-treated control rice plants had significantly lower amounts of ABA during this experiment. However, the ABA content was significantly higher after 6 and 12 h with Si (0.5 and 1.0 mM) application under salinity stress. After 6 and 24 h of 2.0 mM Si and non-Si treatments, the ABA contents were not significantly different from each other under salinity stress. Application of 0.5 mM Si after 6, 12, and 24 h under salinity stress resulted in significantly higher ABA content compared to that after other Si concentrations and salinity-alone-treated plants (Fig. 2).

Our results showed that ABA content decreased with Si treatments in a dose-dependent manner but not in a time-dependent manner.

This could be attributed to the changes in the expression pattern of ABA biosynthesis-related genes and enzymes. Various biosynthetic genes have been identified and reported in different crop plant species (Seo and Koshiba 2002). The ABA biosynthesis-related genes are upregulated during abiotic stress such as that caused by salinity, drought, and heat (Xiong and Zhu 2003; Tuteja 2007). The assessment of ABA biosynthesis is crucial for understanding its actions in plant physiology. Previous studies indicated that zeaxanthin epoxidase (ZEP or ABA1) and

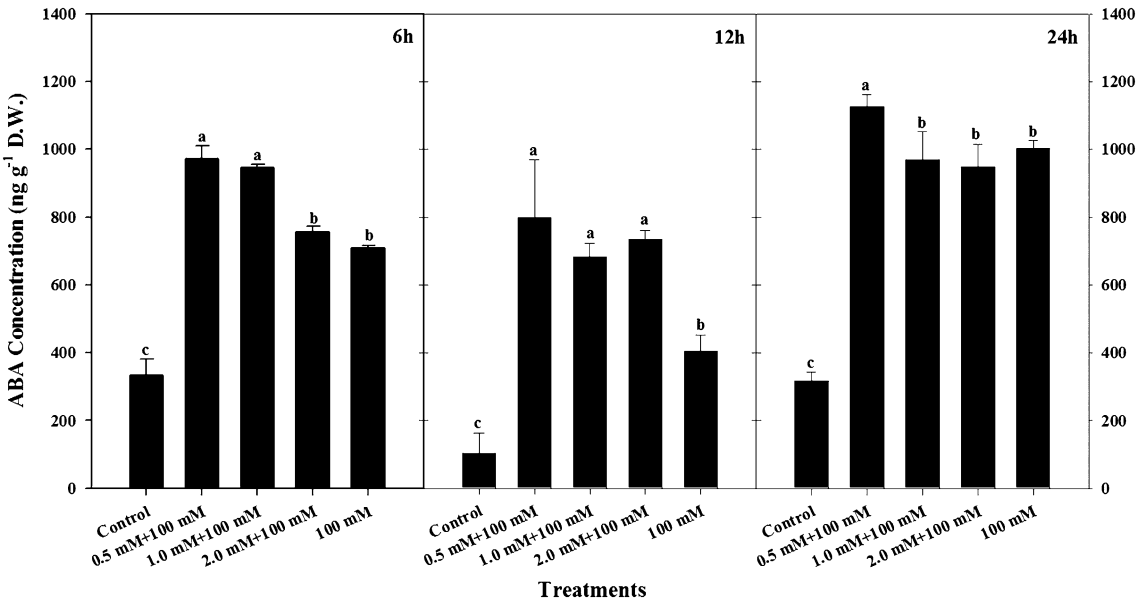

Fig. 2 Quantification of abscisic acid (ABA) after 6, 12, and 24 h of Si and NaCl application to rice plants. *Error bars* represent the standard error of the means within each treatment. For each duration set (6, 12, and 24 h), the *different letters* indicate significant differences ($P < 0.05$) between treatments as evaluated by Duncan's multiple-range test

9-*cis*-epoxycarotenoid dioxygenase (NCED1 and 3) are key enzymes that regulate the ABA biosynthesis pathway in *Arabidopsis* and other plant species, given that expression of the corresponding genes is induced either by drought or exogenous ABA (Qin and Zeevaart 1999; Seo and others 2000; Xiong and others 2002; Xiong and Zhu 2003). In ABA formation, ZEP (Xiong and others 2002), encoded by the *ZEP* gene, is the first oxygenated precursor of the ABA biosynthetic pathway. We assessed with RT-PCR the relative expression of *OsZEP* of rice plants treated with Si and exposed to salinity.

Salinity stress resulted in higher *OsZEP* expression compared to that in control plants. This level was more significant in Si-treated plants under salinity stress than non-Si-treated plants. The relative mRNA expression of *OsZEP* was slightly higher in salinity-only-stressed plants than in DW-treated plants after 6 and 12 h. Si application in combination with salinity caused significantly higher *OsZEP* expression compared to that with salinity alone and the control plants after 6 h. *OsZEP* expression was highly significant in 0.5 mM Si+ salinity-treated plants, whereas the expression level of *OsZEP* decreased with increasing Si from 0.5 to 2.0 mM after 6, 12, and 24 h. However, after 24 h, the NaCl-treated rice plants had a significantly higher mRNA expression of *OsZEP* than rice plants treated with Si + NaCl and DW (Fig. 3).

Si application and salinity stress also resulted in higher mRNA expression of *OsNCED1* compared to salinity alone and control. Plants treated with 0.5 mM Si had a significantly higher expression of *OsNCED1* under salinity stress than plants treated with other concentrations and control plants. The *OsNCED1* expression was not significantly different in 1.0- and 2.0-mM Si-treated plants under salinity stress after 6 and 12 h (Fig. 3). However, this expression was significantly different at the various Si concentrations and NaCl stress after 24 h compared to that of non-NaCl-treated plants. It was noted that with increasing Si concentration the expression of decreased after 6, 12, and 24 h. The expression of *OsNCED1* was significantly lower in DW-treated plants than in plants subjected to all other treatments after 6, 12, and 24 h. An almost similar pattern of expression was observed for *OsNCED3* (Fig. 3) and *OsNCED4* (Fig. 3).

Si and Salinity Dynamics with Other Stress-Related Hormones

We assessed the effect of Si (0.5, 1.0, and 2.0 mM) application on two antagonistically related stress hormones, that is, salicylic and jasmonic acid, under salinity stress. The endogenous JA content was significantly decreased with the application of Si under salinity stress. Salt-treated rice plants, on the other hand, had significantly higher JA content than control and Si-treated plants. JA content was significantly lower in plants treated with 0.5, 1.0, and 2.0 mM Si for 6, 12, and 24 h than in DW- and NaCl-only-treated plants (Fig. 4). For 12 and 24 h, the JA content first decreased with 0.5 mM Si, then it increased with 1.0 mM Si, and then decreased with 2.0 mM Si. However, the JA was reversed at 6 h of Si application

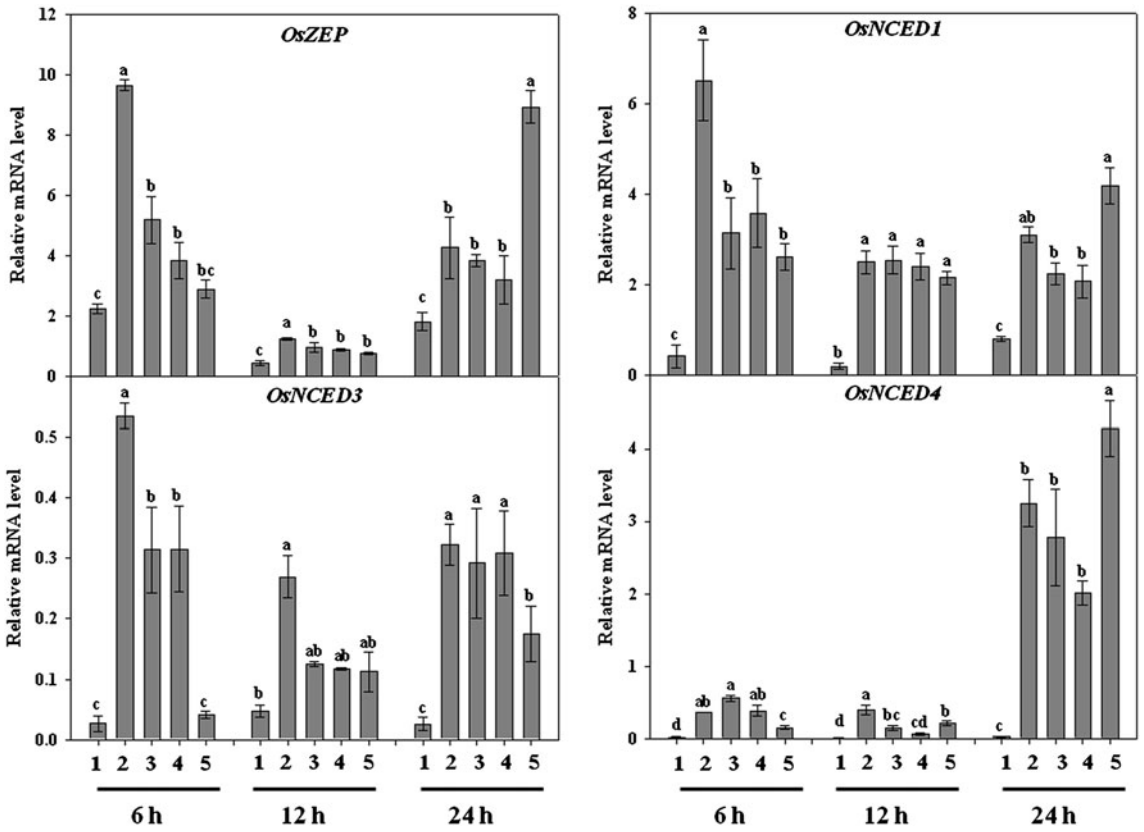

Fig. 3 mRNA gene expression of abscisic acid (ABA) biosynthesis-related genes after 6, 12, and 24 h of Si and NaCl application to rice plants. *OsZEP*, zeaxanthin epoxidase, *OsNCED1*, *3*, and *4*, 9-*cis*-epoxycarotenoid dioxygenase. *1*, control (DW treated); *2*, 0.5 mM Si + NaCl; *3*, 1.0 mM Si + NaCl; *4*, 2.0 mM Si + NaCl; and *5*, NaCl (100 mM). *Error bars* represent the standard error of the means within each treatment. For each duration set (6, 12 and 24 h), the *different letters* indicate significant differences ($P < 0.05$) between treatments as evaluated by Duncan's multiple-range test

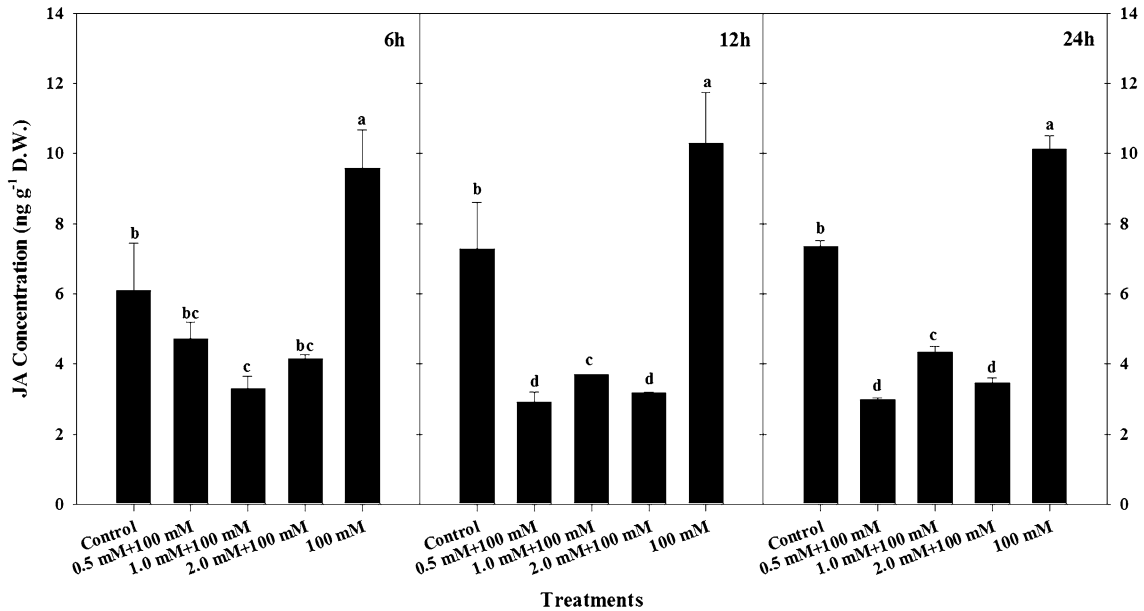

Fig. 4 Endogenous JA content after Si application in saline and nonsaline conditions. *Error bars* represent the standard error of the means within each treatment. For each duration set (6, 12, and 24 h), the *different letters* indicate significant differences ($P < 0.05$) between treatments as evaluated by Duncan's multiple-range test

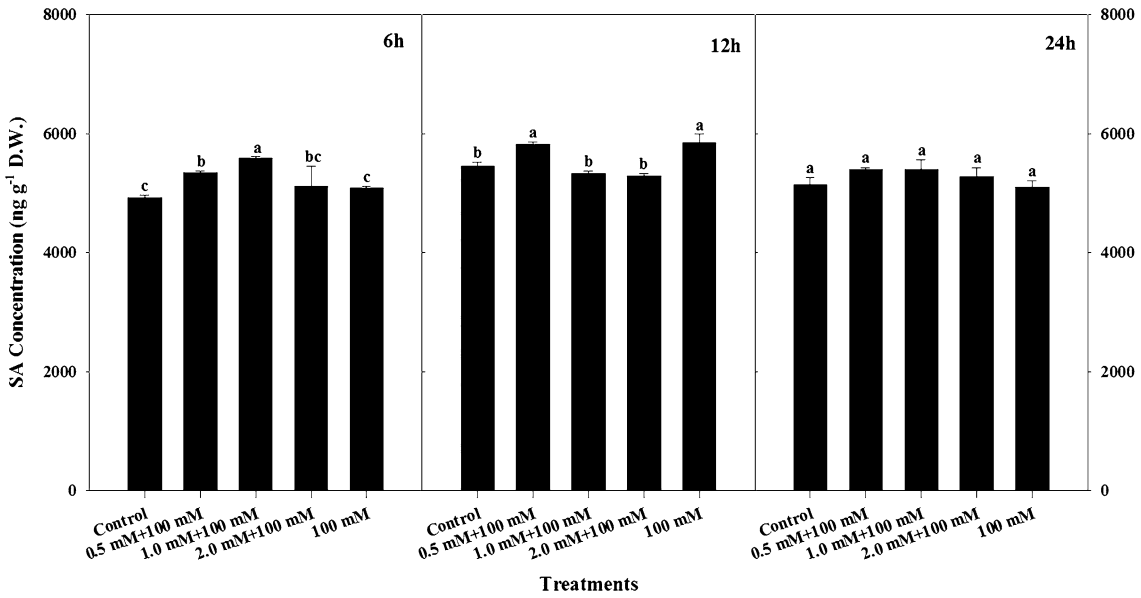

Fig. 5 Endogenous SA content as affected by Si application in saline and nonsaline conditions. *Error bars* represent the standard error of the means within each treatment. For each duration set (6, 12, and 24 h), the *different letters* indicate significant differences ($P < 0.05$) between treatments as evaluated by Duncan's multiple-range test

under salinity stress. The JA content was significantly higher in NaCl-only-treated plants and we found JA contents to almost the same after 6, 12, and 24 h.

The effect of Si on the contents of endogenous salicylic acid (SA) varied with concentration under salinity stress (Fig. 5). After 6 h, the SA content was significantly higher in Si + NaCl-treated plants than in NaCl-only-treated plants. The application of 1.0 mM Si and salinity stress yielded significantly higher SA content than the other Si treatments and control with and without salinity stress (Fig. 5). For the 12-h treatment, plants treated with 0.5 mM Si and the controls under salinity stress had significant amounts of SA. The rest of the Si treatments along with controls without salinity stress had the same level of SA. The application of Si to rice plants in salinity stress and controls with and without salinity stress had no significant effect on SA contents from short-term exposure to 24 h.

Discussion

Silicon (Si) has been known to improve plant growth and development (Ma and Yamaji 2008). This was also shown in the present study. Si application in various concentrations has significantly increased the growth attributes of rice plants. In addition, its application has also regulated the negative effects of salinity stress. Salinity is detrimental to crop growth (Hasegawa and others 2000), and previous studies have shown that exogenous Si application can improve salinity stress tolerance in several grass family crops (Ahmad and others 1992; Alpaslan and others 1998; Hamayun and others 2010). This ability of Si to increase the stress tolerance was also confirmed in the present study as reduced quantities of Na^+ were observed in the rice under salinity stress (Yeo and others 1999; Gong and others 2006). Because the amount of Na^+ was reduced in the presence of Si, a lower rate of EL was observed from the cellular masses of salinity-stressed rice plants (Tables 2 and 3) (Liang and others 2007; Hamayun and others 2010). The Si accumulation in rice provides a physical barrier by forming a 2.5-μm thin layer of silicified cells beneath the cuticle layer (0.1 mm) of leaf blades (Ma and Yamaji 2008). These Si deposits in the root zone of the plant can combat multiple abiotic and biotic stresses, including physical injury or herbivory attack as well as accumulation of cell-degrading ions (for example, Na^+) during salinity stress as these are converted to stable complexes (Gong and others 2006; Saleh and others 2013). Vaculik and others (2012) indicated that Si application to the root zone of *Zea mays* plants can increase plant growth and reduce the accumulation of the toxic cadmium ion flux. This is in agreement with our results. Si-transporter genes are exponentially regulated within a short span of time, ranging from a few minutes to several hours, to accumulate Si inside the plant tissues (Ma and Yamaji 2008). Therefore, in present study, a time-dependent response to Si by rice plants was also assessed. It was observed that with the increase in time and concentration of Si, plant growth is further improved, even during stress. Similar ameliorative effects against stress were observed by Kim and others (2011) and Zhao and others (2013).

The application of Si, on the other hand, regulates the generation of ROS during stress (Zhang and others 2006; Rouhier and Jacquot 2008). Indeed, low ROS concentrations are known to be required for signaling, growth and development, whereas high concentrations are detrimental to the cell and can damage various macromolecules (Rouhier and Jacquot 2008; Choudhury and others 2013). To combat a ROS attack, the plant recruits an impressive array of nonenzymatic and enzymatic antioxidants, whose function is to maintain adequate balance of ROS in their cells (Choudhury and others 2013). These enzymes are involved in the removal of ROS either directly (catalases and peroxidases) or indirectly through the regeneration of the two major redox molecules in the cell, ascorbate and glutathione. Accumulation of these antioxidants reveals that the plant is under higher amounts of stress (Imlay 2003; Mittler and others 2004). The plants have to use more energy to safeguard vital assets. Catalase, peroxidase, and polyphenol peroxidase activities were significantly lower in Si + NaCl-treated plants compared to NaCl-alone-treated plants. These enzymes help the plants eliminate H_2O_2 from mitochondria and microbodies and can regulate plant stress responses. The significantly lower activity of catalases in Si-treated plants under stress indicates less regulation of H_2O_2 at the cellular level (Imlay 2003). Antioxidant enzyme activities increased with salinity, drought, and heat (Mittler and others 2004; Munns and Tester 2008), while some reports have also indicated that these enzymatic activities increased in Si-treated plants exposed to abiotic and biotic stresses (Liang and others 2003, 2005, 2007). Previously, it had been indicated that increased enzymatic activities caused the reduction of plant growth. However, we observed altered levels of growth, which indicated that the plants experienced lower amounts of stress with the application of Si (Fig. 1). This finding is also supported by a previous study which indicated that Si buffers the ROS attack by immobilizing and reducing the uptake of toxic Na^+ (Liang and others 2007).

Higher ROS, on the other hand, autocatalyze peroxidation of lipid membranes and affect membrane semipermeability under high temperature and drought stress (Xu and others 2006; Choudhury and others 2013). Because membrane-bound lipid hydroperoxides are difficult to measure due to their instability, we often estimate the degree of lipid peroxidation by quantifying the secondary breakdown products like MDA (Esterbauer and Cheeseman 1990). Our results showed a decline in lipid peroxidation in plants under Si and NaCl treatments compared to that of controls and NaCl-alone-treated plants (Fig. 1). This decline indicates a decrease in membrane injury and lower oxidative stress in Si-treated plants than in control plants. Changes in the composition of membrane lipids are also associated with solute leakage. For example, higher EL occurs with an increase of linolenic acid (a fatty acid particularly prone to oxidation) content and a decrease in linoleic acid content. The reduced EL and MDA content in Si-treated plants correspond to the minimum damage to the membrane compared to that which occurred with other treatments. Antioxidant scavengers can enhance membrane stability against ROS attack, whereas MDA content can be used to assess the stress injury of plants (Mittler and others 2004; Zhang and others 2006; Munns and Tester 2008). Peroxides of polyunsaturated fatty acids generate MDA and in many cases MDA is the most abundant individual aldehydic lipid breakdown product (Esterbauer and Cheeseman 1990). However, further in-depth analyses are needed at the transcriptome level because the mechanism responsible for Si-mediated regulation of the plant antioxidant enzyme system under stress is still poorly understood.

Stress-responsive ABA can control water relations by regulating stomatal conductance and plant metabolism during stress conditions (Lee and Luan 2012). There are well-established data on the synthesis and genetic regulation of the ABA molecule from various plants such as maize (*Zea mays*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), barley (*Hordeum vulgare*), and *Arabidopsis* (Shinozaki and Yamaguchi-Shinozaki 2000; Xiong and others 2002; Shinozaki and others 2003; Xiong and Zhu 2003; Zhang and others 2006). Previous studies have shown that abiotic stress tends to upregulate the ABA biosynthetic genes such as *ZEP* and *NCED3* (Qin and Zeevaart 1999; Xiong and others 2002), which confirms our results during stress. Various Si concentrations, that is, $0.5 > 1.0 > 2.0$ mM, applied to rice plants during salinity stress gradually decreased the mRNA expressions of *ZEP*, *NCED1*, *NCED3*, and *NCED5*. In current study, with increasing duration of Si treatment (from 6 to 24 h), the mRNA expressions of *ZEP*, *NCED1*, *NCED3*, and *NCED5* in Si-treated plants tended to decrease under salinity stress compared to non-Si-treated salinity-stressed plants (Fig. 3). This shows that with the passage of time the rice plants take up greater amounts of Si to counteract the negative effects of salinity on plant growth. The higher Si concentration lowers the ABA activation during stress. This confirms the results given in Tables 1 and 2. SA, on the other hand, is irregular in its response during stress and with the application of Si (Fig. 5). The JA contents were significantly downregulated while the SA response was irregular at various Si concentrations and durations under salinity stress compared to that under salinity stress alone (Fig. 5).

Jasmonic acid is the key regulator in plant defense. It can act synergistically or antagonistically with other hormones like salicylic acid (Wasternack and others 2006). The results of the present study showed that exogenous Si treatments under salinity can significantly reduce JA compared to that in control plants. This altered level of JA and

antagonistic production of ABA might be correlated with the low level of stress in the Si-treated plants compared to that in control plants (Liang and others 2005; Epstein 2009). Ollas and others (2013) recently observed that stimulated endogenous JA can also enhance the accumulation of ABA during osmotic stress; however, in the present case, the Si application has modulated ABA responses. There is an indirect relationship of the plant's functional membrane and JA synthesis. Si can strengthen the plant membrane, which is an indication of its role in protecting membranes from injury during stress (Liang and others 2007; Ma and Yamaji 2008; Zhao and others 2013). As mentioned previously, this is in agreement with our results with respect to EL and lipid peroxidation. Si accumulation has modulated these interactive signals to synthesize reduced levels of JA compared to those in non-Si-treated plants under salinity stress (Kramell and others 1995). Because small subsets of genes are affected by both ethylene and JA signals, the interaction between these two pathways was likely to be downstream. Elevated levels of JA have been reported in various crop plants after exposure to abiotic stresses like salinity, drought (Hamayun and others 2010), and herbivory (Agrawal and others 2003). The present results coincide with those of Kramell and others (1995), who observed that the endogenous JA level was decreased after abiotic stress (Fig. 4). Thus, the results showed that during Si treatments and salinity stress, the levels of JA are altered. SA, ABA, and JA crosstalking has an important role in regulating the induced defense against stress by exerting antagonistic effects; however, these interactions need further study at the molecular level to understand the underlying mechanisms of stress tolerance, especially in the case of Si.

In conclusion, the application of Si in the root zone of crop plants can ameliorate the adverse effects of salinity stress by increasing plant growth and accumulating more Si and lowering MDA, electrolytes, and Na^+ in rice plants. The higher concentrations of Si extended greater resistance to salinity stress by regulating the genes responsible for ABA biosynthesis while reducing JA synthesis. However, the responses of SA were irregular and need further elucidation at both the biochemical and the molecular level.

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