ROLE OF SILICON IN PLANTS

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ROLE OF SILICON IN PLANTS

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Silicon (Si) is gaining increased attention in the farming sector because of its beneficial effects observed in several crop species, particularly under stress conditions. The magnitude of benefits is predominantly observed in plant species that can accumulate Si above a certain threshold. Therefore, deciphering the molecular mechanisms and genetic factors conferring a plant ability to take up silicon is necessary. Along these lines, several efforts have been made to identify the specific genes regulating Si uptake and distribution in plant tissues. This information finds its usefulness in identifying Si-competent species, and could eventually lead to improving this ability in low-accumulating species. The successful exploitation of Si in agriculture depends highly on the understanding of different Si properties including plant-available Si from the soil, transport within tissues, deposition in planta, and Si effect on different metabolic and physiological processes. In addition, a better comprehension of external factors influencing Si uptake and deposition in plant tissue remains important. A plant can take up Si efficiently only in the form of silicic acid and most soils, despite containing high concentrations of Si, are deficient in plant-available Si. Consequently, soil amendment with fertilizers rich in plant-available Si is now viewed as an affordable option to protect plants from the biotic and abiotic stresses and achieve more sustainable cropping management worldwide. Articles compiled in the present research topic touch upon several aspects of Si properties and functionality in plants. The information will be helpful to further our understanding of the role of Si and contribute to exploit the benefits plants derive from it.

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Silicon dynamics and distribution in plants

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Editorial: Role of Silicon in Plants

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Keywords: silicon uptake, abiotic stress tolerance, biotic stress tolerance, transport dynamics, physiology

Editorial on the Research Topic

Role of Silicon in Plants

Silicon (Si), the second most abundant element on earth surface, is rapidly gaining attention in agriculture because of its many beneficial effects for plants. Hundreds of studies performed with several plant species and under diverse growth conditions have demonstrated the favorable benefits of Si fertilization, particularly in alleviating biotic and abiotic stresses (Fauteux et al., 2005, 2006). Ever since the breakthrough discovery of genes explaining the molecular mechanisms of Si uptake and transport in plants a decade ago (Ma et al., 2006, 2007), many research endeavors have tried to explain how and why Si presence in plants confers advantages. The most challenging aspect consists in defining a mechanistic model explaining the precise mechanisms involved in Si-derived stress tolerance. While many hypotheses have been proposed, there is no conclusive evidence showing exactly how Si plays a role in stress tolerance. Current efforts to resolve this enigma involve comprehensive analyses of the effect of Si supplementation on various abiotic and biotic stresses, biochemical and physiological parameters, mineral co-localization and distribution, and transcriptomic and metabolomic responses. At the same time, research activities are focused on improving Si fertilization and Si sources for crop cultivation. The present research topic compiles many aspects helpful to generate a better understanding required for the optimal utilization of Si to promote sustainable development and climate-adapted cropping.

ROLE OF SILICON IN ABIOTIC STRESS TOLERANCE AND PLANT PHYSIOLOGY

Abiotic stress is one of the most severe constraints for crop cultivation all over the world. Because of climate change and unpredictable weather, abiotic stresses have become more common and challenging. Plants generate reactive oxygen species (ROS) as a first response to most abiotic stresses like salinity, drought, thermal, and heavy metal stress. This response is known to cause severe damages to cell structure and organelles, and to alter normal cell function. A study conducted by Hasanuzzaman et al. has shown that plants growing under heavy metal stress (cadmium) had reduced ROS contents when supplemented with Si compared to control plants. The improved antioxidant defense mechanisms against Cd stress with Si supplementation was found to be associated with an efficient augmentation of antioxidant components, associated with an increased activity of AsA-GSH and glyoxalase pathways. Similarly, Pontigo et al. also observed that Si-derived aluminum (Al) stress tolerance in ryegrass was associated with a change in ROS profile and reduced uptake of Al by plants from the soil.Incidentally, a review article by Kim et al. discusses the role of Si in abiotic stress and its possible implication in regulating the generation of ROS.

Abiotic stress significantly affects physiological processes leading to altered metabolic activities and overall health of plants. Grasses are well-known high accumulators of Si, and, therefore, serve as an excellent model to study the role of Si in plant physiology. To investigate the passive and active regulation of Si transport, McLarnon et al. evaluated some physiological parameters in

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three genotypes of forage grass differing in their ability to accumulate Si. Their results suggest that the varietal differences are attributed to stomatal conductance and transpiration, particularly when plants are grown under control conditions. However, under stress (wounding), an increased level of Si was noticed in all three genotypes, a reaction attributed to a higher expression of Si transporter genes. These results suggest an active mode of regulation of Si uptake under stress conditions. Similarly, Soundararajan et al. observed an improved stomatal development in tissue-cultured carnation plants supplemented with Si. This was correlated with a differential expression of proteins linked to photosynthesis, ribosomes, oxido-reduction, hormone signaling, metal ion binding, and defense responses. Manivannan and Ahn have critically reviewed several such studies suggesting a role of Si in regulating physiological processes in plants. Similarly, based on several studies conducted over the last decade,Rios et al. proposed a model explaining how Si could improve stomatal functioning and enhance root hydraulic conductance through the regulation of aquaporins. The effect of Si on the transcriptional regulation of genes involved in water transport and stress related pathways, including the jasmonic acid pathway, ABA-dependent or independent regulatory pathway, and phenylpropanoid pathway, have been proposed in several studies (Manivannan and Ahn; Rios et al.). Nevertheless, there is still no conclusive evidence for the direct active involvement of Si in any metabolic processes that can explain systematically how Si regulates cellular processes.

ROLE OF SILICON IN BIOTIC STRESS TOLERANCE

The beneficial effects of Si in improving tolerance against diseases and pests are arguably the most commonly described. A review article by Wang et al. provides a catalog of many significant studies and discusses models explaining the role of Si. For a long time, Si-derived resistance to pathogens and insects was thought to be the result of a mechanical barrier formed by the deposition of Si along the cell wall thus hindering their progression. However, studies performed in the 90's associated the presence of Si with specific defense responses *in planta* (Chérif et al., 1992, 1994; Fawe et al., 1998), a phenomenon that has since been shown in many host–pathogen interactions (Fauteux et al., 2005). In a recent study, Si was further shown to interfere with host–pathogen recognition, probably by preventing effectors and signaling molecules from finding their specific targets (Vivancos et al., 2015). Silicon was also suggested to induce indirect defense mechanisms by altering the composition of herbivore-induced plant volatiles (HIPV) (Liu et al.). The HIPV compounds play an important role in attracting parasitoids to infested rice plants. The list of studies reporting prophylactic effects of Si against diseases grow continuously but the underlying molecular mechanisms explaining these properties are not yet fully understood. Nevertheless, these studies offer additional support to explore Si as an environmental-friendly option for sustainable crop management.

The evaluation of different sources of Si is a critical aspect to optimize the practical use of Si fertilization. In this context, Ouellette et al. tested different Si fertilization regimes under high tunnel and field conditions for strawberry production. Under high tunnel, strawberry plants accumulated as much as 3% d.w. Si, which resulted in significant reduction of powdery mildew severity and higher yields. On the other hand, strawberry plants grown in soil, were unable to absorb Si, whether amended in liquid or solid form. Similarly, Keeping tested several sources of Si including fused magnesium (thermo) phosphate, volcanic rock dust, magnesium silicate, calcium silicate slag, and granular potassium silicate for sugarcane plant growth. Only the latter source led to a significant increase in Si accumulation. These studies suggest that Si sources and modes of application will greatly influence Si accumulation in different plant species. Therefore, more extensive efforts are required to better understand the relationship between Si sources and soil properties to obtain higher levels of plant-available Si. Apart from these conventional sources, nano-technological advances are also being used to explore possibilities for the application of Si nanoparticles as a source to elevate stress tolerance in plants (Luyckx et al.).

SILICON DYNAMICS AND DISTRIBUTION IN PLANTS

Understanding the dynamics of molecular movement is essential to explain how plants take up water and mineral elements from the soil and subsequently distribute them to different tissues. While protein polarity and expression regulation of *Lsi1* and *Lsi2* transporter genes are well defined, less is known about investment efficiency and the functionality behind the regulation. A new model simulating the dynamics of Si in the whole rice plant has been developed by Sakurai et al. taking into account Si transport, distribution, and gene expression. Results of the simulation experiments suggest that rice has evolved a system maximizing the investment efficiency of Si uptake. Another study in rice by Hinrichs et al. has shown the involvement of an ATP binding cassette (ABC) transporter (OsABCG25) in the Si-promoted Casparian band formation. This study associates the processes of Si-promoted Casparian-band formation and the role of the exodermis with flux control in the roots.

Deposition of Si mostly occurs in leaf epidermal cells, in outer epidermal cells of inflorescence bracts and in root endodermis. While this influences passive mechanisms like transpiration, Si deposition is not a random process. In a review article, Kumar et al. describe Si depositions patterns, based mostly on observations in grasses, and suggest three dominant modes of Si deposition, namely directed paramural silicification in silica cells, spontaneous cell wall silicification, and directed cell wall silicification.

Silicon deposition has been mostly studied in root and aerial vegetative tissues, while very few efforts have been made to understand Si deposition in seeds. Bokor et al. offered a rare look in this field by exploiting ionomics to study Si in maize kernels.

Ionomics results showed a significant correlation between Si deposition and other elements like Mg, P, S, N, P, Ca, Cl, Zn, and Fe. Bokor et al. observed Si accumulation in the pericarp and embryo but not in the soft endosperm or the scutellum. These studies describing Si dynamics and distribution in plants contribute new findings toward elucidating the role of Si *in planta*.

OUTLOOK

In spite of the ubiquitous presence and effect of Si on numerous plant species, Si research has been mostly conducted on monocots in general, and grasses in particular. This is evidently attributable to the fact that grasses are high Si accumulators, and that rice is a convenient and useful model for Si studies. However, many dicots and primitive plant species are equally known to accumulate high amounts of Si, and recent advents in genomics have confirmed a high level of conservation in Si transporters across the plant kingdom (Deshmukh and Bélanger, 2016). These new finding should stimulate Si research on different plant species and help unravel the complexities of Si properties in plants. Of particular interest, there is a need to dissociate Si from correlative roles, and design experiments aimed at defining with precision whether Si is metabolically or biochemically active or not. In the same manner, the prophylactic properties of Si against biotic and abiotic stress are more than likely linked to a universal phenomenon rather than a multitude of hypotheses, and collaborative efforts should seek to elucidate this mystery. Concerted efforts in Si research can only lead to its accelerated and improved application in the context of sustainable agriculture.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Exogenous Silicon Attenuates Cadmium-Induced Oxidative Stress in *Brassica napus* L. by Modulating AsA-GSH Pathway and Glyoxalase System

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Cadmium (Cd) brings a devastating health hazard to human being as a serious consequence of agricultural and environmental contamination. We demonstrated the protective effect of silicon (Si) on cadmium (Cd)-stressed rapeseed (*Brassica napus* L. cv. BINA Sharisha 3) plants through regulation of antioxidant defense and glyoxalase systems. Twelve-day-old seedlings were exposed to Cd stress (0.5 and 1.0 mM $CdCl_2$) separately and in combination with Si (SiO_2, 1.0 mM) for 2 days. Cadmium toxicity was evident by an obvious oxidative stress through sharp increases in H_2O_2 content and lipid peroxidation (malondialdehyde, MDA content), and visible sign of superoxide and H_2O_2. Cadmium stress also decreased the content of ascorbate (AsA) and glutathione (GSH) as well as their redox pool. The activities of monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and catalase (CAT) were decreased by Cd while ascorbate peroxidase (APX) and glutathione *S*-transferase (GST) activities were increased. The enzymes of glyoxalase system (glyoxalase I, Gly I and glyoxalase II, Gly II) were also inefficient under Cd stress. However, exogenous application of Si in Cd treated seedlings reduced H_2O_2 and MDA contents and improved antioxidant defense mechanism through increasing the AsA and GSH pools and activities of AsA-GSH cycle (APX, MDHAR, DHAR and GR) and glyoxalase system (Gly I and Gly II) enzymes and CAT. Thus Si reduced oxidative damage in plants to make more tolerant under Cd stress through augmentation of different antioxidant components and methylglyoxal detoxification system.

Keywords: antioxidant defense, heavy metals, plant nutrients, ROS, trace element

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INTRODUCTION

Cadmium (Cd) is one of the most toxic elements of the earth releasing from natural and anthropogenic sources which poses detrimental hazardous effects both in plant and animal kingdoms (Wu et al., 2017). Cadmium exposure interrupts nutrient uptake, inhibits enzyme activities, generates reactive oxygen species (ROS) and damages cell components (Wu J. et al., 2016; Rahman et al., 2017). Cadmium possesses various degrees of phytotoxicity and exhibits

potential health problems when accumulated in edible parts of crops (Wu Z. et al., 2016). In plant, Cd threats seed germination and seedling growth (Liu et al., 2012), disrupts photosynthetic machinery (Burzyński and Zurek, 2007) and cellular redox (Wu et al., 2017), damages meristem nucleoli (Qin et al., 2010), and disrupts protein structure (Kabir et al., 2016). Apart from these, Cd-induced growth inhibition, leaf rolling, cholrosis, necrosis, reduced water potential and even death are common phenomena (Sharma and Dubey, 2007; Anjum et al., 2008; Gill and Tuteja, 2011).

Cadmium induces oxidative stress indirectly by enhancing ROS production; such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) (Andresen and Küpper, 2013; Rahman et al., 2016). Plants' antioxidant defense system contains some non-enzymatic antioxidants such as ascorbate (AsA) glutathione (GSH), phenolic compounds, alkaloids, non-protein amino acids, and α-tocopherols as well as a bunch of antioxidant enzymes like catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione *S*-transferase (GST) etc. (Hasanuzzaman et al., 2012a; Rahman et al., 2016). The AsA-GSH cycle enzymes are APX, MDHAR, DHAR and GR and a good coordination among these enzymes can also render better tolerance to Cd or any other metal toxicity (Hasanuzzaman et al., 2012b). Highly cytotoxic methylglyoxal (MG) can also be produced in larger amount in plants if exposed to Cd stress. However, the thiol-dependent glyoxalase I (Gly I) and glyoxalase II (Gly II) enzymes can detoxify it by sequential reactions (Rahman et al., 2016; Hasanuzzaman et al., 2017a,b).

Rapeseed (*Brassica napus* L.) is a plant of Brassicaceae family which is grown as oilseed crop, used as leafy vegetable and feed for cattle. Plants of Brassicaceae family are known as metal accumulators having potential roles in phytoextraction (Gall and Rajakaruna, 2013; Ahmad et al., 2015; Mourato et al., 2015). There are several reports demonstrating the performance of *Brassica* spp. as phytoremediator of heavy metal including Cd (Mourato et al., 2015 and references therein). Reduction of oil content and growth performance was reported in *B. juncea* L. under Cd stress (Ahmad et al., 2015). Effect of Cd stress on oxidative stress tolerance and methylglyoxal detoxification system were not studied extensively in rapeseed plant.

Silicon (Si) is considered to be one of the most common elements of the earth crust by mass which has positive roles in diminishing detrimental effects caused by various heavy metals (Greger et al., 2016; Wu J. et al., 2016; Wu Z. et al., 2016; Rahman et al., 2017; Wu et al., 2017). In a recent study, Kabir et al. (2016) reported the Si-mediated mitigation of Cd toxicity in *Medicago sativa* L. by limiting Fe uptake which involves the mechanism of Fe acquisition downregulation. They also noted that Si might have some roles in protecting plants from oxidative stress through modulating antioxidant enzyme activities. In a similar experiment, Wu et al. (2017) also reported Si induced tolerance to oxidative stress where Si reduced the membrane damage modulating the activities of AsA-GSH enzymes. Considering the above facts, the present study has been executed to investigate the role of exogenous Si application in diminishing Cd-induced oxidative stress through regulating AsA-GSH pathway and glyoxalase system in *B. napus* seedlings.

MATERIALS AND METHODS

Plant Materials, Treatments and Design of Experiment

Sterilized uniform seeds of rapeseed (*B. napus* L. cv. BINA Sharisha 3) were grown under controlled conditions (light, 350 μmol photon $m^{-1}s^{-2}$; temperature, 25 ± 2°C; relative humidity, 65–70%). Hyponex solution (Hyponex, Japan) was applied as nutrient according to necessity after 5,000-fold dilution (EC 0.849 dS m^{-1}; pH 6.0. Twelve-day-old seedlings were treated with 1.0 mM silicon (SiO_2; Wako, Japan) and 0.5 and 1.0 mM Cd ($CdCl_2$; Cadmium Chloride Anhydrous, Wako, Japan). Cadmium concentration of 0.5 and 1.0 mM were considered as mild and severe stress, respectively. Cadmium and Si were applied independently and in combination. The selected dose of Si showed better results under those Cd stresses which were selected after several trial experiments considering oxidative damage or membrane lipid peroxidation level (Supplementary Figure S1) and the phenotypic appearance. Seedlings grown in Hyponex solution only were used as control. Experimental design of this study was completely randomized design (CRD) with three replications. Data were taken after 48 h.

Measurement of Lipid Peroxidation

Malondialdehyde (MDA) content was estimated to measure the level of lipid peroxidation using thiobarbituric acid (TBA) reagent for extraction of leaves (Heath and Packer, 1968; Hasanuzzaman et al., 2017a).

Determination of Hydrogen Peroxide Content

Potassium-phosphate (K-P) buffer (pH 6.5) was used for extracting the leaves and centrifugation was done at 11,500 × *g*. After that, a mixture of titanium tetrachloride ($TiCl_4$) and 20% sulphuric acid (H_2SO_4) (v/v) was added to the supernatant. The final mixture was read spectrophotometrically at 410 nm (Yu et al., 2003).

Histochemical Detection of Hydrogen Peroxide and Superoxide

The H_2O_2 and $O_2^{\bullet-}$ were localized histochemically (Chen et al., 2010) by staining leaves with 1% 3,3-diaminobenzidine (DAB) and 0.1% nitroblue tetrazolium chloride (NBT) solution, respectively.

Extraction and Measurement of Ascorbate and Glutathione

Measurement of ascorbate and glutathione was done by using leaves homogenized in 5% meta-phosphoric acid containing 1 mM ethylenediaminetetraacetic acid (EDTA) and then centrifuging at 11,500 × *g* for 12 min at 4°C. The AsA and

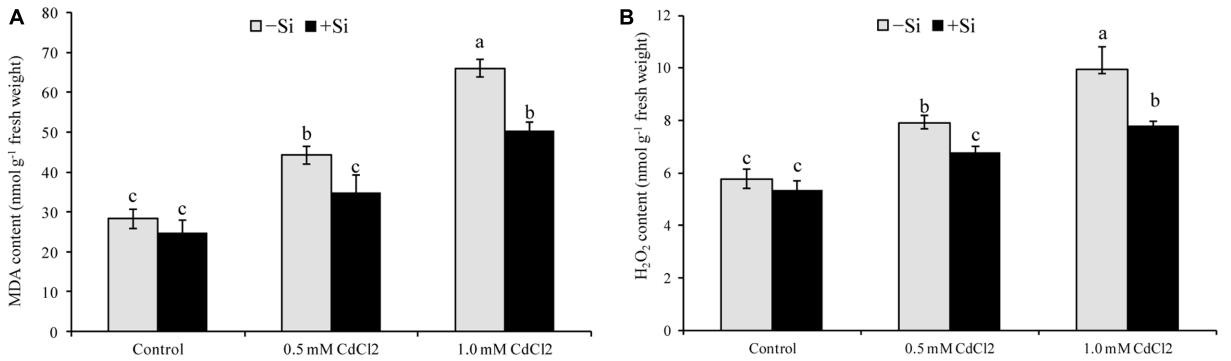

FIGURE 1 | Silicon-induced changes in oxidative stress markers (MDA, **A** and H_2O_2, **B** content) in *Brassica napus* seedlings grown under Cd stress. Values (Mean ± SD) of each treatment are obtained from three replications. Bars with different letters are significantly different at $P < 0.05$ applying Tukey's HSD test.

dehydroascorbate (DHA, oxidized form of AsA) content was assayed following the methods of Huang et al. (2005) and (Nahar et al., 2016a,b). Glutathione and glutathione disulfide (GSSG, oxidized form of GSH) was examined following the method of Yu et al. (2003) and Hasanuzzaman et al. (2017a).

Determination of Protein

The amount of protein from each sample was determined using bovine serum albumin (BSA) as a protein standard (Bradford, 1976). Different concentrations of solution were prepared with BSA to make standard curve which was used to determine the protein concentration of each plant sample.

Enzyme Extraction and Assays

Leaf tissue was homogenized in 1 mL of 50 mM ice-cold K-P buffer (pH 7.0) containing 100 mM KCl, 1 mM ascorbate (AsA), 5 mM β-mercaptoethanol, and 10% (w/v) glycerol. The homogenates were centrifuged at 11,500 × *g* for 10 min, and the supernatants were used to measure enzyme activity (Hasanuzzaman et al., 2017a).

Ascorbate peroxidase (EC: 1.11.1.11) activity was measured according to Nakano and Asada (1981) with a solution mixture of K-P buffer (pH 7.0), AsA, H_2O_2, EDTA, and enzyme extract which was read at 290 nm (Hasanuzzaman et al., 2017a).

Monodehydroascorbate reductase (EC: 1.6.5.4) activity was determined following the method described in Hossain et al. (1984). The reaction mixture contained Tris–HCl buffer (pH 7.5), NADPH, AsA, AO, and enzyme solution which was read at 340 nm (Hasanuzzaman et al., 2017a).

Dehydroascorbate reductase (EC: 1.8.5.1) activity was assayed according to the method of Nakano and Asada (1981). The reaction buffer contained K-P buffer (pH 7.0), GSH, EDTA, and dehydroascorbate (DHA), plant sample and it was read at 265 nm (Hasanuzzaman et al., 2017a).

Glutathione reductase (EC: 1.6.4.2) activity was measured according to the method of Hasanuzzaman et al. (2017a) by monitoring absorbance at 340 nm. The reaction mixture contained K-P buffer (pH 7.0), EDTA, GSSG, NADPH, and enzyme extract.

Glutathione *S*-transferase (EC: 2.5.1.18) activity (Hossain et al., 2006): The reaction mixture contained 100 mM Tris–HCl buffer (pH 6.5), 1.5 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and enzyme solution which was read at 340 nm (Hasanuzzaman et al., 2017a).

Catalase (EC: 1.11.1.6) activity was determined following the method of Hasanuzzaman et al. (2017a) by monitoring absorbance at 240 nm. Enzyme extract was added with the reaction mixture containing K-P buffer (pH 7.0) and H_2O_2.

Gly I (EC: 4.4.1.5) activity was determined following the method of Hasanuzzaman et al. (2017a). The assay mixture consisted of K-P buffer (pH 7.0), $MgSO_4$, GSH, MG, and enzyme extract which was read at 240 nm.

Gly II (EC: 3.1.2.6) activity was determined according to Principato et al. (1987) and Hasanuzzaman et al. (2017a). Reaction mixture contained Tris–HCl buffer (pH 7.2), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB), *S*-D-lactoylglutathione (SLG), enzyme extract, and absorbance was recorded at 412 nm.

Statistical Analysis

The data were subjected to analysis of variance (ANOVA), and the mean differences were compared by Tukey's honest significant difference (HSD) test using XLSTAT v.2017 (Addinsoft, 2017). Differences at $P \leq 0.05$ were considered significant.

RESULTS

Oxidative Damage

Membrane lipid peroxidation increased under Cd stress indicated by increased MDA contents by 56% and 133% in mild and severe stress, respectively, compared with control (**Figure 1A**). Hydrogen peroxide content also rose significantly under Cd stress (**Figure 1B**). However, exogenous Si application reduced both the MDA and H_2O_2 contents (**Figure 1**) in Cd-affected seedlings, compared to Cd alone. As an indicator of oxidative stress, H_2O_2 and $O_2^{\bullet-}$ were determined through histochemical staining. Leaves of the Cd-stressed plants showed brown spots of H_2O_2 and dark blue spots of $O_2^{\bullet-}$ (**Figure 2**) which were prominently

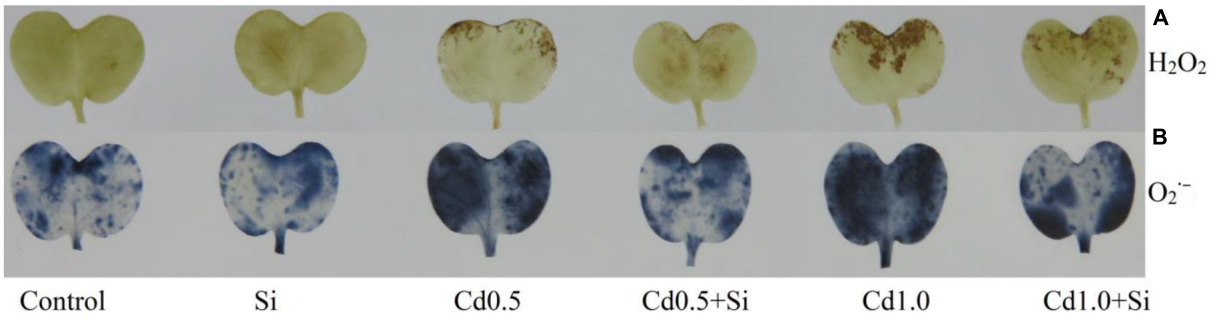

FIGURE 2 | Histochemical detection of H_2O_2 **(A)** and $O_2^{\bullet-}$ **(B)** in leaves of *Brassica napus* seedlings grown under Cd stress induced by exogenous Si. Si, Cd0.5 and Cd1.0 indicate 1.0 mM SiO_2, 0.5 mM $CdCl_2$ and 1.0 M $CdCl_2$, respectively.

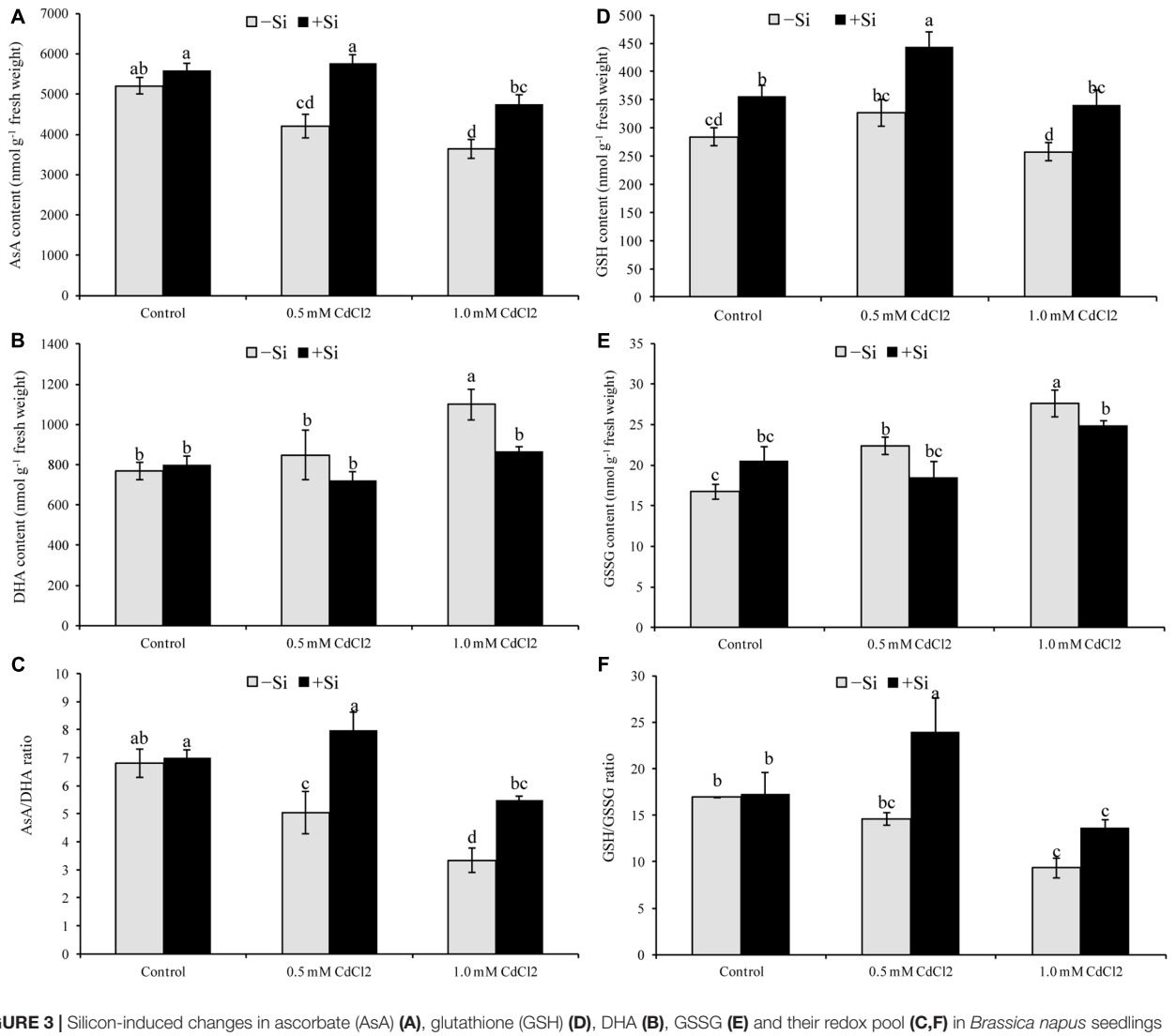

FIGURE 3 | Silicon-induced changes in ascorbate (AsA) **(A)**, glutathione (GSH) **(D)**, DHA **(B)**, GSSG **(E)** and their redox pool **(C,F)** in *Brassica napus* seedlings grown under Cd stress. Values (Mean ± SD) of each treatment are obtained from three replications. Bars with different letters are significantly different at $P < 0.05$ applying Tukey's HSD test.

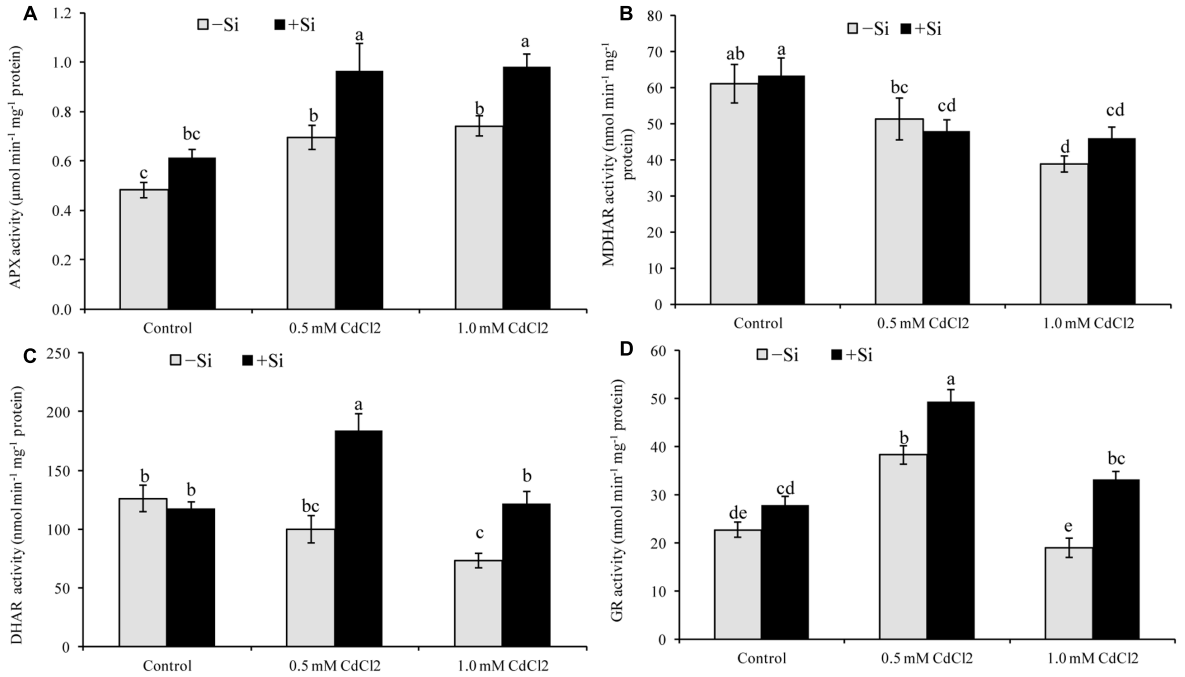

FIGURE 4 | Silicon-induced changes in the activities of AsA-GSH cycle enzymes (APX, MDHAR, DHAR and GR presented in **A–D**, respectively) in *Brassica napus* seedlings grown under Cd stress. Values (Mean ± SD) of each treatment are obtained from three replications. Bars with different letters are significantly different at $P < 0.05$ applying Tukey's HSD test.

evident, compared to control. However, exogenous Si application decreased those spots noticeably from the leaves of Cd affected plants.

Ascorbate and Glutathione Pool

Ascorbate content and AsA/DHA ratio decreased under both levels of Cd stress but DHA content enhanced only in case of higher concentration of Cd (**Figures 3A–C**). Glutathione only decreased with higher level of stress, compared to control. Higher GSSG content was recorded in both levels of stress with a reduction in GSH/GSSG ratio (**Figures 3D–F**). Silicon supplementation decreased DHA content but increased AsA content and AsA/DHA ratio; Si addition with Cd decreased GSSG and increased GSH and GSH/GSSG ratio, compared to the Cd stress alone (**Figures 3A–F**).

Activities of Antioxidant Enzymes

AsA-GSH Cycle Enzymes

The activity of APX increased by 43 and 53% under mild and severe stress, respectively, compared to control. Si supplementation with Cd further increased its activity (**Figure 4A**). Cadmium stress reduced the activities of MDHAR and DHAR in both levels of stress. After Si application, activity of DHAR increased by 84 and 66% in mild and severe stresses, respectively, compared to the non-treated stressed seedlings (**Figures 4B,C**). Comparing with control, GR activity increased and decreased under mild and severe Cd stress, respectively. Silicon addition increased GR activity by 29 and 75% in mild and severe stress, respectively, compared to stress alone (**Figure 4D**).

Other Antioxidant Enzymes

Seedlings exposed to Cd stress reduced CAT activity, compared to control. In contrast, Si addition increased CAT activity by 79 and by 51% for mild and severe stress, respectively, compared to Cd stress alone (**Figure 5B**). Though GST activity upregulated by 108 and 139% under mild and severe stress, respectively (compared with control), Si supplementation didn't change its activity further (**Figure 5A**).

Glyoxalse System Enzymes

Rapeseed seedlings exposed to Cd stress reduced Gly I activity by 16% under mild stress and by 38% under severe stress, compared to control. Cadmium stress also reduced Gly II activity (by 20 and 32% under mild and severe stress, respectively). Silicon addition with Cd increased both Gly I and Gly II activities under both levels of stress (compared to Cd stress alone) (**Figures 6A,B**).

Phenotypic Appearance of Seedlings

Cadmium stress resulted in chlorosis/leaf yellowing symptom, Cd stress also decreased seedlings vigor, compared to control seedlings. Exogenous Si improved phenotypic appearance of seedlings improving the seedlings vigor and alleviating chlorosis symptom (compared to Cd stressed seedlings without Si) (**Figure 7**).

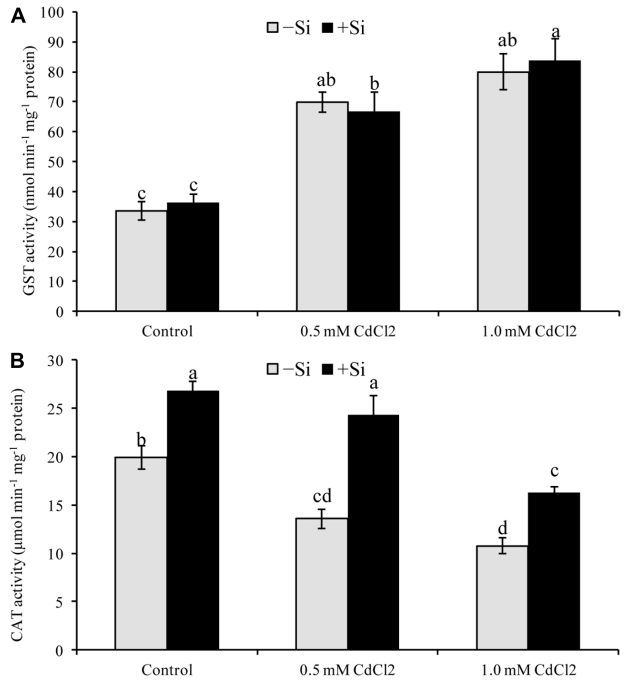

FIGURE 5 | Silicon-induced changes in the activities of glutathione *S*-transferase (GST) **(A)** and catalase (CAT) **(B)** in *Brassica napus* seedlings grown under Cd stress. Values (Mean ± SD) of each treatment are obtained from three replications. Bars with different letters are significantly different at $P < 0.05$ applying Tukey's HSD test.

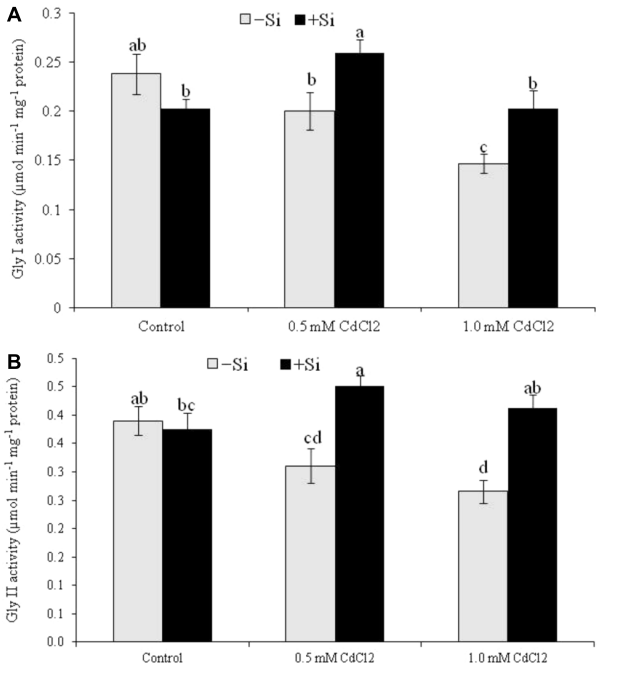

FIGURE 6 | Silicon-induced changes in the activities of glyoxalase enzymes (Gly I and Gly II activity presented in **A** and **B**, respectively) in *Brassica napus* seedlings grown under Cd stress. Values (Mean ± SD) of each treatment are obtained from three replications. Bars with different letters are significantly different at $P < 0.05$ applying Tukey's HSD test.

DISCUSSION

Cadmium does not participate in Fenton reaction (Clemens, 2006). Cd amplifies free Fe^{++} ion by displacing it from active sites which enhances Fenton reaction and ROS production (Romero-Puertas et al., 2004). Cadmium indirectly activates NADPH oxidase activity (Romero-Puertas et al., 2004), impairs stomatal movement, photosynthetic machinery (Islam et al., 2008), CO_2 fixation enzymes (Romero-Puertas et al., 2004) and enhances ROS production. In this study, the *B. napus* seedlings treated with Cd showed oxidative damage (increased H_2O_2 production and MDA content) corroborating the results of previous studies (Andresen and Küpper, 2013; Srivastava et al., 2015). Leaves of the Cd-stressed plants showed brown spots of H_2O_2 and dark blue spots of $O_2^{\bullet-}$. An identical pattern of oxidative stress and damage was noticed in Cd affected mung bean seedlings (Nahar et al., 2016b).

The addition of Si in Cd-treated rapeseed plants reduced the spots of H_2O_2 and $O_2^{\bullet-}$, decreased H_2O_2 and lipid peroxidation/MDA level enhancing the antioxidant defense mechanism (compared to Cd treated plant only). The presence of Si in plant growing medium decreases Cd uptake through root and then decreases the transfer of Cd to shoot which reduces Cd-induced cellular damages (Srivastava et al., 2015; Tang et al., 2015). Decreasing Cd uptake and increasing antioxidant enzymes and photosynthesis Si reduced oxidative stress in cotton plant (Farooq et al., 2013). Results of several other studies are also supportive of the investigation of the present study (Srivastava et al., 2015; Tang et al., 2015).

Ascorbate is potent water soluble ROS scavenger of cell converting H_2O_2 to H_2O by the activity of APX (Hasanuzzaman et al., 2012a; Gill et al., 2015). Ascorbate content and AsA/DHA ratio declined, APX activity increased under Cd stress which was accountable for increasing H_2O_2 level (Hasanuzzaman et al., 2017a). The enzymes MDHAR and DHAR take part in the regeneration of AsA from its oxidative state DHA (Hasanuzzaman et al., 2012a). So, a decrease of AsA content in Cd affected seedlings of this study is corroborating with the decrease activities of MDHAR and DHAR. But Si addition with Cd increased the activities of MDHAR and DHAR, and AsA restoration, AsA/DHA ratio decreasing DHA content (compared to Cd stress alone). When Si was supplemented with Cd treatments the seedlings also showed higher APX activity, compared to Cd treatments alone which is supported by the findings of other studies (Tang et al., 2015). Enhanced activities of AsA-GSH cycle enzymes APX, MDHAR, GR with enhanced levels of AsA and GSH were induced by exogenous Si application in Chilling stressed cucumber leaves which alleviated the oxidative stress (Jiao-jing et al., 2009).

Glutathione having vital biological functions is a water soluble antioxidant of non-protein thiol group, profusely dispersed in the cytosol, chloroplast, cytoplasm, apoplast, mitochondria, and peroxisome. It scavenges a range of ROS viz., H_2O_2, $OH^{\bullet}$, and

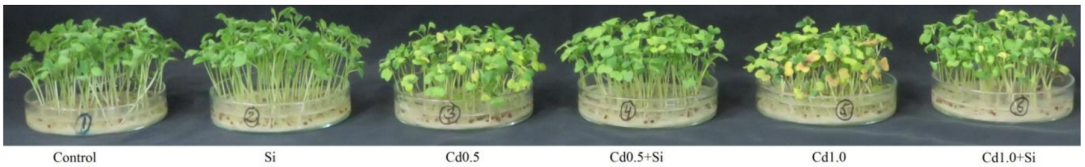

FIGURE 7 | Phenotypic appearance of *Brassica napus* seedlings grown under Cd stress induced by exogenous Si. Si, Cd0.5 and Cd1.0 indicate 1.0 mM SiO_2, 0.5 mM $CdCl_2$ and 1.0 M $CdCl_2$, respectively.

$^1O_2$ (Anjum et al., 2012; Hasanuzzaman et al., 2012a; Gill et al., 2013). In the present study, GSH level did not change but the GSSG level increased highly under Cd stress that resulted in a reduced GSH/GSSG ratio, compared to control. Glutathione reductase catalyzes the reaction involved in transformation of GSSG to GSH. Under mild Cd stress the activity of GR increased but that was not enough to restore and increase GSH content significantly. Under severe Cd stress, both GR activity and GSH level diminished. Similar trend of GSH and GSSG pool, and GR activity were reported previously under Cd stress (Hasanuzzaman et al., 2017a). When Si was co-applied with Cd the activity of GR increased in rapeseed seedlings which renovated and augmented content of GSH, dropped off GSSG level to increase the GSH/GSSG ratio which is comparable with previous studies (Srivastava et al., 2015; Tang et al., 2015).

Catalase presenting in different cell organelles (Garg and Manchanda, 2009) boosts up ROS scavenging process with its highest capacity to scavenge upto six million H_2O_2 in a minute (Gill and Tuteja, 2010). The activity of CAT decreased noticeably due to Cd exposure which is substantiating with the increased H_2O_2 level, compared to control. Exogenous Si supplementation restored and augmented CAT activity of Cd affected rapeseed seedlings which decreased H_2O_2 generation, compared to Cd treatment only which is supported by a similar previous study with rice (Srivastava et al., 2015).

Glutathione *S*-transferases presenting in apoplast, cytosol, chloroplast, mitochondria catalyze the conjugation of xenobiotic substrates and GSH. The activities of GSTs were found to be upregulated in plants under Cd and other stresses as well (Dixon et al., 2010; Hasanuzzaman et al., 2012b, 2017a) that support increased GST activity of Cd affected seedlings of our study. The activity of GST did not increase further in Cd affected seedlings supplemented with exogenous Si. But Debona et al. (2014) demonstrated Si induced enhancement of GST activity in wheat leaves.

Methylglyoxal is an α-oxoaldehyde, highly reactive and cytotoxic compound production of which is spontaneous via different enzymatic and non-enzymatic reactions. Methylglyoxal is amplified 2- to 6-fold under stress condition (than the control) and with its cytotoxic capacity MG damages ultrastructural cellular components including DNA and can cause mutation (Yadav et al., 2005; Hasanuzzaman et al., 2017b). Glyoxalase system poses glyoxalase I (Gly I) and glyoxalase II (Gly II) enzymes which utilize GSH as co-factor to detoxify MG (Hasanuzzaman et al., 2017b). Cadmium stress decreased Gly I and Gly II activity, compared to the control treatment indicating brake down MG detoxification system by Cd toxicity. Mung bean plants (Nahar et al., 2016a) and rapeseed plants (Hasanuzzaman et al., 2017a) demonstrated similar pattern of response of glyoxalase system enzymes under Cd stress. Treatment with Si improved the activities of Gly I and Gly II and also the content of GSH indicating the crucial roles of Si in MG detoxification under Cd stress.

The results reveal that Si alleviated oxidative stress as it decreased H_2O_2 content and membrane lipid peroxidation. The mechanism was Si enhanced components of antioxidant defense system which decreased oxidative stress. Among the studied antioxidant components, Si significantly upregulated AsA and GSH levels, increased activities of APX, DHAR, GR and CAT those scavenged ROS and decreased oxidative damage. The reduction of oxidative damage was also imparted by Si-induced improved glyoxalase system which decreases MG generation and subsequent oxidative damage. The overall advantageous effect of Si was reflected in phenotypic appearance of Cd affected rapeseed seedlings where Si supplementation alleviated chlorosis and improved seedlings vigor.

CONCLUSION

Our results suggest that exogenous Si serves well in regulating antioxidant metabolism in *B. napus* seedlings under Cd stress. Silicon-mediated coordinated actions of AsA-GSH pathway and glyoxalase systems maintained the redox state of AsA and GSH and minimized the Cd-induced oxidative damages. This also indicates a central role of GSH because of its relations with both antioxidant defense systems and glyoxalase systems. The signaling roles of Si in regulating biosynthesis of metabolites and regulation of stress-induced genes and their relation to preventing stress affects and contributing stress tolerances need further inspection. Further research should be focussed on the interrelation of Si with other signaling molecules such as nitric oxide (NO), polyamines and phytohormones.

AUTHOR CONTRIBUTIONS

MH and MF conceived and designed the experiments. MH and KN performed the experiments. MH and TA analyzed the data. MF contributed reagents/materials/analysis tools. MH, KN, and

TA wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01061/full#supplementary-material

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Silicon-Mediated Alleviation of Aluminum Toxicity by Modulation of Al/Si Uptake and Antioxidant Performance in Ryegrass Plants

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Silicon (Si) has been well documented to alleviate aluminum (Al) toxicity in vascular plants. However, the mechanisms underlying these responses remain poorly understood. Here, we assessed the effect of Si on the modulation of Si/Al uptake and the antioxidant performance of ryegrass plants hydroponically cultivated with Al (0 and 0.2 mM) in combination with Si (0, 0.5, and 2.0 mM). Exposure to Al significantly increased Al concentration, mainly in the roots, with a consequent reduction in root growth. However, Si applied to the culture media steadily diminished the Al concentration in ryegrass, which was accompanied by an enhancement in root dry matter production. A reduced concentration of Si in plant tissues was also observed when plants were simultaneously supplied with Al and Si. Interestingly, Si transporter genes (*Lsi1* and *Lsi2*) were down-regulated in roots after Si or Al was applied alone; however, both *Lsi1* and *Lsi2* were up-regulated as a consequence of Si application to Al-treated plants, denoting that there is an increase in Si requirement in order to cope with Al stress in ryegrass. Whereas Al addition triggered lipid peroxidation, Si contributed to an attenuation of Al-induced oxidative stress by increasing phenols concentration and modulating the activities of superoxide dismutase (SOD), catalase, peroxidase, and ascorbate peroxidase antioxidant enzymes. Differential changes in gene expression of SOD isoforms (*Mn-SOD*, *Cu/Zn-SOD,* and *Fe-SOD*) and the profile of peroxide (H_2O_2) generation were also induced by Si in Al-stressed plants. This, to the best of our knowledge, is the first study to present biochemical and molecular evidence supporting the effect of Si on the alleviation of Al toxicity in ryegrass plants.

Keywords: silicon, aluminum, Si transporter genes, phenols, antioxidant enzymes, SOD isoforms genes

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INTRODUCTION

Aluminum (Al) toxicity represents one of the main yield-limiting factors for crops in acid soils (von Uexküll and Mutert, 1995). Under acidic conditions, large and toxic amounts of Al^{3+} become available to plants, thereby affecting a wide range of physical, cellular, and molecular processes, with a consequent reduction in plant growth (Kochian et al., 2005; Mora et al., 2006; Cartes et al., 2010, 2012; Ryan and Delhaize, 2010; Singh et al., 2017). Alterations in the structure and/or functions of

cell wall components (Horst et al., 2010), plasma membrane properties (Yamamoto et al., 2001), nutrient homeostasis (Delhaize and Ryan, 1995; Gupta et al., 2013; Singh et al., 2017), and signal transduction pathways (Matsumoto, 2000; Ma et al., 2002; Sivaguru et al., 2003; Goodwin and Sutter, 2009) can be induced as a consequence of Al binding to numerous cell sites. In most plant species, reactive oxygen species (ROS) production can also be induced by Al toxicity (Kochian et al., 2005), leading to oxidative damage of biomolecules and biological membranes (Yamamoto et al., 2001, 2002, 2003; Singh et al., 2017).

To cope with the deleterious effects of Al, plant species have developed diverse mechanisms, which are generally associated with Al exclusion (also referred to as avoidance or resistance) and/or internal tolerance mechanisms (e.g., Barcelo and Poschenrieder, 2002; Kochian et al., 2005; Poschenrieder et al., 2008). Briefly, exclusion mechanisms involve the root exudation of organic acid anions and/or phenolic compounds, which bind Al^{3+} and limit its uptake into the cytosol. Tolerance mechanisms comprise internal detoxification by forming Al complexes with organic substances in the cytosol, compartmentalization in the vacuole, and enhanced scavenging of ROS (e.g., Barcelo and Poschenrieder, 2002; Kochian et al., 2005; Poschenrieder et al., 2008). Molecular approaches have revealed that Al resistance in several plant species is regulated by genes encoding membrane transporter proteins involved in the efflux of organic acid anions, including members of the ALMT (aluminum-activated malate transporters) and MATE (multidrug and toxic compound extrusion) families (Sasaki et al., 2004; Furukawa et al., 2007; Ryan et al., 2011). In addition, a bacterial-type ATP binding cassette (ABC) transporter (Huang et al., 2009) and antioxidant defense genes (e.g., Milla et al., 2002; Goodwin and Sutter, 2009; Du et al., 2010; Panda and Matsumoto, 2010) have also been implicated in Al tolerance in plants.

Over the last decades, silicon (Si) has become a focus of increasing interest in plant science, since it is considered as a beneficial element for plant growth, particularly under conditions of biotic and abiotic stress (Ma, 2004; Liang et al., 2007; Guntzer et al., 2012; Ma and Yamaji, 2015). To date, several pieces of evidence have indicated that most of the beneficial effects of Si depend on the differential ability of plants to take up Si. Recently, it has been reported that Si accumulation is ascribed to an efficient uptake system mediated by both channel-type and efflux transporters, which perform coordinated functions for effective Si transport from soil to roots and its subsequent distribution within the plants (e.g., Ma et al., 2006, 2007; Yamaji et al., 2008, 2012; Chiba et al., 2009; Mitani et al., 2009a,b, 2011a,b; Yamaji and Ma, 2009; Grégoire et al., 2012; Montpetit et al., 2012; Deshmukh et al., 2013; Ma and Yamaji, 2015). Overall, these transporters appear to be keys features that enable plants to gain an advantage from Si uptake. Nevertheless, the regulation of Si transporters under stress conditions remains poorly understood.

The significant role of Si in the toxicity associated with metals, including manganese (Mn), iron (Fe), cadmium (Cd), arsenic (As), chromium (Cr), copper (Cu), lead (Pb), zinc (Zn), and Al, has been widely reported (Li et al., 2012; Vaculík et al., 2012; Adrees et al., 2015; Liang et al., 2015; Pontigo et al., 2015; Tripathi et al., 2015, 2016). On the basis of the current evidence, Si can regulate plant resistance and/or tolerance to metal toxicity by either external (*ex planta*) or internal (*in planta*) mechanisms (Cocker et al., 1998a; Adrees et al., 2015; Liang et al., 2015; Pontigo et al., 2015; Tripathi et al., 2016). In this regard, it has been proposed that the alleviation of Al stress by Si in plants can mainly be explained by the following events: (i) Si-induced increase in solution pH (Li et al., 1996; Cocker et al., 1998a), (ii) formation of Al-Si complexes in the growth media (Barcelo et al., 1993; Baylis et al., 1994; Ma et al., 1997; Cocker et al., 1998a) or/and within the plant (Corrales et al., 1997; Cocker et al., 1998b; Britez et al., 2002; Zsoldos et al., 2003; Wang et al., 2004; Prabagar et al., 2011), (iii) exudation of organic acid anions and phenolic compounds (Barcelo et al., 1993; Cocker et al., 1998b; Kidd et al., 2001), and (iv) increase in the chlorophyll and carotenoid contents of leaves (Singh et al., 2011). Activation of the plant antioxidant system has also been reported in response to Si supply under Al stress (Shahnaz et al., 2011; Shen et al., 2014; Tripathi et al., 2016). However, to our knowledge, there is a dearth of reports regarding the molecular aspects of the effect of Si on the genes involved in antioxidant defense.

Perennial ryegrass (*Lolium perenne* L.) is a temperate pasture species supporting forage-based intensive dairy and beef production systems in many parts of the world. Due to elevated yields and high nutritional value, ryegrass has become one of the most commonly cultivated species in the permanent pastures of Southern Chile. Nevertheless, large areas of these pastures are sown on acidic soils, which exhibit elevated availability of toxic Al^{+3}, thereby limiting their yield and quality (Mora et al., 2006). Furthermore, our previous studies have demonstrated that toxic levels of Al induced oxidative damage and activated antioxidant enzymes in ryegrass roots, including peroxidase (POD), ascorbate peroxidase (APX), and superoxide dismutase (SOD) (Cartes et al., 2010, 2012). In an attempt to identify new alternatives to alleviate the deleterious effects produced by Al on ryegrass, we aimed in this study to investigate the effect of Si on the modulation of Si/Al uptake and the antioxidant performance of ryegrass plants subjected to Al toxicity.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of ryegrass (*L. perenne* L. cultivar Nui) were soaked with 2% v/v sodium hypochlorite for 10 min, washed repeatedly with distilled water, and then germinated on moist filter paper in a growth chamber at 21°C. After 10 days, seedlings were transferred to 12-L plastic pots containing a continuously aerated basal nutrient solution described by Taylor and Foy (1985). After 10 days in nutrient solution, ryegrass plants were treated with Al and Si. Aluminum (as $AlCl_3$, Merck reagent) was added to the solution at doses of 0 and 0.2 mM. The activity of free Al^{3+} in the nutrient solution, calculated by Geochem-EZ (Shaff et al., 2010), corresponded to 85 μM. Aluminum doses were added in combination with 0, 0.5, and 2 mM Si (as Na_2SiO_3, Merck reagent) in a completely randomized factorial design with three replicates per treatment. During the growth period, the pH of the solution was adjusted daily to 4.5 using dilute HCl

or NaOH, and the nutrient solution was changed every 7 days. Plants were cultured in a greenhouse under controlled growth conditions as follows: 25/20°C day/night temperature, a 16/8 h (light/dark) photoperiod, 350 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF) and 70–80% relative humidity. Plants were harvested 10 days after the initiation of treatments, and shoot and root samples were stored at −20°C or −80°C for subsequent evaluation of biochemical and molecular parameters. In addition, subsamples of fresh material were dried at 65°C for 48 h in order to determinate the dry weight as well as Si and Al concentrations.

Determination of the Mineral Concentration of Al and Si in Plant Tissues

Aluminum analysis was performed on dried roots and shoots. Plant samples were ashed at 500°C for 8 h and treated with 2 M HCl. After filtration of the resulting solution, the total amount of Al was quantified by flame atomic absorption spectrophotometry (FAAS) at 324.7 nm, as described by Sadzawka et al. (2007). Silicon concentration was assayed as described by Pavlovic et al. (2013) with modifications. Dry plant samples were digested with 5 mL concentrated HNO_3 on a hot plate at 70°C for approximately 5 h. Samples were diluted with 10 mL of deionized water, followed by the addition of 1 mL HF (40%), and left overnight. The following day, 5 mL 2% (w/v) H_3BO_3 was added to eliminate excess HF and the volume of the solution was adjusted to 25 mL with deionized water. The Si concentration in the digested samples was determined by FAAS at 251.6 nm. For each chemical analysis, two reference samples were included in each analytical run.

Biochemical Analyses

Lipid Peroxidation Assay

Lipid peroxidation was analyzed using the thiobarbituric acid reactive substances (TBARS) assay, according to the modified method of Du and Bramlage (1992). The absorbance of the samples was measured at 532, 600, and 440 nm in order to correct for interference generated by TBARS-sugar complexes.

Determination of Total Phenols

Total soluble phenols were spectrophotometrically assayed at 765 nm using Folin-Ciocalteu reagent according to the method described by Slinkard and Singleton (1977) with minor modifications (Ribera et al., 2013). Total phenol concentration was calculated using chlorogenic acid as a phenolic compound standard.

Antioxidant Enzyme Assays

SOD (EC. 1.15.1.1), catalase (CAT; EC. 1.11.1.6), peroxidase (POD; EC. 1.11.1.7), and APX (EC. 1.11.1.11) enzyme activities were evaluated from frozen samples stored at −80°C. Plant material was ground in liquid nitrogen and macerated in 50 mM potassium phosphate buffer (K_2HPO_4–KH_2PO_4; pH 7.0). The homogenate was centrifuged at 11,000 × *g* for 15 min at 4°C, and the supernatant was used for assay of enzyme activities. SOD, CAT, APX, and POD activities were calculated on a protein basis. The protein content in the extracts was measured spectrophotometrically using the method described by Bradford (1976), with bovine serum albumin (BSA) used as a standard.

Superoxide dismutase activity was analyzed by measuring inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture contained 400 μL of 0.1 M potassium phosphate buffer pH 7.0, 10 μL of 10 mM ethylenediaminetetraacetic acid (EDTA), 50 μL of 260 mM methionine, 80 μL of 4.2 mM NBT, 170 μL of 130 μM riboflavin, and 300 μL of enzyme extract. The reaction tubes were illuminated for 15 min and the absorbance of samples was measured at 560 nm. Non-illuminated and illuminated reactions without enzyme extract were used as controls. One SOD unit was defined as the amount of enzyme corresponding to 50% inhibition of NBT reduction (Donahue et al., 1997).

Catalase (CAT; EC. 1.11.1.6) activity was measured by monitoring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm for 120 s. A 10-μL aliquot of enzyme extract was added to a reaction mixture containing 1 mL of extraction buffer and 3 μL of H_2O_2 (30% v/v). The enzyme activity was calculated using a molar extinction coefficient of 39.4 mM^{-1} cm^{-1} (Pinhero et al., 1997).

Peroxidase (POD; EC. 1.11.1.7) activity was determined by estimating the formation of tetraguaiacol at 470 nm during 1 min. A 15-μL volume of enzyme extract was added to a reaction mixture containing 1 mL of extraction buffer, 5 μL of H_2O_2 (30% v/v), and 5 μL of guaiacol. A molar extinction coefficient of 26.6 mM^{-1} cm^{-1} was used to calculate the enzymatic activity (Pinhero et al., 1997).

Ascorbate peroxidase (EC. 1.11.1.11) activity was assayed according to the method described by Nakano and Asada (1981), by measuring ascorbate decomposition at 290 nm for 1 min. The coarse extract (40 μL) was diluted in a reaction mixture containing 1 mL of extraction buffer, 5 μL of H_2O_2 (30% v/v), and 40 μL of 10 mM ascorbic acid. Enzyme activity was calculated using a molar extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Gene Expression Analyses

Ryegrass tissues were subjected to RNA extraction using a NucleoSpin® RNA Plant Kit (Macherey-Nagel GmbH and Co., KG, Düren, Germany). First-strand cDNA was synthesized from 1 μg of total RNA using an AffinityScript qPCR cDNA Synthesis Kit (Stratagene, Cedar Creek, TX, USA) following the manufacturer's recommendations. Quantitative real-time polymerase chain (qRT-PCR) reactions were conducted in order to determinate the expression patterns of Si transporter genes (*Lsi1* and *Lsi2*) in roots, as well as those of three SOD isoform genes (*Cu/ZnSOD*, *Fe-SOD,* and *Mn-SOD*) in shoots and roots. All qRT-PCR reactions were performed using Brilliant II SYBR Green qPCR Master mix (Stratagene, Cedar Creek, TX, USA) in an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Cycling conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s. The specific primers used in this study are shown in **Table 1**. The primer sets used for *LpLsi1* (GenBank accession number KY315994) and *LpLsi2* (GenBank accession

TABLE 1 | List of primers sequences used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis of Si transporters and SOD isoforms genes.

Gene name*	Forward primer (5′- > 3′)	Reverse primer (5′- > 3′)
Lsi1	ACGCCCAGCATGTACTACAAC	TCATGAACACCAGCAGGAAC
Lsi2	CTCTGCATGTACTGGAAGGAC	GTTGAGAGGGTTGAGAGTGTG
Fe-SOD	GTTGCCAAGGGAAATCCTGAACCA	AACCCCAGCCGTTTATCTTCAAGC
Cu/Zn-SOD	GTGTTGCTCCCATCAATGTTGT	CCTGCCAAGATCATCAGCATC
Mn-SOD	AATACGAAAATGTGGCTGTGTG	AAAATCTGCATTGTGCATTACG
Actin	CCTTTTCCAGCCATCTTTCA	GAGGTCCTTCCTGATGTCCA
eEF1A (m)	GGCTGATTGTGCTGTGCTTA	CTCACTCCAAGGGTGAAAGC

**Gene name: Lsi1, Low Si transporter 1; Lsi2, Low Si transporter 2; Fe-SOD, iron superoxide dismutase; Cu/Zn-SOD, copper/zinc superoxide dismutase; Mn-SOD, manganese superoxide dismutase; Actin, Actin; eEF1A(m), Eukaryotic elongation factor 1 alpha. Actin or eEF1A(m) were used as housekeeping genes.*

number KY315995) were designed using the Primer3 (v. 0.4.0) and primer BLAST tools. Primers sequences for *LpCu/ZnSOD*, *LpFe-SOD*, and *LpMn-SOD* were obtained from Ribera et al. (2013). Housekeeping genes, *LpActin* or *LpeEF1A (m)*, were used as internal controls (Ribera et al., 2013). All the experiments were performed using three biological replicates, each with three technical replicates.

Detection of H_2O_2 Production by Flow Cytometry

Suspensions of shoot protoplasts were obtained using the method described by Okuno and Furusawa (1977). The protoplasts were centrifuged at 2,500 × *g* for 5 min at 4°C and incubated with the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) to detect intracellular H_2O_2 using the method described by Maxwell et al. (1999) with modifications. H_2O_2 production was analyzed using flow cytometry (BD FACS Canto IISN: V96101286; Becton Dickinson, USA). All measurements were performed using an Ar ion laser excited at 488 nm and emitting at 530 nm. The images were processed through the BD FACSDivaTM, v 6.0 program. A positive control (intact protoplasts plus 100 μM H_2O_2) and negative control (suspension of intact protoplasts without H_2O_2) were used.

Confocal Microscopy

A profile of H_2O_2 generation in protoplast extracts was also examined by Laser Scanning Confocal Microscopy (CLSM). H_2DCFDA fluorescence emission was recorded at excitation/emission of 488/530 nm, and chlorophyll autofluorescence was measured at 633 nm laser excitation and emission of 750 nm. The images were processed using Image Processing software (software FV10-ASW v.0.2c; Arquimed).

Statistical Analysis

Experimental data were analyzed using an analysis of variance (ANOVA) following normality and homoscedasticity tests. Differences among means were separated using the Tukey test at the 0.05 probability level. In addition, the relationship between two response variables was investigated by Pearson correlation.

RESULTS

Concentrations of Al and Si in Plants and Dry Matter Production

Aluminum treatment mostly increased Al concentration in roots, whereas significantly lower amounts of Al accumulated in the shoots (**Table 2**). However, increasing Si doses gradually decreased shoot and root Al concentrations by up to 49 and 56%, respectively, in Al-treated plants (**Table 2**). Interestingly, a negative correlation between Si concentration and Al concentration was observed in shoots ($r = 0.927$, $p \leq 0.01$) and roots ($r = 0.935$, $p \leq 0.01$) of ryegrass grown with Al and Si (**Table 3**). In addition, the Si concentration of ryegrass tissues steadily increased with an increase in Si dose, but this increment was less noticeable when plants were simultaneously supplied

TABLE 2 | Concentration of Al and Si, and dry matter production of ryegrass plants hydroponically cultivated under different Al and Si treatments.

Treatment (mM)	Al concentration (g kg^{-1} DW)		Si concentration (g kg^{-1} DW)		Dry weight (g)	
	Shoots	Roots	Shoots	Roots	Shoots	Roots
0 Al – 0 Si	0.02 ± 0.00cd	0.16 ± 0.02d	0.31 ± 0.09e	0.33 ± 0.03e	6.53 ± 0.29bc	1.37 ± 0.06ab
0 Al – 0.5 Si	0.01 ± 0.00d	0.15 ± 0.00d	5.85 ± 0.44c	6.42 ± 0.20c	7.04 ± 0.29abc	1.37 ± 0.10ab
0 Al – 2 Si	0.01 ± 0.00d	0.13 ± 0.01d	13.78 ± 0.26a	13.47 ± 0.09a	6.69 ± 0.22abc	1.39 ± 0.13a
0.2 Al – 0 Si	0.07 ± 0.00a	3.84 ± 0.24a	0.21 ± 0.03e	0.38 ± 0.10e	6.07 ± 0.42c	0.98 ± 0.06b
0.2 Al – 0.5 Si	0.04 ± 0.00b	2.68 ± 0.10b	4.40 ± 0.13d	4.30 ± 0.15d	7.95 ± 0.42ab	1.48 ± 0.08a
0.2 Al – 2 Si	0.03 ± 0.00bc	1.69 ± 0.11c	10.29 ± 0.19b	11.88 ± 0.20b	8.09 ± 0.32a	1.61 ± 0.06a

Values are means ± standard error of three replicates. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

TABLE 3 | Pearson's correlation among plant growth, chemical and biochemical parameters of ryegrass hydroponically cultivated under different Al and Si treatments.

	Al	Si	Dry weight	TBARS	Total phenols	SOD	CAT	POD	APX
Shoots									
Al	1.00								
Si	−0.927**	1.00							
Dry weight	−0.849**	0.721*	1.00						
TBARS	0.946**	−0.947**	−0.757*	1.00					
Total phenols	−0.904**	0.859**	0.756*	−0.813**	1.00				
SOD	0.693*	−0.827**	−0.432	0.646	−0.721*	1.00			
CAT	−0.099	0.076	−0.118	−0.023	0.418	−0.110	1.00		
POD	0.863**	−0.776*	−0.781*	0.715*	−0.932**	0.666	−0.275	1.00	
APX	0.823**	−0.599	−0.745*	0.657	−0.744*	0.489	−0.073	0.836**	1.00
Roots									
Al	1.00								
Si	−0.935**	1.00							
Dry weight	−0.876**	0.823**	1.00						
TBARS	0.740*	−0.734*	−0.800**	1.00					
Total phenols	−0.825**	0.741*	0.706*	−0.523	1.00				
SOD	0.883**	−0.961**	−0.778*	0.787*	−0.731*	1.00			
CAT	−0.691*	0.838**	0.524	−0.399	0.738*	−0.795*	1.00		
POD	−0.796*	0.925**	0.666	−0.509	0.690*	−0.858**	0.956**	1.00	
APX	−0.925**	0.980**	0.806**	−0.666	0.800**	−0.930**	0.894**	0.962**	1.00

*Asterisks indicate significance as follows: **$p \leq 0.01$,*$p \leq 0.05$.*

with Al and Si (**Table 2**). Of the total amount of Si taken up by plants, over 80% accumulated in the shoots.

No changes in shoot growth were observed in plants treated with Al alone, whereas root dry matter production was reduced by approximately 28.5%. Silicon treatments did not affect ryegrass growth when Si was applied to plants cultivated without Al (**Table 2**). However, root yield was improved by at least 51% when Si was applied to Al-treated plants. Moreover, a positive correlation ($r = 0.823$, $p \leq 0.01$) between Si concentration and dry weight was observed for the roots of Al-treated plants supplied with increasing concentrations of Si (**Table 3**).

Analysis of Si Transporter Gene Expression in Response to Al Toxicity

The relative expression of two putative Si transporter genes (*LpLsi1* and *LpLsi2*) in roots was assessed in ryegrass subjected to different Al and Si supplementation. In plants grown without Al, the expression level of *LpLsi1* and *LpLsi2* was down-regulated by approximately 4.2- and 2.8-fold, respectively, in response to Si addition to the growth media (**Figures 1A,B**). A similar expression pattern was observed when Al was applied alone, with the expression levels of *LpLsi1* and *LpLsi2* being reduced by approximately 7.1- and 2.9-fold, respectively (**Figures 1A,B**). However, when Al was added in combination with Si, the expression level of these Si transporters was significantly enhanced (**Figures 1A,B**). The highest Si dose applied to Al-treated plants increased the expression level of *LpLsi1* by approximately 5.4-fold (**Figure 1A**), whereas that of *LpLsi2* was up-regulated by at least 2.5-fold irrespective of Si dosage (**Figure 1B**).

Lipid Peroxidation

The addition of 0.2 mM Al increased root lipid peroxidation by approximately 29% (**Figure 2B**); however, no differences in oxidative damage were observed in shoots as a consequence of Al supply (**Figure 2A**). Likewise, no significant changes in TBARS accumulation were observed among plants grown with only Si (**Figures 2A,B**). However, Si at the highest concentration supplied diminished lipid peroxidation in Al-treated plants by approximately 32.6 and 27.7% in shoots and roots, respectively (**Figures 2A,B**). Consequently, lipid peroxidation was negatively correlated with Si concentration in shoots ($r = -0.947$, $p \leq 0.01$) and roots ($r = -0.734$, $p \leq 0.05$), as shown in **Table 3**.

Plant Antioxidant Responses

Plants treated with Al showed an evident increment in total phenols (**Figures 3A,B**). A significant increase in total phenol concentration was also observed in the shoots and roots of ryegrass treated with the highest Si dose, with a further increase being observed in plants treated with both Al and Si (**Figures 3A,B**).

In order to investigate the effect of Si on the ROS scavenging enzyme system under Al stress conditions, the activities of SOD, CAT, POD, and APX enzymes were evaluated (**Figures 4A–H**). Aluminum supplied alone significantly increased SOD activity by approximately 37.2% in shoots and 27.5% in roots (**Figures 4A,B**). Likewise, the highest Si dose activated SOD enzyme in non-Al-treated plants (**Figures 4A,B**). However, when Al and Si were simultaneously applied, SOD activity was significantly reduced by 20.08 and 43.8% in shoots and roots, respectively (**Figures 4A,B**).

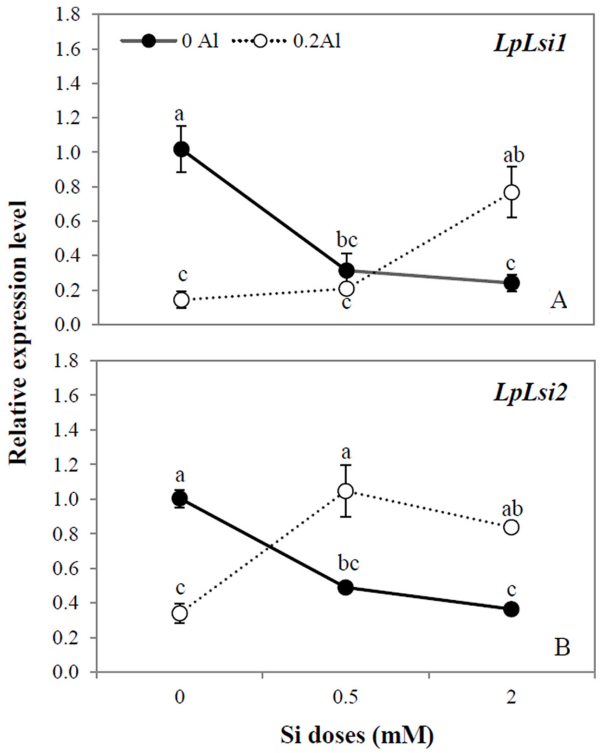

FIGURE 1 | Expression analysis of *LpLsi1* (A) and *LpLsi2* **(B)** genes determined by qRT-PCR in roots of ryegrass hydroponically cultivated under Al and Si treatments. The expression levels were normalized in relation to *Actin* or *eEF1A(m)* gene expression. Data are means of three replicates @ standard error. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

The application of Al alone increased CAT activity in shoots and roots by at least 4.2- and 4.7-fold, respectively (**Figures 4C,D**). In plants grown in the absence of Al, Si enhanced CAT activity by approximately 3.0-fold (shoots) and 5.8-fold (roots) (**Figures 4C,D**). Plants supplied with Al + Si did not show significant differences in CAT activity compared with those supplied with Al alone, the exception being in the roots of plants supplied with the highest Si dose, which exhibited an approximate 60% increase (**Figures 4C,D**).

Shoot POD activity increased by approximately 30% in Al-treated plants compared with non-treated plants, although no significant changes were observed in roots (**Figures 4E,F**). The addition of Si augmented POD activity in plants grown without Al (**Figures 4E,F**). This effect was most evident in roots, in which the activity of this enzyme was increased by 2.1-fold at the highest Si supply (**Figure 4F**). Likewise, root POD was activated by approximately 1.7-fold under combined Al and Si treatments (**Figure 4F**), whereas in shoots the enzyme activity was diminished (**Figure 4E**).

Addition of Al to the growth media considerably increased APX activity by approximately 2.7-fold and 1.8-fold in shoots and roots, respectively (**Figures 4G,H**). Similarly, Si application elevated APX activity in ryegrass (**Figures 4G,H**), and this effect was enhanced by 2.2-fold in the roots of plants receiving the combined Al-Si treatments (**Figure 4H**). Conversely, Si supply decreased shoot APX activity by approximately 25.9% in Al-treated plants (**Figure 4G**).

The changes in antioxidant responses of Al-stressed plants as a consequence of Si uptake were additionally examined by means of Pearson correlation as shown in the **Table 3**. Briefly, we found a negative correlation between Si concentration and SOD activity in shoots ($r = -0.827$, $p \leq 0.01$) and roots ($r = -0.961$, $p \leq 0.01$). Conversely, for roots, we observed positive relationships between Si concentration and either total phenols ($r = 0.741$, $p \leq 0.05$) or the antioxidant enzymes of the second line of defense (CAT, $r = 0.838$, $p \leq 0.01$; POD, $r = 0.925$, $p \leq 0.01$; APX, $r = 0.980$, $p \leq 0.01$).

Analysis of SOD Isoform Gene Expression in Response to Al and Si Treatments

Genes of SOD isoforms (Fe-SOD, Cu/Zn-SOD, and Mn-SOD) were differentially expressed as a consequence of Si and Al supply (**Figures 5A–F**). Aluminum supplied alone reduced the gene expression of Fe-SOD and Cu/Zn-SOD in shoots (**Figures 5A,C**), whereas no changes in the expression pattern of these genes was

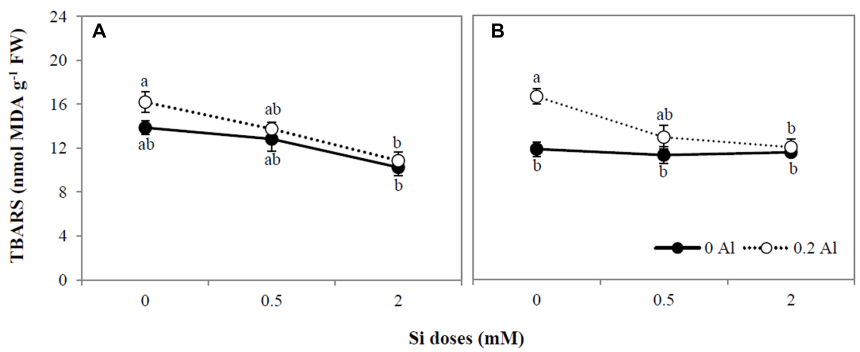

FIGURE 2 | Lipid peroxidation in shoot (A) and root **(B)** of ryegrass hydroponically cultivated under Al and Si treatments. Data are means of three replicates ± standard error. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

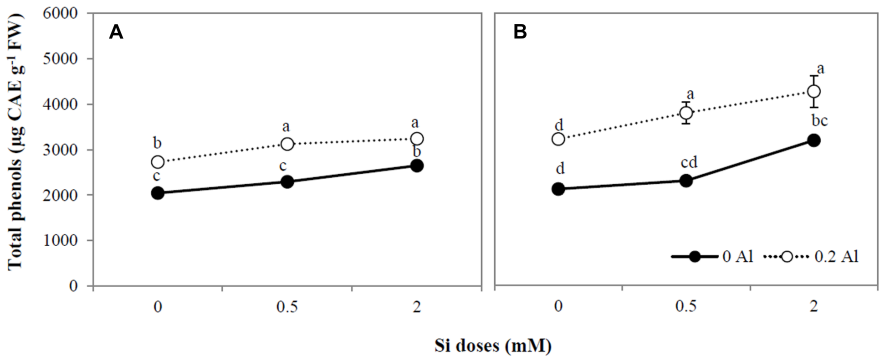

FIGURE 3 | Total phenol concentration in shoot (A) and root **(B)** of ryegrass hydroponically cultivated under Al and Si treatments. Data are means of three replicates ± standard error. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

detected in the roots (**Figures 5B,D**). In addition, expression of the Mn-SOD gene was up-regulated by approximately 1.7-fold in shoots and roots exposed to Al (**Figures 5E,F**). Increasing Si doses lowered the gene expression of Fe-SOD by up to 1.9-fold in the shoots and 2.2-fold in the roots of plants cultivated without Al (**Figures 5A,B**), whereas the transcript levels of Mn-SOD were enhanced in shoots by approximately 1.7-fold by Si addition (**Figure 5E**). In contrast, in plants receiving Si alone, there was no significant changes in the expression level of either shoot Cu/Zn-SOD or root Mn-SOD genes (**Figures 5C,F**). However, in roots, Cu/Zn-SOD was down-regulated by at least 1.8-fold as a consequence of Si supply (**Figure 5D**). In plants simultaneously exposed to Al and Si, the addition of Si did not induce significant changes in the expression level of Fe-SOD in shoots and roots (**Figures 5A,B**). Although a similar expression pattern of Cu/Zn-SOD was observed in the shoots of Al-treated plants under the different Si treatments (**Figure 5C**), the gene expression of this enzyme was down-regulated by up to 1.9-fold in roots (**Figure 5D**). Likewise, Si application to Al-treated plants significantly reduced the transcript level of Mn-SOD by at least 2.2- and 3.8-fold in shoots and roots, respectively (**Figures 5E,F**).

Hydrogen Peroxide Production in Shoot Protoplasts Exposed to Al and Si

Aluminum treatment augmented H_2O_2 generation by approximately 38% in shoot protoplasts (**Figure 6A**). A progressive increase in H_2O_2 production was also observed when Si was added alone, and the accumulation of H_2O_2 was enhanced to an even greater extent in plants simultaneously supplied with Si and Al (**Figure 6A**). This pattern was consistent with the observations made by CLSM analysis (**Figure 6B**), which revealed a progressive increase in the fluorescence of an H_2DCFDA probe generated by Si and Al application.

DISCUSSION

Although several previous studies have reported that Si provide beneficial effects on plants subjected to Al stress, the mechanisms underlying these responses have remained poorly understood. Moreover, only a few studies have examined the effect of Si-mediated amelioration of Al toxicity in terms of the regulation of Al and Si uptake systems (e.g., Britez et al., 2002; Wang et al., 2004; Dorneles et al., 2016) and plant antioxidant performance (e.g., Shahnaz et al., 2011; Shen et al., 2014). Likewise, to date, the effect of Si on Al stress in ryegrass, a forage species belonging to Si-accumulator plants (Jarvis, 1987; Nanayakkara et al., 2008), has yet to be addressed.

The high level of toxic Al in acid soils is an important limiting factor for plant production (Mora et al., 2006). In our study, the exposure of plants to 0.2 mM Al significantly increased Al accumulation, mainly in the roots (**Table 2**), with a consequent reduction of approximately 28.5% in root dry matter production (**Table 2**). These results are consistent with our previous findings for ryegrass (Cartes et al., 2010), since it is well known that Al toxicity involves the rapid inhibition of root growth (e.g., Matsumoto, 2000; Kochian et al., 2005; Horst et al., 2010; Singh et al., 2017). The role played by Si in promoting plant growth under Al toxicity has been widely accepted (e.g., Hara et al., 1999; Singh et al., 2011; Shen et al., 2014; Tripathi et al., 2016). Correspondingly, Si application to Al-treated plants significantly reduced the Al concentration in ryegrass (**Table 2**) and improved root dry weight by at least 51% (**Table 2**). A slight reduction in Si concentration in plant tissues was also found when plants were simultaneously supplied with Al and Si (**Table 2**). Moreover, our results revealed a negative correlation between Si and Al uptake in plants treated with Al and Si, whereas Si concentration and dry matter production were positively related (**Table 3**). The reduction in Al and Si uptake might be attributed to the formation of biologically inactive aluminosilcate (Al-Si) complexes in the growth media, thus lowering Al availability (Barcelo et al., 1993; Baylis et al., 1994; Ma et al., 1997; Cocker et al., 1998a), with the consequent enhancement of root growth. Nevertheless, the formation of Al-Si inside plant tissues could also be involved in the growth-promoting effect of Si under Al stress (Hodson and Sangster, 1993; Cocker et al., 1998b; Wang et al., 2004). Indeed, it has been demonstrated that Al toxicity may be decreased by co-deposition of Al and Si in the root epidermal walls of sorghum (Hodson and

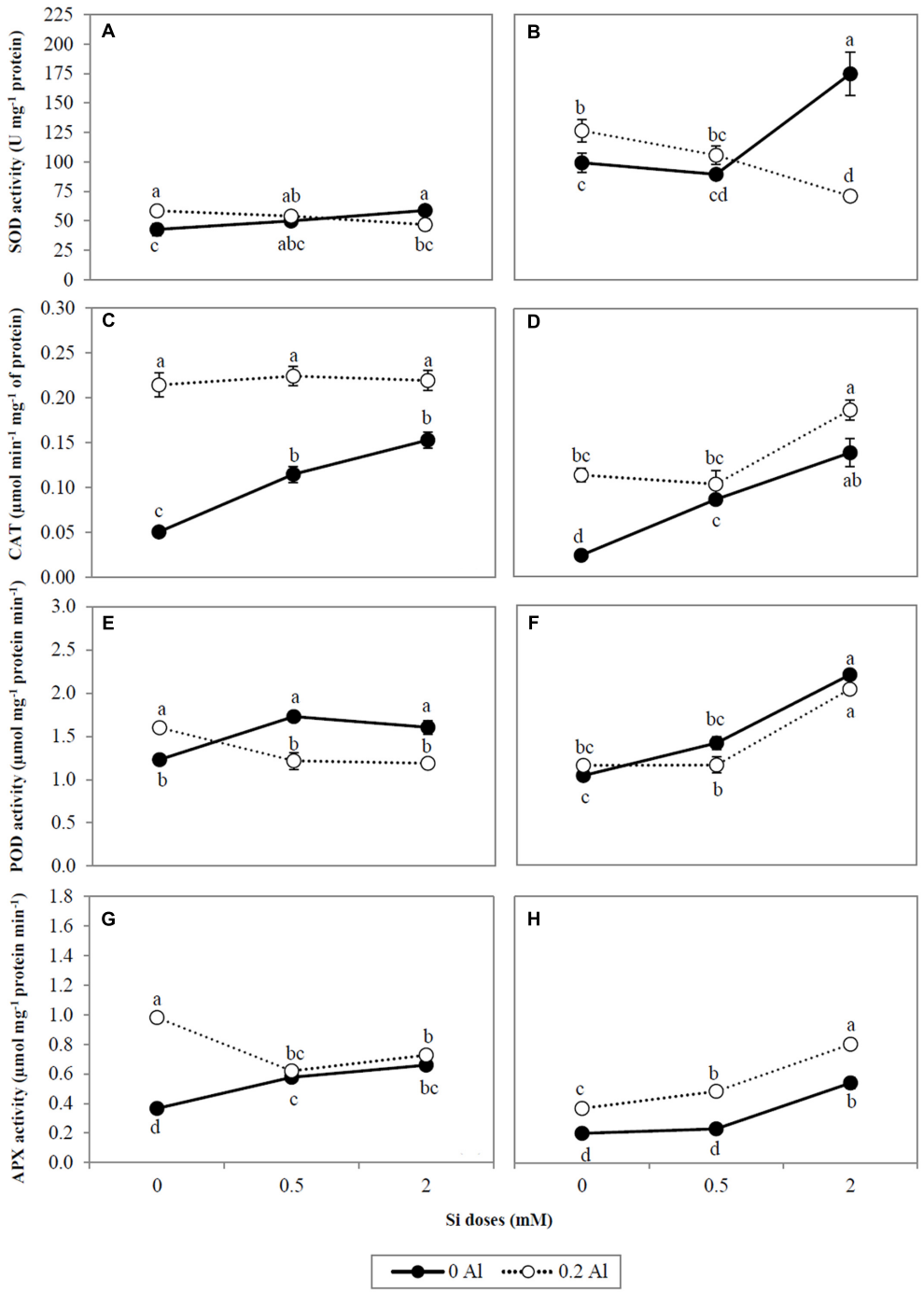

FIGURE 4 | The activity of antioxidant enzyme SOD (A,B), CAT **(C,D)**, POD **(E,F)**, and APX **(G,H)** in shoots and roots of ryegrass hydroponically cultivated under Al and Si treatments. Data are means of three replicates ± standard error. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

Sangster, 1993). Similarly, Cocker et al. (1998b) and Wang et al. (2004) have also suggested that formation of Al-Si complexes in the root apoplast of wheat and maize is a possible mechanism for Al detoxification in plants.

Although all plants contain Si in their tissues, the concentration of this element varies greatly among species, in a range from 0.1 to 10% on a dry weight basis (Epstein, 1999; Ma and Takahashi, 2002), which is indicative of the fact that the benefits of Si to plants grown under stress can also be highly variable. Recent studies have shown that Si accumulation in plants is controlled by influx and efflux Si transporters that could be involved in the differential Si-induced responses to

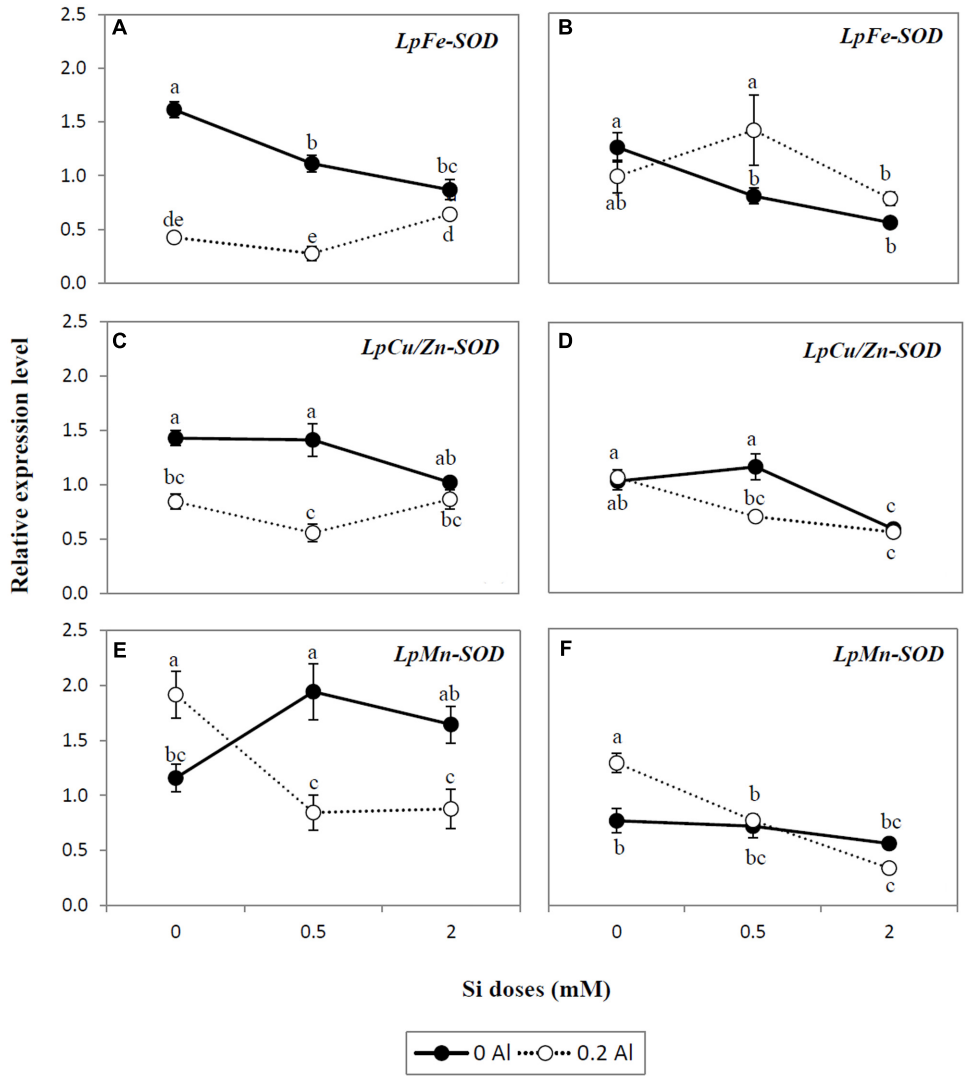

FIGURE 5 | Expression analysis of SOD isoform genes *LpFe-SOD* (A,B), *Cu/Zn-SOD* **(C,D)**, and *Mn-SOD* **(E,F)** determined by qRT-PCR in shoots and roots of ryegrass hydroponically cultivated under Al and Si treatments. The expression levels were normalized in relation to *Actin* or *eEF1A(m)* gene expression. Data are means of three replicates ± standard error. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

cope with different plant stress (e.g., Ma et al., 2006, 2007; Yamaji et al., 2008, 2012; Chiba et al., 2009; Mitani et al., 2009a,b, 2011a,b; Yamaji and Ma, 2009; Grégoire et al., 2012; Montpetit et al., 2012; Deshmukh et al., 2013; Ma and Yamaji, 2015). To further investigate the effect of Si uptake on ryegrass subjected to Al stress, we assessed the gene expression of two Si transporters (*Lsi1* and *Lsi2*) in plants with different Al and Si supply (**Figures 1A,B**). Lsi1 is a channel-type transporter belonging to aquaporin Nodulin26-like intrinsic protein (NIP) III subfamily (Ma et al., 2006), whereas Lsi2 is an Si efflux transporter belonging to the family of putative anion transporters (Ma et al., 2007). Efficient coupling of Lsi1 with Lsi2 controls the uptake of Si in species such as rice, barley, and maize (Ma et al., 2006, 2007; Chiba et al., 2009; Mitani et al., 2009a,b). Our study showed that in plants cultivated without Al, the mRNA expression levels of both *LpLsi1* and *LpLsi2* were down-regulated in plants supplied with Si (**Figures 1A,B**). Some studies have shown that the accumulation of *Lsi1* mRNA in maize (*ZmLsi1)*, barley (*HvLsi1)*, and wheat (*TaLsi1*) is not affected by the addition of Si (Chiba et al., 2009; Mitani et al., 2009a; Montpetit et al., 2012). Nevertheless, Ma et al. (2006, 2007) found that the gene expression of both *OsLsi1* and *OsLsi2* was decreased by approximately 25% in rice, as a consequence of continuous Si application. A similar expression pattern has been detected for *Lsi1* in maize (*ZmLsi1*) (Bokor et al., 2014) as well as for *Lsi2* in barley (*HvLsi2*) (Mitani et al., 2009b) and maize (Bokor et al., 2014). Moreover, a recent study has stated that the Si-induced down-regulation of Si transporter genes is controlled by Si accumulation in the shoots of rice (Mitani et al., 2016).

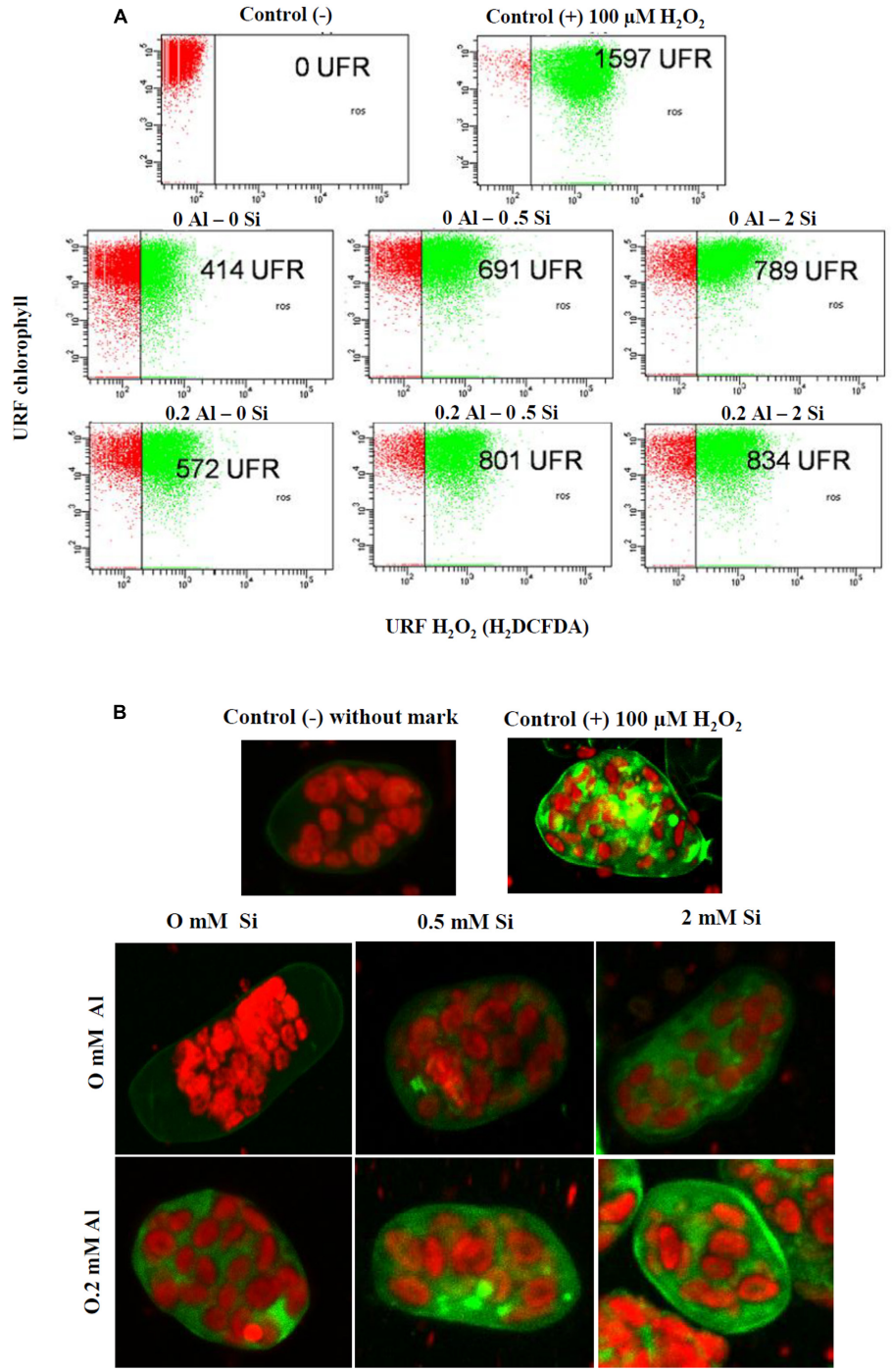

FIGURE 6 | Hydrogen peroxide (H_2O_2) production in shoot protoplasts of ryegrass hydroponically cultivated under Al and Si treatments. (A) Dot plot representation of flow cytometry data. For the positive control, 100 µM H_2O_2 was used. **(B)** Confocal projection images showing the increasing concentration of H_2O_2. Hydrogen peroxide fluorescence were collected by excitation/emission wave lengths 488 nm/530 nm by Confocal Laser Scanning Microscope.

At present, there is little information on the effect of any plant stress on the transcriptional regulation of Si transporters genes. Bokor et al. (2014) observed that Si supply down-regulated the expression of *ZmLsi1* and *ZmLsi2* in the roots of maize subjected to excess zinc (Zn). By contrast, it has been reported that Si increased the expression level of *OsLsi1* and *OsLsi2* under conditions of cadmium (Cd) and copper (Cu) toxicity in rice plants (Kim et al., 2014). Likewise, Vulavala et al. (2016) found that a putative Si transporter in potato (*StLsi1*) was up-regulated in response to Si and drought stress.

Interestingly, we found that the transcript levels of both *LpLsi1* and *LpLsi2* were significantly down-regulated by Al supply, but up-regulated by 5.4-fold (*LpLsi1*) and 2.5-fold (*LpLsi2*) when Al was added in combination with Si (**Figures 1A,B**). Compared with plants cultivated with Si alone, the reduction in Si concentration in plants simultaneously supplied with Al and Si (**Table 2**), could be responsible for the up-regulation of *LpLsi1* and *LpLsi2* (**Figures 1A,B**). This behavior might indicate an increased requirement for Si in ryegrass in order to cope with Al-induced toxicity. Further studies are needed to confirm this assumption.

As a possible alternative mechanism of Si-mediated Al detoxification in plants, enhancement of the antioxidant defense system has also been proposed (Shahnaz et al., 2011; Shen et al., 2014; Liang et al., 2015; Tripathi et al., 2016). As stated above, Al toxicity can lead to the generation of reactive oxygen species (ROS), such as superoxide radicals ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) molecules, which cause oxidative damage to plant cells (e.g., Yamamoto et al., 2001, 2002, 2003; Kochian et al., 2005; Singh et al., 2017). In agreement with previous reports (Cartes et al., 2010, 2012), our results show that 0.2 mM Al increased lipid peroxidation in ryegrass (**Figures 2A,B**), confirming that oxidative stress occurs under Al supply. Nevertheless, 2 mM Si significantly diminished Al-induced lipid peroxidation by approximately 32 and 28% in shoots and roots, respectively (**Figures 2A,B**). Moreover, a negative correlation between Si concentration and lipid peroxidation was detected in Al-treated plants (**Table 3**). Consistent with our findings, Shen et al. (2014) observed a noticeable decrease in lipid peroxidation attributable to Si in peanut grown under Al excess. Similarly, there is increasing evidence showing that oxidative damage to biological membranes decreases as a consequence of Si application to plants subjected to different environmental stresses (e.g., Liang et al., 2003; Zhu et al., 2004; Shi et al., 2005; Gunes et al., 2007, 2008; Li et al., 2012; Khoshgoftarmanesh et al., 2014; Kim et al., 2014; Habibi, 2015; Zia-ur-Rehman et al., 2016).

Whereas Al toxicity enhanced plant phenols concentration (**Figures 3A,B**) and augmented the activities of antioxidant enzymes (**Figures 4A–H**), Si application induced differential responses in the antioxidant system of Al-stressed plants (**Figures 3A,B**, **4A–H**). It has been suggested that Si may enhance Al tolerance by increasing the production of phenolic compounds with Al-chelating ability (Kidd et al., 2001; Shahnaz et al., 2011). Furthermore, it has been reported that Si uptake by plants subjected to certain stresses can lead to increased production of phenolics with antioxidant and/or structural function (Fleck et al., 2010, 2015; Song et al., 2016). Likewise, enzymes and genes involved in the biosynthesis of either soluble phenolics (e.g., flavonoids) or structural polyphenols (e.g., lignin) have also been shown to be induced by Si (Liang et al., 2007; Shetty et al., 2011; Zhang et al., 2013; Song et al., 2016). Here, we found that Si addition (mainly at the highest dose) increased the total phenol concentration in plants treated with Al and Si (**Figures 3A,B**), and that there was a negative relationship between phenols concentration and lipid peroxidation (**Table 3**). Thus, the enhanced phenols accumulation triggered by Si may have contributed to the amelioration of Al-induced oxidative stress in ryegrass.

Differential changes in the activity of antioxidant enzymes, as a consequence of Al and Si treatments, were also observed. SOD constitutes the first line of defense in the enzymatic antioxidant responses by catalyzing the dismutation of $O_2^{\bullet -}$ to H_2O_2 and O_2 (Takahashi and Asada, 1983; Alscher et al., 2002). Our results indicate that the highest Si dose decreased SOD activity in plants subjected to Al stress (**Figures 4A,B**), as supported by the negative correlation between SOD activity and Si concentration (**Table 3**). Likewise, differential gene expression of SOD isoforms occurred in plants exposed to Al and Si (**Figures 5A–F**). The major differences were detected in the roots at the highest Si level, which induced a significant decrease in expression of the *LpCu/Zn-SOD* and *LpMn-SOD* genes in plants grown under Al toxicity (**Figures 5D,F**). A similar expression pattern was observed for the *LpMn-SOD* gene in shoots, which was down-regulated by at least 2.2-fold in combined Al-Si treatments (**Figure 5E**). The decrease in either SOD activity (**Figures 4A,B**) or the gene expression pattern of SOD isoforms (**Figures 5A,D,F**) coincided with a significant reduction in lipid peroxidation at the highest Si dose (**Figures 2A,B**), denoting that 2 mM Si can diminish the requirement for SOD enzyme in Al-treated plants.

It is noteworthy that the antioxidant enzymes responsible for H_2O_2 scavenging (CAT, POD, and APX) were activated by Si in the roots of Al-stressed plants (**Figures 4D,F,H**). Moreover, a direct correlation between Si concentration and the activities of CAT, POD, and APX was found in the roots of plants treated with Al and Si (**Table 3**). The activation of these enzymes was accompanied by a noticeable decrease in lipid peroxidation (**Figures 2A,B**), with a consequent reduction in the oxidative damage of biological membranes induced by Al.

We also detected an apparent increase in intracellular H_2O_2 production in shoot protoplasts of plants simultaneously supplied with Al and Si (**Figures 6A,B**). It is remarkable that there is so little information available regarding the role of Si in H_2O_2 generation under either biotic or abiotic stress conditions. In this context, the only study that has examined the relationship between Si and H_2O_2 production in plants subjected to Al toxicity (Lima et al., 2016) showed an opposite trend when compared with our results. Nevertheless, under freezing stress, Habibi (2015) detected an increase in H_2O_2 levels induced by Si in pistachio plants, which is consistent with the findings of the present study. This significant increase in H_2O_2 production might be related to the reduction in POD activity observed in the shoots of plants simultaneously treated with Al and Si (**Figure 4E**). Indeed, H_2O_2 plays a dual role in vascular plants by either inducing oxidative damage or acting as signaling molecule in several physiological processes, including senescence (Peng et al., 2005), photorespiration and photosynthesis (Noctor and Foyer, 1998), and growth and development (Foreman et al., 2003). H_2O_2 also functions as a second messenger that modulates the expression of antioxidant enzymes and stress responses (Apel and Hirt, 2004). Accordingly, further work should focus on the mechanisms underlying the Si modulation of H_2O_2 production under Al stress.

Finally, taken together, our findings provide the first biochemical and molecular evidence that Si counteracts the negative effects of Al by modulating Al and Si uptake as well as enzymatic and non-enzymatic antioxidant responses in ryegrass plants.

AUTHOR CONTRIBUTIONS

SP and PC conceived the idea and wrote the manuscript. SP performed all the experiments and PC supervised the research. AG-M and HJ contributed to evaluation and discussion regarding aspects of the study related to gene expression analyses. KG assisted with management and analysis of the flow cytometry and laser scanning CLSM data. MM contributed to discussion on aspects associated with the influence of Si on plants subjected to Al toxicity. All authors contributed to the discussion and approved the final manuscript.

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MINI REVIEW
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Silicon Regulates Antioxidant Activities of Crop Plants under Abiotic-Induced Oxidative Stress: A Review

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Silicon (Si) is the second most abundant element in soil, where its availability to plants can exhilarate to 10% of total dry weight of the plant. Si accumulation/transport occurs in the upward direction, and has been identified in several crop plants. Si application has been known to ameliorate plant growth and development during normal and stressful conditions over past two-decades. During abiotic (salinity, drought, thermal, and heavy metal etc) stress, one of the immediate responses by plant is the generation of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH), which cause severe damage to the cell structure, organelles, and functions. To alleviate and repair this damage, plants have developed a complex antioxidant system to maintain homeostasis through non-enzymatic (carotenoids, tocopherols, ascorbate, and glutathione) and enzymatic antioxidants [superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX)]. To this end, the exogenous application of Si has been found to induce stress tolerance by regulating the generation of ROS, reducing electrolytic leakage, and malondialdehyde (MDA) contents, and immobilizing and reducing the uptake of toxic ions like Na, under stressful conditions. However, the interaction of Si and plant antioxidant enzyme system remains poorly understood, and further in-depth analyses at the transcriptomic level are needed to understand the mechanisms responsible for the Si-mediated regulation of stress responses.

Keywords: oxidative stress, stress response, Si fertilization, biochemical and physiological function, stress in plants

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INTRODUCTION

Silicon (Si) has a strong affinity with oxygen; therefore, it usually exists as silica (SiO_2) under natural conditions (Ma and Takahashi, 2002). It also exists in the form of silicic acid [$Si(OH)_4$] and silicate ($xM^1_2OySiO_2$), depending upon the soil pH (Epstein, 1999). Si accumulation/transport occurs in the upward direction, and has been identified in several crop plants. Si transporter genes have been identified in rice, barley, and maize roots, which facilitate its absorption from the soil to the shoot area. Subsequently, it stimulates various physiological responses such as growth, development, and optimization of enzymatic activities. Si accumulation in plant normally

occurs from the root to shoot, and its transport process has been identified in several crops such as rice, maize, and barley (Ma et al., 2006; Mitani et al., 2009; Cooke and Leishman, 2011; Yamaji et al., 2012). Two Si transporter genes were identified by Ma et al. (2006) in rice root and was named as low silicon gene 1 (*Lsi1*) and low silicon gene 2 (*Lsi2*). Following this several Si transport genes have been characterized in other corps such as barley (*HvLsi1*, *HvLsi2*) and maize (*ZmLsi1*, *ZmLsi2*; Ma et al., 2006; Mitani et al., 2009; Yamaji et al., 2012). After the absorption of Si from the soil into the root, it gets translocated to the shoot area, where it can stimulate various physiological responses, such as plant growth and development (Epstein, 1999; Hamayun et al., 2010; Kim et al., 2012; Mateos-Naranjo et al., 2015), enzymatic activity (Epstein, 1999; Liang et al., 2003; Gong et al., 2005; Kim et al., 2014a,b,c; Todorova et al., 2014; Abdel-Haliem et al., 2017), and gene expression (Ma and Yamaji, 2008; Kim et al., 2014a; Vatansever et al., 2017).

To complete a life cycle, plants are continuously exposed to various abiotic stresses and sometime multifaceted stresses. These stresses in turn causing the generation of various reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), or hydroxyl radicals (OH) in cells (Sharma et al., 2012; Das and Roychoudhury, 2014). These ROS can cause serious oxidative damage to the protein, DNA, and lipids of cell components (Apel and Hirt, 2004; Lobo et al., 2010; Tripathi et al., 2017). Therefore, ROS scavenging is most important defense mechanism to cope with stress condition in plants (Sharma et al., 2012; Baxter et al., 2014; Das and Roychoudhury, 2014). According to previous reports, exogenously Si can improve the ability of ROS scavenging by regulation of antioxidants enzyme activity (Torabi et al., 2015; Kim et al., 2016; Tripathi et al., 2017). Furthermore, regulation pattern across various crop plants is different depending upon the exposure time of the stress (Sharma et al., 2012; Kim et al., 2016). Therefore, here, we discussed various possibilities based on previous literature survey and our understanding the role of Si in modulating antioxidant activities in plants during abiotic stress.

DEFENSE MECHANISM AGAINST ROS GENERATION

In natural conditions, plants continuously produce several ROS during photosynthesis and respiration processes in cell organelles such as mitochondria, chloroplast, and peroxisomes. Thus, plants can maintain homeostasis by two different detoxification mechanisms involving non-enzymatic and enzymatic antioxidants (Mittler, 2002; Arbona et al., 2003; Apel and Hirt, 2004; Sytar et al., 2013; Wu et al., 2017). In plants, superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) are the main enzymatic antioxidants, whereas carotenoids, tocopherols, ascorbate, and glutathione are classified as the non-enzymatic antioxidants (Asada, 1999; Racchi, 2013; Kim et al., 2014b,c). Racchi (2013) reported that SOD exists in various forms, such as Cu/ZnSOD, MnSOD, and FeSOD. Depending upon their affinity with the other ions in plants, each SOD are distributed in a different form in various plant organs such as chloroplasts (Cu/ZnSOD, FeSOD), cytosol (Cu/ZnSOD), and mitochondria (MnSOD). Primarily, SOD catalyzes the efficient removal of superoxide free radicals in chloroplasts as they are mainly generated in the photosystem I during the light reaction. CAT is located in the peroxisomes of plant cells, and its main role is the elimination of H_2O_2, which is produced by the SOD reaction. Another antioxidant, APX, also can remove H_2O_2; however, it is distributed in the preoxisomes as well as chloroplasts, cytosol, and mitochondrion (Racchi, 2013). Thus, APX can be found in different forms, such as cAPX (cytosol), mitAPX (mitochondria), sAPX (chloroplast stroma), mAPX (peroxisomes and glyoxisomes), and tAPX (chloroplast thylakoids) depending upon its location (Racchi, 2013). In the chloroplast, APX exist as sAPX and tAPX; the ratio of sAPX and tAPX in chloroplast differs according to the plant species and leaf senescence, and reveals different plant sizes (Sun et al., 2010). The cAPX is located in cytosol; thus, its plays a role in the elimination of H_2O_2, which is generated in cytosol. Therefore, all APXs are different in characteristics such as size, location, role, and amino acid sequences (Caverzan et al., 2012).

Plants can induce defense responses against oxidative stress by activating the non-enzymatic antioxidants, which represent the second line of defense against ROS, hydrophilic molecules (ascorbate, glutathione), and lipophilic metabolites (carotenoids, α-tocopherol; Racchi, 2013; Suzuki et al., 2014; Gowayed et al., 2017) Ascorbate is a water-soluble antioxidant synthesized in mitochondria. It can translocate to other cell compartments by two different pathways. Normally, ascorbate can directly scavenge ROS ($^1O_2$, O_2^-, and OH) in the cell. Furthermore, it is connected with the de-epoxidase enzyme of violaxanthin, and acts as response matrix of APX (Szarka et al., 2013). Due to its various roles, ascorbate is considered as the most powerful antioxidant in the plant cell (Gill and Tuteja, 2010; Racchi, 2013; Suzuki et al., 2014). Glutathione is also an important water-soluble antioxidant, and plays an important role in scavenging $^1O_2$ and OH from chloroplasts (Sharma et al., 2012). In addition, glutathione protects the thiol-groups of enzymes located in the chloroplast stroma and participates in the production of α-tocopherol and ascorbate (Xiang and Oliver, 1998; Hicks et al., 2007; Sharma et al., 2012; Racchi, 2013). Besides its role in detoxification of ROS, glutathione induces physiological responses such as the regulation of sulfur transport and expression of stress defense genes (Noctor et al., 2002; Racchi, 2013). Carotenoids are a class of phenolic compounds distributed in various fruits and vegetables (Racchi, 2013). They can prevent lipid peroxidation by scavenging single oxide radical from chloroplasts (Kühlbrandt et al., 1994). Carotenoids are synthesized in plastids and consist of 40-carbon isoprenoids. According to Lu and Li (2008), carotenoids are classified as carotenes, which include the carbon and hydrogen atoms, and xanthophylls that contain the oxygenated form of carotenes (Wu et al., 2017). The most important role of α-tocopherol is that it can eliminate $^1O_2$, O_2^-, and OH free radicals, which are generated in the thylakoid membranes; thus, it can prevent lipid peroxidation (Fryer, 1992; Kataria, 2017). α-tocopherol has adequate fluidity, enabling it to move easily within the lipid

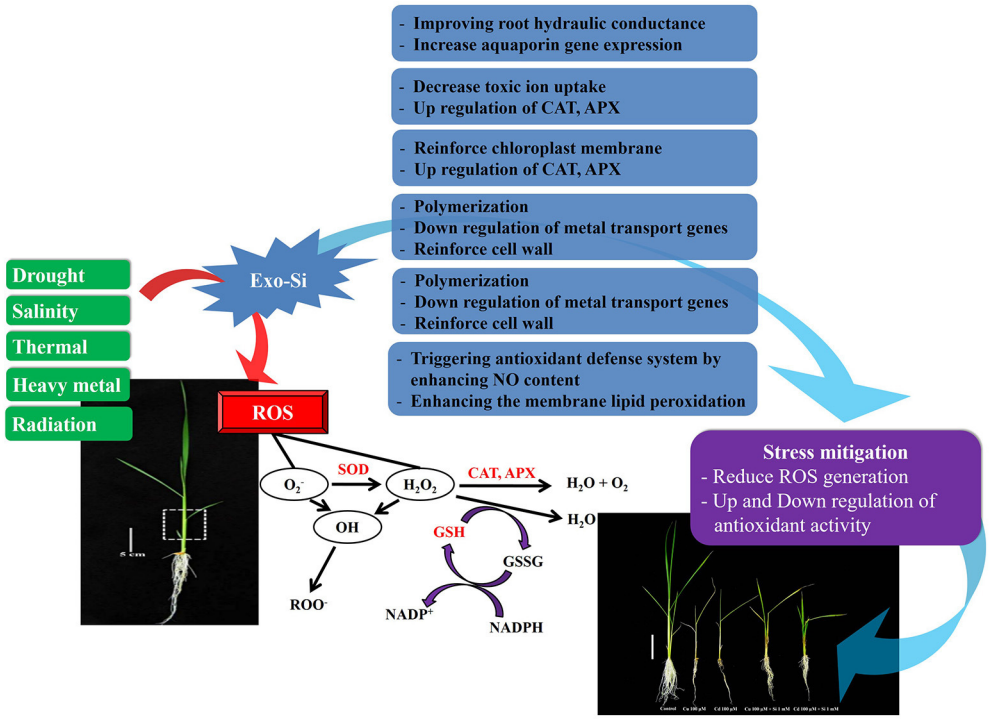

FIGURE 1 | Schematic presentation shows the possible causes that overproduce the reactive oxygen species that could disturb the normal function of cells. The mechanism of antioxidants shown here scavenges the ROS as well as Si effect to mitigate abiotic stress condition.

membrane. Thus, membrane safety is induced by the fluidity of α-tocopherol (Faltin et al., 2010; Racchi, 2013; **Figure 1**).

SI IN ROS SCAVENGING UNDER ABIOTIC STRESS CONDITIONS

The enzymatic/non-enzymatic antioxidants are involved in the removal of ROS either directly (catalases and peroxidases) or indirectly through the regeneration of the two major redox molecules (ascorbate and glutathione) in the cells (**Figure 1**). Accumulation of these antioxidants suggests a high level of stress convened to the plants (Sharma et al., 2012). This could be also assumed that the plant has defended itself from ROS by producing high amount of antioxidant/enzymes. Accoring to Rouhier and Jacquot (2008), Si application in crops during abiotic stress conditions can regulate ROS generation. Here, we investigated via past and current reports that how antioxidant enzymes could regulate after exogenously Si to different plant species during some of the common abiotic stresses (salinity, drought, temperature, wounding, UV, and heavy metal stress).

SALINITY STRESS

Decrease in water potential due to high concentration of sodium and chloride ions inhibit plant growth and development (Torabi et al., 2015). According to Kim et al. (2014c), the application of Si in rice plants under salinity significantly decreased the activities of non-enzymatic MDA and enzymatic antioxidants POD, PPO, and CAT on the other hands, Torabi et al. (2015) observed that when they applied Si to borage plant, SOD activity was significantly increased in Si treatment but activity of CAT and APX was slightly decreased in Si application (**Table 1**). However, Shekari et al. (in press) found that activities of CAT, APX, SOD, and POD were highly increased under Si application with NaCl to herbal *Anethum graveolens* plants. Same pattern of SOD, GPX, APX, GR, and CAT activities was observed by Al-aghabary et al. (2005); Liang et al. (2003), and Zhu et al. (2004). The activities were significantly increased in Si applied barley, cucumber, and tomato plants (**Table 1**).

DROUGHT STRESS

Drought condition cause damage the photosynthetic pigments and disturb balance between ROS production and antioxidants thus overall affecting crop productivity (Iturbe-Ormaetxe et al., 1998; Gong et al., 2005). According to Gong et al. (2005), Si treatment in wheat plants caused high drought tolerance by up-regulating antioxidant activities of CAT, SOD, and GR (**Table 1**). Ma et al. (2016) also suggested that Si supplement wheat plant showed lower lipid peroxidation, glutathione and total flavonoid content whereas increased ascorbate content was observed. Similarly, Shi et al. (2014, 2016) reported that Si supplementation in tomato plants under PEG induced drought

TABLE 1 | Modulation of antioxidant activities by Si application under various abiotic stresses.

Abiotic stresses	Silicon effect	Crop plant	References
Salinity	Increased activity of LPO	Barley	Liang et al., 2003
Salinity	Increased activity of SOD, GPX, APX, and GR Decreased activity of ELP and LPO	Cucumber	Zhu et al., 2004
Salinity	Increased activity of SOD and CAT Decreased activity of APX and MDA	Tomato	Al-aghabary et al., 2005
Salinity	Decreased activity of CAT, MDA, POD, and PPO	Rice	Kim et al., 2014c
Salinity	Increased activity of SOD Decreased activity of CAT and APX	Borago	Torabi et al., 2015
Salinity	Increased activity of CAT, APX, SOD, and POD	Dill	Shekari et al., in press
Drought	Increased activity of CAT, SOD, and GR	Wheat	Gong et al., 2005
Drought	Increased ascorbate contents Reduced glutathione and flavonoid contents	Wheat	Ma et al., 2016
Drought	Decreased activity of APX and MDA	Sunflower	Gunes et al., 2008
Drought	Increased activity of SOD and CAT Decreased activity of POD	Tomato	Shi et al., 2014
Drought	Increased activity of SOD and CAT	Tomato	Shi et al., 2016
High Tem.	Increased activity of SOD, APX, and GPX Decreased activity of CAT	*Salvia splendens*	Soundararajan et al., 2014
Low Tem.	Increased activity of SOD, GSH, APX, MDHAR, GR, and AsA Decreased activity of MDA	Cucumber	Liu et al., 2009
Low Tem.	Increased activity of GSH and AsA Decreased activity of MDA	Maize	Habibi, 2016
Low Tem.	Increased activity of SOD, CAT, and POD Decreased activity of MDA	Turfgrass	He et al., 2010
Mechanical Wounding	Increased activity of CAT, POD and PPO Decreased activity of MDA	Rice	Kim et al., 2016
Ultraviolet-B	Decreased activity of CAT and POD	Soybean	Shen et al., 2010
Ultraviolet-B	Increased activity of SOD and APX Decreased activity of CAT and GPX	Wheat	Tripathi et al., 2017
Heavy metal (Cd)	Decreased activity of MDA	Rice	Kim et al., 2014a
Heavy metal (Mn)	Decreased activity of POD	Cucumber	Maksimović et al., 2012
Heavy metal (Cr)	Increased activity of SOD, GR, and CAT Decreased activity of APX	Pea	Tripathi et al., 2015

APX, ascorbate peroxidase; CAT, catalase; GSH, glutathione reduced form; GR, glutathione reductase; GPX, guaiacol peroxidase; LPO, lipid peroxidase; SOD, superoxide dismutase; ELP, electrolytic leakage percentage; MDA, malondialdehyde; POD, peroxidase; PPO, polyphenol peroxidase; MDHAR, monodehydroascorbate reductase; AsA, ascorbate.

stress caused tolerance via increased SOD and CAT activities as well as improved water uptake ability of roots (**Table 1**). Whilst, Gunes et al. (2008) observed that Si decreased MDA and APX activities in sunflower during drought condition (**Table 1**).

THERMAL STRESS

Like other abiotic stress factors, thermo (cold and heat) stress may also disturb the balance between ROS and antioxidants activity. Soundararajan et al. (2014) treated *Salvia splendens* with Si under high temperature (35°C), and found that the activities of SOD, APX and GPX were increased and contrarily that of CAT was decreased (**Table 1**). Liu et al. (2009) observed that during low temperature (day/night; 15/8°C), Si applied to hydroponically cultivated cucumber plant were more resistant to chilling stress compared to non-Si application and was attributed to more activated antioxidants such as SOD, GSH, APX, GR, MDHAR, and AsA (**Table 1**). Almost same tendency of chilling (day/night; 15/5°C) stress tolerance was observed in turf grass as well, after Na_2SiO_3 fertilization into soil (He et al., 2010; Habibi, 2016; **Table 1**).

MECHANICAL WOUNDING

Normally, natural wounding stress is caused by herbivory or lodging and these events could increase hydrogen peroxide level inside plant tissues (León et al., 2001). According to Kim et al. (2014a), exogenous Si application in rice plants improved mechanical strength to overcome losses from wounding stress (**Table 1**).

ULTRAVIOLET-B

Many studies demonstrated that Si application can induce resistance to UV-B stress via physiological and biochemical

process in plants (Tripathi et al., 2017). In particular, when UV-B applied to tropical plants, MDA, POD, SOD, and anthocyanin contents was increased however, they found decreased activity of CAT was measured (Todorova et al., 2014; **Table 1**). According to Tripathi et al. (2017), UV stress was significantly improved at Si and Si nanoparticle (SiNp) applied wheat seedlings. Especially, mitigation effects between Si and SiNp showed that SiNp applied wheat seedling revealed more strong resistance to UV-B stress (**Table 1**). Other study reported that decreased activity of POD and CAT were measured when they applied Si with UV-B stress to soybean plants (Shen et al., 2010; **Table 1**).

HEAVY METAL STRESS

In addition, during heavy metal stress, Si application can regulate metal transport and prevent damage shown by decreased MDA activity in rice plants (Kim et al., 2016). In cucumber, Si application can ameliorate manganese toxicity observed by decreased POD activity (Maksimović et al., 2012; **Table 1**). Tripathi et al. (2015) applied SiNp with chromium (Cr) to pea seedling after that, they confirmed stress tolerance phenotypes such as enhanced photosynthetic pigments as well as increased activity of SOD, GR, and CAT however, APX activity was decreased (**Table 1**).

CONCLUSIONS

During abiotic stress conditions, the Si application shows varying response to ROS scavenging by activating the defense system plants. In doing so, the activity of antioxidant arsenals (CAT, SOD, PPO, POD, APX, GPX, and GSSH) may also oscillate depending upon the intensity of stress and plant type. Si supplemented plants showed resistance to abiotic stress through, lowering ROS production by (i) enhancing CAT and APX activities as both are involved in conversion of H_2O_2 into H_2O (ii) and decreasing MDA activity.

AUTHOR CONTRIBUTIONS

YK wrote the manuscript; MW and AK contributed in drafting and revising manuscript; IL draw the figure and revised the manuscript.

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Evidence for Active Uptake and Deposition of Si-based Defenses in Tall Fescue

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Silicon (Si) is taken up from the soil as monosilicic acid by plant roots, transported to leaves and deposited as phytoliths, amorphous silica (SiO_2) bodies, which are a key component of anti-herbivore defense in grasses. Silicon transporters have been identified in many plant species, but the mechanisms underpinning Si transport remain poorly understood. Specifically, the extent to which Si uptake is a passive process, driven primarily by transpiration, or has both passive and active components remains disputed. Increases in foliar Si concentration following herbivory suggest plants may exercise some control over Si uptake and distribution. In order to investigate passive and active controls on Si accumulation, we examined both genetic and environmental influences on Si accumulation in the forage grass *Festuca arundinacea.* We studied three *F. arundinacea* varieties that differ in the levels of Si they accumulate. Varieties not only differed in Si concentration, but also in increases in Si accumulation in response to leaf damage. The varietal differences in Si concentration generally reflected differences in stomatal density and stomatal conductance, suggesting passive, transpiration-mediated mechanisms underpin these differences. Bagging plants after damage was employed to minimize differences in stomatal conductance between varieties and in response to damage. This treatment eliminated constitutive differences in leaf Si levels, but did not impair the damage-induced increases in Si uptake: damaged, bagged plants still had more leaf Si than undamaged, bagged plants in all three varieties. Preliminary differential gene expression analysis revealed that the active Si transporter Lsi2 was highly expressed in damaged unbagged plants compared with undamaged unbagged plants, suggesting damage-induced Si defenses are regulated at gene level. Our findings suggest that although differences in transpiration may be partially responsible for varietal differences in Si uptake, they cannot explain damage-induced increases in Si uptake and deposition, suggesting that wounding causes changes in Si uptake, distribution and deposition that likely involve active processes and changes in gene expression.

Keywords: silicon, inducible defense, *Festuca arundinacea*, stomatal conductance, transporter, tall fescue, stress

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INTRODUCTION

Silicon (Si) is considered a non-essential element, but it has many useful functions in plants (Guntzer et al., 2012). Plants take up Si in the form of monosilicic acid [$Si(OH)_4$] via the roots (Ma et al., 2006). It is transported through the xylem and deposited in the leaves to form phytoliths. Phytoliths are solid bodies of silica (SiO_2) found in epidermal layers, both within and between the

plant cells (Piperno, 1988; Currie and Perry, 2007). Trichomes (small hairs found on the leaf surface) may also become enriched with Si and increase the abrasiveness of leaf surfaces. Plants within the grass family (Poaceae) accumulate Si in varying concentrations (up to 10% dry weight) where its primary function is to defend the leaf surface against a range of stresses including drought (Emam et al., 2014; Mitani-Ueno et al., 2016), pathogen attack (Fauteux et al., 2005; Liang et al., 2015) and herbivory (Massey et al., 2006, 2007; Hartley et al., 2015). Many species of grass show diversity in their reported shoot Si concentrations (Ma et al., 2001; Hodson et al., 2005; Massey and Hartley, 2006; Hunt et al., 2008). Differences in the density and efficiency of Si transporters may underpin these differences, as reported in rice (Wu et al., 2006; Ma et al., 2007), whilst environmental conditions such as water availability and herbivory can also drive changes in Si concentration (Quigley and Anderson, 2014; Wieczorek et al., 2015). The relative importance of genotypic, phenotypic and environmental factors for Si uptake remains unclear (Hartley et al., 2015; Hartley and DeGabriel, 2016).

Lsi1 is a root-specific Si transporter involved in the transport of Si from the soil solution [as $Si(OH)_4$] to within the root, first identified in rice (Ma et al., 2006), though orthologues of Lsi1 have now been identified in other crop species (e.g., *Zea mays* L, Mitani et al., 2009a,b; *Hordeum vulgare* L., Chiba et al., 2009; Yamaji et al., 2012 and *Glycine max* (L) Merr., Deshmukh et al., 2013). Lsi1 in rice is a passive aquaporin-like transmembrane protein (Yamaji and Ma, 2007) which transports Si into the root cells, whilst a Si efflux transporter, Lsi2, actively pumps (driven by a proton gradient) Si out of the root cells and into the stele (Ma et al., 2007; Deshmukh and Bélanger, 2016). Aquaporins permit the passage of water through the cell membrane following the gradient in water potential, suggesting that Si can enter the plant cells without the need and use of Si specific transporters (Exley, 2015). Contrary to this, some studies have found that the Si transporters in rice (a hyper-Si accumulator, accumulating up to 10% Si in dry weight) and maize are down-regulated after constitutive Si supply (Yamaji and Ma, 2007; Ma and Yamaji, 2008; Mitani-Ueno et al., 2016), which would not be the case if the transport was purely via water flow into the cells. Furthermore, some studies have reported tissue Si concentrations above that plausible for passive transport only (Faisal et al., 2012; Yamaji and Ma, 2014; Yamaji et al., 2015). Si has been identified in plant parts with low transpiration such as the husk, presumably actively redirected to these locations by Si-mediated transporters (Yamaji and Ma, 2014). Silicon concentrations within specific plant tissues are not always strongly related with transpiration rate, with silicification of silica cells (specific epidermal cells filled with silica) mainly occurring at night (Blackman, 1969) when transpiration rates are low. Silicon deposition has also been found to be independent of water evapotranspiration (Kumar et al., 2016), even when transpiration played a role in the uptake of Si into plants. Further, evidence of silicification of live cells in the absence of transpiration suggests that the cells are actively moving Si into the cells independent of transpiration (Kumar et al., 2016). This may explain the highly organized and distinctive patterns of deposition observed in different species (Hartley et al., 2015).

Increases in Si uptake and changes in Si deposition in response to herbivory may also suggest active redirection of Si within the plant (Hartley et al., 2015). Silicon defenses are now known to be inducible, with up to 400% increases in Si in response to leaf damage (Massey and Hartley, 2006; Massey et al., 2007). Herbivory-induced increases in Si occur in response to a range of herbivores, persist for several months and have been demonstrated in the field (Massey and Hartley, 2006; Massey et al., 2007; Garbuzov et al., 2011; Reynolds et al., 2012; Soininen et al., 2013; Hartley et al., 2015; Wieczorek et al., 2015). To date, no studies have tested whether this increase occurs due to leaf damage leading to higher rates of water loss (i.e., increases in transpiration) and thus subsequent changes in uptake and deposition of Si, or if there is an up-regulation in Si transporter genes in the root, brought on by a damage response from the leaves.

Festuca arundinacea Schreb. (tall fescue) has been classified as both a Si accumulator (Hodson et al., 2005) and a non-accumulator (Ma et al., 2001), suggesting its Si uptake in the natural environment is not uniform. Silicon uptake and deposition is relatively uncharacterized within this species, though previous work (Hartley et al., 2015) has shown it has the ability to take up and deposit Si upon the leaf epidermis, and that the levels of Si within the leaf tissues and the structures it enriches differ amongst breeding varieties within the species (very very soft = 0.43%–0.69% Si, harsh = 0.46%–0.80% Si). Varieties have been described as harsh and soft in terms of their leaf texture, which reflects Si deposition (Hartley et al., 2015). However, how these varieties respond to damage, in terms of Si uptake, and whether any damage-induced increases in Si result from changes in passive Si uptake via transpiration or other more active processes mediated by plant defense responses has not been tested. To date, the studies that have investigated the effects of transpiration on Si uptake have not included an assessment of the effects of damage. Previous studies have focused on the role of transpiration in undamaged plants in cucumber over a short period of time (Faisal et al., 2012) or in detached leaves placed on solution to understand the silicification of cells within the leaf (Kumar et al., 2016). In contrast, our study investigates the effects of herbivore-simulated damage, in an attempt to understand mechanisms driving the induction process. The aim of this study was to determine if damage-induced increases in Si uptake could be explained by environmental variables such as differences in transpiration rates, or if Si-induced defenses are mediated at gene level by changes in Si transporter expression.

This study investigates how altering transpiration rate and simulating herbivory affects the Si concentration of three varieties of *F. arundinacea*. We hypothesize that:

(1) If Si uptake is largely a passive process associated primarily with transpiration rate, varietal differences in Si concentration will be driven by differences in stomatal conductance and stomatal density;
(2) Damage will induce an increase in Si uptake and varieties with a greater rate of Si uptake and deposition will also show a larger induction response and an increased expression of Si transporters;

(3) If damage-induced increases in Si uptake are driven by changes in water relations, then reducing transpiration differences between undamaged and damaged plants will prevent this increase in Si uptake after damage.

MATERIALS AND METHODS

Plant Growth and Experimental Treatments

Three genotypically distinct breeding varieties of *F. arundinacea* contrasting in their ability to accumulate Si (under standard greenhouse conditions, average leaf Si concentrations: very very soft = 0.44%; very soft = 0.43%; and harsh = 0.55%) and varying in leaf texture were provided by the commercial seed company DLF Seeds Ltd., Denmark. The leaf texture is a qualitative trait measured and defined by plant breeders according to how harsh or soft the leaf texture felt on a numerical scale. These were:

- VVS (very very soft leaf texture);
- VS (very soft leaf texture);
- H (harsh leaf texture).

Plants were grown individually in a loam-based compost (John Innes No.2) in 13-cm plastic pots in standard greenhouse conditions: 16 h daylight, 20°C day, 15°C night. Once established, plants were randomly subjected to a combination of bagging and damage treatments:

- Undamaged;
- Damaged;
- Undamaged or damaged, then placed in perforated plastic bags.

The aim of the bagging treatment was to control water flow through the plant; bagging the plants would subject both damaged and undamaged plants to similar levels humidity, thus reducing transpiration (Sellin et al., 2014). Treatments were applied four weeks after sowing, with plants harvested 8 weeks later. There were ten replicate plants of each variety per treatment. Plants were watered twice a week with 100ml of deionized water with 150 mgL^{-1} dissolved sodium metasilicate ($Na_2SiO_3 \cdot 9H_2O$); tap water was added as required. In the treatments where damage was applied, half of the total leaves of each plant were damaged twice a week using a metal file. Damaged and undamaged leaves were separated at harvest and leaf Si concentration analyzed separately.

Epidermal Peel Analysis

During the plant harvest, 5 cm of one leaf from eight replicate plants of each variety per treatment were clipped and painted with clear nail varnish. Transparent sticky tape was placed onto the nail varnish once dried, peeled off and the tape stuck to microscope slides. The slides were analyzed via Nikon Eclipse Ni-U light microscope (Nikon Instruments, Kingston Upon Thames, Surrey) for stomatal, trichome, and phytolith counts.

Si Analysis by Portable X-Ray Fluorescence Spectrometry (P-XRF)

Si was analyzed by portable P-XRF, calibrated using Si-spiked synthetic methyl cellulose and validated using Certified Reference Materials of NCS DC73349 'Bush branches and leaves' obtained from China National Analysis Center for Iron and Steel. Leaf material was ball milled (Retsch MM 400, Haan, Germany) for 2 min at a vibrational frequency of 30 Hz (60 min^{-1}) with 2 cm diameter steel grinding balls in 25 ml grinding jars. Leaf material was pressed at 10 tons into 13 mm diameter pellets with a manual hydraulic press using a 13 mm die (Specac, Orpington, United Kingdom). Si analysis (% Si DW) was performed using a commercial P-XRF instrument (Niton XL3t900 GOLDD analyzer: Thermo Scientific Winchester, United Kingdom) held in a test stand (SmartStand, Thermo Scientific, Winchester, United Kingdom; Reidinger et al., 2012).

Stomatal Conductance Measurements

Stomatal conductance measurements were taken using the Delta –t AP4-UM-3 porometer (Delta-T devices Ltd, Cambridge, United Kingdom). The porometer was calibrated according to the manufacturer's instructions and then the porometer probe was placed on the leaf and the time taken for the leaf to release sufficient water vapor to change the relative humidity in a small chamber by a fixed amount was measured; once stabilized (i.e., the same value was observed for two consecutive readings), the stomatal conductance value was recorded. Five readings per variety, per treatment were taken 1 or 2 days after treatments on five different days. Separate readings of undamaged leaves and damaged leaves of damaged plants were taken.

RNAseq and Differential Gene Expression Analysis (DGEA)

At harvest, three biological replicate samples of unbagged, undamaged, and damaged roots for the VVS and H varieties were flash frozen in liquid nitrogen for RNA extraction. RNA was extracted using TRIzol™ Reagent method from 100 mg of root material according to manufacturer's instructions (Invitrogen, United Kingdom). The RNA quality was checked on a 1% agarose gel to test for degradation and quantified using NanoDrop. DNA digestion and cDNA libraries were prepared and sequenced by Leeds Institute of Molecular Medicine (Leeds, United Kingdom). Sequencing was performed using Illumina HiSeq 3000 (Illumina, Inc., United States) using one lane for all libraries, comprising 2 × 150 bp paired end reads. For library assembly, low quality reads and adapter sequences were removed from the raw FASTQ files using Cutadapt[1] with parameters set to: quality >20 and read length >75 bp. The transcriptome was assembled *de novo* using Trinity RNA-Seq2.1.1 according to the online user-guide[2]. Library reads were aligned to the transcriptome using bowtie2 (Langmead and Salzberg, 2012) and transcript abundance calculated using the RNA-Seq by Expectation Maximization (RSEM) method (Li and

[1]https://github.com/marcelm/cutadapt

[2]https://github.com/trinityrnaseq/trinityrnaseq/wiki

Dewey, 2011). Transcripts were annotated in Trinotate v3.0 using BLAST searches (E value $< 10^{-20}$) against Swissprot. DGEA was carried out on the annotated transcripts using the edgeR package (Robinson et al., 2010; McCarthy et al., 2012) to test for differences in log fold changes (logFC) > 1 with a false discovery rate (FDR) set to <0.05 to correct P-values for multiple testing. To confirm the identity of Lsi2 sequences, the transcripts were searched for sequence similarity to using BLAST and their transmembrane domains were compared to the barley Lsi2 (accession AB447483.1; Mitani et al., 2009a) sequence using TMHMM Server v2.0[3]. The sequences for *Lsi2* (Supplementary Table S1) were only partial sequences, but the transmembrane domains found in these sequences closely matched those in the barley Lsi2 transporter.

Statistical Analyses

All statistical analyses were performed using R (version 3.3.2). Analysis of variance (ANOVA) tests were used to test the main and interactive effects of variety, bagging and damage (using damaged leaves of damaged plants) on leaf Si concentration and stomatal conductance. Paired t-tests were used to test for statistical differences between undamaged leaves and damaged leaves of the same damaged plants, where the aim was to test for localized and systemic responses in Si uptake and differences in stomatal conductance. Bonferroni's correction was applied for t-tests, setting the level of significance to $P < 0.02$. Generalized linear models were used to test the main effects of variety on stomatal, trichome, and phytolith densities. Linear models were used to check for normality and homogeneity of variance following Crawley (2007). Si (%) values were transformed using the arcsine square root transformation to meet the assumptions of the tests. Significance was set at $P < 0.05$ for all analyses other than t-tests. Linear regression was used to test for relationships between stomatal conductance and Si concentration. *Post hoc* Tukey tests were carried out and significance was set at $P < 0.05$. Where models did not meet the assumptions, generalized linear models were applied instead of linear models. Packages used for analyses were as follows: lsmeans package (Lenth, 2016), multcompView (Graves et al., 2015), and ggplot function from ggplot2 package (Wickham, 2009).

RESULTS

Undamaged Plants

Stomatal conductance did not differ significantly between the three varieties but there was a trend for increased stomatal conductance with increasing harshness: VVS displayed the lowest stomatal conductance. Stomatal density, trichome density and Si concentration differed between variety. Stomatal density was higher in the H variety ($F_{2,23} = 4.05$, $P = 0.03$, **Figure 1A**) compared with the VS variety.

The VS and H varieties had more trichomes per mm^2 compared to the VVS variety ($F_{2,23} = 6.02$, $P = 0.008$; **Figure 1B**), but phytolith density did not differ between the varieties (Supplementary Table S2).

The H and VVS varieties differed in their leaf Si concentration ($F_{2,18} = 8.75$, $P = 0.002$; **Figure 1D**). There was a positive relationship between stomatal conductance and Si concentration ($n = 15$, $r = 0.52$, $P = 0.049$; **Figure 2A**) in undamaged, unbagged plants.

The H variety had a higher expression of two *Lsi2* gene isoforms compared to the VVS variety in undamaged conditions (log fold changes = 3.72 and 7.60, FDR < 0.05, TRINITY_DN45085_c2_g1_i1 and TRINITY_DN45085_c2_g2_i2 in Supplementary Table S1).

Damaged Plants

Stomatal conductance was higher in the damaged leaves of damaged plants in the H variety compared with the VVS variety ($F_{2,12} = 6.38$, $P = 0.01$; **Figure 3A**). There were no differences in stomatal conductance between undamaged leaves and damaged leaves of damaged plants in any of the three varieties.

In undamaged leaves of damaged plants, the VVS variety had significantly fewer trichomes per mm^2 compared to the VS and H variety ($F_{2,23} = 5.03$, $P = 0.02$; Supplementary Table S2). In the damaged leaves of damaged plants, no significant varietal differences were observed in terms of trichome density – although there was still a trend for the VVS variety to have fewer trichomes compared to the VS and H variety. Phytolith density was highest in the VS variety for both undamaged leaves of damaged plants ($F_{2,23} = 8.20$, $P = 0.002$) and damaged leaves of damaged plants ($F_{2,23} = 813.83$, $P < 0.001$; Supplementary Table S2).

The damaged leaves of damaged H plants had more Si than both VS and VVS subjected to this treatment ($F_{2,27} = 19.89$, $P < 0.001$; **Figure 3B**). Paired t-tests between undamaged leaves and damaged leaves of damaged plants showed there was a localized response to Si uptake in the H variety only – i.e., the damaged leaves had more Si compared to the undamaged leaves of the same plant ($t = 4.58$, $df = 8$, $P = 0.002$). There was a significant positive linear relationship between leaf Si concentration and stomatal conductance under damaged, unbagged conditions for both undamaged leaves ($n = 15$, $r = 0.62$, $P = 0.02$; **Figure 2B**) and damaged leaves of damaged plants ($n = 15$, $r = 0.75$, $P = 0.001$; **Figure 2C**).

In unbagged, damaged conditions showed that three *Lsi2* gene isoforms were expressed, and these were up-regulated in the H variety compared to the VVS variety (log fold changes = 4.52, 3.51 and 6.78, FDR < 0.05, TRINITY_DN45085_c1_g1_i1, TRINITY_DN45085_c2_g1_i1 and TRINITY_DN45085_c2_g2_i2 in Supplementary Table S1).

Bagged Plants

Under bagged conditions, the patterns of stomatal conductance between varieties were similar to those in unbagged conditions. VVS had significantly lower stomatal conductance compared to VS and H varieties ($F_{2,24} = 19.07$, $P < 0.001$; data not shown).

The VVS variety had significantly fewer trichomes compared to the VS and H varieties under undamaged, bagged conditions ($F_{2,22} = 10.96$, $P < 0.001$). This relationship was the same for

[3]http://www.cbs.dtu.dk/services/TMHMM/

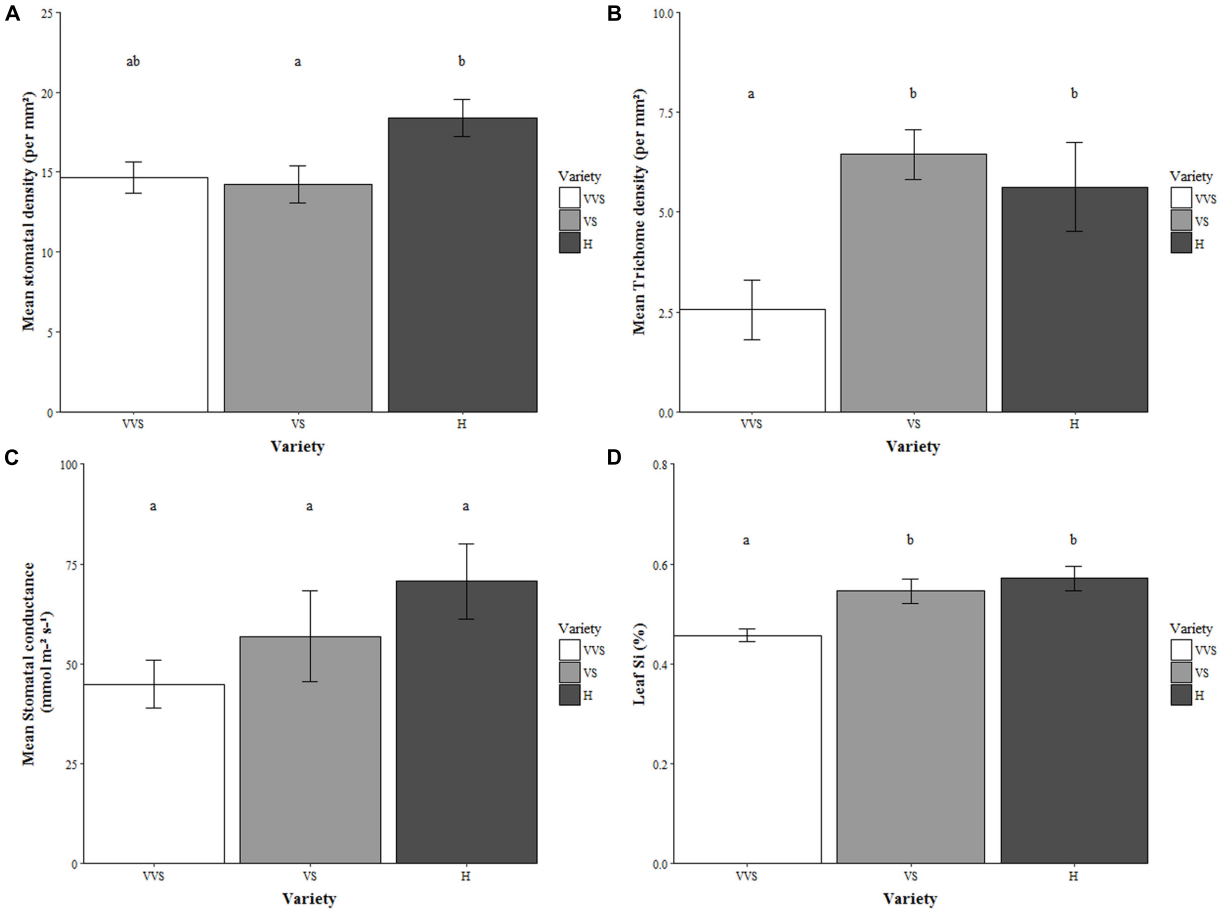

FIGURE 1 | (A) Stomatal density, **(B)** Trichome density, **(C)** Stomatal conductance, and **(D)** Leaf Si concentration. VVS = very very soft, VS = very soft, and H = harsh. Values represent unbagged and undamaged plants. Bars are mean values ± SE. *N* = 9 for stomatal density, *n* = 5 stomatal conductance and *n* = 10 for leaf Si. Letters within bars denote significant differences between treatments (*post hoc* Tukey $p < 0.05$).

both undamaged leaves ($F_{2,22} = 10.07$, $P < 0.001$, Supplementary Table S3) and damaged leaves of damaged plants ($F_{2,22} = 6.39$, $P = 0.007$, Supplementary Table S3). Phytolith density was higher in the VS variety compared with H and VVS in undamaged, bagged plants ($F_{2,22} = 8.63$, $P = 0.002$, Supplementary Table S3) and also in damaged, bagged plants with undamaged leaves ($F_{2,22} = 12.43$, $P < 0.001$, Supplementary Table S3). The H variety had significantly fewer phytoliths on damaged leaves compared with the other two varieties under damaged, bagged conditions ($F_{2,22} = 6.23$, $P = 0.007$, Supplementary Table S3).

In bagged conditions, leaf Si concentration did not differ between varieties in either the damaged leaves or undamaged leaves (**Figure 4**). However, damaged leaves of damaged plants had significantly higher leaf Si than undamaged plants in all three varieties ($F_{1,54} = 11.21$, $P = 0.001$; **Figure 4**). No relationship between leaf Si concentration and stomatal conductance was reported for undamaged, bagged plants or for damaged leaves of damaged, bagged plants. There was a weak relationship between leaf Si concentration and stomatal conductance in the undamaged leaves of damaged, bagged plants ($F_{1,13} = 0.33$, $P = 0.03$).

DISCUSSION

There are clear differences in the accumulation and deposition of Si between the varieties, and in how the varieties respond to damage in terms of induction of Si defenses. In unbagged conditions, these varietal differences tend to reflect similar differences in stomatal density and stomatal conductance, with the H variety tending to have the highest Si concentration and trichome density as well as the highest stomatal density and stomatal conductance, and with VVS having the lowest. Silicon concentration is significantly positively correlated with stomatal conductance in these plants. The H variety also shows higher induction of Si uptake after damage than the two soft varieties, increased expression of the active Si transporter *Lsi2* and shows some evidence of systemic induction the other two varieties do not show. However, in bagged conditions, these varietal differences disappear – undamaged bagged plants have the same Si concentration, regardless of variety, and all varieties respond to damage with induction in Si defenses and we no longer see the systemic induction in H plants. Further, these varieties continue to deposit trichomes and phytoliths on the leaf surface

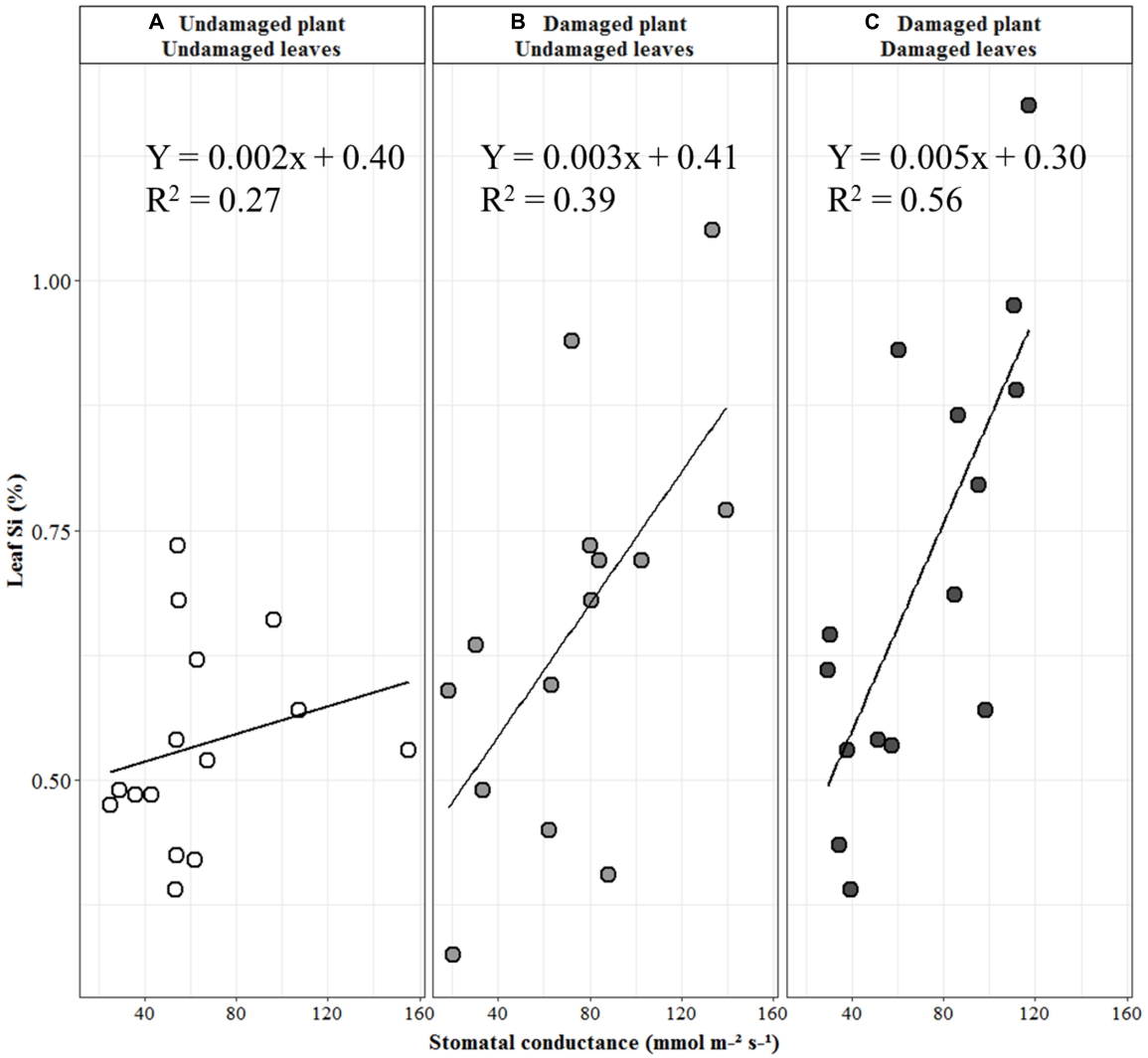

FIGURE 2 | Linear regression between stomatal conductance and leaf Si concentration of unbagged plants. **(A)** Undamaged plants. **(B)** Damaged plants, undamaged leaves. **(C)** Damaged plants, damaged leaves. Regression line equation based on raw Si and stomatal conductance data; statistical analysis based on arcsine transformed Si data (see text for details).

under bagged conditions in similar quantities to the unbagged plants, despite the likely difference in transpiration between these two conditions. These findings cannot be explained purely by passive processes linked to water evapotranspiration, implying that damage-inducted increases in Si deposition require active physiologically regulated processes (Kumar et al., 2016).

Undamaged Plants

We hypothesized that if Si uptake was largely a passive process associated with transpiration rate, varietal differences in Si concentration would be driven by differences in stomatal conductance and stomatal density because Si uptake into the tissues, although mediated by the Si transporters, mainly follows the flow of water from the external environment into the root cells (Raven, 1983; Epstein, 1994, 1999; Exley, 2015). Our findings of a correlation between stomatal conductance and Si support this hypothesis of a strong role of the transpiration stream in Si uptake also found in previous studies (Sangster and Parry, 1971; Henriet et al., 2006; Cornelis et al., 2010; Faisal et al., 2012; Kumar et al., 2016) and suggests a strong role of the transpiration stream in Si uptake. However, the clear differences in Si concentration between the varieties, despite no statistical differences observed in stomatal conductance between them, suggests other factors than transpiration stream may have some influence on Si accumulation and deposition in the undamaged plants. The H variety had a higher expression of the active Si transporter *Lsi2* compared to the VVS variety; varietal differences have also been reported in barley cultivars in expression of *Lsi2*, where Si concentration was positively correlated with *Lsi2* expression (Mitani et al., 2009a). It was reported that constant Si supply led to the down-regulation of *Lsi2* in barley and maize over a period of a week (Mitani et al., 2009a). In our study, Si was

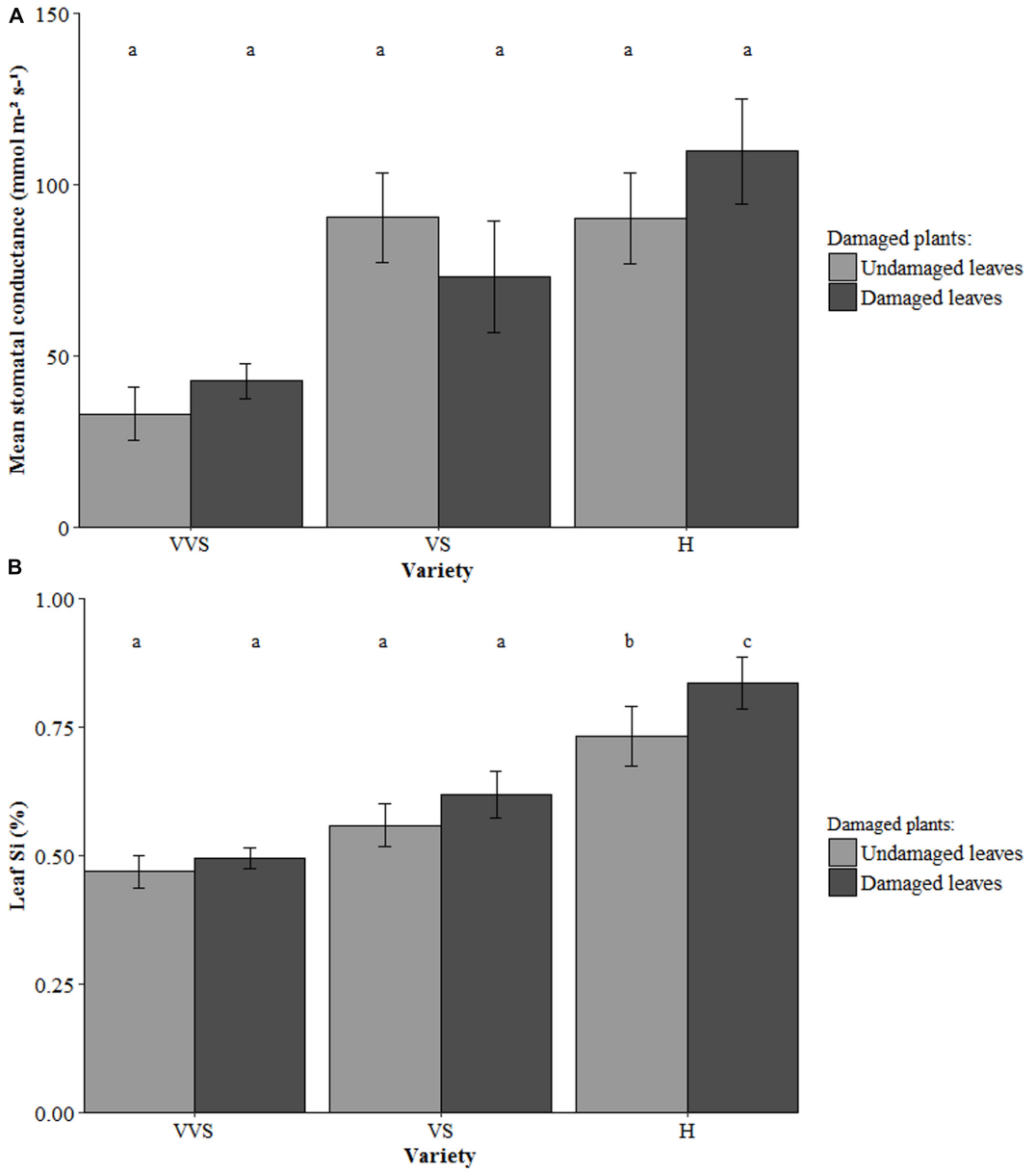

FIGURE 3 | (A) Stomatal conductance, and **(B)** Leaf Si concentration of damaged plants in unbagged conditions. VVS = very very soft, VS = very soft and H = harsh. Bars are mean values ± SE. *N* = 5 for stomatal conductance and *n* = 10 for Si concentration. Letters within bars denote significant differences between treatments (*post hoc* Tukey $p < 0.05$).

constantly supplied over a period of 12 weeks, and it is possible that the VVS variety is less able to upregulate *Lsi2* than the H variety under these conditions. These results suggest Lsi2 has an important role in driving varietal differences in terms of Si concentration in tall fescue.

Damaged Plants

We hypothesized that damaging plants would induce an increase in Si uptake, and that varieties with a greater rate of Si uptake and deposition would show larger induction responses. However, in unbagged conditions damaging leaves only elicited a response from the H variety, both systemically and locally. The undamaged leaves of damaged plants increased leaf Si concentration by 27% and the damaged leaves by 47% compared to the undamaged plants. Such increases in Si after induction have been observed in many other studies (Massey and Hartley, 2006; Massey et al., 2007; Garbuzov et al., 2011; Reynolds et al., 2012; Soininen et al., 2013; Hartley et al., 2015). Although under undamaged conditions varieties did not differ significantly in stomatal conductance (though there was a trend for higher conductance in harsher varieties), in damaged plants the H variety had significantly higher stomatal conductance than the

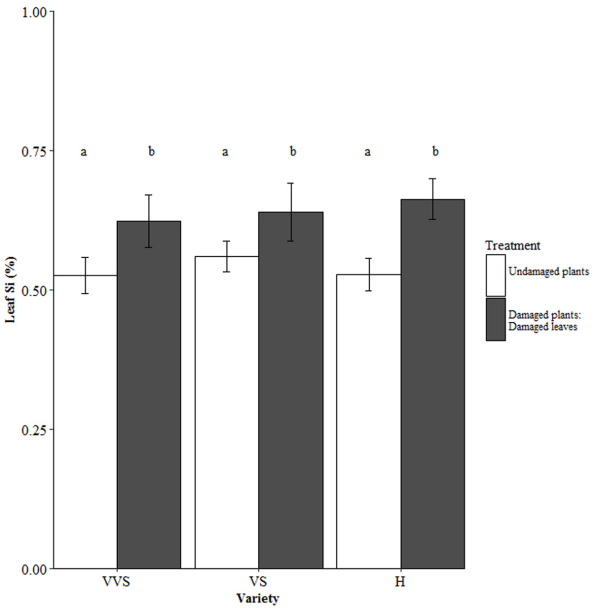

FIGURE 4 | Leaf Si concentration of undamaged plants and damaged leaves of damaged plants under bagged conditions. VVS = very very soft, VS = very soft and H = harsh. Bars are mean values ± SE. *N* = 10. Letters within bars denote significant differences between treatments (*post hoc* Tukey $p < 0.05$).

VVS and VS varieties (see **Figures 1C**, **3A**). This suggests that varietal differences in Si in damaged, unbagged plants may at least in part, be driven by the uptake of water. The lack of response in stomatal conductance, by the VS and VVS varieties is surprising given that most studies (Warrington et al., 1989; Oleksyn et al., 1998; Aldea et al., 2005; Pincebourde et al., 2006) find an increase in stomatal conductance and transpiration when leaves are grazed or perforated, due to damage of the stomata causing impaired function, such as altering the ability of the guard cells to open and close properly. There was also a lack of response to damage in terms of increased Si uptake by VVS and VS varieties, but the VS variety had more phytoliths per mm^2 than in undamaged plants. Thus, although Si concentration did not increase, this variety invested more Si in phytolith production suggesting a shift in allocation patterns of Si under damaged conditions. In damaged plants, there was a greater expression of *Lsi2* gene isoforms compared to the undamaged plants suggesting that this transporter is at least partially responsible for Si-induced defenses in this species. The *Lsi2* transporters were up-regulated in the H variety compared to the VVS variety. Tall fescue is an outbreeding, allohexaploid (Gibson and Newman, 2001) and therefore there may be splice variants of these Si transporters in the different varieties which are only activated upon damage. We were able to see differences between treatments using a small number biological replicates in a species with a complex genome such as tall fescue, providing clear evidence that Si-induced defenses are under molecular control in this species. In barley, Si concentration was positively correlated with *Lsi2* expression (Mitani et al., 2009a), here we also see plants with more Si in the leaves also have a higher expression of *Lsi2*.

Bagged, Undamaged, and Damaged Plants

We hypothesized that if damage induced increases in Si uptake were driven by changes in water relations, bagging plants would prevent this increase in Si uptake after damage. Bagging the plants removed the differences observed between the undamaged and damaged plants in terms of stomatal conductance compared to when plants were not bagged, and also removed the varietal Si differences observed in unbagged plants. However, bagging plants did not remove the Si differences between the undamaged and damaged plants: there was still an induction response to damage, increasing the leaf Si concentration in damaged plants compared to the undamaged plants in all 3 varieties. The systemic induction in the H variety observed in unbagged damaged plants was not found in this treatment, suggesting systemic induction is in part influenced by water relations, but localized responses to damage with increased Si deposition are not. The trend in trichome and phytolith deposition between the varieties remains similar between unbagged and bagged conditions (i.e., that VVS has less trichomes compared to the VS and H variety and that the VS variety has more phytoliths compared with the H and VVS varieties), again suggesting this deposition is not primarily transpiration driven. We also see differences between the varieties in terms of the deposition patterns, even though the stomatal conductance is the same (Supplementary Table S3). Transpiration seems necessary for plants to accumulate Si from the roots to the leaf tissues, but other active means must be at play during the deposition to explain findings in our study. Other work supports this assertion, silica accumulation in silica cells takes place only during leaf development (Sangster, 1970; Motomura et al., 2006; Kumar et al., 2016); if transpiration were the sole cause of Si deposition then all leaves (despite their age) would continue to deposit Si in the silica cells, but this is not the case (Kumar et al., 2016).

Studies that have investigated the relationship between passive/ active uptake of Si in plants have found content of Si both higher (Faisal et al., 2012; Gocke et al., 2013; Kumar et al., 2016) and lower Si than expected for passive uptake (Cornelis et al., 2010), which again goes against the suggestion that Si uptake and distribution is a purely passive process (Exley, 2015). Silicic acid may move freely into the roots but uptake and distribution of Si increases in the presence of the influx and efflux transporters (Farooq and Dietz, 2015; Yamaji et al., 2015). Many studies have shown Si transporters are responsible for the uptake and distribution of Si in different grass species (Ma et al., 2006; Chiba et al., 2009; Mitani et al., 2009a,b; Montpetit et al., 2012). Si transport, both within and between species is variable as is the regulation of the Si transporters – *Lsi1* is down regulated in rice during constant Si supply after 3 days (Ma et al., 2006) whereas in barley and maize for example, the expression is constitutive (Chiba et al., 2009; Mitani et al., 2009a). In terms of inducible plant defenses, plants may only up-regulate expression of Si transporters as needed and rely on their base transcript levels of

Si transporters and transpiration to utilize Si under undamaged, unbagged conditions. Complex interactions between genetic and environmental controls on the expression of transporters may explain why Si levels for the same species are often so variable (Ma et al., 2001; Hodson et al., 2005; Soininen et al., 2013).

Given that Si transporters have been identified in many other species of grass such as rice (Ma et al., 2006) and barley (Mitani et al., 2009a and in some dicotyledons, cucumber, pumpkin, and soybean (Deshmukh et al., 2013) for example (see Deshmukh et al., 2015; Deshmukh and Bélanger, 2016 for others), it is likely that *F. arundinacea* has Si transporters and that differences in these underlie differences in Si uptake and deposition we observe between varieties. Other studies have found intraspecific differences in uptake abilities in rice (Wu et al., 2006; Ma et al., 2007) which revealed that the higher Si accumulating genotypes were able to accumulate more Si due to a higher level upregulation of Si transporters. Perhaps this is also the case for the differences in these varieties and may also be why the high accumulating variety (H) is better able to respond to damage as there is a greater number of Si transporters present. The spacing between the conserved (asparagine-proline-alanine (NPA)) domains in Si transporters is also likely to influence uptake abilities within and between species; the spacing between these amino acids have been shown to determine whether plants are able to import or reject importing Si into the root cells (Deshmukh et al., 2015).

CONCLUSION

Few studies have looked at the relationship between Si accumulation and transpiration, and to date none have looked at these in combination with damage. To date, no studies have looked at differential expression of the Si transporters between undamaged and damaged conditions to test for molecular evidence of Si-induced responses. There were clear differences in the response of the three varieties to the damage treatments within this study, suggesting that damage is an important driver in the accumulation of Si. Removal of differences in stomatal conductance also removed the difference in Si levels between the varieties, suggesting that transpiration has a role in Si accumulation, but the higher Si levels under damaged, bagged conditions show these increases must occur by mechanisms other than just passive movement of Si in the transpiration stream. This gives clear evidence for active Si-induced defenses within this species. Further, we provide the first evidence of molecular based Si-induced defenses by the up-regulation of the active Si transporter, *Lsi2*, in damaged plants. Clearly, further molecular characterization of the mechanisms involved in Si uptake and transport following damage is necessary to fully understand how Si gets from the xylem and into the cells in the leaves. These results not only provide evidence for Si-defenses being regulated at gene level, they also provide insights into target traits for selecting plant genotypes resistant to herbivory for agriculture and other uses.

AUTHOR CONTRIBUTIONS

All authors designed the research. EM conducted the experiments, data analysis, and wrote the manuscript. IL advised on RNA-Seq work. SH and SM-M revised the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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Exogenous Supplementation of Silicon Improved the Recovery of Hyperhydric Shoots in *Dianthus caryophyllus* L. by Stabilizing the Physiology and Protein Expression

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Hyperhydricity is one of the major problems hindering *in vitro* propagation of *Dianthus caryophyllus* L. Silicon (Si) is a well-known beneficial element renowned for its stress amelioration properties in plants. This study has demonstrated the physiological and molecular mechanism behind the Si-mediated recovery from hyperhydricity in *D. caryophyllus* L. 'Green Beauty'. Four weeks old hyperhydric shoots obtained from temporary immersion system were cultured on the Murashige and Skoog medium supplemented with 0 (control), 1.8 mM, or 3.6 mM of potassium silicate (K_2SiO_3). After 2 weeks of culture, we observed only 20% of hyperhydric shoots were recovered in control. On the other hand hyperhydricity, shoot recovery percentage in 1.8 mM and 3.6 mM of Si were 44% and 36%, respectively. Shoots in control possessed higher lipid peroxidation rate compared to the Si treatments. Similarly, damaged stomata were detected in the control, while Si treatments restored the normal stomatal development. Expressions of superoxide dismutase, guaiacol peroxidase, and catalase varied between the control and Si treatments. Furthermore, a proteomic analysis showed that as compared with the control Si up-regulated 17 and 10 protein spots in abundance at 1.8 and 3.6 mM of Si, respectively. In comparison to the 3.6 mM, 1.8 mM of Si treatment up-regulated 19 proteins and down-regulated 7 proteins. Identified proteins were categorized into six groups according to their biological roles such as ribosomal binding, oxido-reduction, hormone/cell signaling, metal/ion binding, defense, and photosynthesis. The proteomic results revealed that Si actively involved in the various metabolisms to accelerate the recovery of the shoots from hyperhydricity. Thus, the outcomes of this study can be utilized for addressing the molecular insight of hyperhydricity and its recovery mechanism by the supplementation of Si. Therefore, we conclude that active involvement of Si in the regulation and signaling process of proteins at 1.8 mM concentration could be efficient to trigger the reclamation process of hyperhydric carnation shoots.

Keywords: antioxidant enzymes, proteomics, silicic acid, tissue culture, vitrification

INTRODUCTION

Silicon (Si) is the second most abundant element in the earth crust (Epstein, 1999) and shows various beneficial effects in the plant growth and development (Ma and Yamaji, 2006). Recently, Si was listed as a "beneficial substance" or "quasi-essential" by International Plant Nutrition Institute. Effect of Si is apparently visible under several abiotic and biotic stress conditions such as drought [2.11 mM sodium silicate (Na_2SiO_3)] (Gong et al., 2005), salinity [1.8 mM potassium silicate (K_2SiO_3)] (Manivannan et al., 2016), temperature (3.6 mM K_2SiO_3) (Soundararajan et al., 2014), powdery mildew (1.7 mM silicic acid) (Fauteux et al., 2006), and herbivory resistance (2.7 mM $NaSiO_3{\cdot}9H_2O$) (Keeping and Kvedaras, 2008), applied in hydroponic solution. Deposition of Si in endodermis and cell walls of root helps in selective nutrient uptake and restricts the transportation of toxic ions to the aerial parts (Yeo et al., 1999). After transpirational bypass flow, polymerized Si forms double layer with cuticle act as the physical barrier against the insects and pathogens (Ma and Yamaji, 2006). Reduction of the transpiration rate and improvement in the stomatal conductance indicate that Si strongly influence the hydraulic adjustment in plants (Ming et al., 2012). Polymerization of Si in the epidermal cells maintains the integrity of the cell membrane and water potential which prevents the electrolyte leakage (Agarie et al., 1998). The physiological improvement by Si is associated with a larger leaf area, enhanced light interception, and improved net photosynthetic assimilation (Ma and Yamaji, 2006). Addition of Si efficiently regulate the activity of the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX) involved in the detoxification of excessively generated reactive oxygen species (ROS) during abnormal conditions (Zhu et al., 2004; Gong et al., 2005; Soundararajan et al., 2014, 2015). Maintenance of low molecular weight antioxidants such as ascorbate and glutathione, and proline, an important osmolyte to provide the osmotic balance (Gunes et al., 2007a). Hence, soluble Si in the xylem sap regulates various enzymatic and non-enzymatic metabolisms in the plants to maintain the proper equilibrium in the osmotic, redox, and ionic status.

Hyperhydricity is the serious problem that intrudes the *in vitro* propagation of several plants. Hyperhydric plants generally possess curled leaves with deformed, glassy, and brittle shoots containing excess amount of water. The accumulation of excessive amounts of water causes severe problems during *in vitro* propagation, organogenesis, germplasm maintenance, cryopreservation, and acclimatization (Ziv, 1991). This causes heavy loss in the medicinal, ornamental, and horticultural industries. Supplementation of Si to the tissue culture medium raise the stability of the cells, tissues, and organs (Sivanesan and Park, 2014). *In vitro* growth, biomass, and anatomy of plants were improved with the supplementation of Si under various forms such as Na_2SiO_3 (1.0 ppm) in *Fragaria* × *ananassa* (Braga et al., 2009), calcium silicate ($CaSiO_3$) (1000 ppm) Musa so. 'Maca' banana (Asmar et al., 2013), and K_2SiO_3 (200 ppm) for *Begonia semperflorens* and *Viola* × *wittrockiana* (Lim et al., 2012), respectively. Oxidative stress created under the hyperhydric condition leads to the generation of ROS such as singlet oxygen (O_2^{-1}), hydrogen peroxide (H_2O_2), and hydroxyl radical (⁻OH) (Muneer et al., 2016). High amounts of lipid peroxidation (LPO) also cause excessive generation of ROS which damage the macromolecules including nucleic acids, proteins, and lipids (Apel and Hirt, 2004). Gunes et al. (2007a) suggested that Si efficiently reduces the LPO rate under stress conditions. During abnormal circumstances, Si assist in the maintenance of stomatal structure (Asmar et al., 2013; Manivannan et al., 2016). Ming et al. (2012) reported that Si either increase or withhold the water potential under water-deficit stress in rice seedlings. Likewise, Si supplementation involved in the maintenance of water balance during the salt stress in the tomato seedlings (Romero-Aranda et al., 2006). Enhanced activities of aquaporins present in the plasma membrane attributes to the reduced H_2O_2 accumulation and increased hydraulic conductance of the Si-treated tomato (Liu et al., 2015). Uptake of Si deposited mostly in the apoplast follows the water transportation (Handreck and Jones, 1968). Maintenance of water status is basis for metabolic activities in tissues (Mali and Aery, 2008). Reduction in the LPO could be caused due to the modulation in antioxidant enzymes activity and deterioration of H_2O_2 in the Si treatments (Gunes et al., 2007b). In cotoneaster, occurrence of hyperhydricity during the shoot multiplication was reduced by the supplementation of Si in the culture medium (Sivanesan et al., 2011). Owing to the beneficial effects of Si in several plants under various stressful environment, the current endeavor has attempted to determine the role of Si in the recovery of hyperhydricity in *Dianthus caryophyllus* L.

Carnation (*D. caryophyllus* L.) is one of the major floricultural crops and is mainly used as the cut flowers and potted plants worldwide. However, the *in vitro* propagation of carnations are highly hindered to its high susceptibility to hyperhydricity (Muneer et al., 2016). Therefore, carnations are considered as an excellent model plants to study the hyperhydricity (Olmos and Hellín, 1998). Carnation is a Si non-accumulator, since the uptake of Si studied under *in vitro* condition is merely lesser (Soundararajan et al., 2015) than the high accumulator (rice) or moderate accumulator (cucumber) (Mitani and Ma, 2005). Till now there is no molecular evidence available to predict the Si accumulation in carnation. In general, Si transporter present in the plant facilitates the massive uptake of Si in the accumulators such as rice, wheat, and sugarcane (Ma and Yamaji, 2006). However, plants with the lesser accumulation of Si could be occurred in passive mode along with the flow of water (Mitani and Ma, 2005).

Transcriptomics and proteomics analysis allows to unearth the dynamic range of changes occurred in the plant system. Transcriptomes study based on the endogenous level of H_2O_2 at different time interval in the hyperhydric shoots on *Allium sativum* L. revealed that genes related to the biosynthetic pathways of phytohormones such as auxin, cytokinin, and ethylene are expressed differentially during the oxidative bursting stage (Liu et al., 2017). Similarly the peach transcriptome study revealed that more than 300 transcripts were altered between the non-hyperhydric and hyperhydric leaves. Most of the transcripts modulated in peach were categorized to play vital roles in posttranscriptional process and photosynthesis,

cellular elimination, cuticle development, and abiotic stress response (Bakir et al., 2016). Moreover, micro-RNA study in peach leaves suggested that around 27 miRNAs were characterized in hyperhydric leaves (Diler et al., 2016). Among them miR5021, ATP binding cassette (ABC) transporters involved in plant cuticle formation and miRnovel2, could involve in the regulation of gibberellin 2-beta-dioxygenase. As a consequence of the several disorders, hyperhydric condition induces the changes in the protein synthesis and affects the interrelated metabolic pathways (Fontes et al., 1999). Expression of Binding Protein (BiP), a member of Hsp70 protein family is higher in the hyperhydric shoots (Picoli et al., 2001). Synthesis of BiP protein is associated with the accumulation of misfolded proteins in the endoplasmic reticulum (Fontes et al., 1999). Under hyperhydric condition, antioxidant enzymes-related proteins were highly up-regulated than the normal carnation (Muneer et al., 2016). Regulation of proteins plays a key role in the developmental process of plants. Increase in the soluble protein in Si-treated cucumber plants helps to overcome the salt stress in cucumber (Zhu et al., 2004). Though, already transcriptome/proteomics studies were carried out to elucidate the influence of Si on proteins expression during the amelioration of abiotic stresses such as osmotic (Liu et al., 2015), salinity (Manivannan et al., 2016), and cadmium (Nwugo and Huerta, 2011), works on the involvement of Si on the amelioration from hyperhydricity was not conducted yet. Hence, to determine the effects of Si during the recovery of hyperhydricity the current report has focused on the physiological, biochemical, and proteomic modifications occurred during the Si-mediated recovery of hyperhydricity in carnation.

MATERIALS AND METHODS

Plant Materials and Culture Condition

Shoots of greenhouse-grown *D. caryophyllus* L. 'Green Beauty' were washed with running tap water for 30 min. The excised nodal explants were soaked in Tween20 (few drops in distilled water) for 10 min and the surfactant were removed using distilled water. Inside the laminar airflow chamber explants were disinfected with 80% (v/v) ethanol for 2 min followed by 2% (v/v) sodium hypochlorite (NaOCl) containing few drops of Tween20 for 10 min. The nodal segments were thoroughly washed with double distilled water (Muneer et al., 2016). Decontaminated nodal explants were cultured on Murashige and Skoog (1962) medium consist of 3.0% sucrose (w/v) supplemented with 1.0 $mg{\cdot}L^{-1}$ of 6-benzyladenine (BA) and 0.5 $mg{\cdot}L^{-1}$ indole-3-acetic acid (IAA) in the temporary immersion system (TIS) (Plantima, A-Tech Bioscientific Company Ltd., Taipei, Taiwan) (350 mL). The immersion frequency was set to 1 min per 90 min (ABL8MEM24012, Schneider Electric, Rueil-Malmaison, France). All the cultures were maintained at 25°C and 80% relative humidity (RH) under a 16 h photoperiod with 50 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$ photosynthetic photon flux density (PPFD) provided by cool white fluorescent lamps (40 W tubes, Philips, The Netherlands).

Silicon Treatments

From our previous experiments, we have found that Si efficiently alleviate abiotic stresses (Soundararajan et al., 2014, 2015; Manivannan et al., 2016) including hyperhydricity (Sivanesan et al., 2011) at 1.8 mM and/or 3.6 mM concentration. Therefore, after 4 weeks, hyperhydric shoots (**Figure 1A**) were cultured on the solid MS medium containing 3.0% (w/v) sucrose and 0.8% agarose with 0, 1.8, and 3.6 mM potassium silicate (K_2SiO_3) as the source of Si. To balance the elements, the excess potassium (K) was deducted from the potassium nitrate (KNO_3) and the loss of nitrate was balanced by the addition of nitric acid (HNO_3). The pH of the medium were adjusted to 5.80 using 1 N NaOH or 1 N HCL before autoclave. Within 4 weeks of planting, shoots were started to dry on the MS medium devoid of Si. Therefore, all the analyses were carried out on 2 weeks old shoots.

Scanning Electron Microscopic (SEM) Analysis of Stomata

Stomatal observations were performed using a scanning electron microscope (SEM) (JSM-6380, JEOL, Tokyo, Japan) operating at 15–25 kV. Briefly, the excised leaves were fixed in 2.5% glutaraldehyde at 4°C for overnight. Staining was done in 1.0% osmium tetroxide solution for 2 h at 4°C. After staining, the samples were dehydrated in graded series of ethanol and final wash with 80% acetone. After fixation and staining, samples were washed with 0.1 M phosphate-buffered saline (PBS, pH 7.0). Finally, the samples were dried and gold coated before the micrograph observation.

Lipid Peroxidation and Estimation of Antioxidant Enzymes

Lipid peroxidation by measuring the content of thiobarbituric acid reactive substances (TBARS) was determined according to Zhu et al. (2004). Samples to analyze antioxidant enzymes activity were prepared according to Soundararajan et al. (2015). Nitro blue tetrazolium (NBT) inhibition method was used to determine the activity of SOD (Giannopolitis and Ries, 1977). The GPX activity was estimated based on the Shah et al. (2001). Cakmak and Marschner (1992) protocol was used to determine the activity of CAT. Enzyme activities were calculated in respective of their protein contents (Bradford, 1976). For the native-polyacrylamide gel electrophoresis (PAGE) analysis, 30 μg of protein from each treatment was mixed with a laemmli buffer (6X) at 5:1 (Laemmli, 1970). Isomers of SOD, GPX, and CAT were observed according to the Shah and Nahakpam (2012).

Determination of Si, Macro- and Micro-Nutrients Content Using Inductively Coupled Plasma Spectrometer

To estimate the content of Si, and macro- and micro-elements, samples dried in an oven at 70°C was finely powdered using a stainless mill (Model 1093, Cytclotec, Tector, Hoganas, Sweden). Samples prepared by ashing at 525°C for 4 h (Model LV 5/11/B180, Naberthern muffle furnace, Lilienthal, Breman,

Germany). The acid digestion was carried out using 20% HCl and the volume was made up to 50 mL using double distilled water. The filtered samples were analyzed using inductively coupled plasma (ICP) spectrometer (Optima 4300 DV/5300 DV, Perkin Elmer, Waltham, MA, USA).

Proteomics

Protein Extraction

The finely powdered lyophilized leaf samples (100 mg) were homogenized with commercial protein extraction kit (Bio-Rad, Hercules, CA, USA) according to Manivannan et al. (2016). The content of protein was quantified by the Bradford assay (Bradford, 1976).

Isoelectric Focusing

For one dimensional (1D) electrophoresis, a 125 μL solubilizing buffer containing 70 μg protein was rehydrated passively for 15 h in 7 cm immobilized pH gradient (IPG) strip (pH 4–7) in the IPGbox (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The focusing was carried out in the Ettan IPGphor 2 isoelectric focusing (IEF) unit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) at 20°C with 50 μA per strip on the following conditions; 300 V for 0:30 (h:min) (Step and Hold), 1000 V for 0:30 (h:min) (Gradient), 5000 V for 1:30 (h:min) (Gradient), and 5000 V for 0.36 (h:min) (Step and Hold). Total time taken until final volt reaches 8.0 KVh was 3.06 (h:min).

Two-Dimensional Electrophoresis

Focused strips were equilibrated in a buffer [8 M urea, 2% SDS, 50 mM Tris-HCl (pH 8.8) 20% (v/v) glycerol] for the reduction with 1.0% DTT and alkylation with 2.5% iodoacetamide for 30 min at RT. Two-dimensional electrophoresis was carried out in 12.5% SDS–PAGE (PROTEAN II, Bio-Rad, Hercules, CA, USA). The proteins were visualized by silver staining and image was taken with a high resolution scanner (Epson, Long Beach, CA, USA).

In-Gel Digestion and MALDI-TOF/MS

By using Progenesis SameSpots 2D software (v. 4.1, Non-linear Dynamics, Newcastle, UK) differentially expressed protein spots (≥1.5-folds) among treatments were identified. The excised spots were cut into small pieces and destained with the potassium ferricyanide (30 mM) and sodium thiosulphate pentahydrate (100 mM) (30 μL) (1:1, v/v) for 30 min at RT. After one time wash with the double distilled H_2O, gels were incubated in ammonium bicarbonate (NH_4HCO_3) (50 mM, v/v) and acetonitrile (ACN) for 15 min at RT, respectively. Followed by the vacuum centrifugation at 20 min, alkylation and reduction were done in 50 mM NH_4HCO_3 containing DTT (10 mM) for 45 min at 56°C and iodoacetamide (55 mM) for 30 min in dark at RT, respectively. After washing with 50 mM NH_4HCO_3, gels were shrunk by the addition of ACN for 15 min at RT and vacuum centrifuge for 20 min. Finally, peptides were digested for 30 min at 37°C using trypsin (5 ng, v/v) (Sigma–Aldrich, St Louis, MO, USA) and the overnight incubation was carried out in NH_4HCO_3 (25 mM, v/v) at 37°C. Aliquots were vacuum centrifuged in new microfuge tubes and the peptides were dissolved in 1–2 μL of a solvent buffer [50% ACN and 0.1% trifluoroacetic acid (TFA)].

In the matrix-assisted laser desorption/ionization time-of-flight/mass spectrometry (MALDI TOF MS)-plate (Applied Biosystems, Franklin Lakes, NJ, USA) in dark along with Angiotensin (Sigma Aldrich, St. Louis, MO, USA) standards for calibration, 1 μL of peptides were mixed with a matrix solution [10 $mg{\cdot}mL^{-1}$ R-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN/0.1% TFA] at 1:1 (v/v). The spots were allowed to dry after wash with the 0.01% TFA and double distilled H_2O. Monoisotopic peaks [10 peaks per spot were selected for peptide mass fingerprint (PMF)] were obtained by the reflection of positive ion mode under the 21 kV voltage between 40 and 3000 Da mass ranges for 100 laser shots.

Functional Identification of Peptides

Mascot software[1] was used to characterize the identified spots. Functions of peptides were identified under the following parameters: SwissProt; enzymes, trypsin; one missed cleavage taxonomy, Viridiplantae (Green Plants); fixed modification, carbamidomethyl cysteine; variable modification; oxidation of methionine; taxonomy, Viridiplantae; peptide mass tolerance of ±50 ppm; and protein mass of 20 KDa. The peaks from the peptide spots producing higher statistically significant ($P < 0.05$) match scores and accounting for the majority of the peaks present in the mass spectra were confirmed as the positively identified proteins. For the gene ontology (GO) annotation AgBase[2] was used. The CIMminer online tool was used to analyze the differential expression of proteins in abundance between the treatments.

Statistical Analysis

All experiments were set up in a completely randomized design. Each treatment was consisted of five explants with five replications per treatment. All assays were performed in three individual biological triplicates. Significant differences among treatments were determined by analysis of variance (ANOVA) followed by the Duncan multiple range test at a 5% probability level by using SAS computer package (SAS Institute Inc., Cary, NC, USA).

RESULTS

Impact of Si on Hyperhydricity Recovery

Recovery percentage of the hyperhydric shoots of carnation grown under the Si non-supplemented condition (control) was lesser on comparison with the Si-supplementation treatments (**Figures 1B,C**). Nevertheless, shoots started to necrotize and dry in the medium devoid of Si from the third week of culture. However, increase in the Si concentration to 3.6 mM decreased the recovery percentage than the 1.8 mM (**Figure 1C**). Higher biomass in the control treatment represents the vitrification of shoots, i.e., excess amount of water in the tissue and the abnormal growth (**Figure 2**).

[1] www.matrixscience.com

[2] http://www.agbase.msstate.edu/

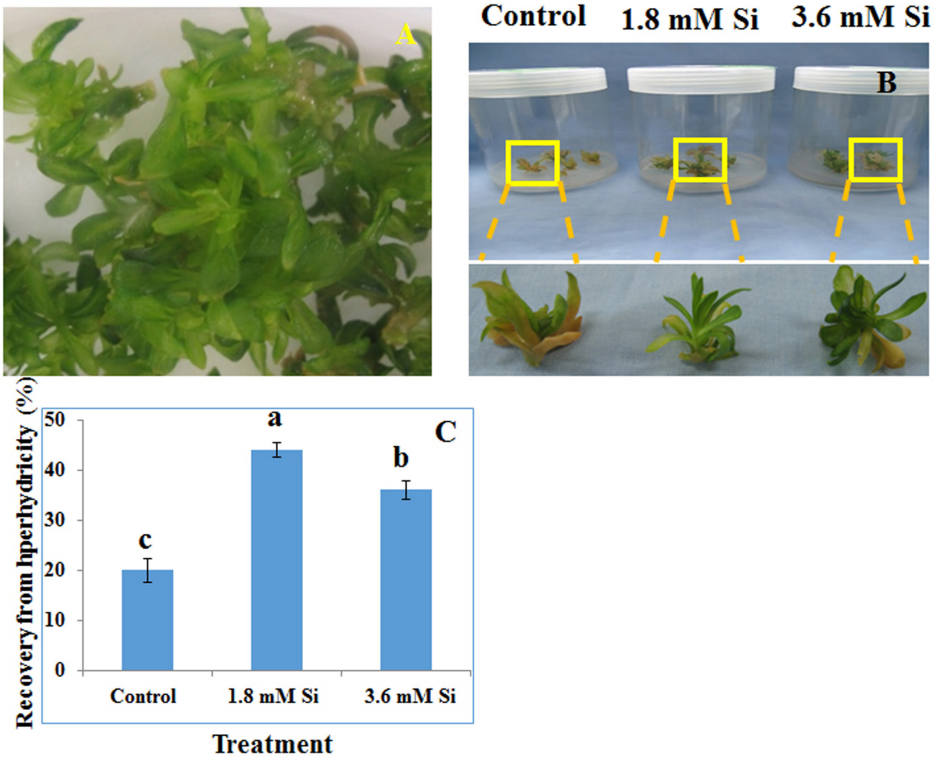

FIGURE 1 | Representative Picture of *Dianthus caryophyllus* L. 'Green Beauty' hyperhydric shoots (A) and its recovery after 15 days of culture in Murashige and Skoog (MS) medium with or without Si supplementation **(B,C)**. **(A)** Induction of hyperhydric shoots in shoot multiplication medium (MS+1.0 mg·L^{-1} BA+0.5 mg·L^{-1} IAA) cultured on temporary immersion system. **(B)** Shoots cultured after 15 days on MS medium with or without Si supplementation. **(C)** Recovery percentage of hyperhydric shoots between the treatments. Different letters indicate significant difference (ANOVA, Duncan, $p \leq 0.05$). Data are the mean ± SD from three replicates.

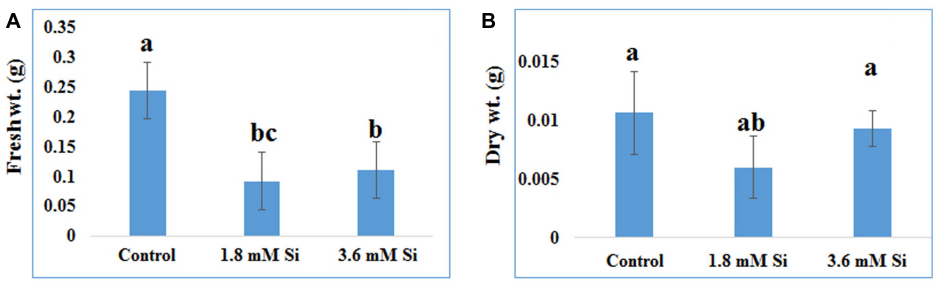

FIGURE 2 | Biomass of recovered hyperhydric shoots cultured on the MS medium with or without Si supplementation. (A) Fresh weight and **(B)** Dry weight. Different letters indicate significant difference (ANOVA, Duncan, $p \leq 0.05$). Data are the mean ± SD from three replicates.

Difference in Stomatal Structure between Control and Si Supplemented Treatments

Deformation of the stomata was observed in the control medium (**Figure 3A**). As anticipated, Si at 1.8 mM showed proper development and compactness of stomata (**Figure 3B**). Nevertheless, density and the formation of stomata were slightly affected in the medium with high concentration of Si (**Figure 3C**).

Lipid Peroxidation and Activities of Antioxidant Enzymes

Decreased LPO content (**Figure 4**) in the Si treatments denoted the reduction of oxidative stress. Expression of SOD in the 1.8 mM Si treatment was greater than the other two treatments. No significance was found between the control and 3.6 mM Si (**Figure 5A**). However, the activity of SOD was lesser in the Si treatments (**Figure 6A**). The increase in the expression and activity of GPX was observed in the control than the Si treatments

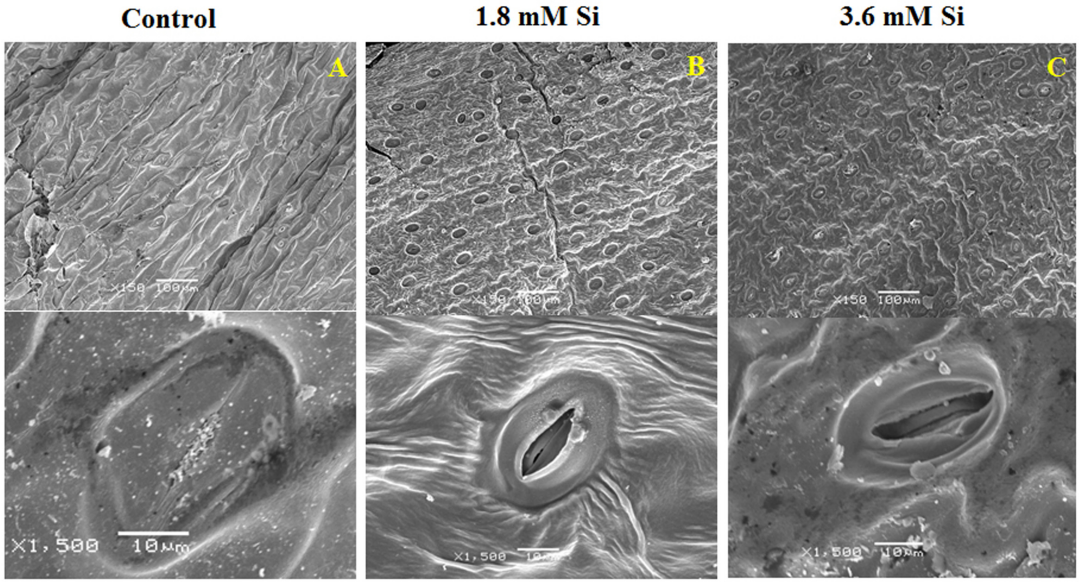

FIGURE 3 | Stomatal development in *D. caryophyllus* L. 'Green Beauty' after 15 days of culture in MS medium with or without Si supplementation. **(A)** Control, **(B)** 1.8 mM Si, and **(C)** 3.6 mM Si.

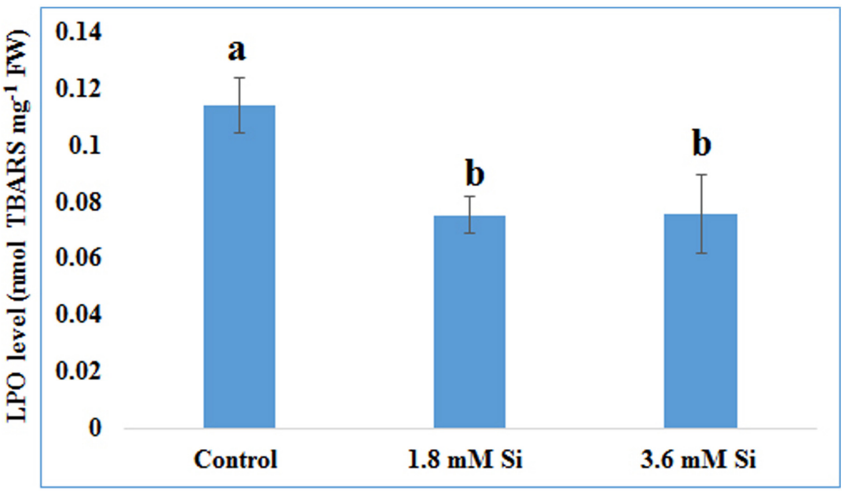

FIGURE 4 | Lipid peroxidation (LPO) of recovered hyperhydric shoots cultured on the MS medium with or without Si supplementation. Different letters indicate significant difference (ANOVA, Duncan, $p \leq 0.05$). Data are the mean ± SD from three replicates. TBARS, thiobarbituric acid reactive substances; FW, fresh weight.

(**Figures 5B**, **6B**). Similarly, on comparison to the control, CAT activity decreased in the Si treatments (**Figure 6C**). However, a native-PAGE analysis of CAT showed no difference in expression among treatments (**Figure 5C**).

Difference in Macro- and Micro-Nutrients Uptake between the Treatments

The delay in the recovery process of the control is correlated with the absence of Si in the medium. The rapid recovery of hyperhydricity was directly proportional to the moderate Si content (6.9 ppm). In contrast, high level uptake of Si (14.3 ppm in 3.6 mM treatment) interrupted the recovery process from hyperhydricity (**Figure 7**). Uptake of K and sodium (Na) were increased to 511.1 and 22.1 ppm, respectively, at the 1.8 mM Si treatment. Nonetheless, availability of K and Na was decreased to 446.5 and 15.29 ppm, respectively, at 3.6 mM. The lower content of calcium (Ca) (57.1 ppm at Si 1.8 mM and 52.6 ppm at Si 3.6 mM) and magnesium (Mg) (19.7 ppm at Si 1.8 mM and 19.6 ppm at Si 3.6 mM) was observed in the Si treatments. Though the sulfur (S) uptake was lower in the Si 1.8 mM

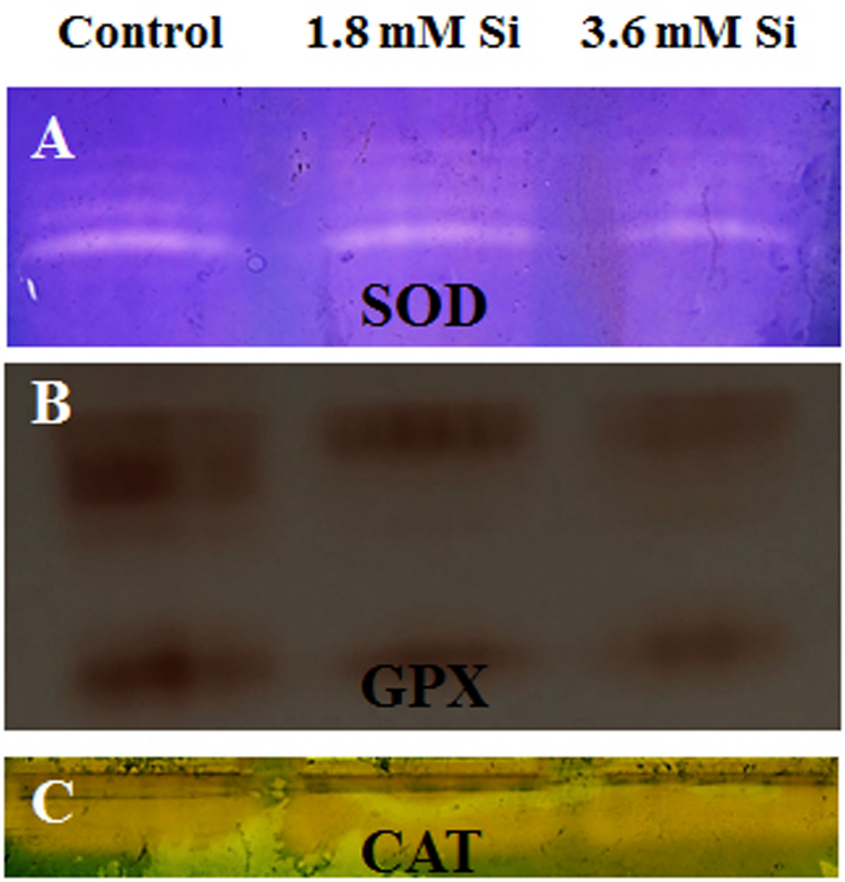

FIGURE 5 | Native-PAGE analysis of antioxidant enzymes during recovery from hyperhydricity with or without Si supplementation. (A) Superoxide dismutase (SOD), **(B)** guaiacol peroxidase (GPX), and **(C)** catalase (CAT).

treatment than the control, phosphorous (P) availability was slightly improved in the former. In contrast, uptake of both S (32.4 ppm) and P (30.6 ppm) was affected by the presence of Si at higher concentration in the medium (**Table 1**). Among micronutrients, except Na (22.1 ppm) and Cu (0.25 ppm), not much significance was observed between control and 1.8 mM Si. Considerable improvement on the Fe uptake (4.26 ppm) was found in 3.6 mM Si treatment, meanwhile Zn content (1.11 ppm) was deeply affected (**Table 2**). Application of Si at both concentrations decreased the Mn, B, and Mo uptake than the control.

Functional Annotation of Differentially Accumulated Proteins in Response to Si Treatments

Totally 120 reproducible spots were detected between the treatments (**Figure 8**). Among them 30 spots significantly showed more than 1.5-fold differences between the treatments were identified using MALDI-TOF MS. Annotated proteins were classified into six groups according to their biological roles such as ribosomal binding, oxido-reduction, hormone/cell signaling, metal/ion binding, defense, and photosynthesis (**Table 3** and **Figure 9A**). Proteomic analysis showed that Si up-regulated 17 and 10 proteins in abundance in the 1.8 and 3.6 mM Si treatment, respectively. On the other hand, among the identified 30 peptides, Si treatment down-regulated 9 spots in 1.8 mM Si and 18 spots in 3.6 mM Si treatments in abundance as compared to the control. In comparison with the 3.6 mM, 19 proteins were up-regulated and 7 proteins were down-regulated in the 1.8 mM (**Figures 9B,C**). Average spot volume of the differentially expressed proteins identified between the treatments were mentioned in the **Figure 10**.

Proteins Involved in Ribosomal Binding

Proteins responsible for the RNA processing during the translation were highly induced in abundance (spots 1, 9, 10, 14, 15, 20, and 22) in the 1.8 mM Si treatment than the control. Despite, Si at a high concentration decreased the up-regulation of proteins in spots 1, 10, and 22.

Proteins Involved in Oxido-Reduction Process

Proteins enhanced during higher oxido-reduction period such as cytochrome b (spot 13), cytochrome b-c1 complex subunit

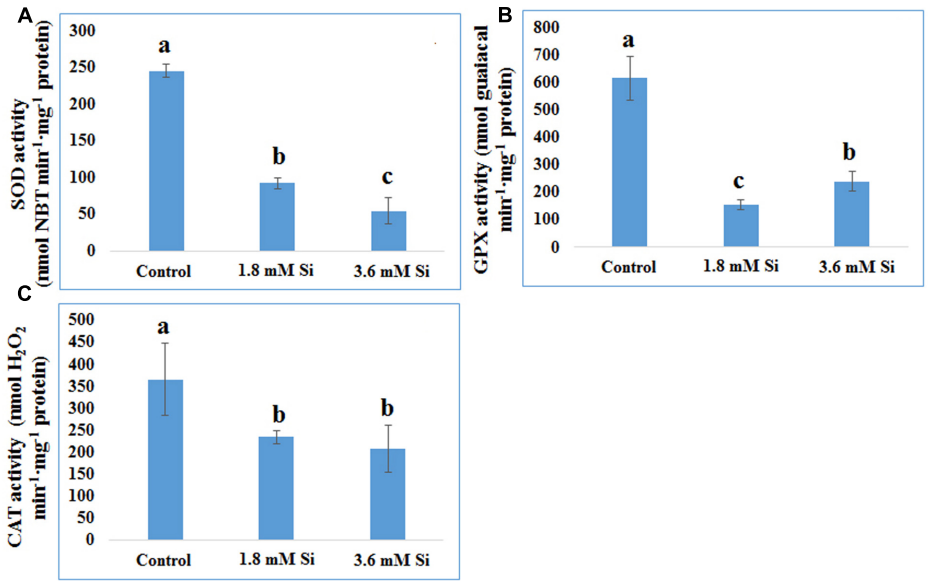

FIGURE 6 | Activity of antioxidant enzymes during recovery from hyperhydricity with or without Si supplementation. (A) SOD, **(B)** GPX, and **(C)** CAT. Different letters indicate significant difference (ANOVA, Duncan, $p \leq 0.05$). Data are the mean ± SD from three replicates.

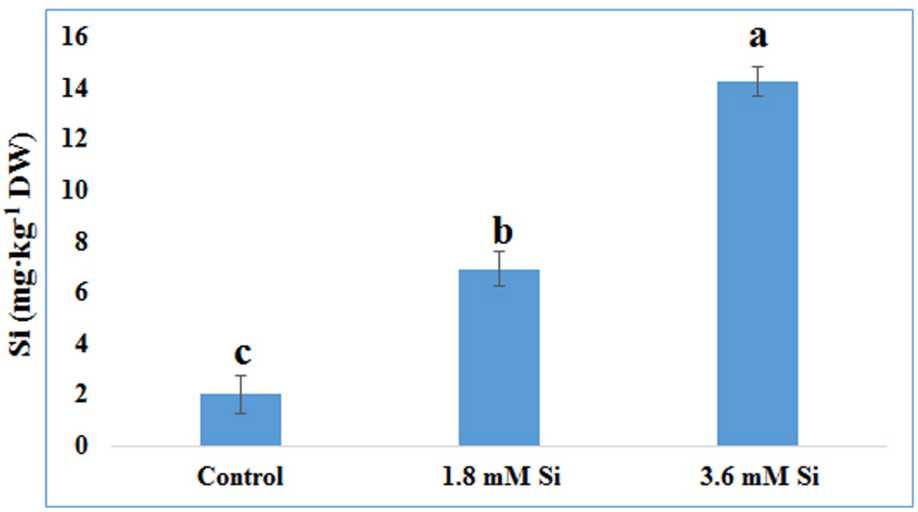

FIGURE 7 | Uptake of Si after 15 days of hyperhydric shoots cultured on the MS medium with or without Si supplementation. Different letters indicate significant difference (ANOVA, Duncan, $p \leq 0.05$). Data are the mean ± SD from three replicates.

8 (spot 29), and thylakoid luminal 11 kDa (spot 23) were down-regulated in abundance on Si treatments. Subsequently, cytochrome c oxidase subunit 5C-3 (spots 8 and 17) was up-regulated in the 1.8 mM Si treatment than the control.

Proteins Involved in Hormone/Cell Signaling

Abundance of alpha-amylase 2 (spot 11) increase in both 1.8 and 3.6 mM Si treatments. The down-regulation of protein domain involved in the metabolism of ABA (spots 16 and 30) was observed in the Si treatments. Decrease in abundance of ethylene receptor protein (spot 19) was observed between control and Si treatments.

Proteins Involved in Metal/Ion Binding Process

Metallothionein-like protein 4A (spot 3) and rhodanese-like domain containing protein 19 (spot 4) were down-regulated in abundance in the Si supplemented MS medium. In contrast, no significant difference was observed between control and 1.8 mM Si treatment in protein asymmetric leaves1/2 enhancer7 (AE7)-like 2 OS protein (spots 6 and 18). Despite, the increase in

Si concentration decreased the expression of protein AE7-like 2 OS (spots 6 and 18) in abundance.

Proteins Involved in Defense Mechanism

All the identified protein spots related to defense mechanism, such as protein Rapid ALkalinization Factor (RALF)-like 9 OS (spot 2), interactor of constitutive active ROPs 5 (spot 5), and defensin-like protein 308 OS (spot 27), were up-regulated in both Si treatments than the control.

Proteins Involved in Photosynthesis

Supplementation of Si up-regulated the ribulose bisphosphate carboxylase small chain (spot 25), ribulose bisphosphate carboxylase large chain (spot 26), and photosystem I assembly protein Ycf4 (spot 28), critical proteins required for photosynthesis. Compared with the 1.8 mM Si, expression of photosynthesis related proteins except PS I-related protein (spot 28) was slightly lesser in 3.6 mM Si treatment.

DISCUSSION

Physiological Improvement Due to Si Supplementation during the Hyperhydricity Recovery Process

Although TIS was considered as a perspective technology to propagate the plants, hyperhydricity in carnation is undesirable during the shoot multiplication process (**Figure 1A**). Majority of loss to the industries occur during the acclimatization process of hyperhydric shoots (Etienne and Berthouly, 2002). Supplementation of Si improved the recovery from hyperhydricity of *D. caryophyllus* L. 'Green Beauty'. Previously, Sivanesan et al. (2011) reported that supplementation of Si along with the shoot multiplication medium reduced the occurrence of hyperhydricity in cotoneaster. Recovery of hyperhydric shoots was higher at 1.8 mM Si treatment than the 3.6 mM. Despite, compared to the control significant improvement in the recovery was observed in high concentration Si treatment (**Figures 1B,C**). Retardation of *in vitro* growth at 3.6 mM supplementation of Si was observed in *D. caryophyllus* L. 'Tula' (Soundararajan et al., 2015). Decrease in growth due to the higher Si content was previously reported in several plants such as *Oryza sativa* (Agarie et al., 1992), *Vigna unguiculata* (Mali and Aery, 2008), and *D. caryophyllus* L. (Soundararajan et al., 2015). According to Yeo et al. (1999) higher deposition of Si cause the cell hardening at the earlier stage of cell elongation. Lu and Neumann (1999) reported that polymerization of Si affects the cell wall extension and lead to the cell rigidity. Deterioration in shoot growth was associated with the malformation of stomata in control (**Figure 3A**). Similar result was observed in hyperhydric shoots of *D. caryophyllus* L. 'Dominga' (Olmos and Hellín, 1998). Though it is not analyzed in this study, the random pattern of Si deposition in/around trichome (Montpetit et al., 2012), stomata (Zuccarini, 2008), and cell wall (Agarie et al., 1998) could resulted in the lesser recovery percentage during higher accumulation of Si (**Figure 7**). Especially, reduction in the growth and necrotic leaves were observed due to the improper regulation on the Si accumulation in *TaLsi1* transformed Arabidopsis (Montpetit et al., 2012). Therefore, delay in the recovery at the higher deposition of Si (∼14 ppm) in carnation could be due to excessive silicification.

According to Werker and Leshem (1987) the lack of recovery from hyperhydricity is linked with the abnormal stomata. Flooding of water in between the intercellular air spaces and substomatal chamber causes the stomata closure (Sibbernsen and Mott, 2010) and interrupt the mesophyll signals (Mott et al., 2008). Previously, Allen et al. (2001) mentioned that the hypertrophic state inhibit the stomatal function. Stomatal closure concurrently brings the water lodging in the apoplast. Supplemented Si could have mediated the signals from mesophyll to guard cells for releasing the blockage in the vapor-phase opening (**Figures 3B,C**). The improper gas exchange process due to deformed guard cells affects the hydrolysis or utilization of water and leads to the osmotic and oxidative stress in control. Nevertheless, shoots failed to survive in the medium devoid of Si by the fourth week of culture (Date not shown). Inadequacy of functional stomata along with hypertrophic mesophyll and protruded cell wall layer cause the death of hyperhydric plants (Fontes et al., 1999). Therefore, abnormal stomata could affect the entire physiology of the plant. Hence the facilitation of stomatal function in the Si treatment could be the prime mechanism behind the accelerated recovery from hyperhydricity in carnation.

TABLE 1 | Concentration of macro-nutrients after 15 days of treatment with different concentrations of Si.

Element (ppm)	Control	Si 1.8 (mM)	Si 3.6 (mM)
K	477.4 ± 7.17	511.1 ± 4.83	446.5 ± 5.00
Ca	65.5 ± 1.00	57.1 ± 0.51	52.6 ± 0.51
Mg	22.3 ± 0.27	19.7 ± 0.19	19.6 ± 0.17
S	40.7 ± 0.36	39.0 ± 0.22	32.4 ± 0.25
P	47.9 ± 0.21	48.4 ± 0.13	30.6 ± 0.36

Data are the mean ± SD from three replicates.

TABLE 2 | Concentration of micro-nutrients after 15 days of treatment with different concentrations of Si.

Element (ppm)	Control	Si 1.8 (mM)	Si 3.6 (mM)
Na	19.40 ± 0.197	22.1 ± 0.260	15.29 ± 0.167
Cu	0.18 ± 0.002	0.25 ± 0.002	0.19 ± 0.002
Zn	1.50 ± 0.003	1.48 ± 0.007	1.11 ± 0.012
Mn	1.65 ± 0.008	1.53 ± 0.006	1.51 ± 0.015
Fe	3.77 ± 0.024	3.77 ± 0.022	4.26 ± 0.044
B	0.66 ± 0.051	0.58 ± 0.002	0.59 ± 0.008
Mo	0.07 ± 0.001	0.06 ± 0.001	0.06 ± 0.005

Data are the mean ± SD from three replicates.

Regulation of Antioxidant Enzymes by Si to Overcome the Oxidative Stress

Impairment in the photosynthetic process and hypoxia condition interfere with the electron transportation during respiration

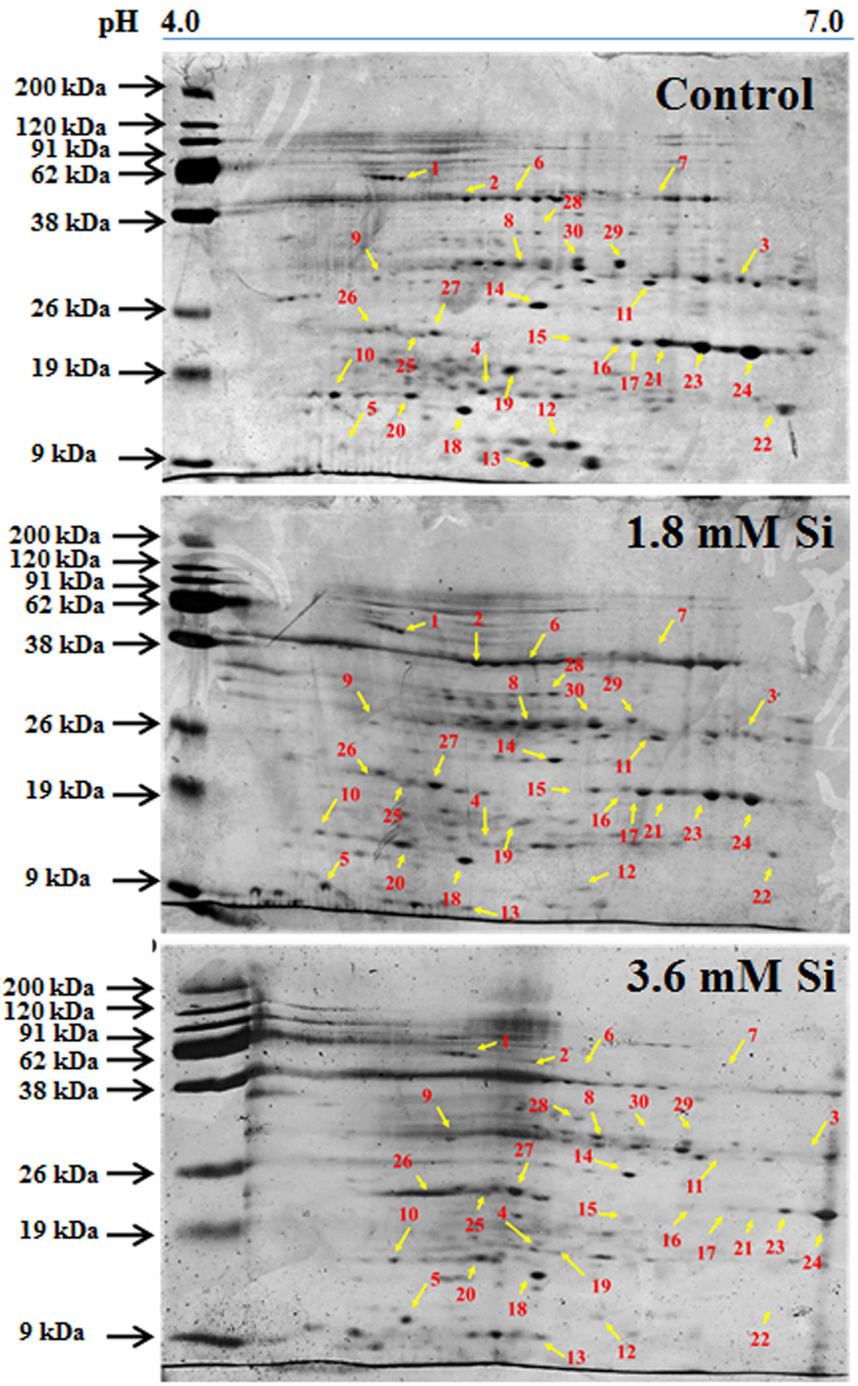

FIGURE 8 | Representative images of two-dimensional gel electrophoresis (2-DE) gel of proteins extracted after 15 days of hyperhydric shoots cultured on the MS medium with or without Si supplementation. Differentially expressed protein spots were analyzed by the Progenesis SameSpot. Proteins with more than 1.5-fold difference among treatments identified by MALDI-TOF were listed in **Table 3**.

and leads to the excessive accumulation of ROS such as O_2^-, H_2O_2, and –OH. Oxidative stress measured with the LPO level (polyunsaturated fatty acids to form conjugated hydroperoxides) (**Figure 4**) correlated with the increased activities of antioxidant enzymes such as SOD, GPX, and CAT in control (**Figure 6**). This denotes the disproportionate presence of ROS in the carnation hyperhydric shoots cultured on Si-devoid treatment. Previously, Muneer et al. (2016) mentioned that activities of antioxidant enzymes were higher under the hyperhydric condition due to the oxidative stress increase in LPO suggested

the accumulation of H_2O_2 and able to produce –OH via Haber–Weiss reaction (Apel and Hirt, 2004). Though antioxidant enzymes activities are substantially known for the reduction in the O_2^- and H_2O_2 level in plants, relationship between the activity of antioxidant enzymes and tolerance was still a matter of controversy. This paradox has been raised probably due to the fact that higher activity of antioxidants correlate with both the enormous presence of ROS groups as well as detoxification of excessively accumulated free radicals (Abogadallah, 2010). For instance, SOD activity was increased under salt stress in *D. caryophyllus* L. (Soundararajan et al., 2015), decreased under prolonged exposure of drought stress in *Agrostis* spp. (DaCosta and Huang, 2007), and no effect was found in SOD under drought stress in *Poa pratensis* (Zhang and Schmidt, 1999). The variation in the expression and activity of SOD (**Figures 5A**, **6A**) could be due to presence of different metal prosthetic group isoforms of SOD and their localization such as Cu/Zn-SOD (cytosol and chloroplasts), Mn-SOD (mitochondria and peroxisomes), and Fe-SOD (chloroplasts). Cavalcanti et al. (2004) reported that peroxidase enzyme was up-regulated under the salinity stress whereas CAT and SOD are either decreased or unchanged, respectively. Activities of antioxidant are mainly involved to

TABLE 3 | Differentially expressed protein spots identified from the two-dimensional gel electrophoresis of *Dianthus caryophyllus* L. 'Green Beauty' shoots during recovery from hyperhydricity in Murashige and Skoog (MS) medium with or without Si supplementation.

Spot no.[a]	Accession number	Nominal mass (M_r)[b]	Theoretical/Exp. *pI*[c]	Protein identification	Species	SC(%)[d]	Score[e]	Fold[f]
Ribosomal binding								
1	Q9B1K8	10809	2.8/11.10	50S ribosomal protein L23	*Lotus japonicus*	39	28	3.0
9	Q06R73	11100	2.5/11.02	30S ribosomal protein S15	*Jasminum nudiflorum*	31	22	2.9
10	Q14FC1	13613	2.05/9.35	50S ribosomal protein L14	*Populus alba*	37	29	4.6
14	Q14FC1	13613	4.1/9.35	50S ribosomal protein L14	*Populus alba*	38	32	3.3
15	B0Z5A0	10376	4.6/10.73	30S ribosomal protein S15	*Oenothera glazioviana*	34	22	2.2
12	Q5XET6	54109	6.9/9.48	RNA pseudouridine synthase 3	*Arabidopsis thaliana*	10	17	4.7
20	Q06R73	11100	3.9/11.02	30S ribosomal protein S15	*Jasminum nudiflorum*	31	23	1.6
22	Q9B1K8	10809	6.8/11.10	50S ribosomal protein L23	*Lotus japonicus*	33	32	2.7
Oxido-reduction								
13	O04354	14604	4.2/5.03	Cytochrome b5	*Borago officinalis*	27	29	2.4
8	Q9FLK2	7088	4.0/8.18	Probable cytochrome c oxidase subunit 5C-3	*Arabidopsis thaliana*	26	12	2.9
17	Q9FLK2	7088	5.2/8.18	Probable cytochrome c oxidase subunit 5C-3	*Arabidopsis thaliana*	33	21	2.5
29	P81247	2336	5.2/9.70	Cytochrome b-c1 complex subunit 8	*Equisetum arvense*	70	13	3.7
23	P82657	2270	6.0/8.48	Thylakoid lumenal 11 kDa	*Spinacia oleracea*	81	26	2.9
Hormone/cell signaling								
7	P80843	1236	5.5/9.98	68 kDa cell wall protein	*Arabidopsis thaliana*	45	15	6.3
11	P86088	1006	4.4/5.58	Alpha-amylase 2	*Capsicum chinense*	87	16	3.0
16	Q9C8Y2	20039	5.1/5.67	Protein C2-DOMAIN ABA-RELATED 2 OS	*Arabidopsis thaliana*	26	38	2.0
19	Q9ZWL6	83315	4.0/7.05	Ethylene receptor	*Passiflora edulis*	31	21	1.8
30	Q9C8Y2	20039	4.6/5.67	Protein C2-DOMAIN ABA-RELATED 2 OS	*Arabidopsis thaliana*	61	71	2.0
Metal/Ion-binding								
3	Q0IMG5	8379	6.5/5.62	Metallothionein-like protein 4A OS	*Oryza sativa*	58	37	2.4
4	Q8RUD6	19312	3.6/6.30	Rhodanese-like domain-containing protein 19	*Arabidopsis thaliana*	25	25	2.2
6	Q9SR25	17150	4.0/6.30	Protein AE7-like 2 OS	*Arabidopsis thaliana*	20	19	2.1
18	Q9SR25	17150	3.4/6.30	Protein AE7-like 2 OS	*Arabidopsis thaliana*	20	19	2.1
21	Q0IMG5	8379	5.6/5.62	Metallothionein-like protein 4A OS	*Oryza sativa*	47	34	3.7
Defense								
2	Q3ECL0	8482	3.5/9.55	Protein RALF-like 9 OS	*Arabidopsis thaliana*	18	20	1.9
5	Q8VYU8	45709	1.9/5.67	Interactor of constitutive active ROPs 5	*Arabidopsis thaliana*	11	18	1.8
27	Q4VP04	9159	3.2/8.53	Defensin-like protein 308 OS	*Arabidopsis thaliana*	45	24	1.6
Photosynthesis								
25	P26985	20439	7.57/0.95	Ribulose bisphosphate carboxylase small chain	*Batophora oerstedii*	53	28	3.3
26	P27063	1647	5.5/9.72	Ribulose bisphosphate carboxylase large chain	*Capsicum annuum*	62	14	2.1
28	A7M975	21497	9.27/4.10	Photosystem I assembly protein Ycf4	*Cuscuta reflexa*	28	36	1.5
Putative								
24	Q9LRQ1	9603	6.6/6.11	Putative BTB/POZ domain-containing protein	*Arabidopsis thaliana*	34	20	1.7

[a]*The spot nos. correspond to the numbers given in protein gel images (**Figure 8**).* [b]*Theoretical molecular mass (Mr) calculated from MASCOT Peptide Mass Fingerprint.* [c]*Isoelectric point (pI) of spots identified from MASCOT Peptide Mass Fingerprint and protein gel images (**Figure 8**).* [d]*Sequence coverage percentage.* [e]*MASCOT score of protein hit.* [f]*Fold change between the treatments calculated from the Progenesis SameSpots 2D software v4.1 (Non-linear Dynamics, Newcastle, UK).*

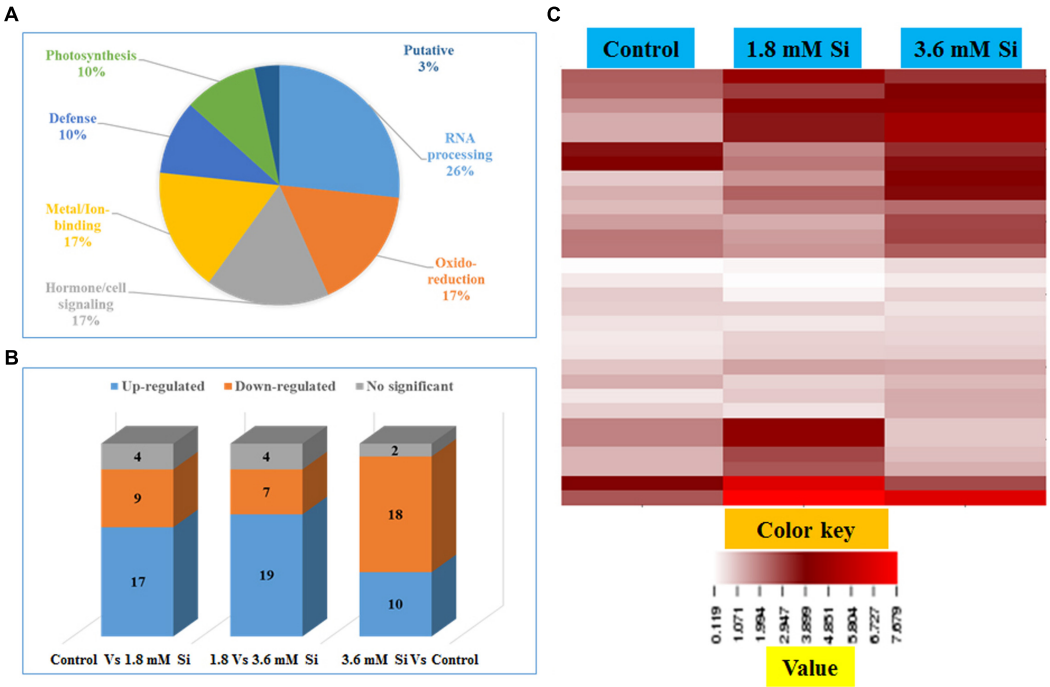

FIGURE 9 | Characterization of differentially expressed proteins in abundance between control and Si treatments. (A) Functional classification of identified proteins by Gene Ontology analysis. **(B)** Bars with varying colors represent the up-(↑), down (↓), or non-significantly (↔) accumulated proteins in recovered hyperhydric shoots with or without Si supplementation. **(C)** Protein expression in abundance illustrated as heat map between the treatments.

maintain the ROS under controlled level (Abogadallah, 2010). In *A. sativum* L. higher concentration of H_2O_2 aggravate the hyperhydricity whereas at the lower concentration it acts as a signaling cascade to alleviate the hyperhydricity (Liu et al., 2017). Decrease in the activities of the antioxidant enzymes found on Si supplemented treatments (**Figure 6**) can be taken as controlled level of ROS maintained in the cells.

Influence of Si on Macro- and Micro-Nutrients Content for Hyperhydricity Recovery

Presence of Si in the medium could affect both the availability and solubility of other elements present in the medium. Macronutrients are important constituents for the building blocks of plant. The improvement of K (**Table 1**) and Na (**Table 2**) uptake at 1.8 mM shows the involvement of Si in the osmoregulatory process. In addition, K also functions in the movement of guard cells for the stomata opening and closure (Siegel et al., 2009). Lesser uptake of K at 3.6 mM Si than 1.8 mM could obstruct the re-adjustment rate. Elevation of Ca content (**Table 1**) in the control is directly correlated with the stomatal deformation (**Figure 3A**) and vice versa on the Si treatments (**Figures 3B,C**). Moreover, the antagonism between the positively charged ions of K at higher level suppressed the Ca level in Si treatments (**Table 1** and **Figure 3**) could have rectified stomatal closure (Siegel et al., 2009; Franz et al., 2011). Addition of ABA elevate the cytosolic Ca^{2+}-level in the guard cell induce the stomatal closure by the activation of Ca^{2+}-permeable channels in the plasma membrane of guard cells (Siegel et al., 2009; Zou et al., 2010). According to the Andrews et al. (1999) under low Mg with high K, shoot growth ratio were increased prior to the root. This condition allows the accumulation of carbohydrates for the shoot development rather than the root induction and/or its development (Niu et al., 2014). Contrary to this, our previous report states that exogenous supplementation of Si to the normal shoot induced the rooting under salt stress in *in vitro* condition (Soundararajan et al., 2015). Photosystem II activity was increased under the S deficiency (Baszynski et al., 1972). Though the interaction between silicic acid and phosphate in anionic form is unlikely to occur, difference in the P level between the two Si treatments probably caused due to change in rate of P-translocation (Ma et al., 2001).

Micronutrients are indispensable components of enzymes and also act as secondary messengers. Recovery from the hyperhydricity mediated by Si are both from the concomitant improvement and restriction in the uptake of micro-nutrients (**Table 2**). Franz et al. (2011) reported that the highest Cu treatment of Zinnia with Si supplementation plants are grown healthier. Silicate ameliorated the excessively presented Zn in the cytoplasm by co-transportation into the extracellular compartments (Neumann and zur Nieden, 2001). Si application increased binding of Mn to cell wall and enhanced distribution prevents Mn to reach its toxic level in *V. unguiculata* (Iwasaki et al., 2002). Modification of cation binding properties of the cell wall in Si treatments lead to the Mn reduction

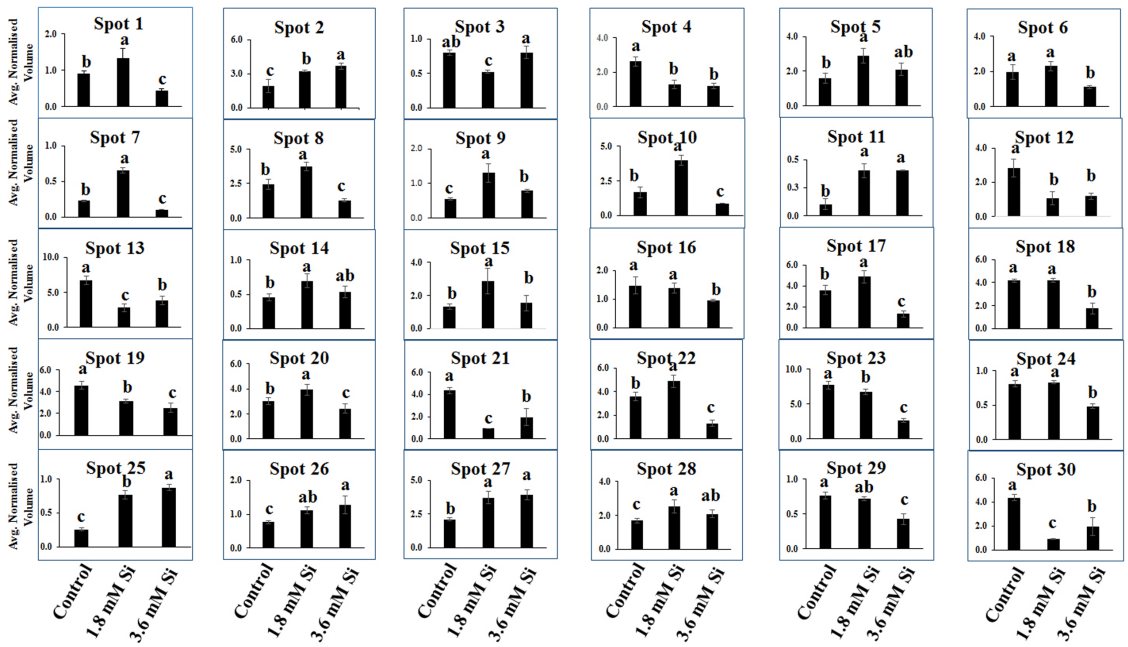

FIGURE 10 | Abundance of differentially expressed proteins of *D. caryophyllus* L. 'Green Beauty' during the recovery from hyperhydricity with or without Si supplementation. Three independent experiments were performed and the mean ± SD were plotted. Different letters indicate average spot volume between the treatments are statistically different at $p \leq 0.05$.

(Rogalla and Römheld, 2002). Boron-silicate (B-Si) complex formation in the soil decreased B uptake in *Spinacia oleracea* L. (Gunes et al., 2007a). Since the pros and cons of organic elements are determined by either lower or excess availability of one or more ions, the varying results obtained in the macro- and micro-element contents could be due to the hyperhydric nature of shoots as well as Si treatments.

Possible Proteomic Regulatory Network of Si for the Adaptation of Hyperhydric Shoots

To plot the critical view point of hyperhydric shoots adaptation in the Si supplemental medium, proteomics analysis was carried out. Functionally categorized peptides details were shown in **Table 3**. Abundance of proteins involved in various metabolisms and their network signals the response of plants to the external stimuli. More than one spots matching to the same protein/peptide could be due to isoforms, subunits, maturation, degradation, and/or post-translational modification of proteins (Nwugo and Huerta, 2011).

Ribosomal binding proteins are depressed under Si devoid condition. Ribosomal proteins plays an important role in the flow of biological information (Bachellerie et al., 2000). Cellular component of the identified 50S and 30S ribosomal proteins (**Table 3**) is chloroplast. Maximum number of proteins in the chloroplast are involved in the photosynthetic electron-transport complexes and ATPase/NADPH complexes. Reduction in the accumulation of chloroplastic ribosomal proteins are directly related with the lesser turnover or abundance of ribulose 1,5-bisphosphate carboxylase (RuBisCo) (spots 25 and 26), which interrupt the physiological process (Pospisilova et al., 1992). Pseudouridine (5-ribosyluracil) is the modified RNA, i.e., polynucleotide chain found in *anti*-configuration. It function as the conformational switch during the low energy requirement for the *syn*/*anti* transition in RNA (Charette and Gray, 2000). The abundance of ribosomal proteins could bring the remarkable changes in the transition of hyperhydric to normal shoots. However, takeoff of this process is delayed during the initial period of 3.6 mM Si treatment (**Figure 10**).

Cytochrome b was proposed as the main producer of O_2^- radicals in the peroxisomes (López-Huertas et al., 2000). Peroxisomal membrane polypeptides uses NADH as an electron donor for O_2^- generation and able to reduce the cytochrome c (del Río et al., 2002). Upregulation of thylakoid protein indicates the higher phosphorylation of light harvest complex II (Jensen et al., 2007) and excessive presence of hydroperoxides (Peltier et al., 2002). This occurred during the excited state transitions induced by the binding of reduced plastoquinone products with cytochrome b_6/f (López-Huertas et al., 2000). Therefore, decreased abundance of cytochrome b complex (spots 13 and 29) and thylakoid protein (spot 29) up-regulated the cytochrome c (spots 8 and 17) (**Table 3** and **Figure 10**) shows the maintenance of equilibrium on ROS generation in Si treatments.

The increase in the abundance of 68 kDa cell wall protein in the 1.8 mM Si treatment depict the accustomed progress in cell development (Irshad et al., 2008) of Si treatments (**Table 3** and **Figure 10**). Meanwhile, lesser expression of cell wall protein acquainted the restriction in the plasticity and cell elongation in control and 3.6 mM Si treatment. Synergistic repression

of ABA signaled by alpha-amylase 2 promoter was found in rice (Xie et al., 2006). Similarly, higher abundance of alpha-amylase (spot 11) and the restraint of ABA-related proteins identified in this study. Augmentation of ABA maximizes the ability of Ca^{2+} to down-regulate the inward K^+ by activating the S-type anion channels (Siegel et al., 2009). Allen et al. (2001) defined that Ca^{2+} program the long-term inhibition of stomata opening. Regulation of stomatal movement by the inward K^+ current could prevent the stomata closure in the Si treatment (**Figures 3B,C**) was correlated with the inhibition of ABA and Ca^{2+}-mediated signaling (**Figure 9C**). Attenuation in the stomatal and excessive wateriness of shoots on control was also corresponds with the higher abundance of ethylene receptor. Increased ROS production activates the ACC synthase, a precursor for ethylene (Kim et al., 2008). Abscission of leaves and shoot death observed in the control can be correlated with the ethylene receptor (spot 19) abundance in the control. Higher H_2O_2 production by the overexpression of Chloroplast Cu/ZnSOD could trigger the ethylene production (Kim et al., 2008). Nevertheless, proper regulation of plant hormone-, ion-, and ROS-signaling mediated by Si could attenuate the abscission rate.

Increase in the metallothionein-like protein in control and 3.6 mM Si treatment indicates the requirement of metal homeostasis. Higher abundance of metallothionein instigate the requirement of metalloenzymes to scavenge the excessively accumulated ROS groups (Zhou et al., 2006). Previously, Okumura et al. (1991) reported that Cu treatment decreased the expression of metallothionein in mRNA level in barely. Similarly, higher Cu content (**Table 2**) observed in the 1.8 mM of Si showed lesser abundance of metallothionein-like protein (spot 3) in carnation. Protein AE7 (spots 6 and 18) participates in the cytosolic Fe-S cluster assembly for the maintenance of genome integrity (Luo et al., 2012). No significance between the control and Si 1.8 mM treatment in Fe and S is correlated with the expression of protein AE7. However, this process could be insubstantial (**Table 2**) in the higher Si supplemented treatment (**Figure 7**). Meanwhile, the higher abundance of rhodanese-like domain containing protein 19 (spot 4) could lead to the formation of sulfite and thiocyanate (Papenbrock et al., 2011). Notably, rhodanese protein upregulation in the control (**Table 3** and **Figure 10**) specifies the oxidization of sulfite in non-enzymatic manner by H_2O_2 (Hänsch et al., 2007). Receptor mediated polypeptide signals (RALF protein, spot 2) regulate the defensive and developmental process on Si treated shoots (Pearce et al., 2001). Similarly, increased in the abundance of interactor of constitutive active ROPs 5 instigate overall physiological development as it is involved in the diverse signaling cascades includes growth and polarity establishment of cell, morphogenesis, cytoskeleton organization, hormone signaling, and other vital cellular processes (Zheng and Yang, 2000). Induced expression of defense-related proteins is an integral part enabling the plant to cope-up instantaneously against the stress.

Reduction in the photosynthesis-related protein in control signifies the poor photosynthetic capacity. RuBisCo plays an important role in the assimilation of CO_2 and conversion of starch (Raines, 2011). Similarly, photosystem complex are vital for light harvesting and the photoprotection (Kozaki and Takeba, 1996). Pospisilova et al. (1992) reported that abnormal development of photosynthetic apparatus is the main reason for the low photosynthetic efficiency. In the Si treatment significant correlation was observed between the improvement in the expression of photosynthesis related proteins such as RuBisCo large (spot 25) and small (spot 26) subunit and photosystem I assembly protein Ycf4 (spot 28) and stomatal developmental (**Figures 3B,C**) (Raines, 2011). Decrease in the respiratory activity in hyperhydric shoots could reflect the lesser reduced and oxidized pyridine nucleotides. Production of NADPH and $NADP^+$ might affected in the control and this process was precisely retrieved in 1.8 mM Si treatment. Therefore, the dignified improvement on the convalescence of hyperhydric shoots in Si treatments depends on the enhanced expression of photosynthetic proteins in abundance and movement of stomata.

CONCLUSIONS

The current endeavor demonstrate various factors related to the amelioration of hyperhydricity with the supplementation of Si in *D. caryophyllus* L. 'Green Beauty'. Facilitation of stomatal opening assisted by the Si supplementation could be the decisive factor for the upregulation of photosynthesis-related proteins. Accelerated re-establishment from the hyperhydricity and progressive physiological development on the Si treatments in *D. caryophyllus* L. was aid by the regulation of nutrient uptake. Minimal LPO content and normal activities of antioxidant enzymes illustrate the equilibrium in redox homeostatic process. Lack of cell elongation and plasticity along with the reduction in the uptake of K under higher concentration of Si could contempt the rapid recovery from hyperhydricity. Active involvement of Si in the regulation and signaling process of proteins in different metabolisms prevents the aggravation of hyperhydricity. From this study, it can be concluded that Si at 1.8 mM could be the optimal concentration to trigger the reclamation process and favorable conditions for the survival of hyperhydric carnation shoots.

AUTHOR CONTRIBUTIONS

PS, AM, and BRJ conceived and designed the experiments; PS and AM conducted the experiment, collected, and analyzed the data; PS wrote the draft of the manuscript; YSC assisted in conducting the experiment, sample collection, and all the analysis; AM and BRJ proofread and finalized the manuscript.

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REVIEW
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Silicon Regulates Potential Genes Involved in Major Physiological Processes in Plants to Combat Stress

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Silicon (Si), the quasi-essential element occurs as the second most abundant element in the earth's crust. Biological importance of Si in plant kingdom has become inevitable particularly under stressed environment. In general, plants are classified as high, medium, and low silicon accumulators based on the ability of roots to absorb Si. The uptake of Si directly influence the positive effects attributed to the plant but Si supplementation proves to mitigate stress and recover plant growth even in low accumulating plants like tomato. The application of Si in soil as well as soil-less cultivation systems have resulted in the enhancement of quantitative and qualitative traits of plants even under stressed environment. Silicon possesses several mechanisms to regulate the physiological, biochemical, and antioxidant metabolism in plants to combat abiotic and biotic stresses. Nevertheless, very few reports are available on the aspect of Si-mediated molecular regulation of genes with potential role in stress tolerance. The recent advancements in the era of genomics and transcriptomics have opened an avenue for the determination of molecular rationale associated with the Si amendment to the stress alleviation in plants. Therefore, the present endeavor has attempted to describe the recent discoveries related to the regulation of vital genes involved in photosynthesis, transcription regulation, defense, water transport, polyamine synthesis, and housekeeping genes during abiotic and biotic stress alleviation by Si. Furthermore, an overview of Si-mediated modulation of multiple genes involved in stress response pathways such as phenylpropanoid pathway, jasmonic acid pathway, ABA-dependent or independent regulatory pathway have been discussed in this review.

Keywords: defense response, gene regulation, photosynthesis, polyamine biosynthesis, regulatory elements

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INTRODUCTION

The surface of earth is covered with 27.70% of silicon (Si) next to oxygen, but the existence of Si in its pure form is rare (Mitra, 2015). Silicon is deposited in the form of quartz (SiO_2), sand, and sand stone in the earth crust (Rédei, 2008). In biological organisms, Si occurs in the form of amorphous silica (SiO_2 nH_2O) and soluble silicic acid ($Si(OH)_4$) (Das and Chattopadhyay, 2000). Moreover in eukaryotes, Si is important for bones, cartilage, connective tissue formation, enzymatic activities, and lymphocyte proliferation (Carlisle, 1988, 1997). In plants, Si is absorbed

as an uncharged monomeric silicic acid in the pH range below 9 (Knight and Kinrade, 2001; Ma and Yamaji, 2006). The level of Si accumulation by plants can be directly correlated with the beneficial effects attributed by Si. Among the plants, monocots like rice, sugarcane, maize, and cereals absorb Si in large quantities on comparison with dicots due to the presence of Si transporters (Ma et al., 2016). The absorption and transportation of Si in plants is a complex process which involves influx and efflux Si transporters belonging to aquaporin family with specific selectivity properties. For instance, the high Si accumulator like rice consists of low silicon rice 1 (Lsi1) transporter, a nodulin 26-like intrinsic protein (NIP) in roots.

Recently, several putative silicon transporters have been identified in monocot and dicot plants by Deshmukh et al. (2015). According to the report, uptake of Si is particularly confined to the plant species consisting of NIP type aquaporins with GSGR selectivity filter along with an exact distance of 108 amino acids between the asparagine-proline-alanine (NPA) domain (Deshmukh et al., 2015). The exogenous supplementation of Si proves to be beneficial for plants particularly under abiotic and biotic stress conditions (Supplementary Table 1). Silicon nutrition resulted in the improvement of growth and development (Eneji et al., 2008; Soundararajan et al., 2014; Zhang et al., 2015), increase in yield (Epstein, 1999), abiotic and biotic stress tolerance (Ma, 2004; Zhu et al., 2004; Liang et al., 2007; Muneer et al., 2014), management of macro and micro nutrients (Tripathi et al., 2014), resistance against pest and pathogens (Lanning, 1966; Cookson et al., 2007).

Apart from the abovementioned advantages, Si augmentation in soil-less cultivation of corn salad improved the edible yield, quality, and shelf life of baby leaf vegetable corn salad by the regulation of nutrient acquisition, uptake of nitrate/iron, phenoloxidase gene expression, and protection of chlorophyll degradation (Gottardi et al., 2012). Likewise, Si inclusion in tissue culture medium resulted in the enhancement of axillary shoot induction (Manivannan et al., 2017), alleviation of hyperhydricity (Soundararajan et al., 2017a), callus induction (Islam et al., 2005), and root morphogenesis (Asmar et al., 2013). Even though, the effect of Si in plants was studied for several years, the mechanisms behind the physiological responses or molecular regulation in plants upon Si nutrition under normal and stressed conditions is still under study.

Broadly, Si-mediated tolerance to stress can be interpreted either in the form of mechanical barrier through $Si(OH)_4$ polymerization in cell walls to prevent the penetration of host tissue by pest or pathogen (Yoshida et al., 1962) or by triggering the chemical resistance mechanism (Fawe et al., 1998). According to Chérif et al. (1992), in cucumber the Si treatment increased the activities of chitinases, peroxidases, and polyphenoloxidases against *Pythium ultimum*. Similarly, Si nutrition enhanced the plant growth by the regulation of antioxidant and nutrient uptake in salt stressed in Salvia (Soundararajan et al., 2014). Moreover, Si retarded the Na^+ and Cl^- transportation due to silicon deposition to cope up the plants under salinity stress (Gong et al., 2006; Shi et al., 2013). Likewise, Si supplementation decreased metal toxicity such as toxicity of aluminum (Al) (Wang et al., 2004), boron (B) (Gunes et al., 2007), cadmium (Cd) (Liang et al., 2005), chromium (Cr) (Tripathi et al., 2012) copper (Cu) (Li et al., 2008), and zinc (Zn) (Neumann and Zur Nieden, 2001). Recently Debona et al. (2017), has elaborately reviewed the possible stress tolerance mechanisms attributed by Si upon abiotic and biotic stresses. According to the review, upon metal toxicity, silicon tends to modulate the pH range of soil, changes the metal speciation, compartmentalization and co-precipitation of metals, and sequestration strategies to combat the metal stress (Debona et al., 2017). In addition, the Si-fortified fertilizers are gaining interest in recent days due to its beneficial results particularly in the improvement of growth, photosynthesis, and maintenance of electrolyte leakage even under stressed conditions (Chen et al., 2011).

Overall, the inclusion of Si is important for plant growth and numerous reports and reviews illustrated the Si dependent modulations of antioxidant enzymes, nutrient contents, homeostasis in reactive oxygen species however, very few studies have dealt with the Si-mediated molecular regulation of genes in plants under abiotic and biotic stresses (Brunings et al., 2009; Song et al., 2014; Yin et al., 2016). The modern high-throughput approaches can aid in deciphering the important genes involved in the Si-mediated stress response in plants (**Tables 1–3**). The Si-dependent expression of genes was first investigated in rice using the microarray approach by Watanabe et al. (2004). According to the results, the addition of Si up-regulated the abundance of a zinc finger protein homolog and down-regulated the expressions of chlorophyll *a*/*b* binding protein, metallothione-like protein, *Xa21* gene family member, and carbonic anhydrase homolog (Watanabe et al., 2004). In general, the zinc finger proteins act as the major transcription factors for stress responsible genes and the enhancement of its expression can increase the regulation of stress responsible genes which might increase the stress tolerance in Si treated plants (Watanabe et al., 2004). In the following sections, the Si-mediated regulations of genes involved in several physiological processes have been discussed.

SILICON REGULATED THE GENES INVOLVED IN PHOTOSYNTHESIS UPON METAL TOXICITY

Among several mechanism of Si-mediated stress amelioration, the primary stress-combating strategies utilized by Si is the enhancement of photosynthesis process in the stressed plants. Broadly, the oxidative stress resulting from both abiotic and biotic stress target photosynthesis by affecting the major enzymes in calvin cycle and photosynthetic electron transport chain (Nwugo and Huerta, 2008; Gong and Chen, 2012; Muneer et al., 2014). Even though, various studies have evidenced the beneficial effects of Si on photosynthesis, only a few have examined the molecular rationale behind the gene expression upon Si addition, particularly in rice. The report by Song et al. (2014) illustrated the transcriptional regulation of photosynthesis related genes under Si amendment and zinc stress. Supplementation of Si increased the transcript levels of *PsbY* (Os08g02630), a vital polyprotein involved in photosystem II (PSII) whereas, the Zn in higher concentration retarded the *PsbY* expression. In detail, the *PsbY* is

TABLE 1 | List of genes up regulated upon the supplementation of Si under abiotic stress.

Abiotic stress	Gene identifier	Functional annotation	Process	Organism	References
Metal toxicity	*Os08g02630*	Subunit of oxygen evolving complex-PSII	Photosynthesis	*Oryza sativa*	Song et al., 2014
Metal toxicity	*Os05g48630*	Photosynthetic co8y stability maintenance	Photosynthesis	*Oryza sativa*	Song et al., 2014
Metal toxicity	*Os07g37030*	Maintenance of cytochrome	Photosynthesis	*Oryza sativa*	Song et al., 2014
Metal toxicity	*Os03g57120*	Ferrodoxin $NADP^+$ reductase	Photosynthesis	*Oryza sativa*	Song et al., 2014
Metal toxicity	*Os09g26810*	Subunit of LHC II complex	Photosynthesis	*Oryza sativa*	Song et al., 2014
Metal toxicity	*Os04g38410*	Subunit of LHC II complex	Photosynthesis	*Oryza sativa*	Song et al., 2014
Drought	*AK070732*	Member of RING domain containing protein family	Regulatory gene	*Oryza sativa*	Khattab et al., 2014
Drought	*AF300971*	Dehydration responsive element binding protein	Regulatory gene	*Oryza sativa*	Khattab et al., 2014
Drought	*AJ578494*	Choline monooxygenase	Regulatory gene	*Oryza sativa*	Khattab et al., 2014
Drought	*AB028184*	NAC regulons (No apical meristem(NAM), *Arabidopsis thaliana* activating factor [ATAF], and cup-shaped cotyledon [CUC])	Regulatory gene	*Oryza sativa*	Khattab et al., 2014
Drought	*NM_001074375*	Dehydrin	Regulatory gene	*Oryza sativa*	Khattab et al., 2014
Salt stress	*Sb02g025110*	S-Adenosyl-L-methionine decarboxylase	Polyamine synthesis	*Sorghum bicolor*	Yin et al., 2016
Salt stress	*Sb04g025720*	S-Adenosyl-Metdecarboxylase	Polyamine synthesis	*Sorghum bicolor*	Yin et al., 2016
Salt stress	*Sb06g021540*	S-Adenosyl-Metdecarboxylase	Polyamine synthesis	*Sorghum bicolor*	Yin et al., 2016
Salt stress	*Sb10g002070*	Arginine decarboxylase	Polyamine synthesis	*Sorghum bicolor*	Yin et al., 2016
Salt stress	*Sb04g021790*	N-Carbamoyl putrescine amidohydrolase	Polyamine synthesis	*Sorghum bicolor*	Yin et al., 2016
Metal toxicity	*At5g22460*	Esterase lipase thioesterase family protein	Transporter gene	*Arabidopsis thaliana*	Li et al., 2008
Metal toxicity	*At5g59030*	Copper transporter	Transporter gene	*Arabidopsis thaliana*	Li et al., 2008

a subunit of oxygen-evolving complex of PSII with manganese-binding polypeptide consisting L-arginine metabolizing enzyme activity (Kawakami et al., 2007). Furthermore, the Si-mediated increase in the level of *PsbY* transcripts could activate the manganese-binding capacity, oxidation of water that might improve the efficiency of PS II and electron transfer rate (Song et al., 2014). Likewise, the application of Si has improved the abundance of *PsaH* which encodes the vital polypeptide subunits in the PSI dimer (Pfannschmidt and Yang, 2012). The *PsaH* knockout mutant damaged the LCH-II complex resulting in the energy transition delay between PS II and PS I (Lunde et al., 2000).

Similarly, the Zn toxicity resulted in the down regulation of *PetC* which has been recovered by Si supplementation. In general, *PetC* codes Rieske Fe-S center-binding polypeptide of cytochrome *bf* complex which is responsible for the proper functioning of cytochrome (Breyton et al., 1994). Hence, the Si mediated up-regulation of *PetC* could augment the structural integrity of the chloroplast (Song et al., 2014). Moreover, Si treatment increased the expression of *PetH* in similar manner with *PetC*. The product of *PetH* is ferredoxin NADP+ reductase, an important enzyme involved in the synthesis of NADPH via photosynthetic electron transport chain. Furthermore, reducible glutathione content in the cells is maintained by *PetH* (Song et al., 2014). In addition to the above listed genes, the supplementation of Si resulted in the up-regulation of genes (*Os03g57120* and *Os09g26810*) involved in the light harvesting complex. Thus, the molecular insight into Si-dependent up-regulation of genes associated with PS I and PS II illustrate the positive effects rendered by Si on photosynthesis process. The physiological improvement of photosynthetic apparatus and reduction in the degradation of chlorophyll pigmentation reported by several researches can be correlated with the genic regulation of photosynthetic genes by Si at molecular level. A putative model representing the Si-mediated regulation of photosynthesis associated genes discussed above have been illustrated in **Figure 1**. Overall, the augmentation of Si instigated the expression levels of important genes in both photosystems to increase the efficiency of photosynthesis particularly under stressful environment.

SILICON MODULATED THE EXPRESSION OF HOUSEKEEPING GENES UPON PATHOGEN INFECTION

In general, housekeeping genes are expressed constitutively in all cells regardless of its patho-physiological state and these genes are vital for the maintenance of proper functioning of cells. Although, the expression of housekeeping genes is constant, several studies illustrated their loss of stability under stressed conditions (Nicot et al., 2005; Jain et al., 2006). According to Brunings et al. (2009), the supplementation of Si down regulated the expression of important housekeeping genes in rice under normal condition however, upon pathogen infection Si up-regulated the housekeeping genes to maintain the cellular functions. Similarly, Ghareeb et al. (2011a) observed the Si-mediated up-regulation of housekeeping genes such as actin (*ACT*), alpha-tubulin (*TUB*), and phosphoglycerate kinase (*PGK*) in *Ralstonia solanacearum* infected tomato. According to Jarosch et al. (2005) actin cytoskeleton provided the basal resistance against the *R. solanacearum*. Therefore,

TABLE 2 | List of genes up regulated upon the supplementation of Si under biotic stress.

Biotic stress	Gene identifier	Functional annotation	Biological process	Organism	References
Rice blast disease	*Os01g0713200*	β-1,3-Glucanase precursor	Defense	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os02g0584800.*	Heavy metal transport/detoxification protein domain-containing protein	Defense	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os02g0585100*	Heavy metal transport/detoxification protein domain-containing protein	Defense	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os04g0469000*	Heavy metal transport/detoxification protein domain-containing protein	Defense	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os04g0610400*	Pathogenesis-related transcriptional factor and ERF domain-containing protein	Defense	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os07g0104100*	Peroxidase precursor	Defense	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os11g0692500*	Bacterial blight resistance Protein	Defense	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os01g0963200*	Peroxidase BP 1 precursor	Defense	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os01g0378100*	Peroxidase precursor	Defense	*Oryza sativa*	Brunings et al., 2009
Bacterial wilt	*AF494201*	Tomato stress-responsive factor	Defense	*Solanum lycopersicum*	Ghareeb et al., 2011b
Bacterial wilt	*M69247*	Pathogenesis-related protein 1	Defense	*Solanum lycopersicum*	Ghareeb et al., 2011b
Bacterial wilt	*M80604*	β-Glucanase	Defense	*Solanum lycopersicum*	Ghareeb et al., 2011b
Bacterial wilt	*U30465*	Chitinase class II	Defense	*Solanum lycopersicum*	Ghareeb et al., 2011b
Bacterial wilt	*X94943*	Peroxidase	Defense	*Solanum lycopersicum*	Ghareeb et al., 2011b
Bacterial wilt	*M83314*	Phenylalanine ammonia lyase	Defense	*Solanum lycopersicum*	Ghareeb et al., 2011b
Bacterial wilt	*X99147*	Arabinogalactan protein	Defense	*Solanum lycopersicum*	Ghareeb et al., 2011b
Bacterial wilt	*L26529*	Polygalacturonase inhibitor protein	Defense	*Solanum lycopersicum*	Ghareeb et al., 2011b
Rice blast disease	*Os02g0807000*	Phosphoenolpyruvate carboxylase kinase	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os01g0554100*	RNA-directed DNA polymerase (reverse transcriptase) domain containing protein	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os03g0803500*	2OG-Fe(II) oxygenase domain-containing protein	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os10g0559500*	2OG-Fe(II) oxygenase domain-containing protein	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os09g0432300*	AAA ATPase, central region domain-containing protein	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os06g0676700*	High pI α-glucosidase	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os08g0190100*	Oxalate oxidase-like protein	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os05g0495600*	P-type ATPase	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os03g0405500*	PDI-like protein	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Bacterial wilt	*AY157064*	WRKY group II transcription Factor	Regulatory gene	*Solanum lycopersicum*	Ghareeb et al., 2011b
Bacterial wilt	*AY383630*	Jasmonate and ethylene responsive factor 3	Regulatory gene	*Solanum lycopersicum*	Ghareeb et al., 2011b
Bacterial wilt	*Z75520*	Ferredoxin I	Photosynthesis	*Solanum lycopersicum*	Ghareeb et al., 2011b

the Si dependent up-regulation of actin in tomato plants induced the host resistance (Ghareeb et al., 2011a). Tomato is considered as the low-level silicon accumulator (∼0.2% dry weight) because of the lack of high density Si transporter (Ma and Yamaji, 2006). Moreover, the impermeability of Si by nodulin 26-like intrinsic protein (NIP) in tomato (SINIP2-1) has been postulated due to the difference in the spacing between two NPA domains which forms the half helix inserts in SINIP2-1. However, the meager uptake of Si by low silicon accumulating plants is unclear but it might be possible that the lesser uptake of Si by tomato plants particularly under stressed environment might be due to the existence of a passive uptake mechanism. Moreover, the application of Si even in less biological concentration in the low accumulating species such as tomato (Romero-Aranda et al., 2006), capsicum (Jayawardana et al., 2015), and roses (Soundararajan et al., 2017b) has rendered abiotic and biotic stress tolerance. Despite the constant expression nature of housekeeping genes, variation in the expression levels upon Si amendment and pathogen infection could induce the basal defense mechanism in the host plant to protect from the pathogen. Taken together, the silicon amendment regulated the expression of vital housekeeping genes to alleviate the biotic stress.

SILICON ALTERED THE EXPRESSION OF REGULATORY ELEMENTS ASSOCIATED WITH STRESS RESPONSE GENES

Stressful environment can induce the expression of myriads of genes involved in stress tolerance, metabolic processes, and signal transduction, etc. in plants (Shinozaki and Yamaguchi-Shinozaki, 2000; Xiong et al., 2002; Rabbani et al., 2003; Shinozaki

TABLE 3 | List of genes down regulated upon the supplementation of Si under abiotic and biotic stresses.

Stress	Gene identifier	Functional annotation	Biological process	Organism	References
Rice blast disease	*Os11g0608300*	Barley stem rust resistance protein	Defense	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os11g0673600*	Disease resistance protein family protein	Defense	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os03g0266300*	Heat shock protein Hsp20 domain-containing protein	Defense	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os03g0235000*	Peroxidase	Defense	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os12g0491800*	Terpene synthase-like domain-containing protein	Defense	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os10g0191300*	Type 1 pathogenesis-related protein	Defense	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os09g0417800*	DNA-binding WRKY domain-containing protein	Regulatory gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os08g0332700*	Trans-acting transcriptional protein ICP0	Regulatory gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os02g0695200*	P-type R2R3 Myb protein	Regulatory gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os09g0110300*	Putative cyclase family protein	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os08g0112300*	Transferase family protein	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os10g0154700*	Cyclophilin Dicyp-2	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os08g0155700*	DNA-directed RNA polymerase largest chain (isoform B1)-like protein	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os11g0194800*	DNA-directed RNA polymerase II	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os11g0106700*	Ferritin 1, chloroplast precursor	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os12g0258700*	Multi copper oxidase, type 1 domain-containing protein	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os01g0770200*	Tyrosine decarboxylase 1	Housekeeping gene	*Oryza sativa*	Brunings et al., 2009
Rice blast disease	*Os01g0627800*	Cytochrome P450 monooxygenase	Photosynthesis	*Oryza sativa*	Brunings et al., 2009
Salt stress	*Sb01g009450*	1-Aminocyclopropane-1-carboxylic acid synthase	Polyamine synthesis	*Sorghum bicolor*	Yin et al., 2016

et al., 2003). Amongst the stress induced genes, transcription factors (TF) are the primary regulators of the downstream genes important for plant tolerance against biotic and abiotic stresses (Gao et al., 2007; Lucas et al., 2011). In general, TFs are facilitated by particular cis-elements called regulons that are located in the promoter section of the target gene (Nakashima et al., 2009; Qin et al., 2011). Broadly, plants consists of a diverse number of regulons responding to stress, for example dehydration-responsive element binding protein (*DREB2*) are triggered by temperature and drought stress (Mizoi et al., 2012). Similarly, the *NAC* regulons [no apical meristem (NAM), *Arabidopsis thaliana* activating factor (ATAF), and cup-shaped cotyledon (CUC)] can be activated by osmotic stress in plants (Nakashima et al., 2009; Fujita et al., 2011). Moreover, the increase in the expression levels of TFs can stimulate a wide range of signal transduction pathways resulting in stress tolerance (Chaves and Oliveira, 2004; Umezawa et al., 2006). According to Khattab et al. (2014), in rice the addition of Si resulted in the up-regulation of TFs involved in the expression of *DREB2A*, *NAC5*, *Oryza sativa RING* domain containing protein *(OsRDCP1)*, *Oryza sativa* choline monooxygenase (*OsCMO)*, and dehydrin *OsRAB16b* (**Figure 2**). In rice, the *OsDREB* triggers the expression of stress-responsive genes that impart tolerance against osmotic stress in abscisic acid (ABA)—independent manner (**Figure 2A**) (Dubouzet et al., 2003; Hussain et al., 2011). In addition, the elevated levels of *OsDREB2A* provided drought resistance in rice (Chen et al., 2008; Wang et al., 2008). Similarly, NACs are TFs with various roles in development and stress response of plants (Tran et al., 2010). According to Fang et al., the rice genome consists of ∼140 putative *NAC* or *NAC*-like genes among them 20 genes including *OsNAC5* are classified as stress responsive genes involved detoxification, redox homeostasis, and macromolecule fortification (Hu et al., 2008). Hence, the Si-mediated enhancement of *OsNAC5* transcripts led to prevention of lipid peroxidation and generation of excess hydrogen peroxide (H_2O_2). The abovementioned metabolic modulations shield the plants from dehydration and oxidative damages caused in stressed conditions (Takasaki et al., 2010; Song et al., 2011). Furthermore, in rice the up regulation of the *OsNAC5* stimulated the stress tolerance by increasing the levels of stress inducible rice genes like *LEA3* (Takasaki et al., 2010; **Figure 2B**). Moreover, *OsRAB16b* belongs to *LEA* genes that are expressed in response to abiotic stresses in both somatic and reproductive tissues (Tunnacliffe and Wise, 2007; Bies-Etheve et al., 2008). Broadly, *LEA* proteins encoded by the *LEA* genes render the property of acclimatization to the plants particularly under stressful conditions (Lenka et al., 2011).

In eukaryotes, the protein turnover is maintained by the Ubiquitin (Ub)-26S proteasome pathway. During the process of ubiquitination, the target proteins are linked to multiple Ub chains by ubiquitin ligases such as E1, E2, and E3 (Kraft et al., 2005; Stone et al., 2005). According to previous reports, the RING E3 Ub ligases play a vital role particularly in response to drought stress in rice (Bae et al., 2011; Ning et al., 2011; Park et al.,

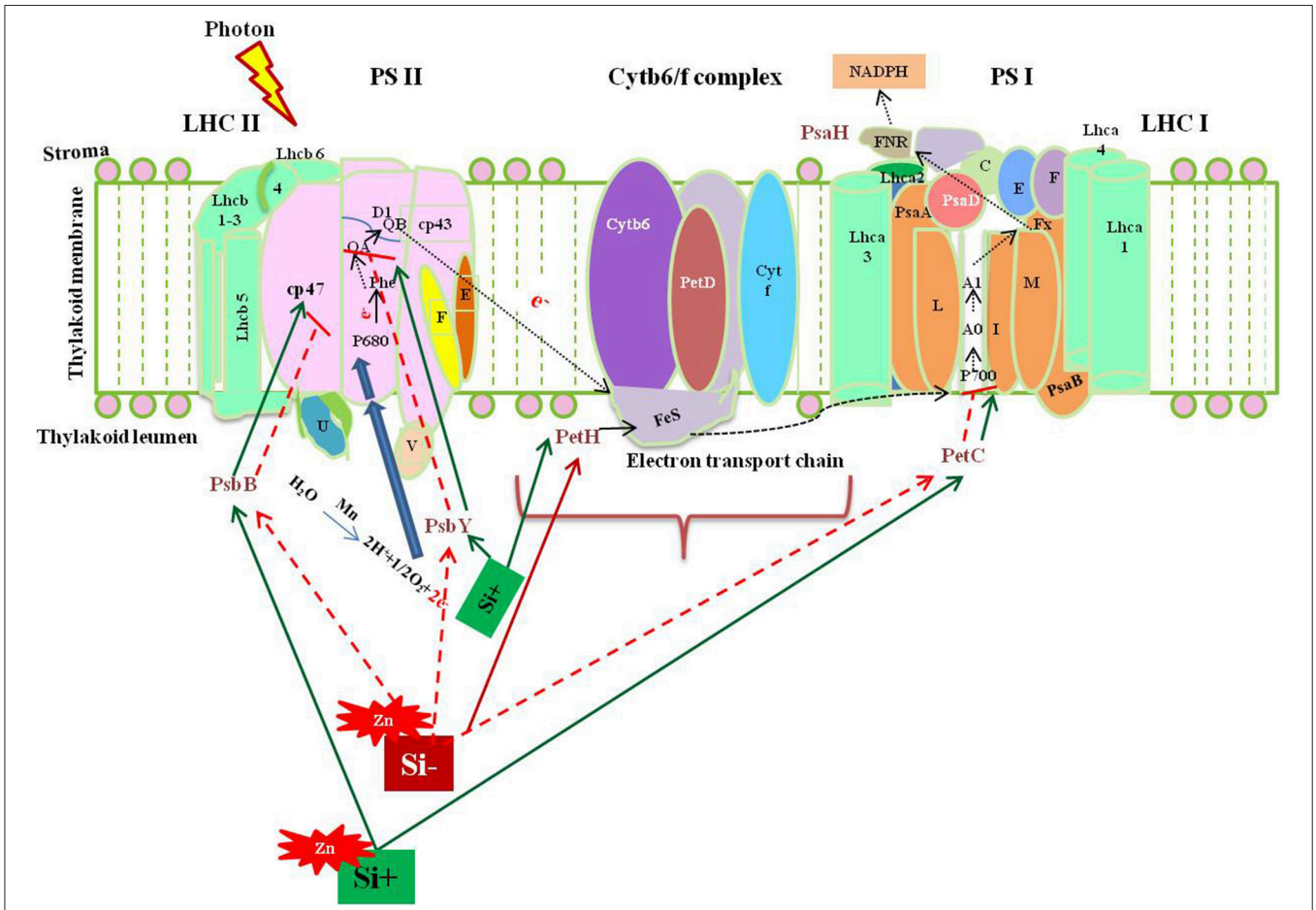

FIGURE 1 | A putative model representing the regulation of photosynthesis related genes upon metal stress with or without Si supplementation. The green standard arrows represent the up-regulation of genes progressing with normal function in the presence of Si, blue standard arrows represent the entry of electrons into the PS II, flow of electron in the photosynthetic cascade has been represented by dotted black arrows, and red dotted arrows indicate the down-regulation of photosynthetic genes and corresponding gene functions upon metal stress in Si- plant. PSII, Photosystem II; Cytb6/f, cytochrome b6/f complex; PSI, photosystem 1; LHC I, light harvesting complex I. The diagram was conceived based on the interpretation from the following literatures (Lunde et al., 2000; Kawakami et al., 2007; Song et al., 2014).

2011). Till date, five homologs of *OsRDCP* were identified in rice which possesses a single *RING* motif in their N-terminal regions (Khattab et al., 2014). The *OsRDCP1* is one among the five homologs which combated the dehydration stress in rice was up-regulated by the application of Si (Bae et al., 2011; Khattab et al., 2014; **Figure 2C**). Similarly, choline mono oxygenase the product of *OsCMO* is a primary enzyme involved in the biosynthesis of glycine betaine (Burnet et al., 1995). The glycine betaine is widely known for its osmolytic property that renders abiotic stress tolerance in several plants. Hence, the Si-mediated enhancement of *OsCMO* gene levels improved the stress tolerance in rice (Burnet et al., 1995; **Figure 2D**). The silicon-dependent up-regulation of transcription factors could interact with the cis-elements located in the promoter region of genes involved in stress resistance and trigger the stress tolerance against abiotic and biotic stresses. These regulatory genes might also induce the transcription of genes associated with the defense related or stress responsive pathways such as phenylpropanoid pathway, ABA-dependent or ABA-independent regulatory pathways to protect the plants from stress.

MODULATION OF GENES INVOLVED IN WATER UPTAKE AND TRANSPORTATION UPON Si NUTRITION

Aquaporins are essential transmembrane proteins that maintain the uptake and movement of water molecules across cell membranes, particularly under abiotic stress condition (Boursiac et al., 2005). However, the function of aquaporin has been implicated by several factors such as abscisic acid, level of calcium ions, free radicals, and ethylene (Azad et al., 2004; Parent et al., 2009; Hu et al., 2012). According to Boursiac et al. (2008) the activity of aquaporin is susceptible to a mere change in the level of ROS, for instance the H_2O_2 stimulated by salt stress resulted in the prevention of aquaporin function by

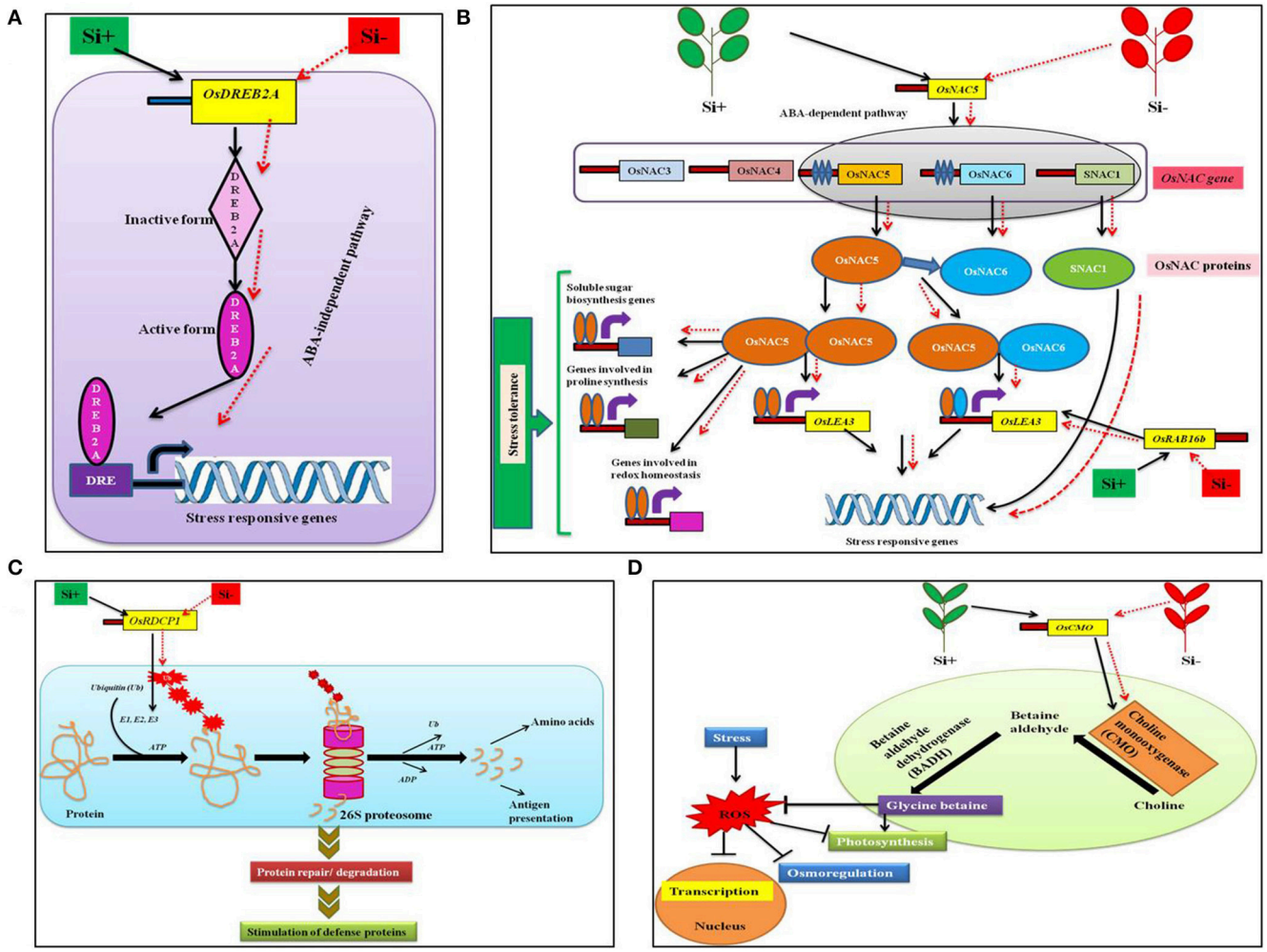

FIGURE 2 | A schematic representation of the regulation of transcription factors under abiotic stress condition with or without Si supplementation. **(A)** Model displaying the *OsDREB2A* regulation in ABA-independent pathway to combat stress. **(B)** Regulation of *OsNAC5* transcription factor in ABA-dependent pathway to trigger stress tolerance related genes. **(C)** The *OsRDCP1* mediated stress tolerance response via the ubiquitin-proteosome degradation pathway. **(D)** Improvement of glycine betaine biosynthesis by *OsCMO* to combat ROS generation. The black standard arrows represent the up-regulation of gene in the presence of Si and red dotted arrows indicate the down-regulation of gene and corresponding functions upon stress in Si- plant. DRE, Dehydration responsive element; DREB2A; dehydration-responsive element binding protein 2A; NAC, no apical meristem (NAM), *Arabidopsis thaliana* activating factor (ATAF), and cup-shaped cotyledon (CUC) regulons; *OsRDCP1, Oryza sativa RING* domain containing protein; *OsCMO, Oryza sativa* choline monooxygenase; *SNAC1,* stress-responsive NAC protein, OsLEA3, *Oryza sativa* late embryogenesis abundant protein; E1, ubiquitin activating; E2, ubiquitin conjugating; E3,ubiquitin ligating enzymes, ATP, adenosine triphosphate; ADP, adenosine diphosphate. The diagram was conceived based on the interpretation from the following literatures (Mizoi et al., 2012; Nakashima et al., 2012; Khattab et al., 2014).

modulating the oxidant gating, phosphorylation condition, and re-localization of aquaporin. The amendment of Si enhanced the uptake of water particularly under salinity stress in several plants however the molecular mechanism behind the improvement of water status is unclear until recently. In *Sorghum bicolor*, the application of Si enhanced water uptake by increasing the activity of aquaporin by the up-regulation of *SbPIP1;6*, *SbPIP2;2*, and *SbPIP2;6* encoding plasma membrane intrinsic protein (PIP), the copious aquaporin in root (Liu et al., 2015; **Figure 3**). In addition, the higher expression of genes related to aquaporin results in the rapid water uptake which also dilutes the excess concentration of Na^+ ions lethal for the plants (Gao et al., 2010). In accordance with the existing reports on the uses of aquaporin up regulation, the findings of Sutka et al. (2011) illustrated that the abundance of aquaporin genes in the roots balance the water uptake by the plants even under water-deficit conditions. In both normal and stressful environment the regulation of aquaporins plays a vital role in maintaining the proper uptake and transportation of water and solutes in plants. The enhancement of aquaporin related genes by silicon might substantiate the improvement of water status in plants treated with Si in salinity and drought stressed plants. The improvement of water status and ion balance aid in the reclamation of plants from stress.

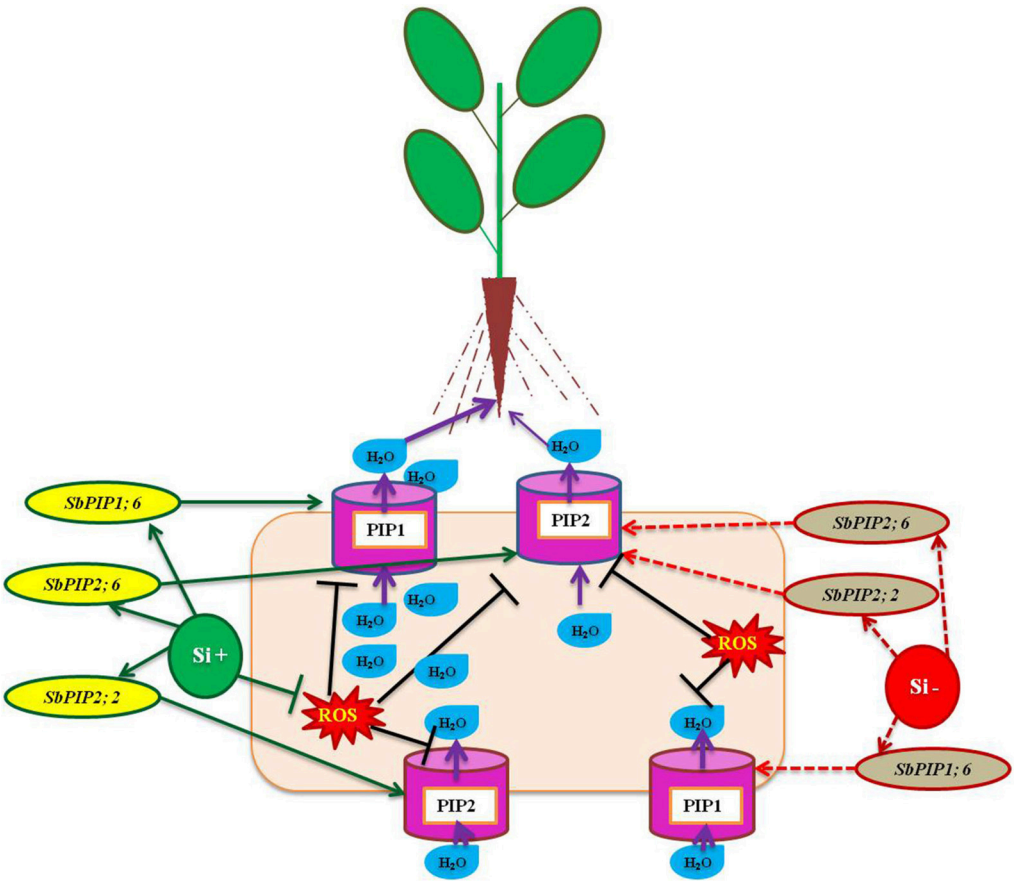

FIGURE 3 | A model representation of aquaporin related genes regulation under osmotic stress condition with or without Si supplementation. The green standard arrows represent the up-regulation of genes in Si+ and red dotted arrows indicate the down-regulation of genes and corresponding functions upon stress in Si- conditions. The down regulation of PIP genes could result in the lesser activity of Aquaporin mediated transportation however upon Si augmentation the up-regulation of PIP genes improve the water status of the plants under stress. *SbPIP1*, Sorghum plasma membrane intrinsic protein, and PIP (plasma membrane intrinsic protein). The model was conceived based on the interpretation from the following literatures (Liu et al., 2015; Maurel et al., 2015).

REGULATION OF POLYAMINE BIOSYNTHESIS GENES BY Si SUPPLEMENTATION

Plants with higher levels of polyamines like putrescine, spermidine, and spermine reported to possess more resistance against environmental onslaughts like salinity (Liu et al., 2006; Chai et al., 2010; Quinet et al., 2010; Pottosin and Shabala, 2014). Furthermore, the elevated levels of genes responsible for the synthesis of polyamines mitigates the negative effects of oxidative stress (Roy and Wu, 2001; Tang et al., 2007). Therefore, the role of polyamines in stress resistance is becoming inevitable and the molecular insight into the Si dependent modulation of polyamines has been reported in *Sorghum bicolor* (Yin et al., 2016). The augmentation of Si elevated the expression level of S-adenosyl-L-methionine decarboxylase (*SAMDC*) gene which encodes a vital enzyme involved in the biosynthesis of polyamines. In addition, the report also hypothesized that the Si-mediated salt tolerance in sorghum has been associated with the polyamines and ethylene synthesis. On the contrary to *SAMDC,* the Si application retarded the synthesis of ethylene in sorghum under salinity stress. Since the polyamines such as spermidine and spermine share a common precursor, S-adenosyl-L-methionine (SAM) with ethylene, it is considered as the presence of a competitive environment amongst the polyamines and ethylene (Pandey et al., 2000). Therefore, in order to reduce the competitive condition, Si could have reduced the ethylene level by the inhibition, 1-aminocyclopropane-1-1-carboxylic acid (ACC) an important ethylene precursor (Yin et al., 2016). The supplementation of Si balanced the metabolism of polyamines and ethylene to mitigate abiotic stress (**Figure 4**). Polyamines are involved in various vital processes such as replication, transcription and translation, stabilization of membranes, and modulation of enzyme activities in addition to stress tolerance. Hence, the regulation of polyamine biosynthesis genes by Si could not only helps in the stress alleviation but also improves the fundamental processes in cells upon stress and increase the growth and development of plants.

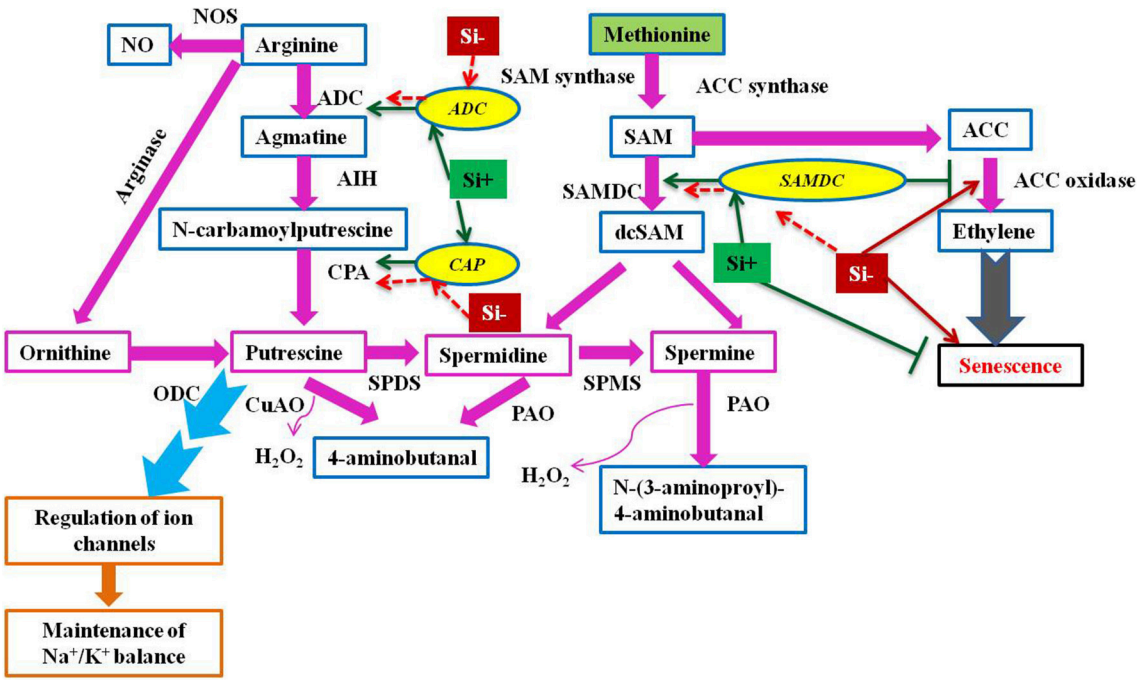

FIGURE 4 | A schematic illustration of polyamine biosynthesis gene regulation under stress condition with or without Si supplementation. The green standard arrows represent the up-regulation of genes in Si+ and red dotted arrows indicate the down-regulation of genes and corresponding functions upon stress in Si- conditions. *SAMDC*, S-adenosyl-L-methionine decarboxylase; *ADC*, arginine decarboxylase; *CAP*, N-carbamoylputrescine amidohydrolase; ACC, 1-aminocyclopropane-1-carboxylic acid; SAM, S-adenosyl-L-methionine; ODC, ornithine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase; CuAO, copper amine oxidase; PAO, polyamine oxidase; AIH, agmatine iminohydrolase; PAO, polyamine oxidase; NOS, nitric oxide synthase. The model was conceived based on the interpretation from the following literatures (Mizoi et al., 2012; Khattab et al., 2014; Shi and Chan, 2014; Kurepin et al., 2015; and Yin et al., 2016).

SILICON-MEDIATED EXPRESSION OF DEFENSE RESPONSIVE GENES

The defensive role of Si against biotic and abiotic stresses has been evidenced by several plant biologists. Especially, the Si-mediated protection against potential plant diseases such as powdery mildew and rice blast disease has been studied widely (**Figure 5**). The extensive study by Rodrigues et al. (2004) elucidated the positive regulation of genes related to the defense mechanism such as chalcone synthase (CHS), phenylalanine-ammonia lyase (PAL), pathogenesis related protein (PR1), peroxidase (POX), chitinases, and β-1, 3-glucanases by Si upon *Magnaporthe grisea* infection. Among the listed genes, CHS is a rate limiting enzyme in the flavonoid biosynthesis pathway and PAL plays a vital role in the synthesis of secondary metabolites with potential chemical defense property via phenylpropanoid pathway (Rodrigues et al., 2004). Furthermore, the peroxidases enzymes are important for lignin biosynthesis which acts as the potential mechanical barrier against pathogens (Rhodes, 1994). Similarly, the pathogenesis related (PR-1) protein in combination with genes related to secondary metabolism acts as the primary outcome of the plant defense response (Zeier et al., 2004). Moreover, the supplementation of Si altered the expression pattern of defense genes in rice to render resistance against *Magnaporthe oryzae* (Brunings et al., 2009). In addition, Si application in rice also induced differential expression of heavy metal transport and detoxification related genes to mitigate the heavy metal toxicity (Brunings et al., 2009). Altogether, Si regulates the genes responsible for vital physiological functions in plants particularly under stressed environment. Among the several mechanism of stress tolerance reported to exhibit by silicon, instigation of defense mechanism is considered as the pivotal one. Particularly, Si-mediated induction of cascade of reactions via phenypropanoid pathway is responsible for the synthesis and accumulation of chemical defense molecules such as phenols, and flavanoids against pathogens. Apart from the phenypropanoid pathway, Si can also induce the systemic acquired resistance (SAR) by the regulation of genes involved in hypersensitivity response and jasmonic acid mediated defense pathway to protect against pathogen attack.

CONCLUSIONS

Silicon is the modest and a major element of soil with enormous benefits to plants especially in the mitigation of abiotic and biotic stress. Owing to its numerous advantages, the inclusion of Si in modern cultivation systems likes soil-less cultivation system has been blooming in several areas. In recent days, the modernization of technology allows us to investigate the molecular level

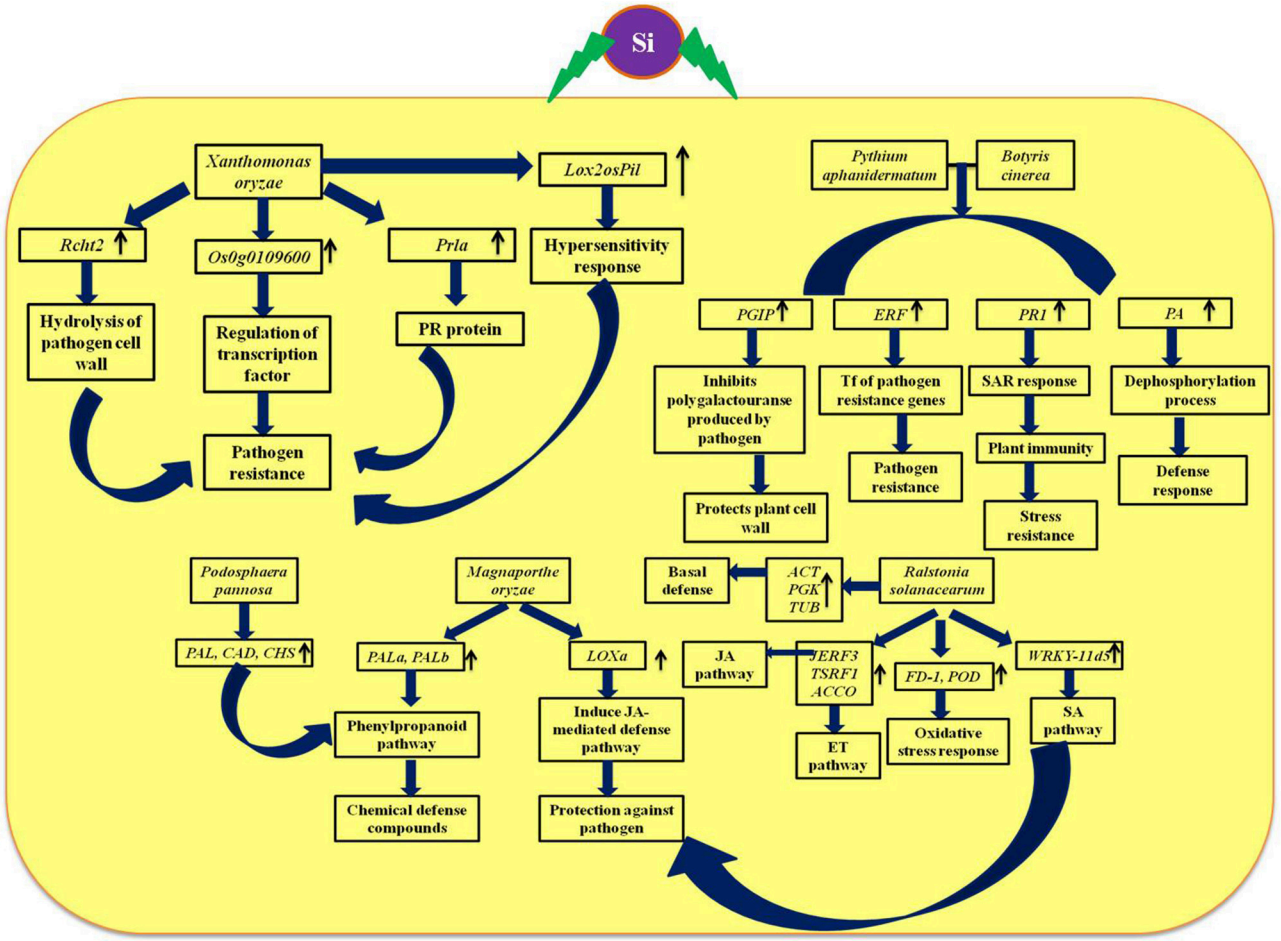

FIGURE 5 | Schematic representation of Si-mediated regulation of vital genes associated with defense and phytohormones upon biotic stress. *Rcht2*, Chitinase; *Prla,* PR-1; *Lox*, Lipoxygenase; *PAL*, phenylalanine ammonia lyase; *CAD*, cinnamyl alcohol dehydrogenase; *CHS*, Chalcone synthase; *PGIP*, Polygalactouranase inhibitor protein; *PA*, phosphatase associated to defense; *PR-1*, pathogenesis-related protein; *ERF*, Ethylene response factor; *JERF*, Jasmonate and ethylene responsive factor 3; *TSRF,* Tomato stress-responsive factor; *ACCO*, 1-aminocyclopropane-1-carboxylate oxidase; *FD-1*, Ferredoxin-I; *POD*, Peroxidase; *WRKY II*, WRKY group II transcription factor; SA, Salicylic acid; JA, Jasmonic acid. The diagram was conceived based on the interpretation from the following literatures (Ghareeb et al., 2011b; Shetty et al., 2011; Rahman et al., 2015; El-Garhy et al., 2016; Song et al., 2016).

regulation of compounds which has been extended to study the role of silicon in gene level by plant biologists under different stress conditions. Even though, the research on understanding of molecular rationale behind the Si-mediated stress tolerance is in rudimentary stage, upcoming outcomes from the recent studies have shed light into several possible ways of Si-dependent stress tolerance in plants. Based on the current reports it is evident that silicon possess multifaceted role in the regulation of genes involved in photosynthesis, secondary metabolism, polyamine biosynthesis, transcription, and water uptake. The molecular level modulations triggered by Si supplementation under stressed environment corresponded to the physiological improvement of plant growth and recovery from stress. In addition, several other novel molecular mechanisms behind the stress alleviation by Si have to be unraveled in the future.

AUTHOR CONTRIBUTIONS

AM, collected the literatures and wrote the manuscript; YA proof-read, finalized, and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01346/full#supplementary-material

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REVIEW
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Silicon-mediated Improvement in Plant Salinity Tolerance: The Role of Aquaporins

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Silicon (Si) is an abundant and differentially distributed element in soils that is believed to have important biological functions. However, the benefits of Si and its essentiality in plants are controversial due to differences among species in their ability to take up this element. Despite this, there is a consensus that the application of Si improves the water status of plants under abiotic stress conditions. Hence, plants treated with Si are able to maintain a high stomatal conductance and transpiration rate under salt stress, suggesting that a reduction in Na^+ uptake occurs due to deposition of Si in the root. In addition, root hydraulic conductivity increases when Si is applied. As a result, a Si-mediated upregulation of aquaporin (PIP) gene expression is observed in relation to increased root hydraulic conductivity and water uptake. Aquaporins of the subclass nodulin 26-like intrinsic proteins are further involved in allowing Si entry into the cell. Therefore, on the basis of available published results and recent developments, we propose a model to explain how Si absorption alleviates stress in plants grown under saline conditions through the conjugated action of different aquaporins.

Keywords: silicon, aquaporins, nutrient uptake, abiotic stress, salinity stress, water relations, water use efficiency

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INTRODUCTION

The uptake of mineral nutrients is regulated by transporters in the root plasma membranes. In general, there is a strong interaction between the uptake of ions and water uptake, since both are dependent on each other. Therefore, the interactions between water transporters (aquaporins) and nutrients transporters need to be determined in root cells. Nutrient deprivation or excess due to changing environmental conditions usually involves fundamental parameters, including the water relations in plants, in which aquaporins play an important role. One of the first pieces of evidence regarding water-nutrient connections was found in plants deprived of nitrogen and phosphorus, in which there was a reversible reduction of cell and root hydraulic conductivity involving aquaporins (Carvajal et al., 1996). It was also reported that, when a plant is subjected to nutrient deficiency, alterations in aquaporins slow the movement of water through the plant (Clarkson et al., 2000; Shaw et al., 2002). The balance of nutrient supply received by roots can be regulated by aquaporins and ATPase and Ca-ATPase activities (Martínez-Ballesta et al., 2003; Cabañero et al., 2006). Therefore, it was suggested that aquaporins can play a central role in nutrient homeostasis, which is likely to comprise (i) support for ion fluxes through provision of an accompanying water flow and (ii) active re-direction of apoplastic/symplastic water flow within tissues and the whole plant (Maathuis et al., 2003).

Plant aquaporins belonging to the MIP (membrane intrinsic proteins) family are mainly homotetrameric transmembrane proteins that facilitate water transport through membranes, but

they can also form heterotetramers (for review see Martínez-Ballesta and Carvajal, 2016). The classification of aquaporins into seven subfamilies is mostly based on phylogenetic distribution, while their localization in different membranes will be used for the nomenclature (e.g., PIP, TIP, and NIP). In addition to facilitating water diffusion, a number of aquaporins have also been shown to transport other molecules (Gerbeau et al., 1999; Bienert et al., 2011). During the last decade it was reported that aquaporins transport specific solutes, like urea (Liu et al., 2003), ammonia (Bertl and Kaldenhoff, 2007), carbon dioxide (Uehlein et al., 2003), hydrogen peroxide (Bienert et al., 2007), lactic acid (Choi and Roberts, 2007), boric acid (Takano et al., 2006), and silicic acid (Ma et al., 2006). The trafficking and subcellular relocalization of aquaporins could be the critical point in the regulation of the transport of mineral nutrients to the cytoplasm, since aquaporins are translocated from the endoplasmic reticulum (ER) to the plasma membrane via the Golgi apparatus (Maurel et al., 2009). However, the molecular and cellular mechanisms underlying the interactions of aquaporins and mineral nutrients still need to be investigated.

Silicon (Si), the second most abundant element in the earth's crust but its essentiality in plant growth and development remains debated since plants differ widely in their ability to take up Si (Sommer et al., 2006). Silicic acid, $Si(OH)_4$, is the only form known to be absorbed by plants (Ma and Yamaji, 2006). It will enter plant roots mainly by diffusion via the apoplastic pathway but requires the presence of specific aquaporins, NIP2s, to enter the symplastic pathways and be eventually translocated to aerial organs via the xylem (Guerriero et al., 2016).

Although Si is generally considered non-essential for plants, some species will accumulate between 1 and 5% on a dry weight basis. Families such as the Poaceae (grasses), most species of monocotyledons, aquatic macrophytes, and some dicotyledons, including the Cucurbitaceae (Rogalla and Römheld, 2002; Piperno, 2006; Schoelynck et al., 2012) have all been shown to accumulate high concentrations and benefit from Si presence. However, even in non-accumulating plants, the presence of Si in nutrient solutions or soils has been reported to be beneficial against abiotic stress (e.g., NaCl; for a review see Zhu and Gong, 2014), but the mechanisms of Si action in relation to water uptake and aquaporins are poorly understood. In this review, the improvement of plant salinity tolerance by Si through enhancement of root water uptake, including the regulation of aquaporin activity and gene expression, is discussed.

Si NUTRITION AND SALINITY STRESS

Si is generally considered non-essential for plant development, but many authors consider Si a 'quasi-essential' element for higher plants, since plant growth may be stimulated by the supply of Si and Si-starved plants may display physical abnormalities (Rafi and Epstein, 1997; Epstein and Bloom, 2005; Ma and Yamaji, 2008). It can enhance growth, yield, and crop quality, particularly under biotic and abiotic stresses, such as herbivory, leaf microbial pathogens, UV radiation, gravity, extreme temperatures, lodging, metal toxicity, nutrient deficiency and toxicity, drought, and salinity (Epstein, 1994, 1999, 2009; Ma and Takahashi, 2002; Richmond and Sussman, 2003; Cooke and Leishman, 2011; Van Bockhaven et al., 2013; Liang et al., 2015).

Salinity stress is an important factor that limits crop yields and productivity worldwide, affecting approximately 800 million hectares (ha) of arable land (FAO, 2008). Although our understanding of the role of Si in abiotic stress resistance is still limited, important advances with regard to salinity stress have been made (Rengasamy, 2010). In fact, it has been widely reported that the provision of Si increases salt tolerance and hence biomass in many important crops grown under different conditions, such as barley (Liang et al., 2005a), wheat (Tuna et al., 2008; Ali et al., 2012; Bibordy, 2014), rice (Gong et al., 2006; Mahdieh et al., 2015), soybean (Lee et al., 2010) sugarcane (Ashraf et al., 2010), tomato (Romero-Aranda et al., 2006; Muneer et al., 2014; Li et al., 2015), and cucumber (Khoshgoftarmanesh et al., 2014), among others.

Na^+ and K^+ Homeostasis

At high salt concentrations, one of the main salt-tolerance mechanisms is the maintenance of low intracellular Na^+ concentration by the reduction of Na^+ influx and/or the increase of Na^+ efflux. Na^+ enters roots passively, via non-selective cation channels and trough other Na^+ transporters such as HKT (high-affinity K^+ transporter) family; consequently, Na^+ is critical to maintain intracellular K^+ concentration (Blumwald, 2000; Munns and Tester, 2008; Kronzucker et al., 2013). It has been shown that Si may alleviate salinity stress by affecting Na^+ and K^+ concentrations (Ashraf et al., 2010). They found interactive effects of NaCl, Si, and genotype, on Na^+, K^+, and the K^+/Na^+ ratio (a salt-stress indicator) in sugarcane. In this study, the addition of Si reduced Na^+ uptake and transport to the shoots and increased the shoot K^+ concentration, with a resultant increase in the K^+/Na^+ ratio. Similarly, Xu et al. (2015) and Garg and Bhandari (2016) reported that Si application to salt-stressed aloe plants, and sensitive and tolerant genotypes of *Cicer arietinum* L., significantly decreased the Na^+ content in roots and its translocation to leaves, while improving K^+ uptake, consequently raising the K^+/Na^+ ratio.

Effects on Nutritional Balance

It is important to point out that one of the main deleterious effects of salinity is an imbalance in essential nutrients. Recent studies on the plant ionome have shown that salinity causes modifications of the tissue levels of macronutrients like N, Ca, P, S, and Mg, and micronutrients such as Zn, Mn, Fe, and B. Hellal et al. (2012) reported increased N, P, and Ca concentrations in the shoots and seeds of fava bean grown under salt stress when Si was supplied. Similarly, Si enhanced the P, Ca, and Mg contents in leaves and roots of aloe and tomato plants (Li et al., 2015; Xu et al., 2015), and maintained higher P and Fe contents in salt-stressed canola plants (Farshidi et al., 2012). Application of Si significantly increased the Ca concentration in shoots of cucumber plants exposed to salinity, while it had no effect on the shoot Ca concentration of plants grown under non-saline conditions (Khoshgoftarmanesh et al., 2014). By contrast, the supply of Si decreased the S content in *Zinnia elegans* exposed

to salinity stress. However, the salinity-induced reduction of micronutrients such as Zn, Mn, Fe, and B was alleviated by Si addition (Manivannan et al., 2015). In previous reports, NaCl stress was found to increase Cu levels in several plant species (Wang and Han, 2007), but in the study by Manivannan et al. (2015), the level of Cu was not affected. These studies provided evidence that Si might induce salt tolerance in many crops, not only via inhibition of Na^+ uptake and translocation, and improvement of the plant K^+ content, but also by affecting the plant status of some other essential nutrients in order to maintain normal physiological conditions. A summary of the relationships between Si and different inorganic ions in plants grown under salinity stress is shown in **Table 1**.

Protection from Oxidative Damage

Plants produce low levels of reactive oxygen species (ROS), which form part of the chemical communication in cells. However, salinity also inhibits plant growth via an overproduction of ROS that can damage macromolecules essential for plant growth and development, such as DNA or lipid membranes. It has been demonstrated recently that Si mitigates oxidative stress by stimulation of antioxidants, both enzymatic and non-enzymatic (Savvas and Ntatsi, 2015), such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APx), peroxidases (POD), glutathione (GSH), and ascorbate (AA). Many authors have reported the beneficial effects of Si with regard to amelioration of salt-induced oxidative stress. Li et al. (2016) showed that the provision of Si in Hoagland's solution at 1, 2, 4, or 6 mM increased the POD activity of *Glycyrrhiza uralensis* seedlings grown under salt stress, after 20 days of treatment. In this study, SOD activity was intensified only at 4 mM Si and the malondialdehyde (MDA) concentration was significantly decreased at all Si levels, compared with the saline control (50 mM NaCl). Garg and Bhandari (2016) showed that the oxidative markers O_2^-, H_2O_2, and MDA were more abundant in *Cicer arietinum* genotypes subjected to long-term salinity, but their levels declined when 4 mM Si was supplied. Additionally, SOD, CAT, guaiacol peroxidase (GPOX), APx, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and AA were increased in salt-stressed plants of both genotypes by Si supplementation. Likewise, Li et al. (2015) reported increased MDA and H_2O_2 concentrations and decreases in SOD and CAT activities in salt-stressed tomato seedlings grown under sand culture; however, Si application reversed all these stress-induced changes. In contrast, Bibordy (2016) found that SOD and CAT activities were suppressed by the supply of Si (2 or 4 g L^{-1}) to canola plants grown under saline conditions. Although differing plant responses to salt stress have been demonstrated, Si supplementation, generally, seems to lead to a decline in ROS production and an increase in ROS scavenging enzymes and antioxidant compounds. Hence, at the cellular level, Si might ameliorate salinity-induced oxidative stress due to more efficient use of ROS-scavenging metabolic pathways, which may increase membrane integrity. This also might be related with a better Na^+-K^+ cellular status and an improvement of the plant ionome.

TABLE 1 | Summary of the relationship between Si and different inorganic ions in plants subjected to salt stress.

Inorganic ions	Relation	Plant species	Reference
Na	Antagonism	*Saccharum officinarum*	Ashraf et al., 2010
		Aloe vera	Xu et al., 2015
		Cicer arietinum	Garg and Bhandari, 2016
K	Synergism	*Saccharum officinarum*	Ashraf et al., 2010
		Aloe vera	Xu et al., 2015
		Cicer arietinum	Garg and Bhandari, 2016
N	Synergism	*Vicia faba*	Hellal et al., 2012
P	Synergism	*Vicia faba*	Hellal et al., 2012
		Aloe vera	Xu et al., 2015
		Solanum lycopersicum	Li et al., 2015
Ca	Synergism	*Vicia faba*	Hellal et al., 2012
		Aloe vera	Xu et al., 2015
		Solanum lycopersicum	Li et al., 2015
		Cucumis sativus	Khoshgoftarmanesh et al., 2014
Mg	Synergism	*Vicia faba*	Hellal et al., 2012
		Ale vera	Xu et al., 2015
S	Antagonism	*Zinnia elegans*	Manivannan et al., 2015
Zn	Synergism	*Zinnia elegans*	Manivannan et al., 2015
Mn	Synergism	*Zinnia elegans*	Manivannan et al., 2015
Fe	Synergism	*Zinnia elegans*	Manivannan et al., 2015
		Brassica napus	Farshidi et al., 2012
B	Synergism	*Zinnia elegans*	Manivannan et al., 2015
Cu	No relation	*Zinnia elegans*	Manivannan et al., 2015

Photosynthesis and Osmoregulation

Salt stress decreases the physiological cell activities involved in photosynthesis (Garg and Bhandari, 2016), mostly due to osmotic stress, nutritional imbalance, and/or nutritional toxicity combined with later oxidative stress. However, recent evidence indicates that Si influences photosynthesis through effects on water uptake and transport. Mateos-Naranjo et al. (2013) showed that the negative effect of high salinity on gas exchange, water-use efficiency (WUE), pigment concentrations (Chl*a* and Chl*b*), and PSII efficiency, was reversed by Si supply for the halophytic grass *Spartina densiflora*. On the other hand, Abbas et al. (2015) reported that Si application enhanced the stomatal conductance, transpiration rate, number of stomata, and stomatal size in salt-sensitive and salt-tolerant okra plants. A complementary protective mechanism of plants growing under saline conditions is the synthesis and accumulation of different osmolytes and compatible solutes. Although this is dependent on the plant species, Si has been found to enhance the contents of proline (Tuna et al., 2008; Soylemezoglu et al., 2009; Siddiqui et al., 2014), soluble protein (Li et al., 2015), polyamines (Wang S. et al., 2015; Yin et al., 2016), glycine betaine, total free amino acids, soluble sugars, and phenolic compounds (Abbas et al., 2015).

In summary, the potential correlation between the application of Si and benefits for plants under saline conditions are: (i) maintenance of the status of essential nutrients, by reduction of Na^+ content and improvement of K^+ content, (ii) greater efficiency of ROS-scavenging metabolic pathways and (iii) increase of gas exchange. All these mechanism are related to

water relations and water-use efficiency as it will be reviewed as follows.

EFFECT OF Si ON WATER UPTAKE AND TRANSPORT

In accumulating species, Si has been assigned an unspecific function in crop protection, since it seems to be involved in structural and dynamic aspects of plant responses that help diminish the deleterious effect (Epstein, 2001). In fact, it is generally agreed that the positive effects of Si are more manifest under conditions of stress. For example, Yeo et al. (1999) indicated that Si could decrease Na uptake by plants under salinity stress. Studies with toxic metals such as Al indicated that silicified tissues may give protection against these metals through co-deposition of Al with Si in some monocotyledons (Sangster et al., 2001).

Accumulation of Si could occur actively (Liang et al., 2005a; Rains et al., 2006) or passively, the latter depending on the transpiration rate as described formerly (Takahashi et al., 1990). However, there are some plants that are excluders (Henriet et al., 2006; Carey and Fulweiler, 2014). The Si/Ca ratio has been reported to be indicative of the Si uptake mechanism (Carey and Fulweiler, 2014): ratios exceeding 1 indicate active uptake, ratios of 0.5–1 suggest passive uptake, and ratios below 0.5 could show exclusion. Also, another indicator proposed is the relationship between the Si availability around the root and the Si concentration inside the plant (Carey and Fulweiler, 2014). However, these indicators could change under saline conditions that alter Ca uptake and transpiration.

Recent studies have clearly established that Si uptake in plants is dependent on an influx channel-type transport, the Lsi1 channel, responsible for Si movement from the external solution into the internal cells. The first Si transporter was identified in rice (Ma et al., 2006), and subsequent studies have shown that this transporter was present in all Si-accumulating species including monocots such as barley, wheat, and maize (Yamaji et al., 2008, 2012; Chiba et al., 2009; Mitani et al., 2009; Montpetit et al., 2012) – and dicots, such as cucumber, pumpkin and soybean (Mitani-Ueno et al., 2011; Deshmukh et al., 2013; Wang H.S. et al., 2015). In addition, another transporter found in rice (Ma et al., 2007) and in a few other species, termed Lsi2, acts as an active efflux transporter carrying Si to the xylem (Ma and Yamaji, 2015). It is important in the long-distance transport of Si through the plant. Much less is known about the nature and properties of Lsi2s and they have only been described so far in monocotyledons and horsetail (Vivancos et al., 2016). Although, in general, Lsi1 and Lsi2 are localized in the plasma membrane, the distribution differs among species; in fact, in rice they are found in the exodermis and endodermis in the mature regions of the main and lateral roots (Ma et al., 2006), while in other monocotyledons such as maize and barley they are localized in epidermal, hypodermal, and cortical cells (Chiba et al., 2009; Mitani et al., 2009). In dicots, the pumpkin CmLsi1 is found in all root cells (Mitani et al., 2011) and the cucumber, CsLsi1 has been recently localized in endodermal and cortical cells in root tips and in root hairs (Sun et al., 2017). Therefore, the localization of Si transporters in the roots could be an important factor that determines how Si influences water uptake and, therefore, the sensitivity of the plant to salinity.

Silicon has been described as a protective element against abiotic stress like salinity, on the basis of its induction of changes in lignin and suberin processing and deposition, which reduces the rates of water loss and evapotranspiration (Cruz et al., 1992; Sonobe et al., 2009; Amin et al., 2016). Along the same lines, Si has been reported to increase lignification in sorghum, thereby increasing xylem resistance to water loss (Hattori et al., 2005). Some studies have also suggested that Si could induce a thicker cuticle in leaves of rice and sorghum, reducing stomatal conductance and decreasing water loss through the epidermal layer and thus maintaining the water potential in leaves (Matoh et al., 1991; Hattori et al., 2005). Furthermore, Si has been reported to improve the regulation of stomatal opening, although the mechanism behind this has not been resolved (Gao et al., 2006). Also, Gao et al. (2004) found that Si increased the water use efficiency in maize due to induction of root hydraulic conductance.

Other results have shown that Si improved the response to abiotic stress when water availability was reduced. In sorghum plants for instance, when Si was applied to the nutrient solution, there was an increase of water uptake and water flow from roots to leaves, together with an increase of stomatal conductance (Sonobe et al., 2009). Hattori et al. (2008) indicated that Si application enhanced root hydraulic conductance, and Sonobe et al. (2009) suggested that the improvement of this parameter could occur in a radial direction in the root (by modification of osmotic characteristics or expression of aquaporins) rather than axially (via modification of the number or diameter of xylem vessels). Therefore, the influence of Si on transpiration and its role in the physiology of the stomata are controversial (Agarie et al., 1998; Gao et al., 2006). It is clear that, under water deficiency, if Si only reduced transpiration, an increase in water use efficiency followed by protection against wilting would occur (Gao et al., 2004). However, if transpiration is increased, accompanied by higher root hydraulic conductance (Sonobe et al., 2009), the water use efficiency will also increase. The role of Si in water relations thus seems to be associated with the maintenance of water use efficiency, but at the moment there is not enough evidence to propose a model that clarifies this response. Therefore, future studies should focus on this matter.

The link between water stress and Si has been well studied in tomato where Romero-Aranda et al. (2006) showed that Si enhanced drought resistance in tomato plants as a result of an increase in leaf water content. Shi et al. (2016) showed that the effects of Si not only increased root hydraulic conductance in tomato, but also maintained membrane integrity and protected it against oxidative damage by an increase of the antioxidant metabolism. Furthermore, several studies on Si and drought stress in different species, including sorghum, maize, and tomato, concluded that Si alleviated the effect of the stress (Agarie et al., 1998; Hattori et al., 2008; Sonobe et al., 2009; Shi et al., 2016). However, although insights into its mechanism of action have not been provided yet, the fact that all reported results concluded

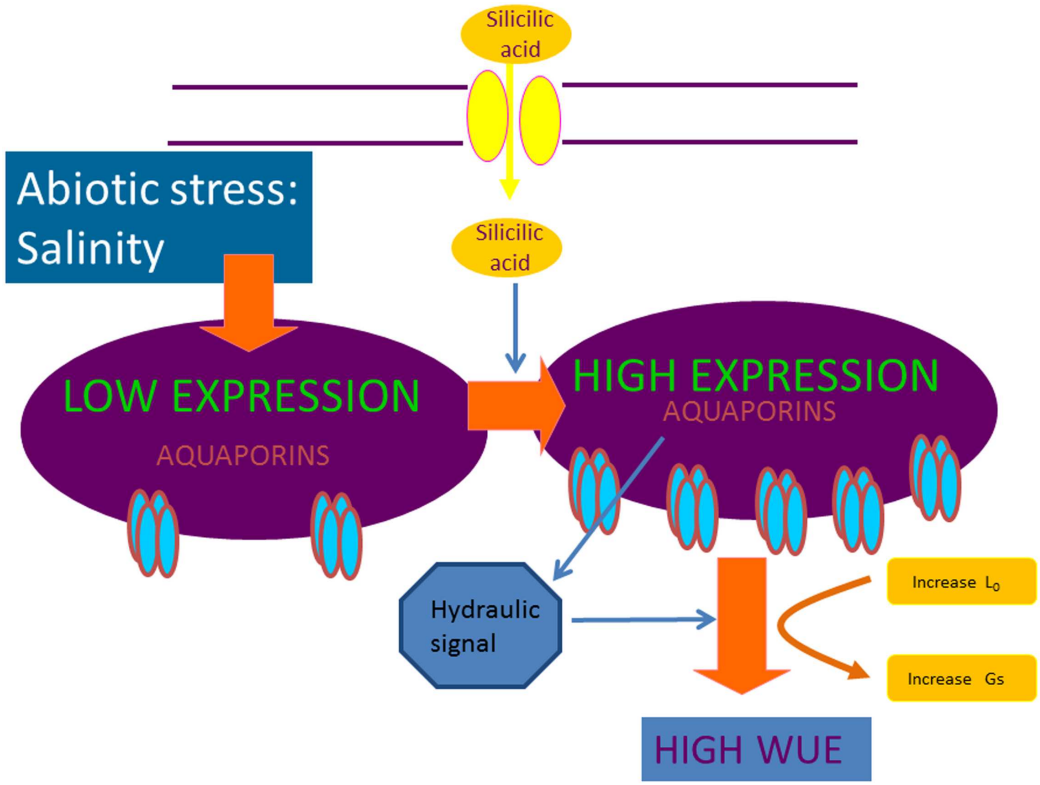

FIGURE 1 | Under salinity stress, Si has been reported to improve stomatal functioning and enhance root hydraulic conductance. The physiological mechanism could involve hydraulic signaling through aquaporin expression, leading to higher water-use efficiency.

that water relations are improved, supports the notion that aquaporins and hydraulic signals are involved (**Figure 1**).

AQUAPORINS AND Si

Aquaporins belong to the major intrinsic protein (MIP) family and allow the transport of water and small solutes through biological membranes (Chrispeels and Maurel, 1994; Kruse et al., 2006). In plants, they are classified into different subfamilies: the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the nodulin26-like intrinsic proteins (NIPs), the small basic intrinsic proteins (SIPs), the uncategorized intrinsic proteins (XIPs), the GIPs, and the hybrid intrinsic proteins (HIPs) (Danielson and Johanson, 2008), according to their subcellular localization, function, sequence length, and substrate selectivity (for review, see Maurel et al., 2015).

Aquaporins have been reported to transport distinct types of substrates, such as ammonia, antimony, arsenite, boron, carbon dioxide, formamide, glycerol, hydrogen peroxide, lactic acid, silicon, and urea (Bienert and Chaumont, 2011; Hove and Bhave, 2011). The substrate selectivity regarding the transported molecule is determined by two factors: the NPA motifs, responsible for proton exclusion, and the aromatic/arginine (ar/R) region, that functions as the main filter in the pore (Wu and Beitz, 2007). Because of this, MIPs facilitate the transport of the widest range of solutes, including several metalloids. Different isoforms belong to the NIP subfamily: the NIP1 subgroup, that is more permeable to water and glycerol, the NIP2 subgroup, that transports metalloids and is the only aquaporin subgroup able to transport Si (Mitani-Ueno et al., 2011), and the NIP3 subgroup, that is notable for its biological function in boric-acid transport. All subgroups are permeable to formamide (Dean et al., 1999; Wallace and Roberts, 2005).

It has been reported that Si plays an important role in the mechanisms that enable plants to cope with biotic and abiotic stresses (Vivancos et al., 2016). However, the capacity to transport Si depends on the plant genotype. A mechanism combining efflux (Lsi2) and influx (Lsi1) Si transporters has been reported to regulate Si accumulation in different cell compartments and plant organs and tissues (Deshmukh et al., 2015). Lsi1, a NIP2 homolog aquaporin and Si-influx transporter, was first identified in rice (Ma et al., 2006), and is conserved among different plant species. Also in rice, NIP2;2 (Lsi6) was classified as a Si transporter which enables silicic acid to pass from the xylem to leaves (Yamaji et al., 2008). Furthermore, NIP2 aquaporins have been identified only in plants where Si has a beneficial role in plant nutrition.

A peculiarity of NIPs that transport Si is their expression profile, in which they are situated on the distal side of the root endodermis plasma membrane (Ma et al., 2006; Chiba et al., 2009; Mitani et al., 2009; Yamaji and Ma, 2009). This allows cooperation with other Si transporters, such as the active Lsi2 efflux

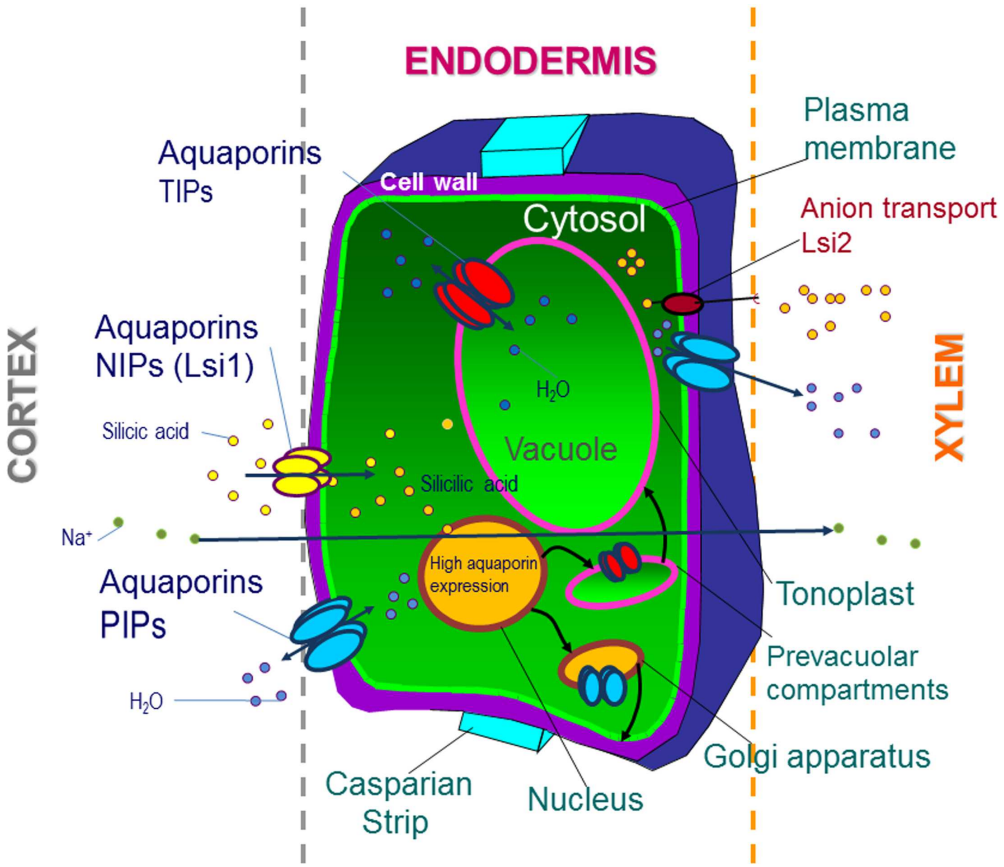

FIGURE 2 | Aquaporin regulation by Si under salt stress in the root endodermis. Silicic acid enters the plant roots by water flow via the apoplastic and symplastic pathways: the symplastic pathway involves the action of aquaporins, mainly NIPs (Lsi1) localized on the distal membrane side of the efflux transporter Lsi2. NIPs allow Si accumulation in the transpiration stream, impeding Na^+ accumulation. In addition, Si increases the expression of the root PIP aquaporin subfamily and therefore enhances root hydraulic conductance under salinity, optimizing water transport in the cell. This, together with a decreased osmotic potential of the root sap due to Si-dependent osmolyte accumulation, allows for an increase in water uptake under stress. Coordination between PIPs and TIPs is responsible for the water balance during osmotic adjustment.

transporters, situated on the proximal side of the membrane. It has been postulated that this cell polar localization of NIPs takes place when these aquaporins have a direct role in Si uptake and translocation (Pommerrenig et al., 2015). Therefore, localization of Si transporters on different sides of the cell membrane may allow optimization of the directional Si flux. Deshmukh et al. (2015) determined that the ability to transport Si was determined by a GSGR amino-acid motif in the selectivity filter of the NIP subfamily, and when this amino-acid group was confined within a specific distance from the NPA domains. This may explain the lack of Si accumulation in some plant species such as tomato (Deshmukh et al., 2015), where an inadequate amino-acid distance between the NPA motifs is observed (Deshmukh et al., 2015). Also, a larger constriction size in the pore of NIP2 proteins, relative to other NIP subgroups, is responsible for Si transport. Furthermore, in graminaceous plants, *NIP2:1* orthologs have been described as Si transporters involved in its distribution and reallocation within these plants (Chiba et al., 2009; Mitani et al., 2009). In horsetail (*Equisetum arvense*), one of the species in the plant kingdom that accumulates high amounts of Si, Si channels of the NIP subfamily were identified. These results point out the complexity of Si uptake and distribution in the whole plant, since the ability to take up Si does not depend solely on the aquaporins, but also on the presence of active transporters (Deshmukh and Bélanger, 2016).

A dual role of aquaporins under salt stress, in the presence of Si, can be described. On the one hand, members of the PIP subfamily may act as regulators of plant water balance, and, on the other hand, the NIP subfamily can participate in Si uptake and cell levels. However, the mechanisms by which Si alleviates salinity stress via aquaporin regulation need a deeper investigation. It has been reported that Si is able to reduce Na^+ and Cl^- uptake and translocation to the shoot in barley (Liang et al., 2005b), alfalfa (Wang and Han, 2007), wheat (Tuna et al., 2008), soybean (Lee et al., 2010), and rice (Gong et al., 2006; Shi et al., 2013) under salinity. In rice, a typical Si-accumulating species, inhibition of Na^+ and Cl^- accumulation by Si may not involve a reduction of the transpiration stream, since Gong et al. (2006) found that stomatal conductance and transpiration were increased. Silicon formed

a physical barrier in the endodermal and exodermal Casparian bands, reducing the translocation of these ions. But, whether a similar mechanism occurs in other Si accumulators must be elucidated. In tomato, a Si-excluder, the levels of Na^+ and Cl^- were maintained in the plant in the presence of Si, despite the reduction of the adverse effects produced by salinity (Romero-Aranda et al., 2006). The ameliorative effect of Si on NaCl stress has been related to osmotic stress alleviation, with the involvement of aquaporins as regulators of plant water status, rather than a palliative effect on ion toxicity (Liu et al., 2015). Therefore similar beneficial effects appear to be observed in both Si-accumulating and non-accumulating plants.

In *Sorghum bicolor* L., Si regulated the expression of the PIP aquaporins, under short-term salt-stress exposure, which restored the root hydraulic conductance, L_p, lost due to salinity. This allowed the plants to maintain their water content and rate of photosynthesis. Silicon alleviated the osmotic effect of salinity without the appearance of symptoms of Na^+ toxicity in the plants (Liu et al., 2014; 2015). In addition, it has been observed that salt stress may reduce L_p through the inactivation of aquaporins by H_2O_2 (Boursiac et al., 2008). Silicon may enhance L_p by decreasing H_2O_2, which affects not only expression, but also PIP activity (Liu et al., 2015). Since H_2O_2 promotes the internalization of PIPs from the plasma membrane under salinity (Boursiac et al., 2008), an influence of Si on PIPs trafficking cannot be discounted.

It has been reported that Si may promote the development of suberized structures in the root endodermis and exodermis (Fleck et al., 2015). Apoplastic Na^+ transport would be thus reduced, preventing the accumulation of this ion in the plant shoot (Krishnamurthy et al., 2011). This may lead to a reestablishment of the expression of PIPs, in order to maintain the water flux through the symplastic route.

Zhu et al. (2015) observed the effect of Si on two cucumber (*Cucumis sativus* L.) cultivars under salinity. In their work, Si increased the expression of the root PIP2 subfamily and decreased the osmotic potential by an increase in the root sugar content, which favored water uptake. The authors concluded that osmotic adjustment by the plants, to acquire water under salt stress, was a mechanism initiated after Si addition that developed differentially in the two cucumber genotypes.

Members of the NIP subfamily may influence plant responses to salinity through controlled Si uptake and transport. For two varieties of rice, the expression of the *OsLsi1* gene, a NIP2 homolog, increased under salt stress, but was higher in the tolerant cultivar compared to the sensitive cultivar, inducing greater Si uptake in the former (Senadheera et al., 2009). In this case, the authors described Si accumulation via the transpiration stream as a mechanism to reduce NaCl transport to the aerial parts of the plant. However, the response of *OsLsi1* expression to the addition of Si alone was the opposite of that which was observed with salinity alone, and so the study of the combination of these two factors in species with high water demand is critical (Senadheera et al., 2009).

Reduction of water uptake and transport induced by salinity stress appears to be alleviated by Si as a function of aquaporin activity. Indeed, NIP aquaporins promotes Si entrance into the cell, which increases the expression of root PIP aquaporin subfamily. This effect enhances root hydraulic conductance enabling an optimal water transport and reduction of Na^+ accumulation (**Figure 2**). On the other hand, this possible mode of action is not found to be directly associated with other secondary effects such as osmotical adjustment (Pei et al., 2010; Ming et al., 2012; Liu et al., 2014) or oxidative-stress amelioration (Shi et al., 2016). Therefore, the direct role of Si on the regulation of aquaporin functionality needs further validation.

CONCLUDING REMARKS

The evidence that Si promotes salinity tolerance via enhancement of root hydraulic conductance and water uptake, thereby contributing to increased water use efficiency, underlines the importance of studying Si uptake mechanisms and their regulation. Furthermore, if the beneficial effects of Si, in both monocotyledons and dicotyledons, are linked to the passage of water through membranes, future studies should concentrate on the influence of Si on aquaporin expression, particularly under abiotic stress conditions. Recent findings suggest that water relations involving aquaporins are the key point in the amelioration of the adverse effects of salinity stress. Considering that Si transport is also mediated by aquaporins (NIP2), this suggests that stimulation of the Si uptake system in plants could lead to a new approach to PIP aquaporin up-regulation, which in turn will reduce Na^+ conglomeration in membranes and increase water uptake and transport. However, to further elucidate Si accumulation and understand its critical role at the whole plant level, molecular and physiological characterization of Si-transporting aquaporins in different plant species is required. The importance of the NIP2 aquaporins subgroup as Si transporters in plants highlights that aquaporins could be the subject of biotechnological intervention to produce salinity tolerant plants and cultivars biofortified with Si.

AUTHOR CONTRIBUTIONS

JJR, MM-B, JMR, and BB contribute to writing. MC contribute to writing, correction and production of figures.

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REVIEW
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Role of Silicon on Plant–Pathogen Interactions

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Although silicon (Si) is not recognized as an essential element for general higher plants, it has beneficial effects on the growth and production of a wide range of plant species. Si is known to effectively mitigate various environmental stresses and enhance plant resistance against both fungal and bacterial pathogens. In this review, the effects of Si on plant–pathogen interactions are analyzed, mainly on physical, biochemical, and molecular aspects. In most cases, the Si-induced biochemical/molecular resistance during plant–pathogen interactions were dominated as joint resistance, involving activating defense-related enzymes activates, stimulating antimicrobial compound production, regulating the complex network of signal pathways, and activating of the expression of defense-related genes. The most previous studies described an independent process, however, the whole plant resistances were rarely considered, especially the interaction of different process in higher plants. Si can act as a modulator influencing plant defense responses and interacting with key components of plant stress signaling systems leading to induced resistance. Priming of plant defense responses, alterations in phytohormone homeostasis, and networking by defense signaling components are all potential mechanisms involved in Si-triggered resistance responses. This review summarizes the roles of Si in plant–microbe interactions, evaluates the potential for improving plant resistance by modifying Si fertilizer inputs, and highlights future research concerning the role of Si in agriculture.

Keywords: silicon, plant–pathogen interactions, physical, biochemical, molecular, defense response

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INTRODUCTION

Silicon (Si) is the second most abundant element after oxygen in the earth's crust, and comprises up to 70% of soil mass (Epstein, 1994; Savant et al., 1997; Ma and Yamaji, 2006). Si was initially not recognized as an essential element for higher plants, although it was known to be beneficial for plant growth and production. Its accumulation among plant species differs greatly, due to differences in root Si uptake capacity (Takahashi et al., 1990). Generally, Si uptake takes place through plant roots as silicic acid [$Si(OH)_4$], an uncharged molecule (Ma and Yamaji, 2006), and passes through the plasma membrane via two Si transporters, Lsi1 and Lsi2, which function as influx transporters and efflux transporters, respectively (Ma et al., 2006, 2007, 2008).

Numerous studies show that Si accumulates in plants and exerts various beneficial effects for many plant species, especially gramineous plants such as rice and sugarcane and some cyperaceous plants (Epstein, 1994, 1999; Liang, 1999; Liang et al., 2005b). Absorbed Si is mainly deposited in

cell walls, and is also involved with stress-related signaling systems (Fauteux et al., 2005). Si is helpful for improving the mechanical and physiological properties of plants and contributes to plants overcoming many biotic and abiotic stresses (Epstein, 1999; Richmond and Sussman, 2003; Ma, 2004; Ma and Yamaji, 2006). For example, Si enhances resistance to diseases caused by fungi, bacteria, and pests (Fauteux et al., 2005; Marschner, 2012), as well as exerting alleviative effects on various abiotic stresses including lodging, drought stress, salt stress, water logging, metal toxicity, nutrient imbalance, radiation damage, high temperature, freezing, and UV in a wide variety of plant species (Epstein, 1994, 1999; Savant et al., 1997; Ma, 2004; Ma and Yamaji, 2006; Liu et al., 2014; Coskun et al., 2016).

Many studies have focused on the role of Si on plant–microbe interactions and enhanced host resistance to a range of microbial pathogens by stimulating defense reactions (Remus-Borel et al., 2005; Cai et al., 2008; Ghareeb et al., 2011; Ye et al., 2013). However, the mechanistic basis and regulation of Si-mediated disease resistance are still poorly understood. Furthermore, the underlying mechanisms of Si regulated plant–microbe interactions have not been identified so far in higher plants. In this review, the effect of Si on plant–microbe interactions are discussed, and the physical, biochemical, and molecular regulatory mechanisms of Si on plant disease resistance are extensively reviewed.

Plant diseases are a major threat to agricultural production as they cause serious loss of crop yield and quality. Numerous studies have reported that Si is effective in controlling diseases caused by both fungal and bacterial pathogens in different plant species (Fauteux et al., 2005; Rodrigues and Datnof, 2015). A priming role of Si has been demonstrated in plant-pathogen interactions and the regulation of Si in plant diseases is summarized in **Table 1**. Si plays a positive role in plant–pathogen interactions and increases plant resistance to disease caused by fungi, bacteria, viruses, and nematodes.

Silicon could alleviate plant disease through preventing pathogen penetration (1) via structural reinforcement (Epstein, 1999; Epstein, 2001; Rodrigues et al., 2015b), (2) by inhibiting pathogen colonization through stimulating systemic acquired resistance, (3) through antimicrobial compound production (Fauteux et al., 2005; Datnoff et al., 2007; Fortunato et al., 2012b; Van et al., 2013), as well as (4) through increasing plant resistance by activating multiple signaling pathways and defense-related gene expression (Fauteux et al., 2005; Chen et al., 2014; Vivancos et al., 2015). The beneficial effects of Si with regard to plant resistance to disease are attributed to Si accumulation in epidermal tissue, the formation of complexes with organic compounds in cell walls, the induction of phenolic compounds, phytolexin/glucanase/peroxidase production, and regulating pathogenicity or stress-related gene expression to limit pathogen invasion and colonization (Belanger et al., 2003; Brunings et al., 2009; Chain et al., 2009; Sakr, 2016). The effect of Si on plant–microbe interactions and related physical, biochemical, and molecular resistance mechanisms have been demonstrated in **Table 1** and will be detailed discussed in the following section.

SILICON-MEDIATED DISEASE RESISTANCE

Physical Mechanisms

The beneficial effects of Si on plant growth are attributed to improved overall mechanical strength and an outer protective layer (Epstein, 1999, 2001; Sun et al., 2010). Successful infection requires plant pathogens to enter the host plant by penetrating physical barriers including wax, cuticles, and cell walls (Schmelzer, 2002; Nawrath, 2006; Łaźniewska et al., 2012).

Silicon-enhanced resistance is associated with the density of silicified long and short epidermal cells, the thick layer of silica under the cuticle, the double cuticular layer, the thickened Si-cellulose membrane, formation of papilla, and complexes formed with organic compounds in epidermal cell walls that strengthen plants mechanically. The physical barriers inhibit pathogen penetration and make plant cells less susceptible to enzymatic degradation caused by fungal pathogen invasion (Inanaga et al., 1995; Fauteux et al., 2005; Datnoff et al., 2007; Van et al., 2013).

Silicon accumulates and, when deposited beneath the cuticle, can form a cuticle-Si double layer to prevent pathogen penetration, thereby decreasing disease incidence (**Figure 1**) (Ma and Yamaji, 2006, 2008). Most Si is cross-linked with hemicellulose in cell walls, which improves mechanical properties and regeneration (He et al., 2015; Guerriero et al., 2016). Si contributes not only to cell-wall rigidity and reinforcement, it also increases cell-wall elasticity during extension growth (Marschner, 2012). In primary cell walls, Si interacts with cell-wall constituents such as pectins and polyphenols, which increase cell-wall elasticity during extension growth (Emadian and Newton, 1989). In rice, Si-induced epidermal cell-wall fortification is associated with reduced severity of blast disease (Kim et al., 2002). Si application restricted hyphael entry to the first-invaded epidermal cell for wheat leaves infected with *Pyricularia oryzae*, while hyphae successfully invaded several neighboring leaf cells when there was no Si treatment (Sousa et al., 2013). A similar result was found in wheat (*Bipolaris sorokiniana*) pathosystem (Domiciano et al., 2013), in which Si supply delayed pathogen ingress into epidermal cells and reduced fungal colonization in foliar tissue. For rice infected with *Pyricularia grisea* and *Rhizoctonia solani*, a decrease in the number of leaf blade lesions was associated with an increased incubation period when Si was deposited on tissue surfaces (Rodrigues et al., 2001; Seebold et al., 2004). Moreover, the number of successful penetrative appressorial sites for *P. oryzae* was decreased in rice supplied with Si, suggesting that the denser Si layer contributed to preventing or delaying pathogen penetration (Hayasaka et al., 2008).

Besides the reinforcement of cell walls by Si, the formation of papillae has also been stimulated by Si during pathogen infection. Silicon accumulation was found to occur in the haustorial neck and collar area of fungus as well as in papillae, which contributed to preventing pathogen invasion (Samuels et al., 1994). Zeyen et al. (1993) demonstrated that

TABLE 1 | Effects of silicon on plant disease and related resistance mechanisms.

Hosts	Diseases	Pathogens	Effects	Reference	Resistance mechanisms
Arabidopsis	Powdery mildew	*Erysiphe cichoracearum, Agrobacterium tumefaciens*	+	Ghanmi et al., 2004; Fauteux et al., 2006; Vivancos et al., 2015	Physical, biochemical and molecular
Banana	Black sigatoka	*Mycosphaerella fijiensis*	+	Kablan et al., 2012	Physical and biochemical
	Fusarium wilt	*Fusarium oxysporum* f. sp. *cubense*	+	Fortunato et al., 2012a	Physical and biochemical
	Root rot	*Cylindrocladium spathiphylli*	+	Vermeire et al., 2011	Biochemical
	Xanthomonas wilt	*Xanthomonas campestris*	+	Mburu et al., 2015	Physical and biochemical
Barley	Powdery mildew	*Blumeria graminis*	+	Wiese et al., 2005	Physical
Bean	Angular leaf spot	*Pseudocercospora griseola*	+	Rodrigues et al., 2010	Physical
Belle pepper	Phytophthora blight	*Phytophthora capsici*	+	French-Monar et al., 2010	Physical
Bentgrass	Dollar spot	*Sclerotinia homoeocarpa*	+	Uriarte et al., 2004; Zhang et al., 2006	Physical and biochemical ?
Bitter gourd	Powdery mildew	*Erysiphe* sp.	+	Ratnayake et al., 2016	Biochemical
Capsicum	Anthracnose	*Colletotrichum gloeosporioides*	+	Jayawardana et al., 2016	Physical and biochemical
Cherry	Fruit decay	*Penicillium expansum, Monilinia fructicola*	+	Qin and Tian, 2005	Biochemical
Chinese cantaloupe	Fusarium root rot	*Fusarium* spp.	+	Liu et al., 2009	Physical and biochemical
	Postharvest pink rot	*Trichothecium roseum*	+	Guo et al., 2007	Physical and biochemical
Coffee	Leaf rust	*Hemileia vastatrix*	+	Carré-Missio et al., 2014	Physical
	Root-knot Nematode	*Meloidogyne exigua*	+	Silva R. et al., 2010	Biochemical
Common bean	Anthracnose	*Colletotrichum lindemuthianum*	+	Polanco et al., 2014; Rodrigues et al., 2015a	Biochemical
Cotton	Fusarium wilt	*Fusarium oxysporum* f. sp. *vasinfectum*	+	Whan et al., 2016	Physical and biochemical
Creeping, turf grass	Brown patch	*Rhizoctonia solani*	+	Uriarte et al., 2004; Zhang et al., 2006	Physical and biochemical?
Cucumber	Crown and root rot	*Pythium ultimum*	+	Chérif et al., 1994	Biochemical
	Fusarium wilt	*Fusarium oxysporum* f. sp. *cucumerinum*	+	Miyake and Takahashi, 1983	Physical and biochemical?
	Powdery mildew	*Sphaerotheca fuliginea, Podosphaera xanthii*	+	Menzies et al., 1991, 1992; Fawe et al., 1998; Liang et al., 2005a	Physical and biochemical
Gerbera daisy	Powdery mildew	*Erysiphe cichoracearum, Podosphaera fusca*	/	Moyer et al., 2008	/
Hami melons	Decay	*Alternaria alternate, Fusarium semitectum, Trichothecium roseum*	+	Bi et al., 2006	Biochemical
Lettuce	Downy mildew	*Bremia lactucae*	+	Garibaldi et al., 2011	Physical and biochemical?
Melon	Bacterial fruit blotch	*Acidovorax citrulli*	+	Conceição et al., 2014	Biochemical
	Powdery mildew	*Podosphaera xanthii*	+	Dallagnol et al., 2015	Biochemical
Muskmelon	Pink rot disease	*Trichothecium roseum*	+	Li et al., 2011	Biochemical
	Powdery mildew	*Sphaerotheca fuliginea*	+	Menzies et al., 1992	Physical and biochemical
Oil palm	Basal stem rot	*Ganoderma boninense*	+	Najihah et al., 2015	Physical
Pea	Brown spot	*Mycosphaerella pinodes*	+	Dann and Muir, 2002	Biochemical
Pearl millet	Downy mildew	*Sclerospora graminicola*	+	Deepak et al., 2008	Physical and biochemical
Perennial ryegrass	Fusarium patch	*Microdochium nivale*	+	McDonagh and Hunter, 2010	Physical
	Gray leaf spot	*Magnaporthe oryzae*	+	Rahman et al., 2015	Biochemical
Potato	Dry rot	*Fusarium sulphureum*	+	Li et al., 2009	Biochemical
Pumpkin	Powdery mildew	*Podosphaera xanthii*	+	Lepolu Torlon et al., 2016	Physical and biochemical?
Rice	Blast	*Pyricularia oryzae, Magnaporthe grisea, Magnaporthe oryzae*	+	Seebold et al., 2000; Kim et al., 2002; Rodrigues et al., 2003; Cai et al., 2008, Hayasaka et al., 2008; Brunings et al., 2009; Domiciano et al., 2015	Physical, biochemical and molecular

(Continued)

TABLE 1 | Continued

Hosts	Diseases	Pathogens	Effects	Reference	Resistance mechanisms
	Brown spot	*Bipolaris oryzae, Cochliobolus miyabeanus*	+	Dallagnol et al., 2011, 2013; Prabhu et al., 2012; Van et al., 2015a	Physical, biochemical and molecular
	Grain discoloration	*Bipolaris oryzae*	+	Prabhu et al., 2012	Molecular
	Leaf scald	*Monographella albescens, Microdochium oryzae*	+	Tatagiba et al., 2016; Araujo et al., 2015	Physical and biochemical
	Sheath blight	*Rhizoctonia solani*	+	Peters et al., 2001; Schurt et al., 2014	Physical and biochemical
Rose	Powdery mildew	*Podosphaera pannosa*	+	Shetty et al., 2012	Physical
Sorghum	Anthracnose	*Colletotrichum sublineolum*	+	Resende et al., 2013	Physical and biochemical ?
Soybean	Phytophthora stem and root rot	*Phytophthora sojae*	+	Guérin et al., 2014	Molecular
	Rust	*Phakopsora pachyrhizi*	+	Cruz et al., 2014; Lemes et al., 2011	Biochemical
St. Augustinegrass	Gray leaf spot	*Magnaporthe grisea*	+	Brecht et al., 2007	Physical and biochemical
Strawberry	Powdery mildew	*Sphaerotheca aphanis*	+	Kanto et al., 2006	Physical and biochemical
Sugarcane	Brown rust	*Puccinia melanocephala*	+	Ramouthar et al., 2015	Physical and biochemical ?
Tall fescue	Brown patch	*Rhizoctonia solani*	–	Zhang et al., 2006	/
Tobacco	Viral infection	*Tobacco ringspot virus*	+	Zellner et al., 2011	Molecular
		Tobacco mosaic virus	/	Zellner et al., 2011	/
Tomato	Bacterial speck	*Pseudomonas syringae*	+	Andrade et al., 2013	Biochemical
	Bacterial wilt	*Ralstonia solanacearum*	+	Ghareeb et al., 2011; Chen et al., 2014	Molecular
	Fusarium crown and root rot	*Fusarium oxysporum* f. sp *radicis-lycopersici*	+	Huang et al., 2011	Physical
Tomato, bitter gourd	Root rot	*Pythium aphanidermatum*	+	Heine et al., 2007	Biochemical and molecular?
Wheat	Blast	*Pyricularia grisea*	+	Filha et al., 2011	Physical and biochemical
	Leaf blast	*Pyricularia oryzae*	+	Silva et al., 2015	Biochemical
	Leaf streak	*Xanthomonas translucens*	+	Silva I.T. et al., 2010	Physical and biochemical
	Powdery mildew	*Blumeria graminis*	+	Chain et al., 2009; Guével et al., 2007; Moldes et al., 2016	Physical, biochemical and molecular
	Spot blotch	*Bipolaris sorokiniana*	+	Domiciano et al., 2010	Physical and biochemical
Zucchini squash	Powdery mildew	*Erysiphe cichoracearum, Podosphaera xanthii*	+	Menzies et al., 1992; Savvas et al., 2009	Physical and biochemical

Positive (+), negative (–) or no effect (/) of silicon on plant resistance to disease. ?, indicates possible defense mechanisms are involved.

barley epidermal cells could produce papillae in response to *Blumeria graminis* f. sp. *hordei* infection during Si application. A similar result has been found in the rose, in which Si supply increased the number of papillae in leaf cells in response to *Podosphaera pannosa* infection (Shetty et al., 2012). The prevalence of papillae after Si treatment could increase rice resistance to blast (Cai et al., 2008), wheat and barley resistance to powdery mildew (Zeyen et al., 1993; Belanger et al., 2003).

Heine et al. (2007) reported that the ability of Si to inhibit fungal spread in root apices is dependent on the uptake of Si into root symplasts. Further, the accumulation of Si on root cell walls did not represent a physical barrier to the spread of *Pythium aphanidermatum* in tomato or bitter gourd roots. In cucumber plants, Si foliar application could increase cucumber resistance to powdery mildew via physical barrier and osmotic effects, but Si root application can induce systemic resistance (Liang et al., 2005a). Taken together, Si, which is deposited in the wax, cuticle, and cell wall, as well as papillae, contributes in part to increased physical resistance against pathogen penetration. However, it is suggested that biochemical resistance to pathogens, as regulated by Si, is more complex than physical resistance alone; this has been strongly contested in recent years.

Biochemical Mechanisms

Silicon-enhanced biochemical resistance is associated with (1) increasing the activity of defense-related enzymes, such as polyphenoloxidase, glucanase, peroxidase, and phenylalanine ammonia-lyase (PAL); (2) inducing antimicrobial compounds production, such as phenolic, flavonoids, phytoalexins and pathogenesis-related (PR) proteins in plants; and (3) regulating systemic signals, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET; Fauteux et al., 2005; Datnoff et al., 2007; Fortunato et al., 2012b; Van et al., 2013).

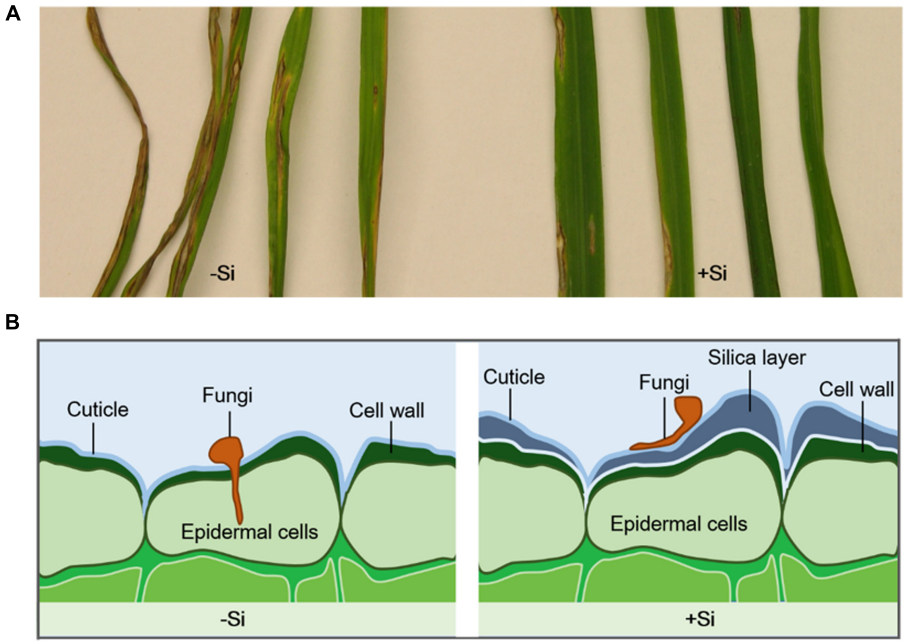

FIGURE 1 | (A) Leaf blast symptoms in rice after inoculated with *Magnaporthe grisea* for 10 days (Sun et al., 2010). Rice plants were continuously treated with (+Si) or without silicon (–Si). **(B)** Silica layer was formed in the cell wall of Si-treated plants and enhanced plant resistance to fungi infection by physical barriers.

Defense-Related Enzymes and Antimicrobial Compounds

Defense-related enzymes are closely linked with disease resistance, and Si has been reported to stimulate the activity of these enzymes during plant–pathogen interactions (Fauteux et al., 2005; Datnoff et al., 2007; Van et al., 2013). Several studies have reported the role of Si in disease resistance by activating defense-related enzyme activities such as chitinase, peroxidases, polyphenoloxidases, β-1,3-glucanase, phenylalanine ammonia-lyase, uperoxide dismutase, ascorbate peroxidase, glutathione reductase, catalase, lipoxygenase, and glucanase. PAL, involved in the synthesis of plant secondary antimicrobial substances, is essential for plant disease resistance responses (Waewthongrak et al., 2015). The higher PAL activity after Si treatment contributes to an accumulation of total soluble phenolic and lignin-thioglycolic acid derivatives in the leaves of banana and coffee plants, and this corresponds with low disease incidence (Silva R. et al., 2010; Fortunato et al., 2012b). Polyphenol oxidase (PPO), which mainly exists in cytoplasm in a free form or bound in chloroplasts, mitochondria, and other subcellular organelles, is the main enzyme of phenolic substance oxidation (Quarta et al., 2013); its activity has been positively correlated with plant disease resistance (Piperno, 2006). Furthermore, PPO was found to be involved in the synthesis of lignin and to increase the antibacterial ability of host plants (Song et al., 2016). Si application could also increase peroxidase (POD) and chitinase (CHT) activities, which play important roles in host–pathogen interactions. POD is involved in cell-wall reinforcement and the final steps of lignin biosynthesis, as well as the cross-linking of cell-wall proteins (Brisson et al., 1994), while CHT is one of the PR proteins that contribute to hydrolyze the cell walls of many phytopathogenic fungi (Pan and Ye, 1992; Shewry and Lucas, 1997).

Defense-related enzyme activities induced by Si may regulate gene expression related to enzyme synthesis; for example, the expression of genes encoding phenylalanine ammonia-lyase (*PALa* and *PALb*) and lipoxygenase (*LOXa*) were significantly up-regulated in Si-treated perennial ryegrass plants, associated with suppression of gray leaf spot (Rahman et al., 2015). Si could elevate the activities of defense-related enzymes (e.g., peroxidase and polyphenol oxidase) via enhancing or priming JA-inducible responses to herbivory in rice (Ye et al., 2013). The beneficial effects of Si for suppressing pathogen infections via an increase in the activities of defense-related enzymes have been found in the pathosystems of cucumber (*Pythium* spp. and *Podosphaera xanthii*), pea (*Mycosphaerella pinodes*), wheat (*Pyricularia oryzae*), rice (*Magnaporthe oryzae*, *Bipolaris oryzae*, *Rhizoctonia solani,* and *Pyricularia oryzae*), melon (*Trichothecium roseum* and *Podosphaera xanthii*), Chinese cantaloupe (*Trichothecium roseum*), bean (*Colletotrichum lindemuthianum*), perennial ryegrass (*Magnaporthe oryzae*), and soybean (*Corynespora cassiicola*; **Table 2**).

A substantial response to defense-related enzymes is the change in antimicrobial substances; generally, lower disease incidence in plants after Si application are associated with a higher activity of defense-related enzymes, which induce the production and accumulation of antimicrobial compounds, such as phenols, flavonoids, phytoalexins, and PR proteins in plants

TABLE 2 | Defense-related enzymes regulated by silicon in plant–pathogen interactions.

Hosts	Diseases	Pathogen	Defense-related enzymes	Reference
Bean	Anthracnose	*Colletotrichum lindemuthianum*	Superoxide dismutase, ascorbate peroxidase, glutathione reductase	Polanco et al., 2014
Cucumber	Crown and root rot	*Pythium* spp.	Chitinase, peroxidases, polyphenoloxidases	Chérif et al., 1994
	Powdery mildew	*Podosphaera xanthii*	Peroxidases, polyphenoloxidases, chitinases	Liang et al., 2005a
Melon	Pink rot	*Trichothecium roseum*	Peroxidase	Bi et al., 2006
	Powdery mildew	*Podosphaera xanthii*	Chitinases, superoxide dismutase, β-1,3-glucanase	Dallagnol et al., 2015
Chinese cantaloupe	Pink rot	*Trichothecium roseum*	Peroxidases, phenylalanine ammonia-lyase	Guo et al., 2007
Pea	Leaf spot	*Mycosphaerella pinodes*	Chitinase, β-1,3-glucanase	Dann and Muir, 2002
Perennial ryegrass	Gray leaf spot	*Magnaporthe oryzae*	Peroxidase, polyphenol oxidase	Rahman et al., 2015
Rice	Blast	*Magnaporthe oryzae, Pyricularia oryzae*	Glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase, superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, lipoxygenase	Rodrigues et al., 2003, 2004, 2005; Cai et al., 2008; Domiciano et al., 2015
	Brown spot	*Bipolaris oryzae*	Chitinase, peroxidase	Dallagnol et al., 2011
	Sheath blight	*Rhizoctonia solani*	Phenylalanine ammonia-lyases, peroxidases, polyphenoloxidases, chitinases	Schurt et al., 2014
Soybean	Target spot	*Corynespora cassiicola*	Chitinases, β-1-3-glucanases, phenylalanine ammonia-lyases, peroxidases, polyphenol oxidases	Fortunato et al., 2015
Wheat	Blast	*Pyricularia oryzae*	Chitinases, peroxidases	Filha et al., 2011

after pathogen penetration (Chérif et al., 1994; Fawe et al., 1998; Rodrigues et al., 2004; Remus-Borel et al., 2005). However, the opposite effect was found in soybeans, in which Si application reduced the basal antioxidant enzyme activity of leaves during *Cercospora sojina* infection, leading to an increase in host susceptibility to frogeye leaf spot. These findings suggest that Si-induced resistance to plant disease was most likely due to the less than optimal conditioning of the antioxidant system (Telles Nascimento et al., 2016).

Antimicrobial compounds help higher plants to combat disease (Fauteux et al., 2005; Datnoff et al., 2007; Van et al., 2013), and Si has been documented to stimulate the accumulation of antimicrobial compounds, such as phenols, flavonoids, and phytoalexins during pathogen infection (Chérif et al., 1994; Fawe et al., 1998; Rodrigues et al., 2004; Remus-Borel et al., 2005); this may therefore contribute to the enhancement of defense-related enzyme activities. Defense-related antimicrobial phenols or lignin-associated polyphenolic compounds increased by Si resulted from the inducing activities of PAL and PPO following pathogen invasion (Rahman et al., 2015). Si-enhanced lignin and flavonoid production is attributed to higher PAL activity induced by Si; PAL converts *L*-phenylalanine into *trans*-cinnamic acid, which in turn is the precursor of lignin and flavonoids (Dixon et al., 2002; Hao et al., 2011).

Lignin and phenolic secondary metabolism play important roles in plant disease resistance. Si is involved in phenolic metabolism and lignin biosynthesis in plant cell walls (Marschner, 2012). It also increases lignin-carbohydrate complexes and lignin content in the epidermal cell wall of rice, and enhances plant resistance to blast disease (Inanaga et al., 1995; Cai et al., 2008). Si supply could increase the total concentration of soluble phenolic compounds in host plants and enhance plant disease resistance through delaying the growth of invading pathogens (Dallagnol et al., 2011; Fortunato et al., 2015). Flavonoids, another phenolic compound, are also induced by Si and enhanced rose plant resistance to *Podosphaera pannosa* (Shetty et al., 2012), and wheat resistance to *Pyricularia oryzae* (Silva et al., 2015).

Higher accumulation of phenolic and lignin or lignin-thioglycolic acid derivatives, due to Si treatment, fortified cucumber plants against damping-off (*Pythium ultimum*) (Chérif et al., 1994), wheat against powdery mildew (*Blumeria graminis*) (Belanger et al., 2003) and blast (*Pyricularia oryzae*) (Filha et al., 2011), *Arabidopsis* against powdery mildew (*Erysiphe cichoracearum*) (Ghanmi et al., 2004), soybean against target spot (*Corynespora cassiicola*) (Fortunato et al., 2015), melon against powdery mildew (*Podosphaera xanthii*) (Dallagnol et al., 2015), rice against blast disease (*Magnaporthe grisea*) (Cai et al., 2008), brown spot (*Bipolaris oryzae*) (Dallagnol et al., 2011), and sheath blight (*Rhizoctonia solani*) (Zhang et al., 2013).

Phytoalexins is recognized to be critical in plant defense against pathogen infection. Enhanced production of phytoalexins reduces the incidence of powdery mildew caused by *Podosphaera xanthii* in cucumber plants (Fawe et al., 1998), as well as blast caused by *M. grisea* in rice (Rodrigues et al., 2004, 2005). Si supply is reported to increase accumulation of the flavonoid phytoalexins in cucumber plants during *Podosphaera xanthii* infection (Fawe et al., 1998). Similar results have been found in rice, in which Si increased resistance to blast by stimulating the production of phytoalexins, such as momilactones A and B (Rodrigues et al., 2004, 2005). With regard to perennial ryegrass (*Magnaporthe oryzae*) pathosystems, Si-induced enhancement of phenolic acids, including chlorogenic acid and flavonoids, and relative levels of genes encoding PAL and lipoxygenase contributed to improved resistance to gray leaf spot disease (Rahman et al., 2015).

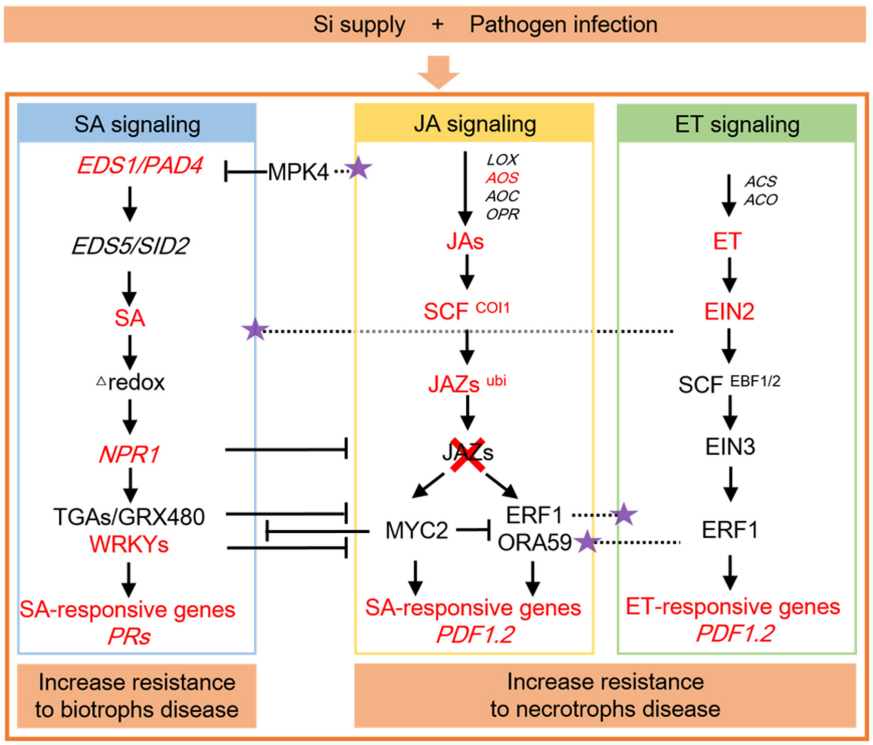

FIGURE 2 | Signaling pathways in the plant defense response regulated by silicon (Si). Crosstalk between signaling pathways in plant defense originating from the actions of salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are demonstrated, as well as their interactions in modulating the defensive response regulated by Si. The SA signaling pathway mainly involves in biotrophs disease, whereas JA and ET signaling pathways attribute to necrotrophs disease. T, negative effect; purple stars, positive effect; red, increased or up-regulated by Si supply. The networking of signaling pathways are modified from Pieterse et al. (2009).

Systemic Signals

To prevent pathogen infection, host plants have developed a complicated immune system providing several layers of constitutive and inducible defense mechanisms, which are regulated by a complex network of signal transduction pathways (Grant et al., 2013). SA, JA, and ET play key roles in plant immunity networks and regulate plant defense responses (Clarke et al., 2000; Devadas et al., 2002). SA is mainly active against biotrophic and hemibiotrophic pathogens, whereas JA and ET are predominantly involved against necrotrophic pathogens (Pieterse et al., 2012).

Several studies have suggested that Si may regulate plant stress responses by modulating phytohormone homeostasis and signaling pathways (Zhang et al., 2004; Fauteux et al., 2006; Iwai et al., 2006; De Vleesschauwer et al., 2008; Brunings et al., 2009; Chen et al., 2009; Ghareeb et al., 2011; Reynolds et al., 2016). Plant phytohormones accumulate in Si-treated plants in response to pathogen invasion, wounding, or herbivory (Fauteux et al., 2006; Ye et al., 2013; Kim et al., 2014); for example, Si-induced rice defense against insect herbivores through JA accumulation (Ye et al., 2013) and regulated wound-induced JA biosynthesis (Kim et al., 2014). In Si-treated *Arabidopsis* plants infected with powdery mildew pathogen (*Erysiphe cichoracearum*), the biosynthesis of SA, JA, and ET in leaves was stimulated, leading to increased resistance (Fauteux et al., 2006). Similarly, tomato infected with *Ralstonia solanacearum* showed that Si triggers activation of the JA and ET signaling pathways (Zhang et al., 2004; Chen et al., 2009; Ghareeb et al., 2011). The stimulating effects of Si on the JA and ET signaling pathways in rice challenged with *Magnaporthe oryzae* demonstrate that the Si-mediated signaling pathway is critical for enhancing rice resistance to blast disease (Iwai et al., 2006; De Vleesschauwer et al., 2008; Brunings et al., 2009). However, Van et al. (2015a) suggest that Si-induced rice resistance to *Cochliobolus miyabeanus* is regulated independently of the classic hormones SA and JA, but that it does interfere with the synthesis and/or action of fungal ET. In the defense of *Arabidopsis* against powdery mildew, although Si increases the expression of genes encoding enzymes involved in the SA pathway, resistant phenotypes show a significantly decreased production of SA and expression of defense genes compared with susceptible controls, implying that Si-mediated resistance involves mechanisms other than SA-dependent defense responses (Vivancos et al., 2015).

The signaling pathways in the plant defense response regulated by Si were demonstrated in **Figure 2**. The *EDS1* and *PAD4* genes are required for SA biosynthesis, whereas the *EDS5* and *SID2* genes involve in regulating SA biosynthesis (Shah, 2003). In *Arabidopsis*, the *TaLsi* plant, which contained higher Si, were more resistance to *Golovinomyces cichoracearum* infection than control plants when treated with Si, and corresponded with

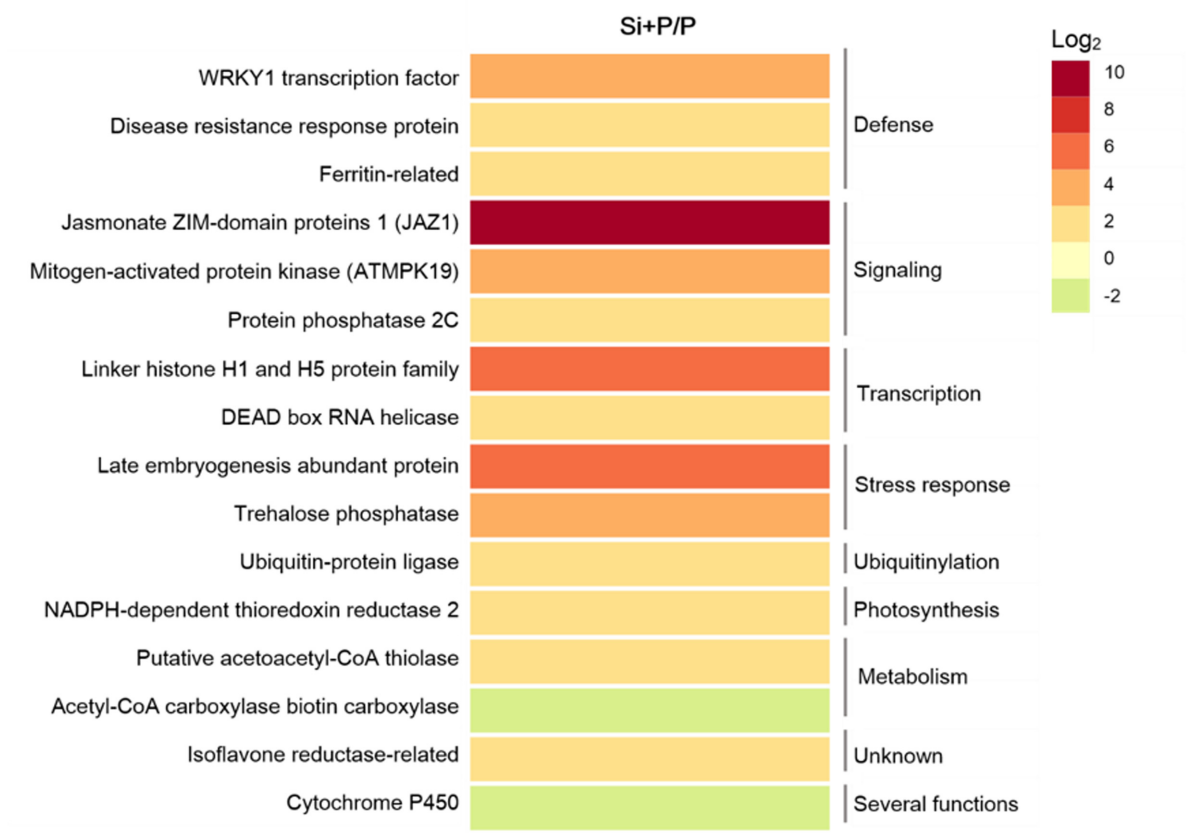

FIGURE 3 | Significantly regulated genes in response to silicon in tomato plants after infected with *Ralstonia solanacearum* for 72 h (Ghareeb et al., 2011). The heatmap represents the ratio of genes expression between plants with silicon and inoculated with *R. solanacearum* (Si + P) and plants without silicon and inoculated with *R. solanacearum* (P).

higher expressions of *EDS1* and *PAD4* genes, as well as *NPR1* and three SA-induced *PR* defense genes *PR1*, *PR2,* and *PR5* (Vivancos et al., 2015). Moreover, the mutants of *TaLsi1 sid2* and *TaLsi1 pad4*, which crossed mutants *pad4* and *sid2* with the line *TaLsi1*, showed lower area under the disease progress curve (AUDPC) after Si supply, suggesting that Si-enhanced resistance to *Golovinomyces cichoracearum* infection in *Arabidopsis* is maintained in *pad4* and *sid2* mutants engineered to better absorb Si (Vivancos et al., 2015). The regulatory protein NPR1 is critical for activation of *PR* gene expression in response to SA, and *NPR1* itself is positively regulated by some SA-inducible WRKY proteins (Li et al., 2004). During tomato plant infected with *R. solanacearum*, the gene expression of transcription factor *WRKY1* was upregulated in response to Si (Ghareeb et al., 2011). Si induced defense related genes and transcripts belong to the SA dependent pathway, which accompanied by an increase in the level of endogenous SA and subsequent *PRs* expression (Durrant and Dong, 2004; Kurabachew et al., 2013).

Silicon can induce expression of a large spectrum of inducible defense responses and amplifies the JA-mediated induced defense response by serving as a priming agent for the JA pathway (**Figure 2**), for example, the enhanced induction of defense-related enzymes and proteins, as well as enhanced induction of transcripts encoding proteins involved in JA signaling, whereas JA promotes overall leaf silicification and the maturation of phytolith-bearing silica cells by increase Si accumulation (Fauteux et al., 2006; Ye et al., 2013). During rice attacked by caterpillar *Cnaphalocrocis medinalis* (leaffolder, LF), significant decreases in Si deposition and an apparent loss of Si-induced LF resistance were observed in transgenic events that silenced the expression of either allene oxide synthase (OsAOS) or CORONATINE INSENSITIVE1 (OsCOI1), which is involved in JA biosynthesis or perception, suggesting that Si primes JA-mediated antiherbivore defense responses (Ye et al., 2013). Ubiquitin-protein ligase is suggested to be involved in the fine-tuning of JA-related response by degrading the JA-negative regulator, JAZ1 (Thines et al., 2007). Dreher and Callis (2007) demonstrated that up-regulation of ubiquitin-protein ligase by Si application in plants after pathogen infection may contribute to tuning the signaling of a defense response.

JERF3, *TSRF1* and *ACCO* are ET marker genes, *JERF3* is a transcription factor which is activated in response to ET and JA signaling, *ACCO* involved in ethylene biosynthesis, and

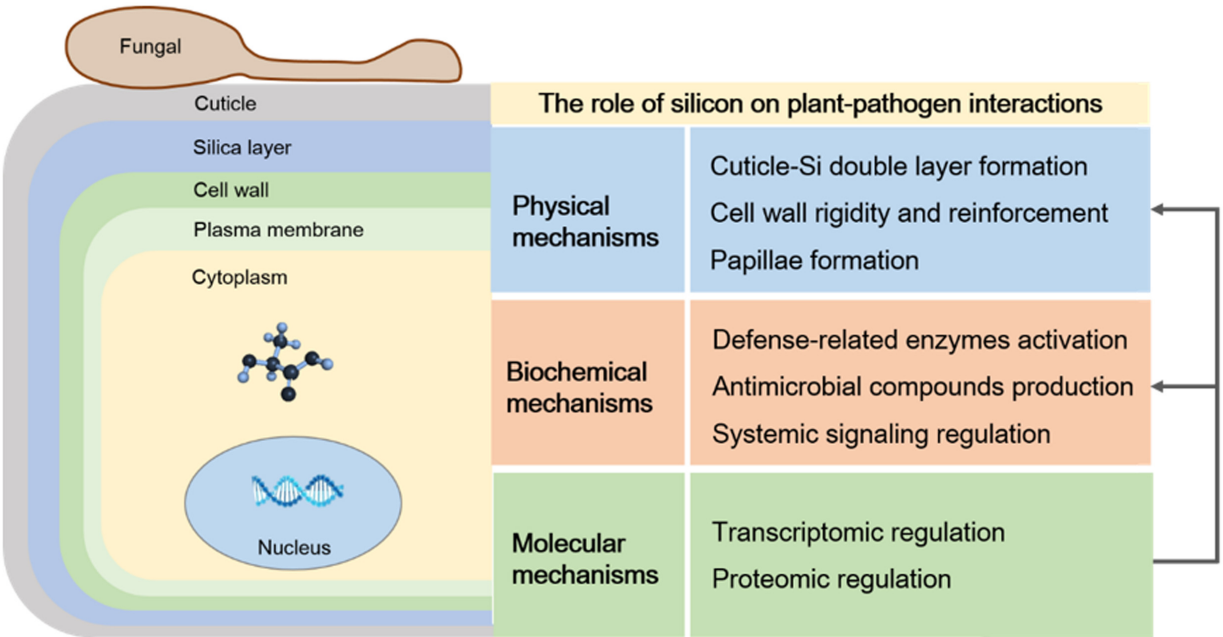

FIGURE 4 | The role of silicon (Si) on plant–pathogen interactions. Si mediated plant defense responses were classified as physical, biochemical and molecular mechanisms. Physical mechanisms involved in cell wall reinforcement and papillae deposition, biochemical mechanisms were attributed to activating defense-related enzymes, stimulating antimicrobial compounds production as well as regulating the complex network of signals pathways, and the molecular mechanisms mainly contained the regulation of genes and protein related to defense responses.

TSRF1 is an ET-responsive transcription factor (Pirrello et al., 2012). In tomato plants, the expression of *JERF3*, *TSRF1* and *ACCO* genes were upregulated by Si when challenging with *R. solanacearum*, supporting that Si induced resistance were mediated via ET and JA signaling pathways (Ghareeb et al., 2011). ET and JA interact to regulate the expression of particular defense-related genes such as *PDF1.2* upon pathogen perception (Pieterse et al., 2009) (**Figure 2**). In *Arabidopsis*, Si increased the *PDF1.2* expression during *Botrytis cinerea* infection, suggesting its role as a modulator of the signaling pathways involved in the plant's response to fungal infection (Cabot et al., 2013). In rice-*C. miyabeanus* pathosystems, ET-insensitive *OsEIN2a* antisense plants were more resistance to brown spot than wildtype plants, and Si treatment of the *OsEIN2a* antisense transgenics or coapplication of Si and ET signaling blocker silver thiosulfate (STS) had no additive effect on brown spot resistance, suggesting that Si specifically targets the ET signaling pathway to defense resistance (Van et al., 2015a).

Three classes of active defense mechanisms are distinguished in plant–pathogen interactions regulated by Si application: the primary response comes in cells infected by pathogens; the secondary response is induced by elicitors and restricted to cells near to the initial infection site; and thirdly, the systemic acquired response is transported hormonally to all tissues of the infected plant (Hutcheson, 1998).

Molecular Mechanisms

Silicon is involved in the metabolic processes of plant–pathogen interaction, activating defense genes of host plants via a series of physiological and biochemical reactions and signal transductions, as well as inducing the resistance response in plants to prevent plant diseases (Fauteux et al., 2005; Vivancos et al., 2015). Si may act in the primary response and modulate the activity of post-elicitation intracellular signaling systems which regulate the expression of defense genes related to structural modifications of cell walls, hypersensitivity responses, hormone synthesis, antimicrobial compound synthesis, and PR proteins (Fauteux et al., 2005).

Transcriptomic and proteomic studies have been conducted to illustrate the defense responses of Si in various pathosystems (Fauteux et al., 2006; Chain et al., 2009; Majeed Zargar et al., 2010; Ghareeb et al., 2011; Nwugo and Huerta, 2011). Si could induce tomato resistance to *Ralstonia solanacearum* via up-regulating the expression of genes involved in defense and stress responses, such as WRKY1 transcription factor, disease resistance response protein, ferritin, late embryogenesis abundant protein, and trehalose phosphatase (**Figure 3**) (Ghareeb et al., 2011). The similar result have been found in tomato stems of rhizobacteria and silicon treated-tomato genotypes upon inoculation with *R. solanacearum* compared to the non-treated, pathogen inoculated control, in which most of the up-regulated genes are involved in signal transduction, defense, protein synthesis and metabolism, while a large proportion of down regulated genes were involved in photosynthesis, lipid metabolism (Kurabachew et al., 2013). Crosstalk between signaling pathways in plant defense regulated by Si and related transcription factor have been detailed discussed in the Section of "Systemic Signals" and **Figure 2**. During the induction of systemic acquired

resistance in cucumber mediated with Si, the expression of gene encoding a novel proline-rich protein (PRP1) was enhanced, which contributed to cell-wall reinforcement at the site of attempted penetration of fungi into epidermal cells (Kauss et al., 2003). During pathogen interactions in tomato plants (*R. solanacearum*), the expression of *CHI-II*, *GLU*, *PGIP*, and *POD*, which are attributed to virulence factors released by the pathogen to inhibit host resistance and facilitate host invasion, were down-regulated by Si application (Ghareeb et al., 2011). In tomato plants inoculated with *R. solanacearum*, 26 proteins were markedly changed by Si supply, suggesting that Si-mediated disease resistance may be related to change at a protein level (Chen et al., 2014).

Silicon could negate many transcriptional changes induced by pathogen infection, for example, *Arabidopsis* infected with the fungus *Erysiphe cichoracearum* results in alteration of the expression of a set of nearly 4000 genes, and the number or expression level of up-regulated genes, which are defense-related, were not changed compared with control and Si-treated plants, whereas the magnitude of the down-regulated genes, which are involved in primary metabolism, were attenuated when treated with Si (Fauteux et al., 2006). In wheat plants infected with *Blumeria graminis* f. sp. *tritici*, about 900 genes responding to pathogen infection were altered in control leaves, while few genes were changed by the pathogen in Si-supplied plants, suggesting that Si almost eliminated the stress imposed by the pathogen invasion (Chain et al., 2009). Similar findings were obtained by Brunings et al. (2009), the impact of *Magnaporthe oryzae* inoculation on the transcriptome of rice is diminished by Si application. Therefore, rather than inducing resistance by transcriptional reprogramming of defense-related genes, Si seems to eliminate the impact of pathogen infection on the transcriptome of host plants, probably through preventing the exploitation of pathogen virulence factors (Van et al., 2015b).

CONCLUSION AND PERSPECTIVES

By combining available information on the interaction of plant–microbes mediated by Si, the physical, biochemical, and molecular mechanisms that can be attributed to Si-mediated plant defense responses have been summarized in this review (**Figure 4**). Firstly, Si induces resistance against a wide range of diseases by acting as a physical barrier, which is based on pre-formed defense barriers before pathogen infection, for example, wax, cuticle, and cell-wall protection, and post-formed defense barriers after pathogen infection, for example, cell-wall reinforcement and papillae deposition at infection sites. Secondly, Si-induced biochemical resistance during plant–pathogen interactions involves activating defense-related enzymes activates, stimulating antimicrobial compound production, and regulating the complex network of signal pathways. Finally, Si may act at a molecular level to regulate the expression of genes involved in the defense response. Understanding plant–microbe interactions regulated by Si will be helpful in the effective use of this mineral to increase crop yield and enhance resistance to plant diseases. Although numerous studies have elucidated the possible mechanism of Si-mediated resistance at the physical, biochemical, and molecular levels, detailed mechanisms of Si regulated plant–microbe interactions, such as plant signaling transduction and transcriptome regulation of defense-related pathways, are needed for further study.

AUTHOR CONTRIBUTIONS

MW and SG wrote the manuscript; LG contributed in the tables; SD and YS contributed in the figures; QS and SG revised the manuscript.

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Silicon Supplementation Alters the Composition of Herbivore Induced Plant Volatiles and Enhances Attraction of Parasitoids to Infested Rice Plants

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Silicon (Si) is important in plant defenses that operate in a direct manner against herbivores, and work in rice (*Oryza sativa*) has established that this is mediated by the jasmonate signaling pathway. Plant defenses also operate indirectly, by the production of herbivore induced plant volatiles (HIPVs) that attract predators and parasitoids of herbivores. These indirect defenses too are mediated by the jasmonate pathway but no earlier work has demonstrated an effect of Si on HIPVs. In this study, we tested the effect of Si supplementation versus Si deprivation to rice plants on subsequent HIPV production following feeding by the important pest, rice leaffolder (*Cnaphalocrocis medinalis*). Gas chromatography–mass spectrometry analyses showed lower production of α-bergamotene, β-sesquiohellandrene, hexanal 2-ethyl, and cedrol from +Si herbivore-infested plants compared with −Si infested plants. These changes in plant chemistry were ecologically significant in altering the extent to which parasitoids were attracted to infested plants. Adult females of *Trathala flavo-orbitalis* and *Microplitis mediator* both exhibited greater attraction to the HIPV blend of +Si plants infested with their respective insect hosts compared to −Si infested plants. In equivalent studies using RNAi rice plants in which jasmonate perception was silenced there was no equivalent change to the HIPV blend associated with Si treatment; indicating that the effects of Si on HIPVs are modulated by the jasmonate pathway. Further, this work demonstrates that silicon alters the HIPV blend of herbivore-infested rice plants. The significance of this finding is that there are no earlier-published studies of this phenomenon in rice or any other plant species. Si treatment to crops offers scope for enhancing induced, indirect defenses and associated biological control of pests because parasitoids are more strongly attracted by the HIPVs produced by +Si plants.

Keywords: HIPV, induced plant defense, biological control, jasmonate, hexanal 2-ethyl, α-bergamotene, β-sesquiohellandrene, cedrol

INTRODUCTION

Silicon (Si) has not historically been considered an essential plant nutrient though it has been termed 'quasi-essential' (Epstein, 1994; Luyckx et al., 2017). Evidence has mounted in the last decade that Si plays important roles in plant defense against biotic and abiotic stress (Ma et al., 2001; Ma, 2004; Ahmed et al., 2013; Balakhnina and Borkowska, 2013; Pontigo et al., 2015; Cooke and Leishman, 2016) including against insect herbivores and pathogens in agriculture (Ye et al., 2013; Wang et al., 2017). Among studies of the effects of Si on plant defense against herbivores, two primary modes of action have emerged as important. The first of these is the mechanical mode afforded by the deposition of inorganic amorphous oxide (SiO_2) phytoliths in the epidermis of foliage and in spines and trichomes (Ma, 2004; Hartley et al., 2015). These defenses are often constitutive ('always on') but can also be induced by damage such that the plant is responding to herbivory with greater deposition of silicon in defense structures (Hartley et al., 2015). Silicon deposition provides structural rigidity to plants and the resulting physical toughness also makes the plant surface less vulnerable to penetration and colonization by fungal pathogens (Wang et al., 2017) and tougher for herbivores to masticate and digest (Kvedaras and Keeping, 2007; Reynolds et al., 2016). The second mode of action of silicon in plant defense is enhancement of the induced production of defense chemicals (Wu and Baldwin, 2010) including enzymes such as polyphenol oxidase and inhibitors such as trypsin protease inhibitor. For both of the foregoing mechanisms, there is strong evidence that the influence of Si is mediated by the jasmonate signaling pathway (Ye et al., 2013). The lipid-derived plant hormone, jasmonate, plays several important roles in plant metabolism including prioritization of defense over plant growth when a plant is under biotic stress (Yang et al., 2012). Ye et al. (2013) demonstrated Si elevated rice plant defense against rice leaffolder (*Cnaphalocrocis medinalis*) by promoting phytolith accumulation in leaves as well as polyphenol oxidase, peroxidase, trypsin protease inhibitor, and Bowman–Birk protease inhibitor activity. The significance of the jasmonate pathway in these effects was established by use of RNAi to silence expression of the CORONATINE INSENSITIVE1 gene which is involved in jasmonate perception. For the RNAi rice plants, the positive effects of Si on plant defenses were negated, leading to normal development of the herbivore. By logical extension, it is expected that the influence of Si on plant defense will also apply to mechanisms other than the two broad types described above, provided that these mechanisms too are mediated by the jasmonate pathway.

Plant defenses are not always direct in nature; they can also operate indirectly via the third trophic level, the natural enemies of herbivores. Work in recent decades has amply demonstrated that plants are able to manipulate the composition of the volatiles they emit, such that they serve as synomones, semiochemicals that benefit both the emitter and receiver (Becker et al., 2015). In this mechanism, a plant responds to herbivore attack by producing a particular herbivore induced plant volatiles (HIPVs) blend to which natural enemies such as predators and parasitoids are attracted, leading them to attack the herbivores (Mumm and Dicke, 2010; Schuman et al., 2012). Crucially, the jasmonate pathway is the most important of the three known signal-transduction pathways that underlie the induction of HIPVs (Ament et al., 2004; Wei et al., 2011; Zhang et al., 2013). Accordingly, the positive effects of Si on direct plant defenses described above may apply to indirect plant defense based on HIPV production.

To date, no work has been published on the effects of Si on HIPVs in any plant system. There is, however, indirect evidence that HIPVs may be affected by Si treatment to plants. Using a cucumber system, Kvedaras et al. (2010) demonstrated that Si enhanced the attraction of the predator, *Dicranolaius bellulus*, to plants infested by *Helicoverpa armigera*. An associated field study showed that Si-treated plants were more attractive to 'wild' predators than Si deficient control plants (Kvedaras et al., 2010). Whilst those results are consistent with Si altering the HIPV blend of pest-infested plants, that paper did not include any results for the volatiles emitted by the plants. This leads to the hypothesis that Si alters the HIPV blend thus enhancing the attraction of natural enemies. To test our hypothesis, we studied the volatiles of rice plants grown under Si deficient conditions versus with Si supplementation, and determined the effects on volatiles when infested by a major economic insect pest. We then complemented volatile studies with behavioral assays to determine whether attraction of biologically relevant parasitoid species was affected by changes in volatiles. Finally, RNAi plants were used to explore whether silenced perception affected the influence of the Si regime on plant volatiles.

MATERIALS AND METHODS

Plants

Rice plants of wild type (WT, var. Shishoubaimao) and a CORONATINE INSENSITIVE1 (OsCOI1) RNAi line deficient in jasmonate perception were used in this study. The OsCOI1 line was generated as described by Ye et al. (2013). Plants were hydroponically grown in climate control chambers (27 ± 1°C, 75 ± 5% RH) using a modified Hoagland's solution (Hoagland and Arnon, 1950). For +Si rice plants, a hydroponic solution with 2.11 mM sodium metasilicate (Na_2SiO_3) was used. For −Si rice plants, 4.22 mM of Na^+ was added to the hydroponic solution to equalize Na^+ concentrations with +Si rice plants. The pH of solutions was mediated to 5.6–6.0 by adding hydrochloride acid. Hydroponic solution was renewed weekly and plants were grown to 6–8 weeks of age for all studies.

Herbivores and Parasitoids

Folded rice leaves with middle-aged to mature larvae of *C. medinalis* feeding inside were collected from the experimental farm of South China Agricultural University (23°17′N, 113°36′E). Foliage was kept turgid by immersing the cut ends in water within insect rearing cages (45 cm × 45 cm × 45 cm) placed in climate controlled rooms (27 ± 1°C, 75 ± 5% RH) to obtain adult *C. medinalis*. Newly emerged adults were transferred onto 2- to 3-week-old maize (*Zea mays* L.) plants to generate

the larvae used in experiments. The rice leaffolder parasitoid, *T. flavo-orbitalis*, was reared from hosts collected from the experimental farm of Fujian Agriculture and Forestry University (26°29′N, 118°48′E). Army worm (*Mythimna separata* (Walker)) and its parasitoid, *Microplitis mediator* Haliday, were supplied by Keyun Biocontrol (Henan, China) and Hebei Academy of Agricultural and Forestry Sciences (Hebei, China), respectively.

Plant Volatile Analysis

Rice volatile organic compounds were collected by dynamic headspace collection (Zhang et al., 2009). One rice plant was placed in a 15-L glass vessel. Purified air was pumped into the vessel at 200 mL/min. The system was purged for 1 h before attaching a tube filled with 80 mg matrix Porapak Q (Sigma-Aldrich) to the air outlet to adsorb the volatiles. Headspace collections were carried out at 27 ± 1°C, 70 ± 5% RH, and lasted for 4 h. Samples were collected from plants in each of the following experimental treatments for WT and *OsCOI1* rice: +Si +Herbivore; +Si −Herbivore; −Si +Herbivore; and −Si −Herbivore. All plant treatments were prepared concurrently with individual plants laid out in separate insect proof cages (50 cm × 50 cm × 50 cm). Volatile collection commenced 12 h after the plants were infested with four 3rd instar larvae per plant. Each treatment had nine replicates.

Headspace samples were eluted by 500 μL dichloromethane into 2-mL glass vials, then mixed with 5 μL internal standard nonyl acetate at 100 ng/μL. Volatiles samples were then analyzed by GC-MS (Agilent 7890B-5977A). The temperature was held at 40°C for 3 min then increased at 5°C min^{-1} to 220°C. Compounds were identified by comparing the mass spectra with the instrument's internal NIST 2011 spectra database and Wiley Spectra Lab (John Wiley & Sons, New York, NY, United States). The quantity of each compound was calculated by comparing the peak area of each compound with internal standard.

Olfactometer Studies of Parasitoids on WT Rice Plants

Two rice-herbivore-parasitoid systems were used: WT-*C. medinalis*–*T. flavo-orbitalis* and WT-*M. separata*–*M. mediator*. To obtain herbivore attacked plants, four 3rd instar larvae of either herbivore species were allowed to feed on one plant for 12 h. All larvae were then removed carefully without further mechanical damage to the plant before connecting to the Y-tube olfactometer. Pairs of plants were set up as: −Si +Herbivore vs. −Si −Herbivore; +Si +Herbivore vs. +Si −Herbivore; and −Si +Herbivore vs. +Si +Herbivore. Each pair of plants was connected to the arms of a Y-tube olfactometer to test parasitoid response to treatments. Plants were placed individually into the volatile collection apparatus and purified air supplied at 100 mL/min. Preference responses of parasitic wasps to the pairs of plants described above were tested in a Y-tube olfactometer. Individual female adult wasps were released at the downwind side of the tube and 2 min were allowed for the wasp to pass the Y-tube junction and remain in that arm, otherwise the wasp was recorded as a no choice. Each odor comparison was repeated on 3–4 days with 10–20 wasps per day.

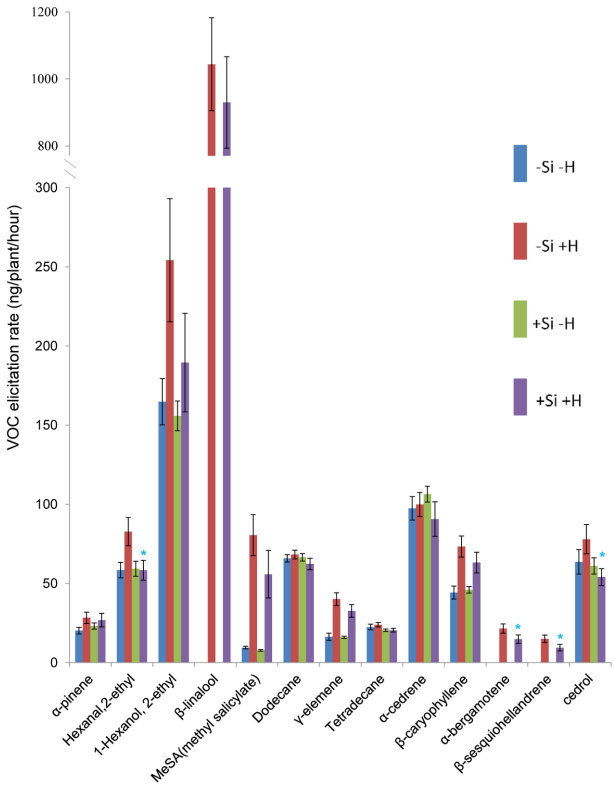

FIGURE 1 | Effect of herbivore (H) (*C. medinalis*) infestation and silicon (Si) treatment on volatile organic compound (VOC) production by wild type rice plants. [Mean ± SE; *, significant effect of silicon within herbivore treatment ($P < 0.05$) by Fisher's Least Significant Difference test of one-way analysis of variance (ANOVA)].

Statistical Analysis

Data analysis was undertaken using SPSS software (IBM SPSS Statistics version 24.0, Armonk, NY, United States). For plant volatile analysis, the average elicitation rate (ng/plant/h) was calculated for each identified compound from nine replicates of each treatment (with exception of eight for treatment +Si + Herbivore). Fisher protected least significant difference (LSD) tests of analysis of variance (ANOVA) was used to compare the difference level between treatments.

Y-tube olfactometer data were analyzed using a Chi-square test. Wasps that did not make a choice were excluded from the analysis.

RESULTS

Effect of Silicon on HIPVs

Thirteen of 60 compounds were identified from the volatile blend collected as head space samples of rice plants. The composition of volatile blends was affected by *C. medinalis* infestation. For WT plants, β-linalool, α-bergamotene, and β-sesquiohellandrene were detected only in samples from +Herbivore plants, whether +Si or −Si (**Figures 1**, **2**). Production of five other compounds,

hexanal 2-ethyl, 1-hexanol 2-ethyl, methyl salicylate (MeSA), γ-elemene, and β-caryophyllene, was significantly elevated (*P*-value: 0.011, 0.020, <0.001, <0.001, <0.001, respectively) for −Si +Herbivore plants (82, 254, 81, 40, 73 ng/plant/h, respectively) compared with −Si −Herbivore plants (58, 164, 10, 16, 44 ng/plant/h, respectively). Infested +Si plants produced significantly lower amounts (compared with −Si +Herbivore plants) of four compounds: hexanal 2-ethyl, α-bergamotene, β-sesquiohellandrene and cedrol (**Figure 1**) (58, 15, 9, 54 ng/plant/h, respectively, vs. 83, 22, 15, 78 ng/plant/h, respectively) (*P*-value: 0.013, 0.022, 0.015, 0.027, respectively).

For *OsCOI1* plants, as in WT plants, β-linalool, α-bergamotene, and β-sesquiohellandrene were detected only in samples from +Herbivore plants, whether +Si or −Si (**Figure 2**). Production of MeSA, γ-elemene, and β-caryophyllene was significantly (*P*-value: 0.030, 0.016, 0.020, respectively) elevated for +Herbivore plants compared with −Herbivore plants (−Si +Herbivore: 54, 27, 59 ng/plant/h, respectively, vs. −Si −Herbivore: 7, 14, 40 ng/plant/h, respectively; +Si +Herbivore: 68, 37, 71 ng/plant/h, respectively, vs. +Si −Herbivore: 3, 16, 44 ng/plant/h, respectively) (**Figure 2**). However, the effect of Si on *OsCOI1* plants differed from that observed for WT plants. Infested +Si plants produced higher (rather than lower) amounts of α-bergamotene and β-sesquiohellandrene compared with −Si +Herbivore plants (18, 14 ng/plant/h, respectively, vs. 10, 7 ng/plant/h, respectively) (*P*-value: 0.034, 0.034, respectively); whilst production of hexanal 2-ethyl and cedrol was unaffected by Si treatment (rather than being suppressed by Si as observed in WT plants).

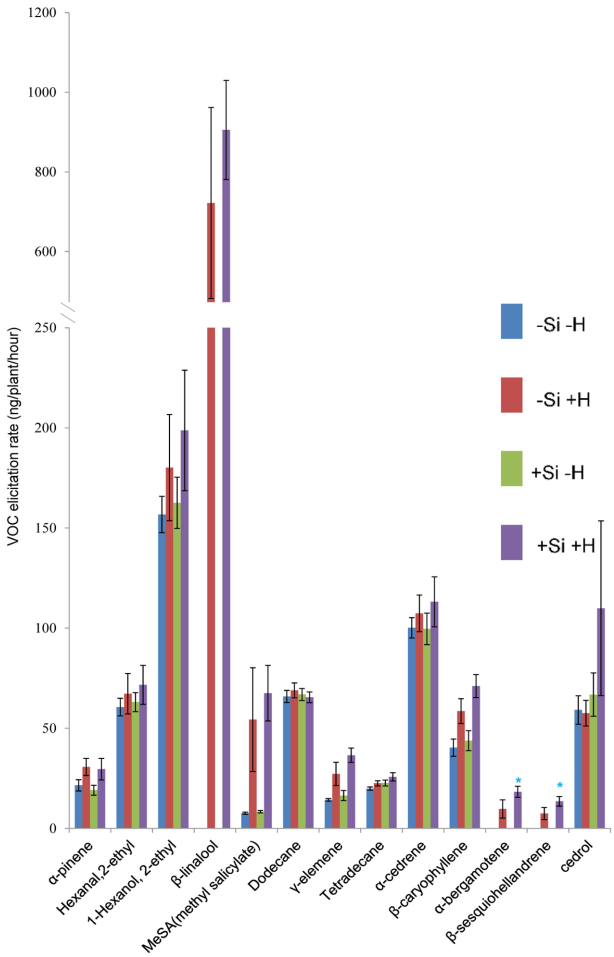

FIGURE 2 | Effect of herbivore (H) (*C. medinalis*) infestation and silicon (Si) treatment on volatile organic compound (VOC) production by *OsCOI1* RNAi rice plants. [Mean ± SE; *, significant effect of silicon within herbivore treatment ($P < 0.05$) by Fisher's Least Significant Difference test of one-way analysis of variance (ANOVA)].

Parasitoid Response to HIPVs

The responses of both parasitoids *T. flavo-orbitalis* and *M. mediator* to experimental treatments was consistent across species. The volatile blend of +Herbivore plants was significantly more attractive than the blend from −Herbivore plants, regardless of Si regime (**Figures 3**, **4**). Both parasitoids also responded significantly more strongly to the volatile blend of +Si +Herbivore than to −Si +Herbivore plants.

DISCUSSION

Silicon is the second most abundant element in the earth's crust, yet the vast majority of this is not available to plants (Epstein, 1994; Savant et al., 1997; Ma and Yamaji, 2006), existing predominantly as feldspars and quartz minerals. Plants take up Si from the soil solution as silicic acid [$Si(OH)_4$], or $Si(OH)_3O^-$ at high pH, so although the Si content of soils can be as high as 45% (Currie and Perry, 2007), availability can be low. Deficiencies are especially important for plants such as rice that require this element in large amounts (Horuz et al., 2013). Evidence of this is the fact that plants have evolved influx, efflux and channel-type transporters to actively uptake Si (Ma and Yamaji, 2006, 2015; Trembath-Reichert et al., 2015; Vivancos et al., 2016) as well as that yield increases when plant-available Si is added to the growing medium (Tavakkoli et al., 2011). The beneficial roles of Si to various plants have been the subject of extensive research and include alleviation of abiotic stresses such as drought (Marques et al., 2016), metal toxicity, and micronutrient deficiency (Hernandez-Apaolaza, 2014); and biotic stress from pathogens (Winslow, 1992; Vivancos et al., 2015) and herbivores (Reynolds et al., 2009, 2016; Ye et al., 2013). To date, there is increasing evidence of Si priming plants for defense against herbivore attack (Reynolds et al., 2009; Hartley and DeGabriel, 2016). But, a conspicuous gap in knowledge about the effects of Si on plant biology is for the volatiles that are produced by plants and that are often key semiochemicals that drive ecological interactions including induced, indirect plant defense against herbivores enemies (Pare and Tumlinson, 1999; Dicke and Loon, 2000; Degenhardt et al., 2003).

The present results support our hypothesis and show, for the first time in any plant species, that plants grown with available Si have a different HIPV profile compared to plants grown under Si deficiency. Earlier work by Kvedaras et al. (2010) showed that

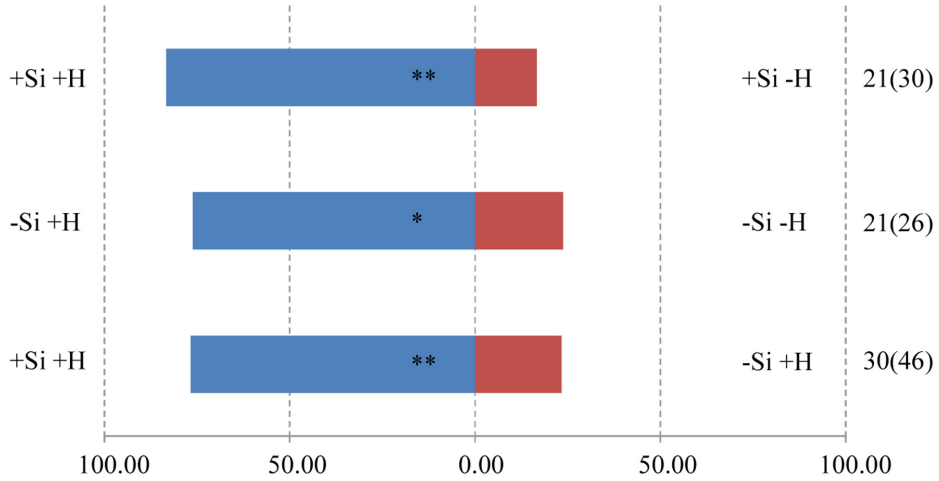

FIGURE 3 | Y-tube olfactometer response of the parasitic wasp, *T. flavo-orbitalis* to volatile profiles of WT rice plants, showing the effect of herbivore (H) (*C. medinalis*) infestation and silicon (Si) treatment. Asterisks indicate significant difference from a 50:50 distribution (Chi-square test; $*P < 0.05$; $**P < 0.01$; *n*, number of wasp individuals responding to odor source, number in parentheses means the number of wasp individuals used for test). (Statistic comparison, +Si +H vs. +Si –H, $\chi^2 = 8.000$, $P = 0.005$; –Si +H vs. –Si –H, $\chi^2 = 5.762$, $P = 0.016$; +Si +H vs. –Si +H, $\chi^2 = 8.533$, $P = 0.003$). % parasitic wasps to plant odour source.

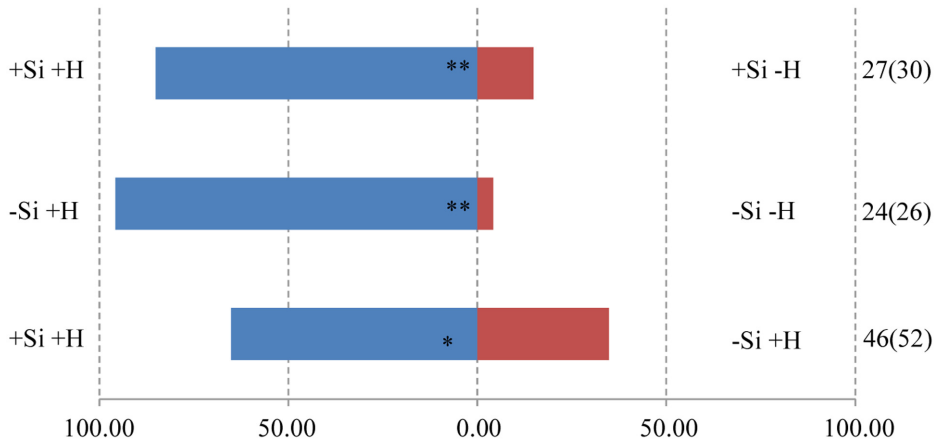

FIGURE 4 | Y-tube olfactometer response of the parasitic wasp, *M. mediator* to volatile profiles of WT rice plants, showing the effect of herbivore (H) (*M. separata*) infestation and silicon (Si) treatment. Asterisks indicate significant difference from a 50:50 distribution (Chi-square test; $*P < 0.05$; $**P < 0.01$; *n*, number of wasp individuals responding to odor source, number in parentheses means the number of wasp individuals used for test). (Statistic comparison, +Si +H vs. +Si −H, $\chi^2 = 13.370$, $P = 0.000256$; −Si +H vs. −Si −H, $\chi^2 = 20.167$, $P = 0.000007$; +Si +H vs. −Si +H, $\chi^2 = 4.261$, $P = 0.039$). % parasitic wasps to plant odour source.

+Si plants were more attractive than −Si plants to a generalist predator in olfactometer tests and to wild predators (of unknown identity) in a field experiment. That study did not, however, include work on plant volatiles so the mechanism underpinning the apparent effects on predators remained unclear. In the present study, several of the volatile compounds that were influenced by herbivore infested rice plants were shown to be HIPVs, since they were not produced by uninfested plants, irrespective of the Si status of the plant. These were β-linalool, α-bergamotene, and β-sesquiohellandrene, each of which have been identified as HIPVs in other studies (Hare, 2011; De Backer et al., 2016; Pinto-Zevallos et al., 2016). Whilst it is possible to record antennal responses for parasitoids to individual HIPVs (Takemoto and Takabayashi, 2015), it is known that blends of HIPVs and the relative quantity of different compounds is more important than any single compound (Van Wijk et al., 2010, 2011). Accordingly, in addition to the three compounds that were produced only by infested plants, several others were produced in greater quantities by infested rather than healthy plants: hexanal 2-ethyl, 1-hexanol 2-ethyl, methyl salicylate, γ-elemene and β-caryophyllene. Most crucially, the differences in blends between plants with a different Si regime (within herbivore treatment) involved a significant change in the composition of volatiles produced, with lower production of two of the HIPVs (α-bergamotene and β-sesquiohellandrene) and of hexanal 2-ethyl and cedrol. Hexanal 2-ethyl (synonym, 2-ethyl hexanol) has been reported as

a volatile compound released by *Rutaceae* spp. foliage (Robbins et al., 2012) and is produced by insect-damaged roots of carrot (*Daucus carota*) (Weissteiner and Schütz, 2006). Cedrol has been reported as a foliar and fruit volatile produced by *Ficus carica* L. (Soltana et al., 2017) and, though it has not been reported to be a HIPV, exhibits bioactivity against arthropod pests (Eller et al., 2014).

The foregoing differences in volatile blends between experimental treatments were demonstrated to have ecological relevance by the behavior of two parasitoid wasp species. Both *T. flavo-orbitalis* and *M. mediator* responded more strongly to the volatile blend from +Herbivore plants than −Herbivore plants within each of the Si regimes, demonstrating that the experimental conditions were suitable for these parasitoids to exhibit biologically appropriate behavior consistent with HIPVs guiding them to infested plants. Crucially, both parasitoids also exhibited a significant preference to the odor blend from +Si over −Si when both of these treatments were herbivore-infested.

The nature of the change in volatile blend brought about by Si pre-treatment (**Figures 1**, **2**) was not a clear cut switching-on, or -off, for the release of a given compound. Rather, the results are consistent with earlier studies showing that it is the ratio of compounds in blends that is crucial for attraction to natural enemies (Pare and Tumlinson, 1999; Dicke and Loon, 2000; Degenhardt et al., 2003). Specifically, our results are consistent with the heuristic that both parasitoids were attracted to a blend (**Figures 3**, **4**) in which the major HIPVs, especially linalool, were present but in which more minor volatiles (hexanal-2-ethyl and cedrol) were at background levels, equivalent to those emitted by herbivore-free plants.

The present study establishes a stronger evidence base for exploring scope to promote biological control of pest herbivores by ensuring that crops have an optimal supply of Si so that they are able to mount a strong induced, indirect defense based on HIPV production. Fully exploiting the apparent priming effects of Si on plant defense demands a more comprehensive understanding of the underlying metabolic pathways. Work by Ye et al. (2013) demonstrated the importance of the jasmonate signaling pathway for positive effects of Si on rice defenses. The present study demonstrates that Si can enhance the attractiveness of the HIPV blends produced by WT rice when attacked by a herbivore but that this effect of Si does not occur among RNAi plants with silenced jasmonate perception. The emission of HIPVs is known to be influenced by the jasmonate pathway, particularly in the case of chewing herbivores such as those used in the present study (Dicke and Baldwin, 2010). Accordingly, the present results support the finding of Ye et al. (2013) that Si promotes plant defenses via the jasmonate pathway, but extends the range of effects to include HIPVs production. Further studies are required to determine whether Si also interacts with the salicylic acid and ethylene defense pathways of plants (Ruther and Kleier, 2005; Zarate et al., 2007; Koornneef et al., 2008; Dicke et al., 2009). Each of these, as well as the jasmonate pathway, can lead to the induction of HIPVs including by feeding of sucking pests such as aphids (Ozawa et al., 2000; van Poecke and Dicke, 2002). It will, therefore, be important to determine the extent of interactions between Si and varying plant defense mechanisms in order to fully exploit the potential of this element in novel plant protection approaches.

AUTHOR CONTRIBUTIONS

JL and GG designed the experiments. JL, JZ, LH, JW, and YS conducted the experiment. JL, PZ, and GG analyzed and interpreted the data. JL, OR, MY, and GG drafted and revised the paper. All authors read and approved the final manuscript.

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Silicon Transporters and Effects of Silicon Amendments in Strawberry under High Tunnel and Field Conditions

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Together with longer production periods, the commercial transition to day-neutral strawberry (*Fragaria* × *ananassa*) varieties has favored the development of diseases such as powdery mildew (*Podosphaera aphanis*) that thrives in late summer-early fall. In an attempt to find alternative solutions to fungicides currently employed to curb the disease, we wanted to investigate the potential of silicon (Si) amendments that have been associated with prophylactic properties against powdery mildews. To this end, our first objective was to determine if strawberry was a Si-competent species following the recent characterization of the properties of Si transporters that plants must carry to uptake silicic acid. Based on genomic data, we were able to conclude that strawberry contained both functional influx (Lsi1) and efflux (Lsi2) transporters for Si uptake. Subsequently commercial experiments under high tunnel and field conditions were conducted with different Si fertilization regimes: constant soluble Si feeding in high tunnel, and bi-weekly soluble Si feeding or three concentrations of calcium silicate fertilization in the field. Results from high tunnel experiments showed that strawberry could accumulate as much as 3% Si on a dry-weight basis, the highest concentration ever reported for this species. All six tested cultivars contained roughly the same concentration, thereby confirming the limited genetic variability, also observed in other species, associated with the trait. Silicon fertilization under high tunnel led to a significant reduction of powdery mildew severity in both years and on all cultivars, and a significant increase in yield of marketable fruits reaching as much as 300% with cv. Monterey. By contrast, Si fertilization under field conditions in soils deficient in plant available Si, either in soluble or solid form, did not result in significant accumulation of Si in plants, regardless of the cultivars, year or concentrations. Our results have thus provided both genotypic and phenotypic proof that strawberry can greatly benefit from Si fertilization, but have also highlighted the importance of validating the fertilization regime to ensure that Si is properly absorbed and/or available to the plant.

Keywords: silicon, powdery mildew, day-neutral strawberry, *Podosphaera aphanis*, silicon absorption, Lsi1, Lsi2, silicon fertilization

INTRODUCTION

Strawberry has been exploited for its flavorful red fruits around the globe for centuries (Darrow, 1966). It is a perennial, dicotyledonous plant from the *Rosaceae* family (Maas, 1998). The commercial strawberry plant (*Fragaria* × *ananassa* Duchesne), is a hybrid species that came from the cross between *Fragaria chiloensis* and *Fragaria virginiana* (Hancock et al., 1999). There are three types of strawberry being cultivated today, and they are classified by their response to photoperiod. In June-bearing varieties, flowering is initiated by short days in the fall and a crop is produced the following year during spring-summer. They have been the standard strawberry type for many years in Canada until more productive varieties were developed. For instance, the everbearing type produces two crops, one in the spring and the other in the fall. More recently, the advent of day-neutral type, which corresponds to photoperiod insensitive plants, allowed fruit production through the fall as long as the temperatures are between 4° and 29°C (Dodgson, 2007). Since the 1990s, most strawberry growers use day-neutral varieties to produce fruits on longer periods and even on a year-round basis in Florida and California (Hancock and Simpson, 1995; Darnell et al., 2003).

Together with the longer production periods, the commercial transition to day-neutral types has favored the development of new diseases on strawberry. For instance, powdery mildew, a disease influenced by fluctuating humidity conditions in the late summer and fall months, has become a major problem (Dodgson, 2007). Powdery mildew on strawberry plants is caused by the fungus *Podosphaera aphanis*. It reduces the number of marketable fruits by decreasing fruit set, inducing cracks, and decreasing flavor and storage time (Pertot et al., 2008). It can reduce marketable yield from 20 to 70% (Dodgson, 2007). The optimal conditions for *P. aphanis* to thrive are long periods of temperatures between 18° and 22.5°C and an alternation between low and high relative humidity common in late summer- early fall (Amsalem et al., 2006). Since most day-neutral cultivars are susceptible to powdery mildew, additional treatments of fungicides are required to prevent and reduce the disease, leading to higher costs of production and potentially more pesticide residues on fruits (Xu et al., 2008). Also, several fungicides used against powdery mildew are part of quinone outside inhibitors (QoI) and sterol demethylation inhibitors (DMI). Both are known for their high resistance risk (Fernández-Ortuño et al., 2006; Sombardier et al., 2010). To circumvent this problem, other control methods are being sought to manage powdery mildew.

Over the last few years, several reports have highlighted the positive effects of silicon (Si) fertilization in agriculture (Liang et al., 2015a). Incidentally, the International Plant Nutrition Institute (IPNI) has recently added Si to its list of beneficial nutrients. For instance, several studies have shown that Si has prophylactic properties against a number of biotic and abiotic stresses on multiple crop species (Bélanger et al., 1995; Fauteux et al., 2005). Interestingly, Si is reported to be particularly efficient against diseases caused by biotrophic fungi such as powdery mildews (Vivancos et al., 2015). In the case of strawberry, very few studies have looked into the potential of Si either under experimental or commercial conditions. Wang and Galletta (1998) reported that foliar applications of Si significantly reduced strawberry powdery mildew (*P. aphanis*) and increased biomass but those results were contradicted by Palmer et al. (2006) who did not find any significant reduction of powdery mildew using foliar applications. On the other hand, Kanto et al. (2004, 2006) found that Si amendments in nutrient solutions and soils reduced the incidence of powdery mildew on strawberry.

Currently, Si fertilization remains marginal in strawberry in particular and in agriculture in general because there are still controversial and lingering questions regarding how plants can benefit from Si amendments, and methods of fertilization that optimize its effects. For instance, it is well recognized that silicic acid is the only soluble form a plant can absorb, but it is still unclear what plant species possess the adequate transport system, i.e., an influx and efflux transporter, to take up Si from the soil. No studies have ever looked into the presence of such transporters in strawberry so it remains unknown whether strawberry plants are good accumulators of Si. Accordingly, guidelines for Si fertilization in strawberry crops are lacking.

Considering the increasing presence and pressure of powdery mildew on day-neutral strawberry varieties, and the potential to exploit Si as an alternative to synthetic fungicides to control the disease, we were interested to determine if strawberry was a potential accumulator of Si and the fertilizing conditions that would optimize its absorption. In this context, our objectives were: (1) to analyze genomic data from strawberry for the presence of *bona fide* Si influx transporter, belonging to the family of NIP-III aquaporins as per Deshmukh et al. (2015), and Si efflux transporter; (2) to evaluate soluble and/or solid forms of Si fertilizers under both high tunnel and field commercial productions on several day-neutral varieties; and (3) to determine and compare Si accumulation, powdery mildew incidence and fruit yield for all varieties under the different fertilization treatments. Our results show for the first time that strawberry has the genetic and phenotypic predisposition to absorb Si but that Si fertilization regimes will influence the amount absorbed by the plants and thus the benefits they derive from Si.

MATERIALS AND METHODS

Presence of Si Transporters in Strawberry

Plant species capable to accumulate Si must contain influx transporters (Lsi1) of the type NIP-III aquaporins and efflux transporters (Lsi2), known as putative ion transporters (Ma and Yamaji, 2015; Deshmukh and Bélanger, 2016). Genomic data were analyzed for the presence of such transporters as previously described in Shivaraj et al. (2017). Protein sequences from the FAN_r1.1 and FANhybrid_r1.2 versions of *Fragaria* × *ananassa* reference genome were retrieved from the GDR (Genome Database for Rosaceae, https://www.rosaceae.org/). Blast search performed using the query sequences of known NIP-IIIs and Lsi2 homologs from soybean, poplar, wheat and rice against both versions only identified partial NIP-III and Lsi2 homologs. Consequently, assembly of available RNA-seq data for *Fragaria* × *ananassa* tissues from SRA database (https://www.ncbi.nlm.nih.gov/sra) along with FAN_r1.1 were used to obtain full sequences.

Construction of Phylogenetic Tree, Protein 3D Structure and Transmembrane Domain Profiling

Phylogenetic trees for NIP-IIIs (Lsi1s) and Lsi2s were constructed using MEGA (version 6) software tool. Protein sequences were aligned using ClustalW and subjected to construct phylogenetic tree using Maximum likelihood method with 1000 bootstrap iterations. The protein 3D structures were constructed using the SWISS-MODEL server with an automated protein homology-modeling option (https://swissmodel.expasy.org/). The transmembrane domain profiling was performed using TMHMM tool (www.cbs.dtu.dk/services/TMHMM/). Functional annotation of Lsi2 was performed with Conserved Domain Database (CDD, www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

Location of Commercial Experiments

The experiments under high tunnel were conducted at the farm of Les Fraises de L'Île d'Orléans inc. (St. Laurent, Ile d'Orléans, Québec, Canada) from May to October 2014, and 2015. A high tunnel of 30 × 8 m with a simple polyethylene plastic membrane was used and the sides of the tunnel were open to allow for ventilation.

The field experiments were conducted at the Ferme François Gosselin (St. Laurent, Ile d'Orléans, Québec, Canada) from May to October 2015, and 2016. For both seasons, plants were placed on raised beds covered with a plastic mulch in mid-May. Space between rows was 1.3 m while space between plants was 28 cm.

Plant Material and Soils

High Tunnel Experiments (2014 and 2015)

A set of 6 day-neutral (DN) cultivars namely Charlotte, Seascape, Monterey, Albion, Amandine and Verity were used in this study. The first three were used in 2014 and all six in 2015. Charlotte is a cross from Mara des bois and CAL 19 and was created in 1995 in France. It has good hardiness and resistance to powdery mildew. Seascape is a favorite among commercial growers but is considered susceptible to powdery mildew. Albion is a cultivar known for its large, glossy, and tasty fruits, but there are no reports on its susceptibility to powdery mildew. The offspring of Albion, Monterey, is a relatively new cultivar with large fruits, but with fairly high susceptibility to powdery mildew. Amandine is a new cultivar produced in Spain with a high-yielding potential and a good resistance to powdery mildew. Verity is also a new cultivar bred in the United Kingdom. Its interaction with powdery mildew is unknown but it has a high yield potential with glossy fruits. All plants were transferred to production in mid-May for both years and were grown in a soil-less substrate, Mélange Bio (Fafard & Frères, Qc).

Field Experiments (2015 and 2016)

In 2015, the experiments were conducted at l'Ile d'Orléans at La Ferme François Gosselin on an Orleans type soil being in the brunisolic order and the great group dystric brunisol (Marcoux, 1980). To determine Si levels in soil, the calcium chloride extraction method was used (Liang et al., 2015b). The soil pH was 6.4 and the plant available Si ranged between 19 and 21 mg/kg, a concentration considered very low since concentrations below 100 mg/kg are considered deficient (Liang et al., 2015b). The cultivars Seascape (DN), San Andreas (DN) and Jewel (June bearing) were tested.

In 2016, the experiments were conducted in fields from the same grower where the soils were in the podzolic order and in the great group of humo-ferric podzols. Their pH was 6.7 and their plant available Si content was measured between 15 and 16 mg/kg. The cultivar San Andreas was replaced by Albion (DN). For each of the 2 years, bare-root plants obtained from Lassen Canyon Nursery Inc. (Redding, CA) were transferred into the field in early May.

Experimental Design

High Tunnel Experiments (2014 and 2015)

The experimental design was a randomized block design. For the first year (2014), we tested three cultivars (Seascape, Charlotte, and Monterey) in combination with the two fertilization regimes (control and Si+) that were distributed in four blocks as repetitions of the six treatments. In the second year (2015), we had the same two fertilization regimes (control and Si+) in combination with six cultivars (Seascape, Charlotte, Monterey, Albion, Amandine, and Verity) in four blocks. In both experiments, each gutter supporting the pots was treated as a block. For each Si treatment and cultivar in each block, five pots containing three strawberry plants were used.

Field Experiments (2015 and 2016)

In 2015, two fertilization regimes (control and Si+) were used in combination with three cultivars in a split-plot design where three blocks enclosed the Si treatment as the main plot and cultivars (3) as subplots. Plant number for each combination was 20 and statistical analysis was conducted on three (N) independent values for yield calculation. In 2016, four Si treatments were used with three cultivars. The experimental design was randomized complete blocks (6) with eight plants for each cultivar, each treatment in each block.

Silicon Treatments and Fertilization

High-Tunnel Experiments (2014 and 2015)

Two 1,000-l containers were used for holding the different solutions that fed the plants by a drip feed irrigation. The rate of the drip irrigation was 4 l per pot per hour. The water tension was continuously recorded with a tensiometer. The irrigation was manually activated when the water tension was below −3.0 kPa. For the Si+ treatment, liquid potassium silicate (Kasil®, PQ Corporation) was used in one of the 1,000-l container to obtain the maximum soluble concentration of 1.7 mM Si while the other had the control treatment (Si-) and an additional K (0-0-52) to compensate for the addition of K in the container with Kasil®, Since the Si amendment was directly added to the nutrient solution, Si was fed to the plants whenever irrigation was applied (typically 20 min a day). Over the experimental period, each plant received on average 800 ml of solution per week.

During the experiments, volume, pH, and EC of the drainage solutions were measured daily in order to correct the nutrient solutions in case of mismanagement. Air temperature,

soil temperature and air relative humidity were recorded continuously with a HOBO™ data logger.

Field Experiments (2015 and 2016)

In 2015, soluble Si in the form of Kasil® was used as Si source. Briefly, a 1,000-l tank was used to prepare a solution 1.7 mM Si for which the pH was adjusted to 7 with nitric acid. The control solution (Si-) was amended with (13-0-46) to counterbalance K and N brought by Kasil® and the nitric acid in Si+ solution. Based on the grower's estimate of fungicide costs, Si applications were made so that the cost of Si fertilization would not exceed that of fungicides. Accordingly, both the Si and control treatments were applied twice a week by drenching with a drip tape system (AquaTraxx) previously installed. Each fertigation, with a duration of 60 min, gave *ca*. 620 ml of solution per plant per week.

In 2016, wollastonite (58% SiO_2, 23% CaO, 6% MgO) (Canadian Wollastonite, Kingston, ON), mesh size 400, was used as Si source based on reports (Liang et al., 2015a), preliminary experiments in the greenhouse and its high level of plant available Si. Four doses based on conversions from pot experiments were tested: 0 (control), 12, 24, and 36 g per plant corresponding roughly to 2,000, 4,000, and 6,000 kg per ha, respectively, with the former being a common concentration for field applications (Datnoff et al., 1997). At the planting stage, the wollastonite was incorporated directly into the planting holes and bare root strawberry plants were immediately planted. Fertilization was performed according to the grower's standard regime in all treatments.

Silicon Accumulation in Strawberry Leaves and Fruits

To measure Si concentrations in plants, the oldest leaves of the plants were sampled, dried at 60°C for at least 2 days and pulverized with a bead mill homogenizer (Omni Bead Ruptor 24, Omni International). Silicon concentrations were measured with the X-ray fluorescence spectrometry method (Niton XL3t955 GOLDD+ XRF) adapted from Reidinger et al. (2012). Leaf sampling was made in mid-July and mid-September for high-tunnel experiments and in mid-September for field experiments. In parallel, fruits were sampled regularly over the sampling period and stored for measurements of Si concentrations.

Powdery Mildew Severity

The incidence of powdery mildew was evaluated every week with a disease scale adapted from Horsfall and Barratt (1945). This scale consisted to evaluate a global infection level of the leaves for a plant where 0 meant no powdery mildew and 5 meant more than 75% of the entire plant presenting symptoms and/or signs.

The AUDPC (area under the disease progress curve) was used in order to quantify the disease severity:

$$AUDPC = \sum_{i=1}^{N_{i-1}} \frac{(y_i + y_{i+1})}{2} (t_{i+1} - t_i)$$

where y = disease level
and t = time of record

Yield and Fruit Quality

For both, tunnel and field experiments, yield and fruit quality were measured three times a week from mid-June until the end of September. The variables for measuring yield was the weight of marketable fruits per plant. Fruits were considered unmarketable if too small (less than 6 g) and/or misshapen and/or diseased.

Data Analysis

ANOVA was performed on data with the software JMP version 12.0.1 (SAS Institute Inc.). When significant ($p < 0.05$), additional statistical tests were performed. Tukey was used for multiple comparison when allowed. When indicated in the figure legends, orthogonal contrasts were also used and a $P < 0.05$ was considered statistically significant.

RESULTS

Presence of Influx and Efflux Silicon Transporters in Strawberry

A single influx (Lsi1) Si transporter, FaNIP2-1 belonging to NIP-III, was identified in strawberry. The FaNIP2-1 showed the characteristic features reported to be required for the functionality of the protein with respect to Si permeability (**Figure 1**). The FaNIP2-1 has six transmembrane domains, two conserved NPA motifs, a G-S-G-R Ar/R selectivity filter and, importantly, the 108 AA spacing between NPA motifs (**Figures 1A,C**). Furthermore, homology-based 3D protein model of FaNIP2-1 showed a typical hourglass-like structure (**Figure 1A**). Detailed investigation of FaNIP2-1 protein structure revealed a pore formed at the center of the protein making a transmembrane channel capable to transport Si (Supplementary Figure 1). Phylogenetic analysis clustered FaNIP2-1 along with influx Si transporters from other dicot species including wild strawberry, cucumber, and soybean (**Figure 1B**). Protein sequence alignment of FaNIP2-1 with known Si transporters showed conserved Ar/R selectivity filters, NPA motifs and the spacing between NPA domains (**Figure 1C**).

Similarly, a single efflux (Lsi2) Si-transporter was observed in strawberry. Functional classification of proteins via subfamily domain architectures performed using CDD search classified the protein as a Si transporter (Supplementary Table 1). It showed typical transmembrane domain profile as observed in known Lsi2s from different plant species (**Figure 2**).

Silicon Concentration in Leaves and Fruits

High-Tunnel Experiments

In control nutrient solutions, strawberry plants accumulated 0.42% ± 0.07 Si d.w. in their leaves by mid-July, and 0.72% ± 0.13 by the end of September, regardless of the cultivar or the year (data not shown). By contrast, plants fertilized with Si accumulated close to or more than 1% Si d.w. by July and more than 2% d.w. by mid-September (**Figures 3A,B**). In 2014, Charlotte had significantly more Si than the other cultivars under study at both sampling times (**Figure 3A**).

In 2015, analysis of strawberry leaves of all six cultivars showed that they all accumulated between 1.0 and 1.5% Si d.w. with only a significant difference between Charlotte and Verity by

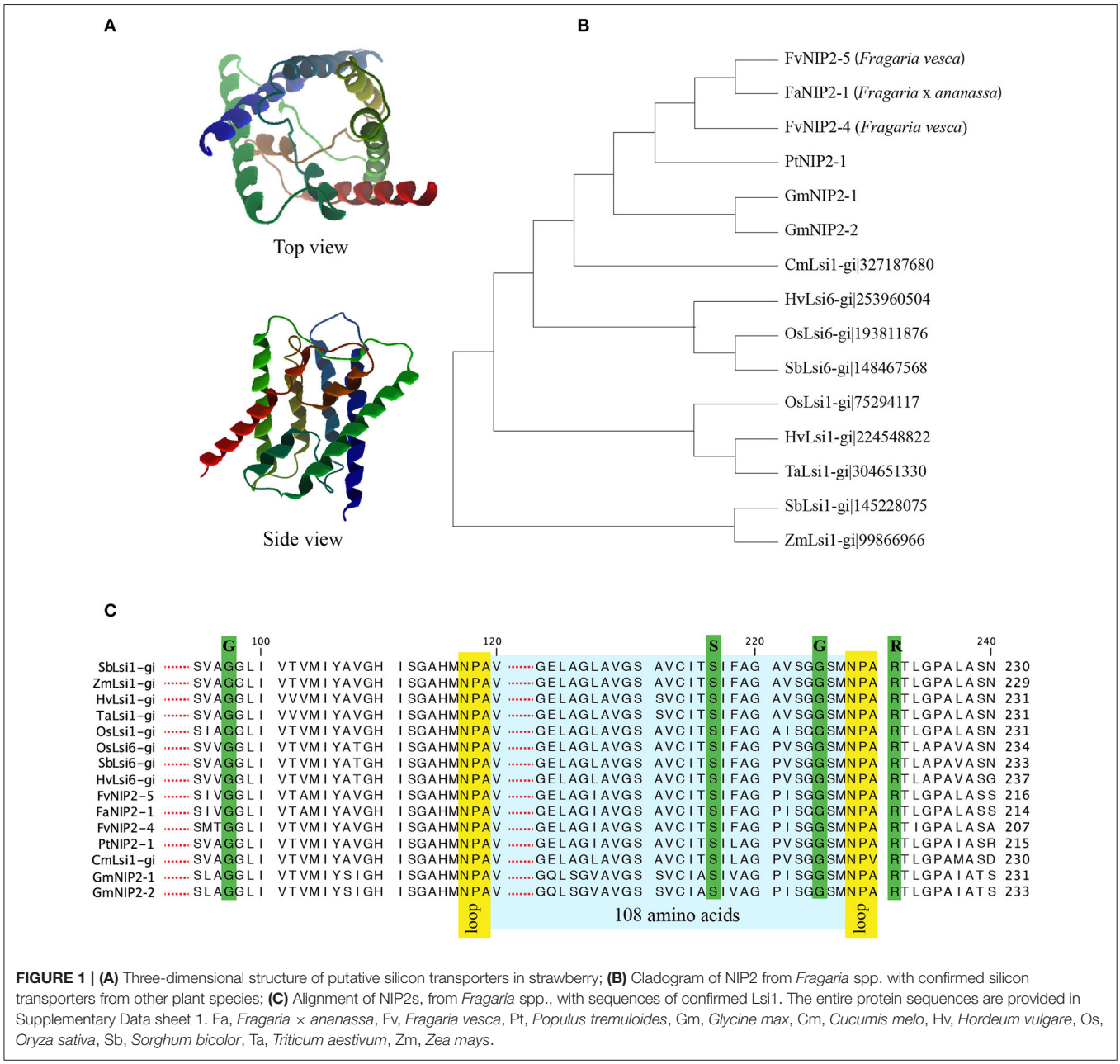

FIGURE 1 | (A) Three-dimensional structure of putative silicon transporters in strawberry; **(B)** Cladogram of NIP2 from *Fragaria* spp. with confirmed silicon transporters from other plant species; **(C)** Alignment of NIP2s, from *Fragaria* spp., with sequences of confirmed Lsi1. The entire protein sequences are provided in Supplementary Data sheet 1. Fa, *Fragaria* × *ananassa*, Fv, *Fragaria vesca*, Pt, *Populus tremuloides*, Gm, *Glycine max*, Cm, *Cucumis melo*, Hv, *Hordeum vulgare*, Os, *Oryza sativa*, Sb, *Sorghum bicolor*, Ta, *Triticum aestivum*, Zm, *Zea mays*.

mid-July (**Figure 3B**). Toward the end of the season, all cultivars had concentrations ranging between 2.5 and 3.0% d.w. No significant differences in Si concentrations were observed among the cultivars in September.

Strawberry fruits harvested throughout the season were measured for Si concentration. In all tested samples, Si concentrations never reached the level of detection (LOD) indicating that Si was never translocated to the fruit (data not shown).

Field Experiments

In the course of field experiments, the Si concentrations were markedly different than the ones observed in high tunnel experiments. Control plants never exceeded a concentration of 0.3% in either 2015 or 2016 for all cultivars tested (**Figures 4A,B**). Surprisingly, a bi-weekly fertilization of Si with potassium silicate did not significantly increase Si concentration in plants by mid-September (Contrast $_{(\text{Si vs. Control})}$; $P = 0.4846$) (**Figure 4A**). Only plants from San Andreas appeared to accumulate more Si as a result of the Si treatment. Plant Si levels obtained in the field were nearly 8 times lower that the ones observed in high tunnel experiments.

In 2016, calcium silicate as a solid source of Si fertilization did not improve Si intake by the plants regardless of the concentrations used

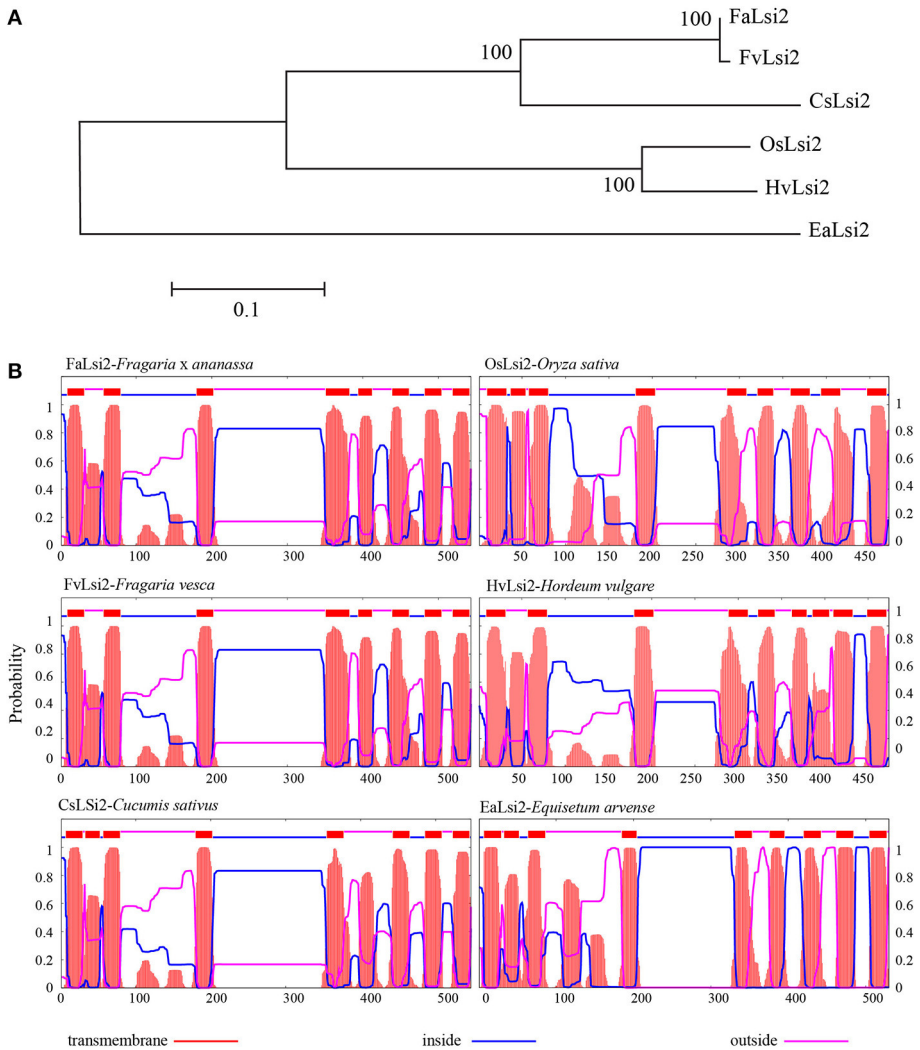

FIGURE 2 | (A) Phylogenetic relationship between the efflux silicon transporter (FaLsi2) identified in *Fragaria* × *ananassa* (Fa) and other plant species and **(B)** distribution of the 11 transmembrane domains predicted in *Fragaria* × *ananassa* protein with those of *Fragaria vesca* (Fv) and the four functional Si efflux transporters previously identified in four plant species, predicted with TMHMM tool (http://www.cbs.dtu.dk/). The entire protein sequences are provided in Supplementary Data sheet 1. Cs, *Cucumis sativus*, Hv, *Hordeum vulgare*, Os, *Oryza sativa*, Ea, *Equisetum arvense*.

(Contrast $_{\text{(calcium silicate doses)}}$; $P = 0.6184$) (**Figure 4B**). The concentrations *in planta* never exceeded 0.3%, in stark contrast once again with observations from the high tunnel experiments.

Powdery Mildew Severity

High Tunnel Experiments

In high tunnel experiments, the first signs of powdery mildew appeared in mid-July and expanded through September in both years. **Figure 5A** shows the average AUDPC calculated from the global infection level values observed on strawberry leaves during the growing season for the three cultivars treated or not with Si in 2014. The Si treatment consistently provided better disease control on all three cultivars (Contrast $_{\text{(Si vs.Control)}}$; $P < 0.0001$) with a reduction of *ca*. three units of AUDPC. Plants from cv. Charlotte were naturally more resistant than those of cvs. Monterey and Seascape (**Figure 5A**).

In 2015, powdery mildew severity was slightly higher than in 2014. As a result, the prophylactic effects of the Si treatment were even more apparent, and significant reduction of powdery mildew severity was observed on all six treated cultivars (Contrast $_{\text{(Si vs.Control)}}$; $P < 0.0001$). In particular, plants from cvs. Charlotte, and Verity responded to Si fertilization with reductions of AUDPC score ranging from 40 to 50% (**Figure 5B**).

Field Experiments

In field experiments, powdery mildew severity was nearly absent in both 2015 and 2016. Therefore, no differences could be observed among the cultivars or between the Si treatments.

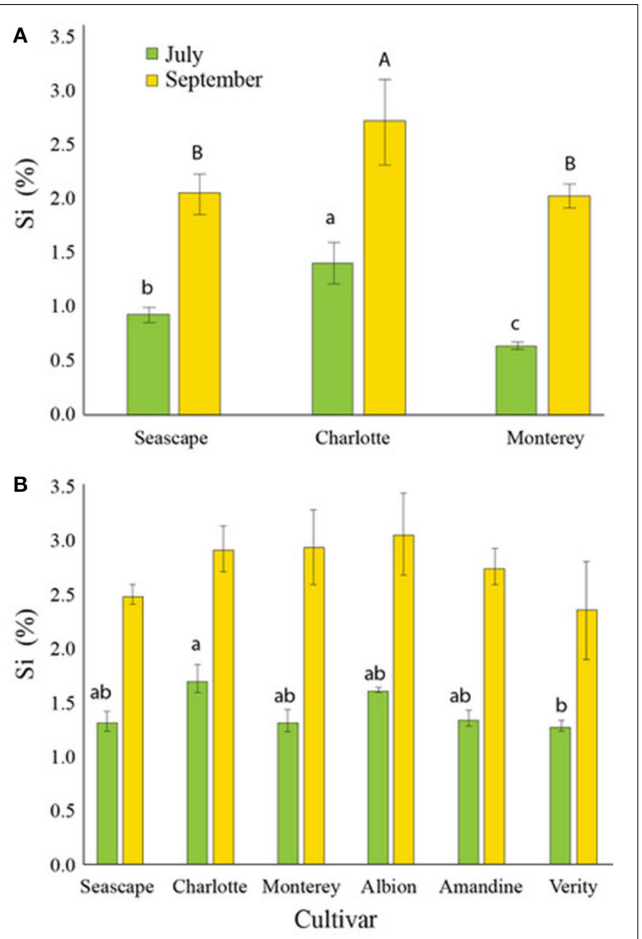

FIGURE 3 | Percent average silicon (Si) content in leaves of strawberry plants treated daily with a 1.7 mM Si solution in a high-tunnel commercial setting for two different dates in summer 2014 **(A)** and in summer 2015 **(B)**. Each value represents the mean ± SE ($n = 4$). Multiple comparisons between cultivars were made each month independently. Means with different letters are significantly different (Tukey HSD).

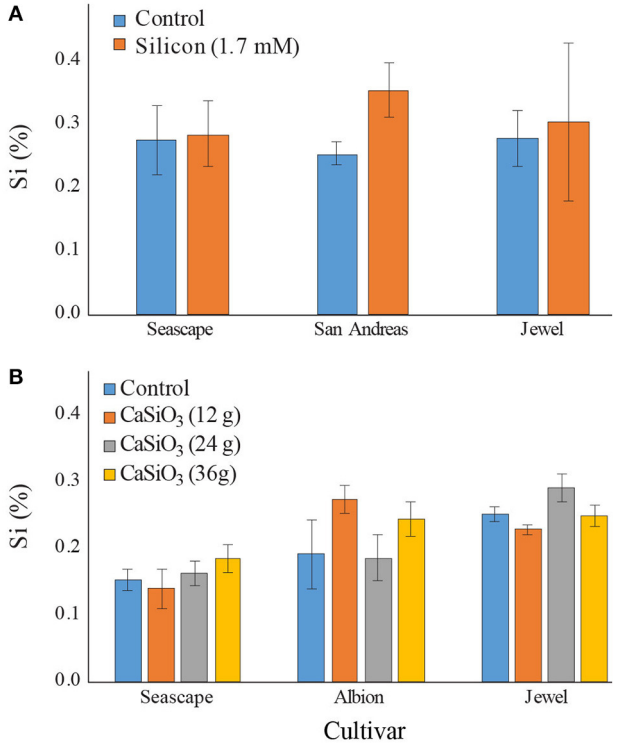

FIGURE 4 | Percent average silicon (Si) content in leaves of strawberry plants grown in field **(A)** treated twice a week with a 1.7 mM Si solution in summer 2015 **(B)** or fertilized with different concentrations of calcium silicate (wollastonite) in summer 2016. Sampling was made in September for both experiments. Each value represents the mean ± SE (n =3 in 2015; n = 6 in 2016).

Yield and Fruit Quality

High Tunnel Experiments

During the course of the high tunnel experiments, fruits were harvested and graded as marketable or not throughout the growing season. In 2014, a significantly higher yield of marketable fruits was obtained as a result of Si fertilization (Contrast $_{(Si\ vs.Control);}$ $P < 0.0001$; **Figure 6A**).

In the 2015 experiments, where powdery mildew severity was higher, the beneficial effects of Si on marketable fruits per plant were strikingly apparent on all cultivars tested (Contrast $_{(Si\ vs.Control)}$; $P < 0.0001$). While yields were generally lower in 2015, differences between control and Si+ plants were sometimes as high as 300%, as in cv. Monterey for example (**Figure 6B**). In general, the cultivars that showed the lowest yields for marketable fruits in control plants in 2015, e.g., Seascape, Charlotte, Monterey, and Albion, were the ones that benefited the most from Si fertilization (**Figure 6B**). Although the Si effect was more modest for Amandine and Verity plants, these two cultivars showed the overall highest yield under Si treatment (**Figure 6B**). No sign of phytotoxicity or albinism on fruits was ever observed during the experiments.

Field Experiments

In field experiments, no significant difference was observed in marketable fruits as a result of a bi-weekly fertilization with soluble Si (**Figure 7A**). On the other hand, plants from cv. Seascape appeared to be slightly more productive than those of cv. San Andreas (Contrast $_{(cvs)}$; $P = 0.0562$). In 2016, when plants were subjected to a solid source of Si fertilization, no difference in yield was observed under Si treatment (**Figure 7B**) but plants from Seascape outperformed those of Albion ($P < 0.0001$). No yield data are available for Jewel because it is a short-day cultivar that only initiates its flower buds in the fall for production the following season.

DISCUSSION

This study provided the first genetic proof that strawberry possesses both influx (Lsi1) and efflux (Lsi2) Si transporters and, as a result, is a receptive plant for Si fertilization. Our results

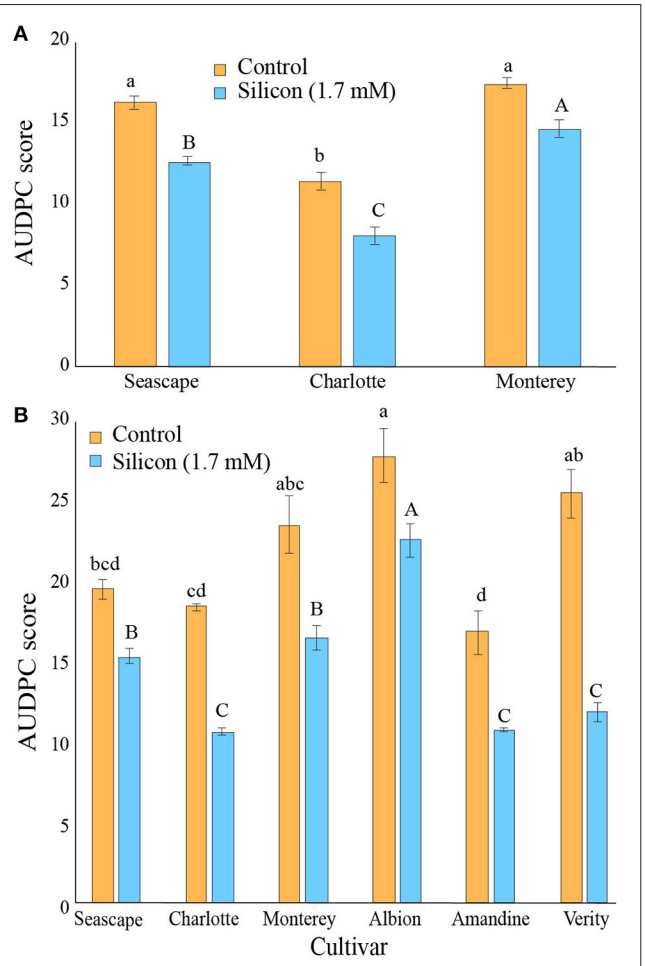

FIGURE 5 | Area Under Disease Progress Curves for *Podosphaera aphanis* on strawberry leaves of plants treated or not with 1.7 mM Si in a high tunnel commercial setting during summer 2014 **(A)** and summer 2015 **(B)**. Each value represents the mean ± SE ($n = 4$). Multiple comparison test (Tukey HSD) has been done on each treatment independently. Means with different letters are significantly different.

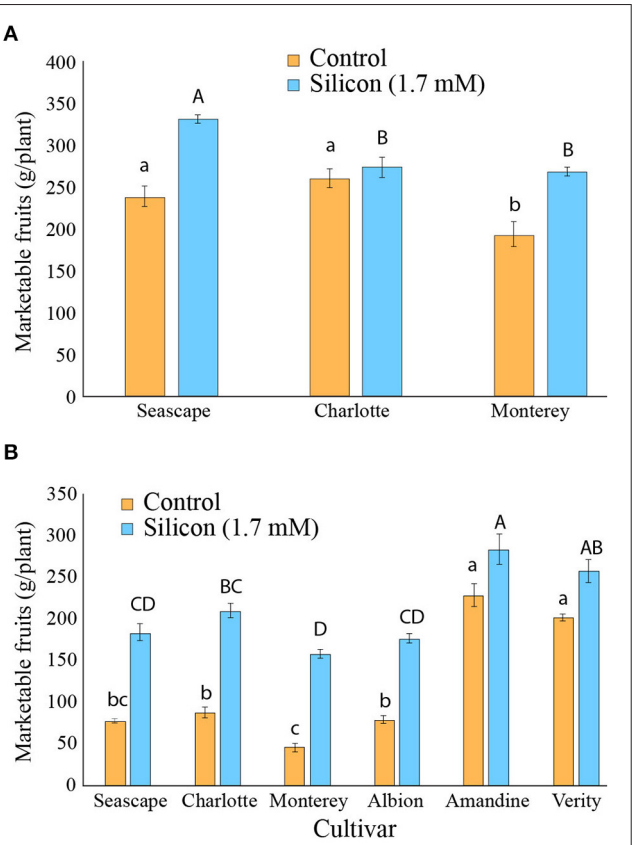

FIGURE 6 | Weight of marketable fruits for strawberry plants treated or not with potassium silicate in a high tunnel commercial setting. **(A)** Summer 2014. **(B)** Summer 2015. Each value represents the mean ± SE ($n = 4$). Multiple comparison test (Tukey HSD) has been done on each treatment independently when the ANOVA was significant. Means with different letters are significantly different.

further showed that the fertilization regime will greatly influence the benefits strawberry derives from the treatment.

In spite of the growing interest for Si fertilization in agriculture, many plant species are unable to absorb Si to concentrations that will yield benefits. For the longest time, plants were categorized as low, intermediate and high Si absorbers based on empirical data (Jones and Handreck, 1967), with the latter two categories being seemingly the only ones where Si was associated with positive effects. The discovery of Si transporters in rice (Ma et al., 2006, 2007) offered the first scientific basis to determine with precision what plant species could absorb Si. Indeed, Deshmukh et al. (2015) recently showed that plants possessing NIP-III aquaporins with the proper configuration for Si were able to absorb Si to concentrations exceeding 1% and that all other species lacking this specific transporter were unable to actively take up Si from the soil. Previous works offered conflicting data about the predisposition of strawberry for Si absorption (Miyake and Takahashi, 1986; Kanto et al., 2006) and the latest classification by Hodson et al. (2005) suggested that strawberry was a poor absorber. We thus took advantage of available genomic data to ascertain if strawberry did possess Si influx and efflux transporters. Our results clearly showed that it contained an influx transporter with a GSGR pore and a 108 aa distance between the NPA loops as essential conditions for Si uptake. We further showed that it also contained a Lsi2 with high-sequence and transmembrane profile similarities to functional Lsi2s reported in rice, barley, cucumber and horsetail (Vivancos et al., 2016). On the basis of this evidence, we concluded that strawberry could be classified as a Si-competent species, which supported our initiatives to conduct field trials.

Large-scale Si fertilization of strawberry under two commercial settings, high tunnel and field, highlighted interesting yet unexpected results. In the high tunnel experiment, where Si was fed to the plant in soluble form on a regular basis, we have shown that strawberry can accumulate Si in concentrations as high as 3%, the highest level ever reported for this species. This result corroborates perfectly our genomic data and should eliminate all confusion with respect to the

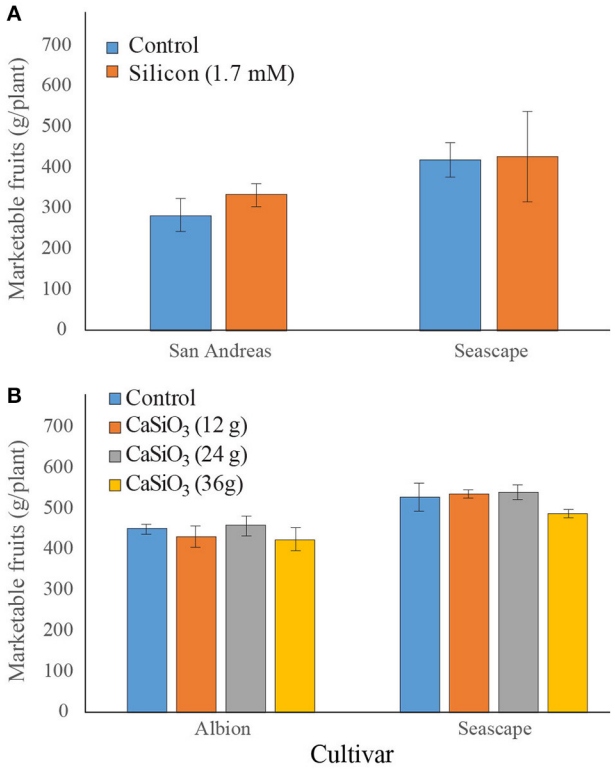

FIGURE 7 | Number of marketable fruits on strawberry plants treated or not twice a week with a 1.7 mM Si solution **(A)** in the field for summer 2015 or treated or not with different concentrations of wollastonite **(B)** in summer 2016. Each value represents the mean ± SE ($n = 3$ in 2015; $n = 6$ in 2016).

classification of strawberry as Si absorber. All six cultivars tested over the course of the two experimental seasons accumulated roughly the same concentration, which would indicate a limited genetic variability for the trait, at least within the germplasm under study. It is noteworthy to mention that, to date, very little within-species variability has been observed among plants in terms of Si accumulation. This suggests that Si permeability is tightly controlled by the transporters Lsi1 and Lsi2, with very little flexibility for intermediate potential, as shown by Deshmukh et al. (2015). Interestingly, our results confirmed that Si was not translocated to the fruit, thereby eliminating concerns about Si potentially affecting fruit firmness or quality.

In contrast to the high-tunnel experiments, we observed concentrations averaging only 0.3% Si in plants of field experiments treated with soluble Si twice a week. Except for a marginal increase of 0.1% with cv. San Andreas, the treatment did not increase Si concentrations in plants over a 4-month period. These results are surprising considering that field plants only received 22.5% less Si solution than high tunnel plants (620 vs. 800 ml). Assuming perfect absorption of over 500 mg Si delivered to the plant over the course of the experiment, this could have translated into over 2–3% Si, on the basis of dry plants averaging between 15 and 20 g. One possible reason can be leaching of the solution although we would have expected at least some increments considering the solution was delivered directly at the base of the plant. Another possibility is that silicic acid became unavailable to the plant because of an interaction with soil particles. It is well known that some soils will release more or less silicic acid depending on texture, pH, conductivity, although this phenomenon has been less described with soluble fertilizers (Liang et al., 1994; Tubana et al., 2016). Nevertheless, further investigations are warranted to determine if our liquid or solid fertilization treatments actually increased plant available Si in the treated soils, and how soil properties can influence this important parameter.

Following the poor results obtained in 2015, we decided to test Si fertilizers that are more affordable and easier to apply. Calcium silicates are by far reputed to be the best source for Si fertilization with wollastonite being a particularly good substrate of plant-available Si (Pereira et al., 2004). At the end of the summer, regardless of the cultivar tested, or the calcium silicate concentration added to the plants, our results once again showed low Si concentrations below 0.25% and no significant differences in line with the Si treatment. These results raise a number of questions while answering some of the inconsistencies reported in the literature with respect to Si fertilization. In terms of questions, it is indeed puzzling that no increase in plant Si concentrations was observed, especially since preliminary experiments in soilless media had suggested that wollastonite would be a good source of Si (unpublished results). From the start, both soils where experiments took place were very low in plant available Si, a result well in line with concentrations found in control plants. This could mean that they had low Si content initially, but more importantly, that they were not conducive for silicic acid solubilization. For instance, light soils, generally sandy soils such as ours, do not have a good content in Si availability. Liang et al. (1994) related that the correlation with Si availability is positive with clay content but negative with sand content. In addition, in the case of a neutral soil (pH > 6.5), the silt content, the sand content and the pH are three factors negatively correlated with Si availability (Liang et al., 1994). In the field experiments, pH was 6.4 (2015) and 6.7 (2016) whereas soil texture was silty-sandy-loam (2015) and sandy-loam (2016), conditions that correlate well with the low Si concentrations found. Our results further suggest that Si amendments in such soils are not conducive to release silicic acid. Incidentally, Kanto et al. (2006) did not find accumulation of Si in strawberry plants fertilized in the field, although soil properties were not reported. On the other hand, in high tunnel experiments and in preliminary greenhouse experiments, plants grew in sphagnum-peat-moss substrate, which is known to be very acidic (Payette and Rochefort, 2001). This would further support that acidic pH conditions are more amenable to Si fertilization as they promote a better release and solubilization of silicic acid.

Our data can serve to explain the inconsistencies and confusion often encountered in the literature with respect to Si benefits. As a first point, the disparities in Si concentrations we observed between high tunnel and field strawberries explain why the classification of strawberry, in terms of Si absorption, has been a source of conflict on the basis of phenotypes. As a second point, it brings into question the reliability of Si fertilizers

and data reporting or not benefits. Unfortunately, most papers studying Si fertilization fail to report the amount absorbed by strawberry plants (Dehghanipoodeh et al., 2016; Yaghubi et al., 2016). The consequences of these oversights are that faulty conclusions can be drawn, leading to negative reports about Si benefits, attributable only to a deficient treatment, or positive results, attributable to other properties of the fertilizers.

In high tunnel experiments where Si fertilization led to high Si concentrations in plants, significant reductions in levels of powdery mildew severity were observed in all cultivars tested in both in 2014 and 2015. Interestingly, cv. Charlotte, reputed for its resistance to *P. aphanis*, did have the lowest powdery mildew severity among tested cultivars in both seasons, and in both the control and the Si+ treatments. These results confirm the multiple reports of the prophylactic properties of Si against powdery mildews on a number of crops (Chérif et al., 1992; Dik et al., 1998; Bélanger et al., 2003; Shetty et al., 2012). In a recent study, Vivancos et al. (2015) suggested that Si was particularly efficient against biotrophic pathogens because of the mode of attack of these pathogens relying on effectors to establish biotrophy; Si deposition would interfere with effectors finding their specific targets. Regardless of the mode of action, our data show convincingly that Si can be an efficient treatment to reduce powdery mildew severity in strawberry. Given that powdery mildew becomes particularly severe in late summer and early fall under our tested conditions, and that strawberry plants continue to absorb Si actively from July to September, there is a positive synchrony between the treatment and disease reduction. Unfortunately, both the absence of powdery mildew infection and the lack of an efficient Si treatment in 2015 and 2016, prevent from drawing conclusions about Si efficacy in the field experiments.

The reduction in powdery mildew severity linked to Si fertilization in high tunnels translated into significantly higher yields in terms of marketable fruits in both seasons and all cultivars. While it is unknown if the yield increase is solely attributable to powdery mildew reduction, or to other stimulating factors linked to Si, these positive effects were quite remarkable in some cultivars (e.g., Monterey) and certainly represent the highest selling point for growers to implement this approach. In addition, our data never showed negative effects of Si on plants or fruits, thus confirming the lack of phytotoxicity of the element. These observations allow to reject claims linking soluble Si fertilization to albino fruits (Lieten et al., 2002), an artifact most likely attributable to improper mixing of Si in the nutrient solution. For a producer, the main objective remains to optimize yield in order to maximize profits. If this can be attained by reducing the negative environmental impact of fungicides used for powdery mildew control, Si fertilization could find a niche as a component of an integrated program for strawberry production.

CONCLUSIONS

In conclusion, the objectives of this project were to determine if strawberry had the proper genetic tools to absorb Si, and to determine the fertilization conditions under which strawberry could benefit from a Si treatment. Our results showed that strawberry has the proper transporters to uptake Si, and that under a constant soluble Si fertilization regime and a peat substrate, it can absorb as much as 3% Si d.w., clearly classifying it as a Si-competent species. This treatment reduced significantly the severity of *P. aphanis* in all tested cultivars and increased the yield of marketable fruits. These results highlight the potential of Si amendments for producers with opportunities to lower fungicide use and increase revenues. Our results further show that the fertilization regimes and soil conditions can greatly influence the benefits strawberry will obtain from Si amendments, and these should be considered carefully before fertilizing with Si.

AUTHOR CONTRIBUTIONS

SO and MG performed all the greenhouse and field experiments, CL participated in greenhouse experiment and silicon quantification, CL and JL involved in data analysis. RD performed the bioinformatic analysis, LG, AG, MD involved in planning the field and greenhouse experiments, RB designed and directed the project. All authors contributed in drafting the MS.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00949/full#supplementary-material

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Uptake of Silicon by Sugarcane from Applied Sources May Not Reflect Plant-Available Soil Silicon and Total Silicon Content of Sources

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Soils of the tropics and sub-tropics are typically acid and depleted of soluble sources of silicon (Si) due to weathering and leaching associated with high rainfall and temperatures. Together with intensive cropping, this leads to marginal or deficient plant Si levels in Si-accumulating crops such as rice and sugarcane. Although such deficiencies can be corrected with exogenous application of Si sources, there is controversy over the effectiveness of sources in relation to their total Si content, and their capacity to raise soil and plant Si concentrations. This study tested the hypothesis that the total Si content and provision of plant-available Si from six sources directly affects subsequent plant Si uptake as reflected in leaf Si concentration. Two trials with potted cane plants were established with the following Si sources as treatments: calcium silicate slag, fused magnesium (thermo) phosphate, volcanic rock dust, magnesium silicate, and granular potassium silicate. Silicon sources were applied at rates intended to achieve equivalent elemental soil Si concentrations; controls were untreated or lime-treated. Analyses were conducted to determine soil and leaf elemental concentrations. Among the sources, calcium silicate produced the highest leaf Si concentrations, yet lower plant-available soil Si concentrations than the thermophosphate. The latter, with slightly higher total Si than the slag, produced substantially greater increases in soil Si than all other products, yet did not significantly raise leaf Si above the controls. All other sources did not significantly increase soil or leaf Si concentrations, despite their high Si content. Hence, the total Si content of sources does not necessarily concur with a product's provision of soluble soil Si and subsequent plant uptake. Furthermore, even where soil pH was raised, plant uptake from thermophosphate was well below expectation, possibly due to its limited liming capacity. The ability of the calcium silicate to provide Si while simultaneously and significantly increasing soil pH, and thereby reducing reaction of Si with exchangeable Al^{3+}, is proposed as a potential explanation for the greater Si uptake into the shoot from this source.

Keywords: acid soils, aluminum, calcium silicate, liming, plant stress, silicon uptake, soil pH, thermophosphate

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INTRODUCTION

Although silicon (Si) is abundant in the Earth's crust (28.8%) (Wedepohl, 1995), it is not considered an essential element for terrestrial plants other than the Equisitaceae (Epstein, 1994). However, there is now considerable evidence for its role in plant health and ecology (Cooke and Leishman, 2011, 2012), and specifically in mitigating numerous abiotic and biotic stresses, including water and salinity stress, metal toxicities, nutrient imbalance, fungal and bacterial pathogens, and insect herbivores (reviews by Ma, 2004; Datnoff et al., 2007; Liang et al., 2007; Epstein, 2009; Reynolds et al., 2009; Zhu and Gong, 2014; Adrees et al., 2015). Among crop species that accumulate Si to levels >1.0% shoot Si dry mass (Ma and Takahashi, 2002), rice (*Oryza sativa* L.) and sugarcane (*Saccharum* spp. hybrids) have been well-studied, and are capable of removing up to 470 and 700 kg Si ha^{-1} $annum^{-1}$, respectively, on Si-rich soils (Ross et al., 1974; Savant et al., 1997b, 1999; Meena et al., 2014). Yield responses in rice and sugarcane to soil Si amendments have frequently been recorded on the weathered tropical or sub-tropical soils on which they are largely grown (e.g., Oxisols, Ultisols, and organic Histosols) (Cheong and Halais, 1969; Elawad et al., 1982; Yamauchi and Winslow, 1989; Savant et al., 1997b, 1999; Alvarez and Datnoff, 2001; Meyer and Keeping, 2001; Berthelsen et al., 2001b; Tsujimoto et al., 2014). Due to high rainfall and temperatures, such soils have typically been depleted (desilicated) of soluble sources of Si (McKeague and Cline, 1963c; Savant et al., 1997a; Epstein, 2001; Meena et al., 2014), leading to marginal or deficient levels of plant Si in these crops (Savant et al., 1997a, 1999; Meena et al., 2014). Besides being low in essential nutrients, these highly weathered soils are also acidic and may therefore be high in soluble forms of aluminum (Al) (where soil pH_w < 5.5) (Sanchez, 1976; Fageria et al., 1988), which in turn can remove soluble Si through reaction to form insoluble hydroxyaluminosilicates (HASs) (Farmer et al., 1979; Doucet et al., 2001; Schneider et al., 2004).

Plants take up Si as monomeric silicic acid (H_4SiO_4), the dominant form of Si in soil solution (Epstein, 1994). The solubility of silicic acid in soil solution is strongly pH-dependent and related to its adsorption/desorption reactions on soil colloids. Solubility and concentration in soil solution is highest at low pH and decreases progressively up to a pH of 9.8, the pK_1 of silicic acid, where the latter dissociates to form $H_3SiO_4^-$. At this pH, the silicate anion is maximally adsorbed to soil surfaces, especially Al and Fe hydrous oxides, causing the concentration of Si in soil solution to decrease (Beckwith and Reeve, 1963; Jones and Handreck, 1963; McKeague and Cline, 1963a,b; Haynes, 2014; Liang et al., 2015). This relationship between Si solubility and pH is one of the major factors accounting for the loss of Si in weathered, acidic soils, and is exacerbated through intensive, long-term cropping and resulting export of Si from the landscape (Berthelsen et al., 2001b; Sommer et al., 2006; Vandevenne et al., 2012; Haynes, 2014).

A further problem in weathered, acid soils is, as noted above, the occurrence of high levels of exchangeable Al^{3+}. Soil acidity and associated Al^{3+} toxicity have long been recognized as significant, and increasing, constraints in sugarcane production in the South African sugar industry (Sumner, 1970; Meyer et al., 1971; Moberly and Meyer, 1975; Schroeder et al., 1994) and indeed wider agricultural production in South Africa (Barnard and du Preez, 2004). For soils under sugarcane production in South Africa, Miles et al. (2014b) showed convincingly that available Si is strongly limited under conditions of high exchangeable $H^+ + Al^{3+}$. Their results indicated that high levels (>40 mg kg^{-1}) of soluble Si occurred only where exchangeable $H^+ + Al^{3+}$ levels were below approximately 0.5 $cmol_c L^{-1}$ and that Al^{3+} is probably a key factor in constraining plant-available Si in acid soils. Differences in available soil Si and pH across regions are strongly reflected in Si uptake by sugarcane, with leaf Si content consistently higher (10–25 g kg^{-1} dry matter) in less acid soils (pH > 6.5), but seldom exceeding the industry threshold (Miles and Rhodes, 2013) of 7.5 g kg^{-1} dry matter in the more weathered acid soils of the coastal and hinterland sugarcane production regions (pH ≤ 5.5) (Van der Laan and Miles, 2010; Miles et al., 2011).

Hence, in these soils there is an urgent need to replenish plant-available soil Si in order to sustain maximum crop production, reduce abiotic stresses (especially water stress and Al toxicity) (Meyer and Keeping, 2001), and as a means to augment plant resistance of more susceptible cultivars to the lepidopteran stalk borer *Eldana saccharina* Walker (Keeping and Meyer, 2006; Kvedaras and Keeping, 2007; Kvedaras et al., 2007; Keeping et al., 2014). Silicon amendment can also reduce infections of brown rust (*Puccinia melanocephala* H. and P. Sydow), which occurs in several rust-susceptible cultivars in South Africa (Ramouthar et al., 2016). With this in mind, recent research efforts on provision of Si for sugarcane production in South Africa have focussed on identifying sources with high plant-available Si, and which can simultaneously correct soil pH and reduce Al toxicity (Rhodes et al., 2013; Keeping et al., 2014, 2017). Calcium (magnesium) silicate, supplied in the form of metallurgical slags, has proven most effective in supplying plant-available Si for sugarcane (Gascho, 2001; Berthelsen et al., 2001a; Meyer and Keeping, 2001; Bokhtiar et al., 2012; McCray and Ji, 2013; Crusciol et al., 2014; Tubana et al., 2016). Keeping et al. (2017) found that alkaline Si sources, such as calcium (Ca) silicate slag, cement, and granulated ground blast furnace slag, produced significantly more plant-available Si and greater plant uptake than sources with little or no pH-corrective capacity, such as potassium (K) silicate, bagasse fly ash, and diatomaceous earth. In line with their ability to increase soil pH, slags and cement also significantly reduced Al saturation, and to an extent equivalent to that of dolomitic lime applied at the same rate (Keeping et al., 2017).

However, other Si sources, such as thermophosphates, sometimes referred to as fused magnesium (Mg) phosphate (see Ma and Takahashi, 2002, p. 18), have also shown significant potential in supplying Si (Korndörfer and Gascho, 1999; Gascho, 2001; Kingston, 2011), as has volcanic rock dust (crushed basalt) when applied to sugarcane on highly weathered soils in Mauritius (D'Hotman De Villiers, 1961, 1962). The latter amendment, applied at rates from 110 to 440 tons ha^{-1}, produced cumulative yield responses of between 49 and 90 tons cane ha^{-1} over five crops. Subsequent studies confirmed that the soluble silicon in

the basalt accounted for the favorable yield increases (Halais and Parish, 1964). However, previous work has shown that the total Si content of a source, its provision of plant-available Si (as determined by soil tests), and uptake of Si, especially in rice and sugarcane, did not necessarily concur (Gascho, 2001; Ma and Takahashi, 2002; Kingston, 2011; Haynes et al., 2013; McCray and Ji, 2013; Elephant et al., 2016; Keeping et al., 2017). This observation, together with the novel opportunity to investigate several new Si sources (volcanic rock dust, magnesium silicate and slow-release potassium silicate) for sugarcane in South Africa, prompted further study of the relationship between total source (or product) Si content, available (calcium chloride ($CaCl_2$) extractable) soil Si following application, and subsequent plant Si accumulation, along with the potential of these sources to reduce acid saturation.

To this end, two trials were conducted using potted sugarcane grown in a low-Si soil, supplied with Si at a single (Trial 1) or two (Trial 2) elemental rates via applications of a Ca silicate slag (Calmasil®, http://www.pbd-lime.co.za/calmasil.htm), thermophosphate (Calsimag-P®), Mg silicate (Prosil Plus®), granular K silicate, volcanic rock (basalt) dust (Turbo-Grow®, www.turbo-grow.co.za), and, in Trial 2, a dolomitic lime control (**Table 1**). Calmasil is a slag by-product of the stainless industry, while Calsimag-P is manufactured by blending and fusing apatite and serpentine in a furnace, and the resulting amorphous Ca/Mg/P/Si complex is milled to a fine powder and granulated. The Mg silicate (Prosil Plus) is also a serpentine mineral source derived from crushed volcanic rock mined from Colombian batholiths. The K silicate consisted of two types: "Type M" slow release granules that contained compounds of Mg, which imparted a "free running" characteristic to the granules and their slow dissolution in water; "Type MC hardened" slow release granules that contained both Mg and Ca for the same reasons, and had been oven dried at 130–140°C to further reduce their solubility. The study tested the hypothesis that the Si content of these sources (as specified by the supplier) and available soil Si following application, directly affects subsequent plant Si uptake as reflected in leaf Si concentration. More specifically, the thermophosphate and especially volcanic rock dust, Mg silicate and K silicate, with higher total Si contents than the slag (>2-fold higher for the rock dust and 3-fold higher for the K silicate; **Table 1**), were predicted to produce significantly higher leaf Si concentrations than the latter. Furthermore, the sources were compared with respect to their ability to ameliorate soil acidity and Al toxicity, and to supply Ca and Mg to the soil and plant.

MATERIALS AND METHODS

The trials were established in a randomized design in a shadehouse with clear polycarbonate roofing and walls of 40% green shade cloth, over the period November 2013 to May 2014 (Trial 1; 27 weeks) and October 2014 to March 2015 (Trial 2; 22 weeks). Treatments were replicated 12 times in each trial, with one pot in each row of pots comprising a single replicate of each treatment. All pots (total of 84 in Trial 1 and 96 in Trial 2) were filled with soil collected from the same site within a sugarcane field (Field 380, Inanda Farm, 29°37′37″S, 30°56′58″E, KwaZulu-Natal (KZN), South Africa). The soil collected for Trial 1 was taken from an area immediately adjacent to that collected for Trial 2. The soil type was an Inceptisol (Soil Survey Staff, 2006), which in the USA is among the soil orders commonly found in humid and sub-humid regions, and known to have limiting plant-available Si (Tubana et al., 2016). In KZN, the soil consists of gray loamy sands, moderately to strongly acid, with a low level of fertility (Beater, 1970), and is typical of the weathered, acidic, low-Si soils of the rainfed regions of the South African sugar industry, as described earlier. The soil for each trial was collected from the top 15 cm layer within an area of ∼400 m^2, air dried, thoroughly mixed, and passed through a 1 mm sieve. Single samples for analysis were taken from the mixed and sieved bulk soil for each trial before it was placed into pots. The soil properties of each bulk collection are summarized in **Table 2**. Although the collection site was specifically chosen due to the acid nature of the soil, it was discovered after analysis that the soil for Trial 1 was of a higher pH, higher Ca and clay content, and

TABLE 1 | Product name (in alphabetical order), supplier (all South Africa based except for Prosil Plus) and silicon content of products used in Trials 1 and 2.

Product	Supplier	Percent Si
Calmasil (calcium silicate)[a]	PDB Lime (Pty) Ltd., Middleburg, Mpumalanga	10.3
Calsimag-P®[b]	Farmsecure Agri Science, Amanzimtoti, KZN	12.6
Kulu dolomitic lime[c]	Geyser's Fertilizer and Lime, Durban, KZN	0.0
Potassium silicate type M	Tangmere Resources (Pty) Ltd., Uvongo, KZN	30.8
Potassium silicate type MC	Tangmere Resources (Pty) Ltd., Uvongo, KZN	30.8
Prosil Plus WP (magnesium silicate)[d]	AgroMatChem Ltd., Ta'Xbiex, Malta	16.3
Turbo-Grow® (volcanic rock dust)[e]	Turbo-Grow (Pty) Ltd., Wendywood, Gauteng	24.9

Note that the Kulu dolomitic lime was used as a control (zero Si) and is listed here for the sake of completeness regarding products used.
[a] *Calmasil: electric arc furnace slag, 24.8% Ca, 6.0% Mg.*
[b] *Calsimag-P: granulated thermophosphate, 21.5% Ca, 8.0% Mg, 8.7% P.*
[c] *Dolomitic lime: 21.0% Ca, 8.1% Mg.*
[d] *Prosil: 18.1% Mg.*
[e] *Turbo-Grow: volcanic rock dust: 5.4% Ca, 3.2% Mg (all Turbo-Grow values based on analysis by SGS Lakefield Research, Booysens, South Africa).*

much lower acid saturation than that for Trial 2 (**Table 2**). The most likely explanation is that the area from which the soil for Trial 1 had been collected had inadvertently been limed or used as a site for dumping lime by the grower some time—possibly several years —previously. Consequently, control treatments incorporating dolomitic lime were not included in Trial 1 (see below). Fortuitously, these differences between the soil in each trial provided an opportunity to compare Si uptake and effect on soil properties of two sources (Calmasil and Calsimag-P) common to both trials.

Treatments and Fertilizer

Plastic pots (6.41 L) were filled with 6,700 g of soil and application rates for all Si treatments (products), lime and fertilizers were converted from kg ha^{-1} to g kg^{-1}, based on the average (disturbed) soil density (1,215 g cm^{-3}) and a top-soil depth of 15 cm; i.e., a soil mass of 1,822,500 kg ha^{-1}. For Trial 1, the five Si sources (Calmasil, Calsimag-P, Prosil Plus, K silicate Type M and Type MC) were all applied at product rates (**Table 3**) intended to provide an elemental Si rate of 300 kg Si ha^{-1} across all treatments. The control was left untreated, as the soil collected for Trial 1 was of a slightly higher pH and lower acid saturation than that for Trial 2 (**Table 2**). Consequently, control treatments incorporating dolomitic lime were considered unnecessary for Trial 1.

For Trial 2, Calmasil, Calsimag-P, and Turbo-Grow, plus a dolomitic lime control with zero Si, were each applied at two rates to produce a total of eight treatments (**Table 4**). The Si sources were applied to provide a lower Si rate (Si 1) of 300 kg Si ha^{-1} and a higher rate (Si 2) of 750 kg Si ha^{-1} (**Table 4**). The rates for the dolomitic (Kulu) lime control were made equivalent to that of Calmasil, given the similar neutralizing capacity of the two materials (Calmasil = 102.8% of pure $CaCO_3$).

The particle size distributions of the Si sources used in Trials 1 and 2 (**Table 5**) were determined by shaking 100 mg samples through 8 sieve sizes for 5 min using a Fritsch (Germany) Pulverisette 03502 mechanical vibrator.

Treatments and fertilizer were simultaneously hand-incorporated into the entire volume of soil following initial moistening with 1,000 ml water pot^{-1}. Basal fertilizers were applied as follows: 88 mg nitrogen kg^{-1} as limestone ammonium nitrate (LAN), 66 mg phosphorous (P) kg^{-1} as Ca di-orthophosphate, 2 mg copper kg^{-1} as copper sulfate, and 8 mg zinc kg^{-1} as zinc sulfate. In both trials, 173 mg K kg^{-1} as K chloride was supplied to all treatments except the K silicate treatments in Trial 1, where top-up K at 35 mg kg^{-1} was provided as K chloride. Top dressings of LAN (54 mg N kg^{-1}) and K chloride (71 mg K kg^{-1}, Trial 1) or K sulfate (Trial 2, 69 mg K kg^{-1} and 28 mg S kg^{-1}) were supplied twice monthly after planting.

Plants

Sugarcane transplants of variety N12 (Anon, 2006) were produced from single-budded setts cut from mature stalks of field-grown cane of the same age and from the same field. Single 1-month old transplants were planted into each pot immediately

TABLE 3 | Treatments and product rates for Trial 1.

Treatment	Product rate	
	kg ha^{-1}	mg kg^{-1}
Control	0	0
Calmasil	2,913	1,598
Calsimag-P	2,459	1,349
Prosil Plus	1,840	1,010
K silicate type M	974	534
K silicate type MC	974	534

Products were applied at rates intended to provide an elemental silicon rate of 300 kg Si ha^{-1} across all treatments except the control, where no product was applied (zero silicon).

TABLE 4 | Treatments, product rates and silicon rates for Trial 2.

Treatment	Product rate		Silicon rate	
	kg ha^{-1}	mg kg^{-1}	kg ha^{-1}	mg kg^{-1}
Dolomitic lime 1	2,913	1,598	0	0
Dolomitic lime 2	7,282	3,996	0	0
Calmasil Si 1	2,913	1,598	300	165
Calmasil Si 2	7,282	3,995	750	412
Calsimag-P Si 1	2,459	1,349	300	165
Calsimag-P Si 2	6,148	3,373	750	412
Turbo-Grow Si 1	1,205	661	300	165
Turbo-Grow Si 2	3,012	1,653	750	412

*Products were applied at two rates to provide a lower (Si 1) and higher (Si 2) elemental silicon rate. The lower rate is the same as that used throughout Trial 1 (see **Table 3**). Dolomitic lime was applied (as a control with zero silicon) at the same product rates as Calmasil due to the equivalent liming capacity of the two products.*

TABLE 2 | Characteristics of soil from Inanda Farm (KwaZulu-Natal, South Africa) collected September 2013 from immediately adjacent areas in the same field for Trials 1 and 2.

Trial	P[a] (mg L^{-1})	K (mg L^{-1})	Ca (mg L^{-1})	Mg (mg L^{-1})	Si (mg L^{-1})	Total cations (cmol L^{-1})	pH ($CaCl_2$)	$H^+ + Al^{3+}$ (cmol L^{-1})	Acid saturation[b] (%)	Organic matter (%)	Clay (%)
1	60	152	635	47	14	4.5	4.5	0.5	10.6	3.8	13.0
2	67	142	282	32	10	3.4	4.0	1.3	38.8	4.1	16.4

[a] *Truog analysis.*

[b] *Acid saturation = [(H + Al)/(H + Al) + Ca + Mg + K + Na] × 100.*

TABLE 5 | Particle size distribution of silicon sources used in Trials 1 and 2.

Source	Particle size distribution (%)					
	>5.0 mm	5.0–2.0 mm	2.0–1.0 mm	1.0–0.5 mm	0.5–0.2 mm	<0.2 mm
Calmasil	0.0	0.0	0.3	5.8	26.4	67.5
Calsimag-P	0.5	94.3	4.3	0.0	0.0	0.9
Turbo-Grow	0.0	0.0	0.5	3.7	4.2	91.6
Prosil Plus	0.1	0.1	0.5	3.3	5.6	90.4
K silicate type M	0.6	97.0	0.8	0.6	0.4	0.6
K silicate type MC	9.0	75.3	9.5	3.6	1.6	1.0

Particle size was determined using a Frisch Analysette equipped with King Test laboratory sieves.

after the first soil sample (see below). Pots were drip irrigated daily to weekly, depending on moisture demand.

Soil and Leaf Analysis

Soil samples were taken with an augur inserted to the base of all pots in each trial at 7 days after application of treatments and again at 7 days after the trials were harvested at age 27 weeks (Trial 1) or 21 weeks (Trial 2). The purpose of the 7-day interval before the first sample was to allow time for equilibration and any sorption of H_4SiO_4 to sesquioxides and soil surfaces that may affect measurement of its availability (Babu et al., 2016a). Samples from 3 (Trial 1) or 4 (Trial 2) adjacent replicates of the same treatment were composited to reduce analysis costs. Plant-available soil Si was determined using 0.01 *M* calcium chloride ($CaCl_2$) extraction, a widely-accepted method that provides a close approximation of the soil environment (Berthelsen and Korndörfer, 2005; Sauer et al., 2006; Haynes et al., 2013; Miles et al., 2014b; Babu et al., 2016b). Soil analyses were performed by the South African Sugarcane Research Institute (SASRI) Fertilizer Advisory Service (FAS), with Si and P concentrations determined using the ammonium molybdate blue colourimetry (Liang et al., 2015) and Truog methods (Miles et al., 2014a), respectively. All soil analyses in the FAS are performed on a volumetric basis. Only results pertinent to the hypotheses tested in this study and for elements provided by the Si sources are reported, i.e., soil concentrations of Si, Ca, Mg, P, as well as pH and acid saturation.

Leaf sampling was conducted once, at harvest. The third fully unfurled or "top visible dewlap" leaf was removed from the major tillers in each pot; leaf blades were stripped from the midrib and the blades dried, ground and analyzed for their elemental nutrient content by the SASRI FAS. Samples from 3 adjacent replicates of the same treatment were composited to produce sufficient material for analysis. Leaf Si content was determined using the dry ashing and molybdenum blue colorimetry method (Liang et al., 2015). As for soil, only results pertinent to the hypotheses tested and for elements provided by the Si sources are reported, i.e., leaf concentrations of Si, Ca, Mg, P.

Data Analysis

All data were tested for univariate normality (Shapiro-Wilk test) and homogeneity of variance (Bartlett's test), and appropriate transformations (log or square root) applied when these conditions were not met, prior to analysis of variance. Where ANOVA yielded significant differences between treatments, planned comparisons of means were performed using the Holm-Sidak multiple-comparisons test. All analyses were carried out using Genstat 14th Edition.

RESULTS

Effects of Si Sources on Soil Properties in Pre-plant and Post-harvest Soil Samples—Trial 1

The soil treatments significantly affected ($P < 0.001$; ANOVA) soil Si concentrations in the pre-plant and post-harvest soil samples. Of the five Si sources applied, only Calmasil and Calsimag-P significantly increased $CaCl_2$-extractable soil Si above that of the untreated control in pre-plant and post-harvest samples (**Figure 1A**). Calsimag-P released greater quantities of Si than Calmasil, but the difference was significant only in the post-harvest soil sample (**Figure 1A**); this was despite application of Calsimag-P at a lower product rate (**Table 3**) to compensate for its higher total Si content. Concentrations of extractable Si diminished between the pre-plant and post-harvest soil samples by 59% (control), 77% (Calmasil), 44% (Calsimag-P), 52% (Prosil Plus), 69% (K silicate type M), and 67% (K silicate type MC) (**Figure 1A**).

The soil treatments significantly affected soil Ca concentrations in the post-harvest sample and Mg and P concentrations in the pre-plant and post-harvest soil samples (**Table 6**). In the post-harvest treatment, Calmasil supplied significantly more Ca than all other treatments except Calsimag-P. None of the other treatments differed in this respect (**Table 6**). Calmasil and Calsimag-P both supplied significantly more Mg than the other treatments in pre-plant and post-harvest samples, and Prosil Plus significantly more Mg than the K silicate treatments, which did not differ from the control (**Table 6**). Calsimag-P increased P significantly compared with the Prosil Plus and K silicate treatments in the pre-plant sample and significantly above all other treatments in the post-harvest sample (**Table 6**).

Although the treatments significantly affected soil pH in pre-plant and post-harvest samples, they had no effect on acid saturation (**Table 6**). In the pre-plant sample, Calmasil raised pH significantly above the control and Prosil

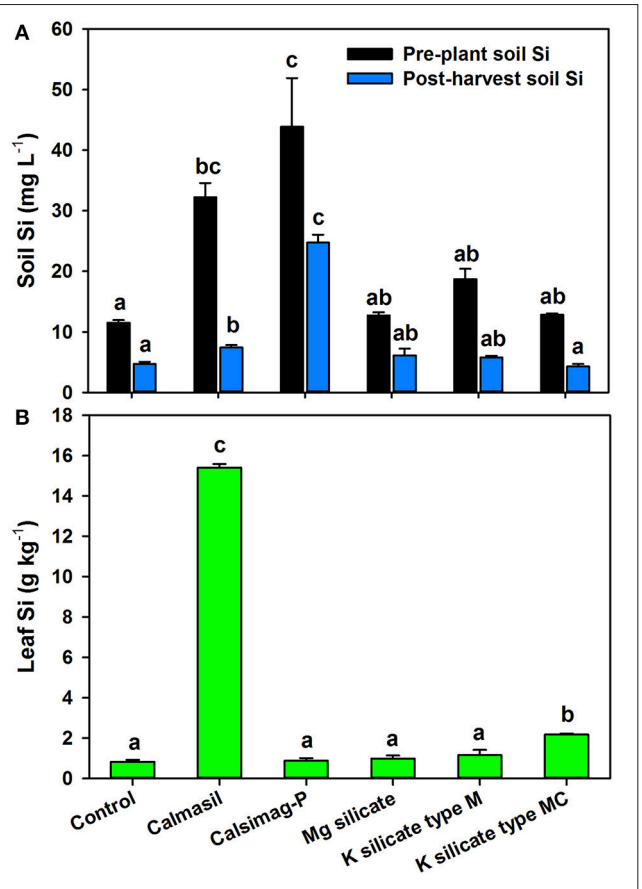

FIGURE 1 | Silicon concentrations in soil (A) and third leaf **(B)** following application at rates specified in **Table 3** of different silicon sources (represented on X-axis) in Trial 1. The control was untreated. In **(A)**, black bars represent pre-plant soil and blue bars represent post-harvest soil; mean values for bars of the same color and with the same letter/s above them are not significantly different. In **(B)**, mean values for bars with the same letter above them are not significantly different (Holm-Sidak test, $P < 0.05$; ANOVA, $P < 0.001$ for soil and leaf Si). Error bars are standard errors. Mg silicate = Prosil Plus

Plus, but not the other treatments (**Table 6**), while in the post-harvest sample, Calmasil increased pH significantly above that of all other treatments except Calsimag-P; the latter was also significantly higher than the control. Prosil Plus and K silicate did not differ from the control (**Table 6**).

Effects of Si Sources on Leaf Si, Ca, Mg, and P Concentrations—Trial 1

The soil treatments significantly affected leaf Si concentrations ($P < 0.001$; ANOVA); however, only Calmasil (by 19-fold) and to a much lesser extent K silicate type MC (by ~3-fold) increased leaf Si above the control (**Figure 1B**). It is clear from **Figure 1** that for Calmasil and Calsimag-P leaf Si did not increase in direct relation to the concentration of extractable soil Si.

The treatments had no significant effects on leaf Ca, Mg, or P concentrations (**Table 7**).

Effects of Si Sources on Soil Properties in Pre-plant and Post-harvest Soil Samples—Trial 2

The soil treatments significantly affected ($P < 0.001$; ANOVA) soil Si concentrations in the pre-plant and post-harvest soil samples. Of the three Si sources applied, only Calmasil and Calsimag-P significantly elevated $CaCl_2$-extractable soil Si above that of the dolomitic lime control (**Figure 2A**). Calsimag-P released significantly greater quantities of Si at the higher and lower elemental Si rates than Calmasil at each rate (**Figure 2A**), even though Calsimag-P was applied at a lower product rate (**Table 4**) to compensate for its higher total Si content. In the post-harvest sample, Calsimag-P at the lower rate (Si 1) produced significantly higher extractable Si than Calmasil at the higher rate (Si 2) (**Figure 2A**). Turbo-Grow did not raise extractable soil Si above that of the lime control in both soil samples (**Figure 2A**). Concentrations of extractable Si decreased between the pre-plant and post-harvest soil samples by 59% (Calmasil Si 1), 57% (Calmasil Si 2), 40% (Calsimag-P Si 1), and 35% (Calsimag-P Si 2), (**Figure 2A**). Soil Si also decreased over the course of the trial in the Lime 1 (29%), Lime 2 (21%), Turbo-Grow Si 1 (49%), and Turbo-Grow Si 2 (31%) treatments (**Figure 2A**).

The soil treatments significantly affected soil Ca and Mg concentrations in the pre-plant and post-harvest soil samples, and P in the pre-plant sample (**Table 8**). In the pre-plant sample, Calmasil Si 2 supplied significantly more Ca than all other treatments and significantly more Mg than all other treatments except Turbo-Grow Si 2 (**Table 8**). Turbo-Grow Si 2 provided significantly more Ca than dolomitic lime 2 and Calsimag-P Si 2 (**Table 8**). Compared with lime (which was applied at the same product rates), Calmasil provided 48 and 65% more Ca, and 47 and 63% more Mg, at Si 1 and Si 2, respectively.

In the post-harvest sample, Calmasil Si 2 still supplied significantly more Ca than all other treatments; however, its supply of Mg was not significantly greater than that of lime 2 or Calsimag-P Si 2 (**Table 8**). The supply of Ca and Mg by Turbo-Grow at both treatment rates diminished to concentrations significantly lower than that of all other treatments, other than Ca provided by Calsimag-P Si 1 (**Table 8**). In the pre-plant sample, Calsimag-P Si 2 raised soil P significantly above all other treatments (and 2.3-fold above that of the control) and Calsimag-P Si 1 above all treatments except Turbo-Grow Si 1 (**Table 8**). Soil P is not presented for post-harvest samples in **Table 8**, because the FAS laboratory employs different soil P test methods depending on soil pH (Truog for pH $\leq$ 5.5 and resin for pH $>$ 5.5; Miles et al., 2014a), thus rendering the data from different treatments non-comparable.

The soil treatments significantly affected soil pH and acid saturation in the pre-plant and post-harvest soil samples (**Table 8**). Calmasil Si 2 raised soil pH significantly above that of all other treatments in the pre-plant and post-harvest samples, with the exception of lime 2 in the post-harvest sample (**Table 8**). In the pre-plant sample, the *lower* rate of Calmasil (i.e., Si 1) was as effective in correcting pH as the *higher* rate of lime (i.e., lime 2) (**Table 8**). Although Calsimag-P was applied at a lower product rate than dolomitic lime (**Table 4**), it nonetheless produced pH

TABLE 6 | Soil concentrations of elements (calcium, magnesium, phosphorus) provided by the silicon sources, soil pH and acid saturation in pre-plant and post-harvest samples from Trial 1.

Treatment	Ca (mg L^{-1})	Mg (mg L^{-1})	P (mg L^{-1})	pH ($CaCl_2$)	Acid Sat[a] (%)
PRE-PLANT SAMPLE					
Control	659	58 a	126 ab	4.5 a	8.6
Calmasil	763	83 b	136 ab	4.9 b	2.7
Calsimag-P	681	82 b	203 b	4.8 ab	5.2
Prosil Plus	609	58 a	125 a	4.6 a	8.6
K silicate type M	700	52 a	114 a	4.6 ab	7.0
K silicate type MC	671	55 a	95 a	4.6 ab	8.4
P-value	0.84	<0.001	0.002	0.014	0.29
POST-HARVEST SAMPLE					
Control	823 a	84 ab	59 a	4.6 a	5.1
Calmasil	1268 b	146 c	54 a	5.3 c	0.2
Calsimag-P	980 ab	131 c	185 b	5.0 bc	1.4
Prosil Plus	804 a	106 b	58 a	4.8 ab	3.7
K silicate type M	820 a	76 a	60 a	4.7 ab	3.7
K silicate type MC	815 a	81 a	57 a	4.6 ab	5.5
P-value	0.013	<0.001	<0.001	<0.001	0.15

*Application rates are specified in **Table 3**; the control was untreated. See **Figure 1** for soil silicon concentrations. Values are means (n = 3 for pre-plant, n = 4 for post-harvest). P-values are from ANOVA. Where ANOVA indicates significant differences, means within a column followed by the same letter are not significantly different (Holm-Sidak test, $p < 0.05$).*
*[a]Acid Sat = Acid saturation (see **Table 2** for definition).*

TABLE 7 | Leaf concentrations of elements (calcium, magnesium, phosphorus) provided by the silicon sources applied in Trial 1 at the rates specified in Table 3.

Treatment	Ca	Mg	P
	g kg^{-1}		
Control	1.7	0.9	1.4
Calmasil	1.7	1.0	1.3
Calsimag-P	2.1	1.2	1.3
Prosil Plus	1.9	1.1	1.3
K silicate type M	1.8	0.9	1.3
K silicate type MC	1.9	1.1	1.4
P-value	0.61	0.28	0.95

*The control was untreated. See **Figure 1** for leaf silicon concentrations. Values are means (n = 4). P-values are from ANOVA.*

levels that were comparable with those of lime at their respective low and high rates (**Table 8**). Turbo-Grow evidently had little effect in raising pH at the product rates applied, with the pre-plant sample pH elevated by only 0.1 unit and the post-harvest sample by 0.5 unit above that of the untreated bulk soil used in the trial (**Tables 2**, **5**).

Calmasil Si 2 significantly reduced acid saturation percent below that of all other treatments in the pre-plant sample, including lime when compared at the same product rates (**Table 8**). The reduction of acid saturation (elimination of reactive Al) by Calmasil at the *lower* application rate (Si 1) was not significantly different from that produced by lime at the *higher* application rate (Si 2) (**Table 8**). Calsimag-P Si 1 and Turbo-Grow Si 1 had similar (and the least) effects on acid saturation, although Calsimag-P Si 2 reduced it to a level not significantly different from that of lime at the lower application rate (dolomitic lime 1) (**Table 8**). The higher application rate of Turbo-Grow (Si 2) had little effect in reducing acid saturation below that of the lower rate (Si 1) of this product (**Table 8**). All products however, lowered acid saturation substantially compared with that of the untreated bulk soil (38.8%, **Table 2**).

Similar differences and similarities between treatments were evident in the post-harvest soil sample; however, it was apparent that acid saturation decreased from pre-plant levels in the lime, Calmasil and Calsimag-P treatments, while that in the Turbo-Grow treatments increased above those of the pre-plant values (values between pre-plant and post-harvest samples were not compared statistically) (**Table 8**). In the post-harvest sample, acid saturation in the Calsimag-P Si 2 treatment did not differ significantly from that in any of the lime or Calmasil treatments (**Table 8**).

Effects of Si Sources on Leaf Si, Ca, Mg, and P Concentrations—Trial 2

The soil treatments significantly affected leaf Si concentration ($P < 0.001$; ANOVA); however, Calmasil Si 2 was the only treatment that significantly increased leaf Si above the dolomitic lime control (**Figure 2B**). Calmasil Si 2 increased leaf Si 2-fold over that of lime applied at the same product rate (lime 2). Calsimag-P - notwithstanding its substantially greater release of soluble Si into the soil, especially at the higher rate (**Figure 2A**)-had a small and non-significant effect in raising leaf Si above the lime controls (**Figure 2B**). Turbo-Grow had no discernible effect on leaf Si content (**Figure 2B**). As for Trial 1, it is evident for Trial 2 (**Figure 2**) that leaf Si in the Calmasil and Calsimag-P

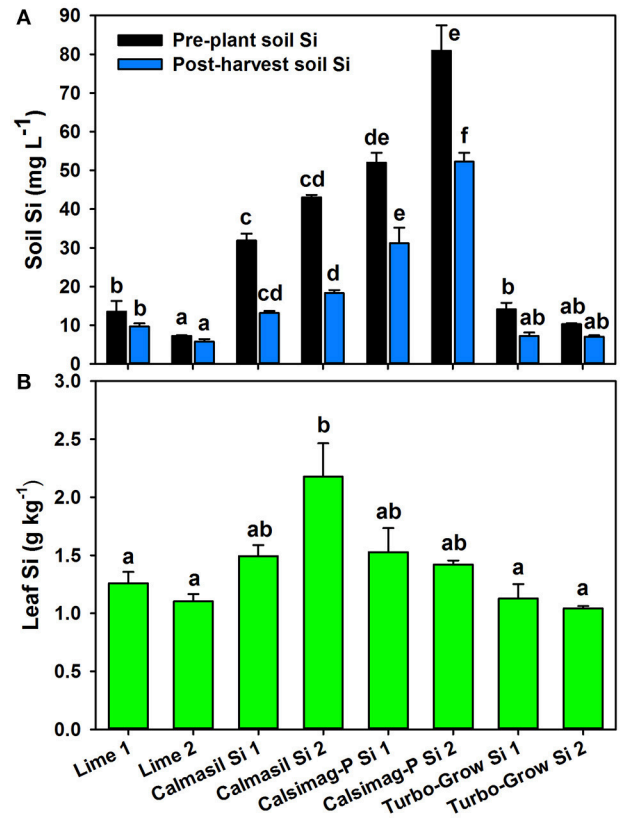

FIGURE 2 | Silicon concentrations in soil (A) and third leaf **(B)** following application of different silicon sources (represented on X-axis) at lower and higher rates (Si 1 and Si 2; see **Table 4**) in Trial 2. Product and silicon application rates are specified in **Table 4**; the lime control was applied at the same product rates as Calmasil. In **(A)**, black bars represent pre-plant soil and blue bars represent post-harvest soil; mean values for bars of the same color and with the same letter/s above them are not significantly different. In **(B)**, mean values for bars with the same letter above them are not significantly different (Holm-Sidak test, $P < 0.05$; ANOVA, $P < 0.001$ for soil and leaf Si). Error bars are standard errors.

TABLE 8 | Soil concentrations of elements (calcium, magnesium, phosphorus) provided by the silicon sources applied at lower and higher rates (Si 1 and Si 2), soil pH and acid saturation in pre-plant and post-harvest samples from Trial 2.

Treatment	Ca (mg L^{-1})	Mg (mg L^{-1})	P (mg L^{-1})	pH ($CaCl_2$)	Acid Sat[a] (%)
PRE-PLANT SAMPLE					
Dolomitic lime 1	638 b	93 ab	153 a	4.5 b	4.8 c
Dolomitic lime 2	829 cd	131 bcd	157 a	5.1 c	1.4 b
Calmasil Si 1	947 de	137 cd	147 a	5.0 c	1.5 b
Calmasil Si 2	1365 f	214 f	154 a	6.0 d	0.5 a
Calsimag-P Si 1	449 a	73 a	234 b	4.4 b	14.4 d
Calsimag-P Si 2	711 bc	166 de	353 c	5.1 c	3.6 c
Turbo-Grow Si 1	660 d	106 abc	168 ab	4.1 a	15.6 d
Turbo-Grow Si 2	1030 e	185 ef	148 a	4.1 a	10.9 d
P-value	<0.001	<0.001	<0.001	<0.001	<0.001
POST-HARVEST SAMPLE					
Dolomitic lime 1	1025 bcd	179 bc	–	5.1 bc	2.8 b
Dolomitic lime 2	1377 d	237 cd	–	5.9 ef	0.5 a
Calmasil Si 1	1247 cd	176 bc	–	5.4 cd	1.0 ab
Calmasil Si 2	1822 e	259 d	–	6.2 f	0.4 a
Calsimag-P Si 1	668 ab	130 b	–	4.8 ab	8.2 c
Calsimag-P Si 2	985 bc	220 cd	–	5.5 de	1.2 ab
Turbo-Grow Si 1	425 a	49 a	–	4.5 a	21.0 c
Turbo-Grow Si 2	478 a	50 a	–	4.5 a	16.0 c
P-value	<0.001	<0.001	–	<0.001	<0.001

*Product application rates are specified in **Table 4**; the lime control was applied at the same product rates as Calmasil. See **Figure 2** for soil silicon concentrations. Values are means ($n = 3$ for pre-plant, $n = 4$ for post-harvest). P-values are from ANOVA. Means within a column followed by the same letter are not significantly different (Holm-Sidak test, $p < 0.05$).*

[a] *Acid Sat = Acid saturation (see **Table 2** for definition). See text for explanation of missing values for P in post-harvest sample.*

treatments did not increase in direct relation to the concentration of extractable soil Si.

Leaf concentrations of Ca and Mg were significantly affected by the soil treatments (**Table 9**). Calmasil Si 1 and Si 2 produced significantly higher leaf Ca than Turbo-Grow Si 2, and Calmasil Si 2 produced significantly higher leaf Ca than lime 2 and Calsimag-P Si 1 and Si 2 (**Table 9**). For Mg, the only significant difference occurred between Calmasil Si 2 and Turbo-Grow Si 2 (**Table 9**). The treatments had no significant effect on leaf P concentration (**Table 9**).

DISCUSSION

A striking outcome of this study was that although Calmasil had the lowest total Si content (10.3% Si; **Table 1**), a larger proportion of the Si it provided was taken up by the plant, as it consistently produced the highest leaf Si concentrations in sugarcane. By contrast, sources with high total Si content (K silicate, Prosil Plus, Turbo-Grow; 16.3–30.8% Si; **Table 1**) produced leaf Si concentrations that were substantially (between 2.2- and 15.4-fold) lower than Calmasil and not significantly different from the lime or untreated controls, other than K silicate type MC (**Figures 1B**, **2B**). The above high-Si-content sources also provided no detectable increases in $CaCl_2$-extractable soil Si compared with the controls in both pre-plant and post-harvest samples (**Figures 1A**, **2A**). The low Si provision (even soon after their application) and plant uptake from these sources indicates that they provided little in the way of plant-available Si, despite their high total Si content. Also striking, was the much lower than expected uptake of Si from the Calsimag-P treatments, especially in Trial 1 (**Figures 1B**, **2B**), in direct contrast with this product's substantial and extended provision of extractable soil Si in pre-plant and post-harvest soil samples (**Figures 1A**, **2A**). Although all Si sources were applied at product rates intended to achieve equivalent Si rates of 300 or 750 kg ha^{-1} (**Tables 3**, **4**), the results of the soil analyses clearly show that the total Si content of sources, as stipulated by the manufacturers, cannot be used as a basis for predicting a product's performance in terms of the release of extractable soil Si following its application. These conclusions are borne out by the results of other studies. For

TABLE 9 | Leaf concentrations of elements (calcium, magnesium, phosphorus) provided by the silicon sources applied at lower and higher rates (Si 1 and Si 2) in Trial 2.

Treatment	Ca	Mg	P
	g kg^{-1}		
Dolomitic lime 1	1.5 abc	1.0 ab	1.5
Dolomitic lime 2	1.3 ab	1.1 ab	1.7
Calmasil Si 1	1.6 bc	1.1 ab	1.6
Calmasil Si 2	1.8 c	1.2 b	1.7
Calsimag-P Si 1	1.3 ab	1.0 ab	1.6
Calsimag-P Si 2	1.4 ab	1.1 ab	1.7
Turbo-Grow Si 1	1.6 abc	1.0 ab	1.5
Turbo-Grow Si 2	1.2 a	0.9 a	1.6
P-value	<0.001	0.005	0.29

*Product application rates are specified in **Table 4**; the lime control was applied at the same product rates as Calmasil. See **Figure 2** for leaf silicon concentrations. Values are means (n = 4). P-values are from ANOVA. Means within a column followed by the same letter are not significantly different (Holm-Sidak test, $p < 0.05$).*

example, Haynes et al. (2013) found that a negligible quantity of the very high total Si content (29.1%) of fly ash was in extractable form (using several extractants) compared with steel slag and processing mud, which had the lowest total Si contents but relatively high extractable Si. Korndörfer and Gascho (1999) reported high Si content in steel slag (29%) and Mg silicate (39%), but low availability and uptake (by rice) from these sources compared with wollastonite and thermophosphate. Notably, Elephant et al. (2016) showed from soil incubation studies that Calmasil produced higher concentrations of $CaCl_2$-extractable soil Si than Langfos® (crushed sedimentary phosphate rock) and quarry dust (crushed dwyka tillite), with 14.2 and 24.4% total Si, respectively.

A further conundrum with respect to the Si supplying capacity of the sources studied here is the role of particle size. Studies have generally shown that Si availability increases as particle size decreases and surface area of dissolution increases (Medina-Gonzales et al., 1988; Datnoff et al., 1992; Gascho, 2001; Ma and Takahashi, 2002; Haynes et al., 2013). However, in the present study, the materials with the finest particle size (Turbo-Grow and Prosil Plus; **Table 5**) did not have high Si supplying capacity, while Calsimag-P, a granular product (**Table 5**), released substantial quantities of Si. The form of Si in the product and its solubility are clearly critical, as emphasized by Kingston (2011), Haynes et al. (2013), Babu et al. (2016b) and Tubana et al. (2016). For example, Babu et al. (2016b) noted that slag is a recently formed polycrystalline material and supplies Si at a relatively fast rate and high concentration, while wollastonite, a geologically formed pure crystalline mineral, releases Si at slower rates and lower concentrations.

The potential of thermophosphates, such as Calsimag-P, to be highly efficacious sources of plant-available Si has been demonstrated in other studies (Gascho and Korndörfer, 1999; Pereira et al., 2004; Kingston, 2011). In line with this, Gascho (2001) pointed out that although the total Si content of thermophosphate may be low compared to certain silicate slags (e.g., electric furnace slag, 18.2% Si), the proportion of soluble Si is high. While Calsimag-P did not significantly raise leaf Si content above controls in the present study, this source has previously produced leaf Si values comparable with those of Calmasil using the same soil and sugarcane variety (Keeping et al., 2017); this indicates that uptake from this source can occur, but may be strongly dependent on specific environmental or soil conditions. Such results support Snyder's (2001) point that laboratory analyses of Si-containing materials can only be used as initial screening procedures to identify promising Si sources, and that glasshouse and field studies of plant uptake are ultimately required to provide certainty about the Si supplying capacity of sources.

In the present study, Calmasil significantly increased soil pH in both trials and above that of the equivalent lime treatments in Trial 2 (**Table 8**); it also reduced acid saturation in pre-plant and post-harvest soil samples, with significantly greater efficiency than dolomitic lime in Trial 2 (**Table 8**). Notably, Calmasil Si 2 in Trial 2 was the only Si treatment that raised soil pH($CaCl_2$) well above 5.0 in the pre-plant sample (**Table 8**), at which point Al would precipitate out (Fageria et al., 1988) and its reaction with Si would be reduced. This emphasizes the value of Ca silicate slags in ameliorating soil acidity and Al toxicity, while also supplying Si, Ca, and Mg (Korndörfer and Gascho, 1999; Meyer and Keeping, 2001; Haynes et al., 2013; Castro et al., 2016; Ning et al., 2016). In Trial 1, pH was not increased above 5.0 in the pre-plant sample or above 5.3 in the post-harvest sample (**Table 6**). Here, Calmasil was applied at only one rate (2,913 kg ha^{-1}, equal to the lower Calmasil Si 1 rate in Trial 2), yet the leaf Si concentration of the Calmasil treatment was substantially higher than that in Trial 2 (10-fold more than Si 1 and 7-fold more than Si 2; **Figures 1B**, **2B**). This was not likely due to differences in available soil Si concentration, which was in fact higher in the Calmasil Si 2 treatment in Trial 2 than in the Calmasil treatment in Trial 1 (**Figures 1A**, **2A**). Possibly the already low acid saturation and $H^+ + Al^{3+}$ levels in the soil used in Trial 1 (**Table 2**) contributed to greater Si uptake and higher leaf Si levels in this trial, given that the soil had probably been limed by the grower many months or even years prior to its use. In contrast to Calmasil, Calsimag-P did not raise soil pH or reduce acid saturation in Trial 2 (**Table 8**); this may have lowered its effectiveness in elevating leaf Si content as a result of rapid complexation of released Si with soluble, reactive Al^{3+} (Farmer et al., 1979; Doucet et al., 2001; Schneider et al., 2004; Exley, 2012).

Where Si sources are high in total Si but nonetheless provide little or no plant-available Si to the soil, as in the case of the Mg silicate (Prosil Plus), K silicate (**Figure 1A**), crushed volcanic rock (Turbo-Grow, **Figure 2A**), diatomaceous earth or fly ash (Kingston, 2011; Haynes et al., 2013; Keeping et al., 2017) and quarry dust (Elephant et al., 2016), the effects of Al^{3+} in reducing their provision of Si to plants would be largely immaterial. However, the situation for slags may be different. Babu et al. (2016a) pointed out that trace amounts of Al^{3+} reduce the equilibrium solubility of Si due to the co-deposition of these elements as hyrdroxyaluminosilicate (HAS) within the soil environment (see Cocker et al., 1998, for a review of this process). They also argued that the presence of Al^{3+} ions on slag particles

can reduce the rate of dissolution of silica and act as catalysts in accelerating the process of polymerization of monomeric H_4SiO_4 to colloidal silica, which cannot be taken up by plants. Adsorption of the silicate anion ($H_3SiO_4^-$) to hydroxides of Al and Fe (sesquioxides) increases at higher pH (especially above pH 9) and is of critical importance in constraining the concentration of Si in soil solution (Beckwith and Reeve, 1963; Jones and Handreck, 1963; McKeague and Cline, 1963b; Kato and Owa, 1996; Tavakkoli et al., 2011; Haynes, 2014). This is especially so when higher rates of calcium silicate slag are applied, wherein the higher pH and concentration of solubilizing Si produced by the slag promotes increasing adsorption of $H_3SiO_4^-$ to sesquioxide surfaces (Haynes et al., 2013; Babu et al., 2016a). While Calmasil contains on average 1.07% Al, the greatest source of this element in the present study, by a very large margin, would be the acid soil used (**Table 2**). Notwithstanding the substantial reductions in acid saturation in the Calmasil treatments in both trials (**Tables 6, 8**), small quantities of native soluble Al^{3+} ions may have reduced equilibrium Si solubility or increased its polymerization in the manner described by Babu et al. (2016a). Under field conditions, where wetting and drying cycles would serve to concentrate Si solubilized from slag, polymerization may be especially important in this regard (Keeping et al., 2013).

The mechanisms discussed above do not, however, satisfactorily account for the low concentrations of Si in leaf tissue from the Calsimag-P treatments, where abundant levels of soluble Si were present in the treated soil (**Figures 1A**, **2A**). This suggests that the Si was either not taken up by the plants or, if taken up, not translocated to the shoot. The substantial reductions in soil Si from pre-plant to post-harvest samples in both trials indicate that appreciable plant uptake occurred and/or that some of the available Si was converted during the course of the trials to forms not readily extractable with 0.01 *M* $CaCl_2$. Under conditions of low pH, high acid saturation (and therefore presence of soluble Al^{3+}), and high Si concentration in the rhizosphere, it is likely that the plant will simultaneously take up Al^{3+} and H_4SiO_4 into the root cells, where they may react and co-precipitate (Hodson and Evans, 1995; Cocker et al., 1998; Hodson and Sangster, 1999). As stated by Hodson (2011), co-deposition *in planta* of Al with Si in solid phytoliths is a relatively widespread phenomenon in higher plants, and in roots Al is often co-deposited with Si in epidermal and cortical cells. An *in planta* mechanism that immobilizes Si and inhibits its translocation from the roots to the shoot, may explain the abundant soil Si levels but low (or relatively low) leaf Si concentrations in the Calsimag-P treatments in this study. On the other hand, the robust liming effect of Calmasil may have been sufficient to reduce solubility and plant uptake of Al^{3+} to the extent that its co-deposition with Si within the plant had a much-reduced effect on translocation of Si to the shoot. As mentioned, the already low acid saturation of the soil used in Trial 1 (**Table 6**) may have accentuated such an effect and promoted the high leaf Si accumulation in the Calmasil treatment (**Figure 1B**). Detailed studies of Al and Si co-deposition in roots of sugarcane, such as those performed by Cocker et al. (1997) in wheat and Prabagar et al. (2011) in Norway spruce, may reveal *in planta* interactions between these elements, their possible effects on Si translocation, and the extent to which low shoot Si accumulation is a reflection of reactions within the soil (which affect uptake) or reactions within the plant (which affect translocation).

Attention by sugarcane growers to addressing problems of Si and other nutrient deficiencies is an important step in avoiding plant stress and reducing infestation by sugarcane borer (White and White, 2013; Keeping et al., 2014; Nikpay et al., 2015). The present study emphasizes a further essential step, which is to improve soil health and root growth by reducing soil acidity and Al toxicity through liming and/or calcium silicate provision. With increasing acidification of soils due to long periods of monocropping and intensive use of nitrogenous fertilizers, not only in South Africa but across many tropical and sub-tropical crop-growing regions (Meyer et al., 1998; Barnard and du Preez, 2004; Ma, 2004; Fageria and Baligar, 2008; Van der Laan and Miles, 2010; Marafon and Endres, 2013; Meena et al., 2014), the use of calcium silicate slags presents a valuable substitute for conventional dolomitic limes. As demonstrated in the present study and previous studies, slags also provide Ca and Mg (**Table 8**); both of these nutrients, along with P, were nutritionally adequate in Trials 1 and 2 (**Tables 7, 9**) (Miles and Rhodes, 2013). Moreover, slags have the additional advantages of supplying Si, being more reactive than lime (and in the current study more effective on a mass for mass basis), and correcting acidity and eliminating Al^{3+} to a greater soil depth (Korndörfer and Gascho, 1999; Pereira and Cabral, 2005; Bokhtiar et al., 2012; Marafon and Endres, 2013; Haynes, 2014; Castro and Crusciol, 2015; Castro et al., 2016). The results presented here indicate that Si sources (such as thermophosphate) that provide ample soluble Si but have limited liming capacity, may not be effective sources in acid soils due to reaction of their solubilized Si with Al^{3+} within the soil and perhaps to a larger extent within the plant. This underscores the advantages of alkaline Si sources that can simultaneously eliminate Al^{3+} in the rhizosphere and reduce its uptake. Future field studies should focus on means to further eliminate Al^{3+} or prevent its reaction with Si, by combining treatments of slag with gypsum and sources of organic matter, such as crop residues, manure or bagasse (the sugarcane stalk residue remaining after juice extraction). Reduction of subsoil acidity, and the retention of soil moisture and improved rainwater infiltration associated with higher soil organic matter, are crucial practices in ensuring root health (Thorburn et al., 1999; Bell et al., 2001; Pankhurst et al., 2005; Sumner, 2011, 2012), and may be equally important in augmenting Si uptake from both native and applied sources.

Finally, there has been increasing focus on the importance of recycling of crop residues, which may contain large quantities of amorphous Si in the form of phytoliths (i.e., phytogenic Si), back into soils in an effort to compensate for the large-scale and ongoing removal of Si from agricultural landscapes when crops are harvested (Struyf et al., 2010; Clymans et al., 2011; Guntzer et al., 2012; Keller et al., 2012; Vandevenne et al., 2012; Cornelis and Delvaux, 2016). In sugarcane, the dry leaf matter is an important potential source of Si, as the concentration of Si in dry leaf may reach 3% DM (Van Dillewijn, 1952), following

the deposition of amorphous Si to form phytoliths in green leaves (Kaufman et al., 1979; Tripathi et al., 2011). A rainfed crop that yields a total biomass of 80 t ha^{-1} at harvest could produce 16 t ha^{-1} dry leaf matter, as dry leaf matter may account for 20% DM of total biomass (Purchase et al., 2008), potentially providing 480 kg Si ha^{-1}, assuming the above 3% Si composition. As in the case for rice (Ma and Takahashi, 1991; Savant et al., 1999; Haynes, 2014; Klotzbücher et al., 2016), this alone could provide much of the Si that would otherwise need to be provided in the form of silicate amendments, and highlights the substantial yearly removal of Si from sugarcane fields effected in the process of crop residue removal and burning. Silicon provision through retention of crop residues should therefore be viewed as a prominent but generally overlooked benefit of crop residue retention along with the many other benefits of this practice (Thorburn et al., 1999; Bell et al., 2001; van Antwerpen et al., 2001; Pankhurst et al., 2005) in sugarcane production.

CONCLUSIONS

Vendors of new Si-bearing materials frequently lay considerable emphasis on the high Si content of their products, without clear evidence as to how much of the total Si is plant-available. Yet this and previous studies have shown that the total Si content of sources is not a reliable indicator of how effective they may be in, firstly, releasing sufficient quantities of plant-available Si into soil solution and, secondly, in facilitating its uptake by the plant. In weathered acid soils dominated by Al and Fe sesquioxides, and which occur across much of the tropical and sub-tropical regions where Si-accumulating crops such as rice and sugarcane are grown, calcium silicate appears to be the most effective source in both respects, as it dissolves readily in an acid soil environment to release silicic acid, and is also an efficient liming agent capable of significantly reducing acid saturation and Al toxicity. The latter factor may be critical in constraining reaction of available Si with Al^{3+}, either in the soil or in the plant roots, and thereby maximizing Si uptake and translocation to the shoot. Other sources with high Si content either provide very small quantities of soluble Si or, if they do provide adequate Si, may have limited or no liming capacity; consequently they are unable to counteract the direct toxic effects on Al^{3+} on roots or its reaction with silicic acid. As a result of these properties and their provision of ample Ca and Mg, calcium silicate slag appears still to offer the most effective and affordable Si source for sugarcane growers, at least in the acid, sandy soils of the dryland production regions of the South African sugar industry. However, attention should also be directed toward practices that promote recycling of phytogenic Si back into soils, principally through retention of crop residues and preservation of soil organic matter, which in themselves may also promote uptake of Si from silicate slags.

AUTHOR CONTRIBUTIONS

MK conceived and conducted the research, performed the statistical analyses, and wrote the manuscript.

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Silicon and Plants: Current Knowledge and Technological Perspectives

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Elemental silicon (Si), after oxygen, is the second most abundant element in the earth's crust, which is mainly composed of silicates. Si is not considered essential for plant growth and development, however, increasing evidence in the literature shows that this metalloid is beneficial to plants, especially under stress conditions. Indeed Si alleviates the toxic effects caused by abiotic stresses, e.g., salt stress, drought, heavy metals, to name a few. Biogenic silica is also a deterrent against herbivores. Additionally, Si ameliorates the vigor of plants and improves their resistance to exogenous stresses. The protective role of Si was initially attributed to a physical barrier fortifying the cell wall (e.g., against fungal hyphae penetration), however, several studies have shown that the action of this element on plants is far more complex, as it involves a cross-talk with the cell interior and an effect on plant metabolism. In this study the beneficial role of Si on plants will be discussed, by reviewing the available data in the literature. Emphasis will be given to the protective role of Si during (a)biotic stresses and in this context both priming and the effects of Si on endogenous phytohormones will be discussed. A whole section will be devoted to the use of silica (SiO_2) nanoparticles, in the light of the interest that nanotechnology has for agriculture. The paper also discusses the potential technological aspects linked to the use of Si in agriculture and to modify/improve the physical parameters of plant fibers. The study indeed provides perspectives on the use of Si to increase the yield of fiber crops and to improve the thermal stability and tensile strength of natural fibers.

Keywords: silicic acid, biosilicification, stresses, priming, cell wall

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INTRODUCTION

Silicon (Si) is considered non-essential (or quasi-essential, Epstein and Bloom, 2005) for plant growth and development. Plants develop well in its absence, although in some cases, e.g., the silicifier horsetail and rice, the absence of Si triggers increased susceptibility to fungal infection (Datnoff and Rodrigues, 2005; Law and Exley, 2011). When supplied to the growth medium (as silicic acid, *vide infra*), plant vigor and resistance to (a)biotic stresses increase (Azeem et al., 2015; Coskun et al., 2016; Guerriero et al., 2016a). Si is taken up by plants as silicic acid $Si(OH)_4$ via aquaporin type channels (Nod26-like intrinsic proteins, NIPs) (Ma et al., 2006; Grégoire et al., 2012; Deshmukh et al., 2013). A specific 108 amino acid spacing between the conserved NPA domains

determines $Si(OH)_4$ permeability (Deshmukh et al., 2015). Plants are classified into accumulators, excluders and intermediate type (Mitani and Ma, 2005), depending on the amount of biogenic silica found in their tissues. Among the accumulators are Equisetales, Cyperales and Poales: in *Graminae*, rice is the highest silicifier where Si (in the form of biogenic silica, *vide infra*) accounts for up to 10% of the shoot dry weight (Ma et al., 2002). Tomato is among the excluders, while *Urtica dioica* (i.e., nettle) is an intermediate type (Trembath-Reichert et al., 2015).

In (some) plants the provision of $Si(OH)_4$ has a latent effect in the absence of an external stimulus (Fauteux et al., 2005, 2006). This has been observed in the *Arabidopsis*-powdery mildew pathosystem (Fauteux et al., 2006). It should, however, be noted that in rice, $Si(OH)_4$ supplementation does trigger major changes, as it induces the upregulation and downregulation of 35 and 121 transcription factors respectively (Van Bockhaven et al., 2012). This difference may be in part due to the different cell wall types (Yokoyama and Nishitani, 2004) and to the structural importance of Si in type II cell walls (i.e., cell walls characterized by the presence of more phenylpropanoids as compared to type I cell walls in dicots).

By precipitating as SiO_2 and being incorporated into biological structures (e.g., the cell wall, *vide infra*), Si exerts its protective action via the formation of a physical barrier. However, this passive role is too simplistic and does not explain why plants supplemented with Si are better suited to face exogenous stresses. Compelling evidence in the literature shows that specific cell wall components trigger SiO_2 precipitation (reviewed by Guerriero et al., 2016a). In rice cell suspension culture, a hemicellulose-bound form of Si has been identified (He et al., 2015), in horsetail mixed-linkage glucans (MLGs) have been proposed to participate in SiO_2 formation (Fry et al., 2008) and this has been recently confirmed in rice where overexpression of a hydrolase acting on MLGs was shown to affect silicification (Kido et al., 2015). In horsetail, callose was shown to template biosilicification (Law and Exley, 2011). Very recently, the role of callose in templating biosilicification has been additionally proven by using *Arabidopsis* plants either overexpressing or lacking the callose synthase gene *PMR4* (Brugiére and Exley, 2017): while the wild-type plants and overexpressors responded to a pathogen-like challenge by accumulating both callose and silica, the mutants did not produce callose and, consequently, deposited significantly less silica.

Si PRIMING

Several papers demonstrated that $Si(OH)_4$ (hereafter referred to as Si for simplicity) acts as a "tonic" by priming plants, i.e., by preparing the defense responses which are then fully deployed at the onset of the stress, as will be discussed in detail in the next sections. The effects of Si under normal conditions are indeed latent, since, for the majority of the studies available, no major modifications, e.g., in gene expression, are observed. Under control conditions Si probably activates the metabolic status of the plant, by making it more efficient in responding to exogenous stimuli.

In rice, a Si-accumulator, Si causes alterations of C/N balance in the source-sink relationship under unstressed conditions, by favoring a remobilization of amino acids to support the increased N demand during grain development (Detmann et al., 2012, 2013). These data support the hypothesis that Si has a signaling role in plant cells. Si was indeed suggested to have a role as second messenger by binding to the hydroxyl groups of proteins involved in cell signaling, thereby partaking in the signal transduction (Fauteux et al., 2005).

It is important to mention that Si primes defense responses also in Si non-accumulators, i.e., tomato (Ghareeb et al., 2011): tomato is protected against *Ralstonia solanacearum* by Si which causes an upregulation, upon infection, of genes involved in ethylene and jasmonic acid signaling, i.e., *JERF3*, *TRSF1*, *ACCO*, as well as genes involved in stress response, i.e., trehalose phosphatase, late embryogenesis abundant protein, ferritin. In this study, the authors also observed an increased expression of a negative regulator of the jasmonic acid signal, *JAZ1*, together with a ubiquitin protein-ligase: the authors propose that *JAZ1* helps in preventing the eventual damage caused by the stimulation of defense-related compounds and that the ubiquitin protein-ligase may degrade JAZ1. In tomato challenged by *R. solanacearum*, Si also upregulates a MAPK (*MAPK19*), a WRKY transcription factor and linker histones (*H1* and *H5*). These findings corroborate the role of Si in intracellular signaling and suggest its involvement in transcription too (Ghareeb et al., 2011).

Silicon was shown to upregulate the expression of a leucine-rich repeat receptor-like kinase (LRR-RLK) in rice (Fleck et al., 2011), which is a protein involved in intracellular signal transduction. High-throughput technologies relying on *–omics* will help shed light on the missing genes/proteins involved in the signal transduction underlying Si priming (the so-called "prime-omics"; Balmer et al., 2015).

Si AND ABIOTIC STRESS ALLEVIATION

Si assumes key functions in the plant response to numerous environmental constraints. Two major processes contributing to stress resistance are commonly considered (i) a physical and mechanical protection afforded by SiO_2 deposits and (ii) a biochemical response triggering metabolic changes. The precise distribution/speciation of accumulated Si in plant tissue allows us to gain additional information regarding its modalities of action and requires the use of biophysical tools, such as laser ablation (LA), extended X-ray fine structure (EXAFS), X-ray absorption near edge structure (XANES) and micro particle-induced X-ray emission (micro-PIXE).

According to Liang et al. (2013), Si improves lodging resistance by strengthening the stem basis in rice. It also enhances UV tolerance due to the protective effect of Si deposition bodies on the leaf epidermis (Goto et al., 2003) or by reducing UVB-induced membrane damages (Shen et al., 2010).

Silicon influences water relations in drought-treated plants: it induces the formation of a silica cuticle double layer under the leaf epidermis which reduces water losses through cuticular

transpiration (Gong et al., 2003). Si also reduces stomatal conductance in relation to turgor loss of guard cells resulting from Si deposition and modified cell wall properties (Zhu and Gong, 2014). Si improvement of drought resistance may also be ascribed to strong abilities to extract water from the soil as a consequence of Si-related promotion of root elongation (Hattori et al., 2005) and up-regulation of aquaporin genes (Liu et al., 2015).

Silicon contributes to salt stress alleviation through inhibition of Na^+ (Zhu and Gong, 2014) and Cl^- (Shi et al., 2013) uptake. Translocation of toxic ions from root to shoot is also reduced by Si supply (Savvas and Ntatsi, 2015). In rice, Si alleviates NaCl toxicity by blocking the transpirational bypass flow through precipitation as SiO_2 in exodermis and endodermis (Yeo et al., 1999). Potassium uptake allowing the maintenance of K/Na is improved by Si nutrition which has a direct stabilizing effect on proton pump activity in salt-treated root tips (Xu et al., 2015).

In metal-polluted soil, Si may influence the bioavailability of toxic elements. The presence of soil sodium metasilicate or alkaline Si-containing material may induce a rise in the rhizospheric pH leading to a decrease in available heavy metal concentration in the soil (Wu et al., 2013). Soluble silicate hydrolyzes to generate gelatinous metasilicic acid (H_2SiO_3) retaining heavy metals (Gu et al., 2011). According to Kidd et al. (2001), Si-treated plants may also exude phenolics such as catechin and quercetin having strong Al-chelating abilities. The formation of hydroxyl-aluminum silicate in the apoplast also contributes to Al detoxification (Wang et al., 2004).

Compartmentation of toxic ions is an important process in heavy metal tolerance. Si improves heavy metal retention by roots, with an obvious accumulation in the endodermis (Keller et al., 2015). At the shoot level, accumulation of Mn was mainly observed in epidermis in response to Si treatment (Doncheva et al., 2009). Iwasaki and Matsumura (1999) reported that Si increases Mn accumulation in the leaf trichomes. Controversial data are available in the literature regarding co-precipitation of Si with heavy metals. Keller et al. (2015) did not detect Cu and Cd in phytoliths and the absence of Cu-Si coprecipitation was also noticed in maize by Collin et al. (2014). He et al. (2013), however, identified a mechanisms of co-deposition of Si and Cd in the rice cell walls via a [Si-wall matrix] Cd complexation, which may explain a Si-induced decrease in the Cd influx in cells. Ma et al. (2015) considered that a hemicellulose bound form of Si with a net negative charge is responsible for inhibition of Cd uptake leading to a downregulation of *Nramp5* coding for a transporter involved in Cd transport. Kim et al. (2014) also reported a downregulation of other heavy metals transporter (*OsHMA2* and *OsHMA3*) when Cu/Cd-treated rice was supplied by Si.

Numerous studies reported that Si induces an improved behavior of heavy metal-treated plants in relation to regulation of antioxidant enzymes (Adrees et al., 2015), oversynthesis of endogenous antioxidants leading to mitigation of oxidative stress (Imtiaz et al., 2016), maintenance of net photosynthesis relying on the stabilization of chloroplast structures, PSII integrity and increased pigment concentration (Nwugo and Huerta, 2008; Tripathi et al., 2015a). Si may thus be of paramount importance for triggering adapted plant response, but the precise molecular cues involved in the adaptative processes still need to be clearly identified.

Si AND BIOTIC STRESS

Si was reported to improve defense against biotic constraints occurring in the form of plant pathogens (fungi, bacteria, and viruses) or animals (vertebrates and arthropod herbivores).

Silicon deposition increases abrasiveness of plant tissues and thus reduces palatability and digestibility for herbivores (Massey and Hartley, 2009). Hartley et al. (2015) demonstrated by Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX) that phytolith morphology inside the tissues has more influence on abrasiveness than the actual Si concentration. Using the same technique, Keeping et al. (2009) demonstrated that the pattern of Si deposition in sugarcane is responsible for enhanced resistance to *Eldana saccharina*. Physical strength of the leaf resulting from Si accumulation may afford mechanical protection and thus lower the rate of infection as reported for *Rhizoctonia solani* (Zhang et al., 2013; Schurt et al., 2014) or *Bipolaris oryzae* (Ning et al., 2014).

Biochemical/molecular mechanisms are also induced or reinforced by Si allowing the plant to improve resistance to biotic stress and include defensive compounds such as phenolics, phytoalexins and momilactones (Remus-Borel et al., 2005), but also to activation of defensive enzymes such as peroxidase, polyphenol oxidase, lipoxygenase and phenylalanine ammonia lyase (Rahman et al., 2015). According to Cai et al. (2008), Si treatments may increase transcripts levels corresponding to those defense-related genes.

Reynolds et al. (2016) reported that Si also operates by attracting predators or parasitoids to plant under herbivore attack. Indeed, soluble Si contributes to increase herbivore-induced plant volatiles to promote predator attraction by pest-infected plants. Moreover, according to James (2003) and Connick (2011), the phenology of insect's life cycle is also slowed down in Si-treated plants, making it more prone to predation.

EFFECTS OF Si ON PHYTOHORMONES

Silicon impacts on endogenous phytohormones are commonly analyzed in response to stress conditions. In rice plants exposed to heavy metals, Si reduced endogenous concentration of jamonic acid (JA) and salicylic acid (SA), while abscisic acid (ABA) first increased and then decreased after 14 days of treatment (Kim et al., 2014): the ABA has an antagonist behavior with JA/SA biosynthesis. The effect of such phytohormonal changes on the expression of genes involved in heavy metal response still needs to be elucidated in Si-treated plants. Kim et al. (2011) also reported that Si reduced JA concentration in response to wounding, while Lee et al. (2010) reported an increase in gibberellins concentration in Si-treated plants exposed to salinity.

Resistance to biotrophic pathogens may be associated with SA whereas JA and ethylene (ET) are generally associated with

resistance to necrotrophic pathogens. Fauteux et al. (2006) showed that Si improved biosynthesis of SA, JA and ET in leaves exposed to *Erysiphe cichoracearum*. Similarly, Si-treated tomato plants exposed to *R. solanacearum* activated JA and ET signaling pathways to increase resistance (Ghareeb et al., 2011). Brunings et al. (2009) also provided evidence that genes controlling ET signaling pathway may be activated by Si treatment. Conversely, Si improves resistance to the fungus *Cochliobolus miyabeanus* by interfering with the production of fungal ET (Van Bockhaven et al., 2015). Data regarding the effect of Si on phytohormone metabolism in the absence of stress are still rare. Markovich et al. (2017), however, recently demonstrated that Si increases cytokinin biosynthesis in *Sorghum* and *Arabidopsis* and that such an increase may strongly contribute to delay senescence. Plant hormones interactions are responsible for a complex biochemical and physiological network: a deep understanding of Si influence on hormonal properties thus requires technical approaches allowing to quantify a wide range of hormonal compounds simultaneously, including minor conjugated forms.

SILICA NANOPARTICLES

The use of nanotechnology in agriculture is gaining importance because it contributes to develop new sustainable strategies. Nanomaterials can for example be engineered to immobilize nutrients or to release them in a controlled manner in the soil (Fraceto et al., 2016).

Some papers in the literature have studied the effects of silica nanoparticles (SNPs) on plant physiology and we will here review some of them.

Mesoporous SNPs (MSNPs, 20 nm in size) coupled to FITC were shown to be taken up by three important crops (lupin, wheat, maize), as well as *Arabidopsis* protoplasts and to be translocated to the aerial parts following the xylematic flow after entering the roots via symplastic/apoplastic routes (Sun et al., 2014). Very interestingly, this study also showed that MSNPs accumulated in the cell walls, therefore highlighting the existence of an affinity with cell wall components. The monodisperse nature of the MSNPs and their size, achieved via a fine-tuning of pH and surfactant concentration, were essential for the efficient uptake by plants: the entry takes place via the pores in the cell walls of the roots cells (Sun et al., 2014).

Mesoporous SNPs were shown to boost the growth, total protein content and photosynthesis of lupin and wheat seedlings and to induce no changes in the activity of antioxidant enzymes (Sun et al., 2016). Interestingly in this study, the authors observed a shift of 14 cm^{-1} and 10 cm^{-1} in the Raman peaks of chlorophyll from wheat and lupin when isolated chloroplasts were incubated with MSNPs suggesting a change in the molecular structure of chlorophyll.

Silica nanoparticles were shown to protect wheat seedlings against UV-B stress by stimulating the antioxidant defense system (Tripathi et al., 2016). In particular, SNPs reduced the adverse effects of the UV-B stress, i.e., low fresh weight, reduction in chlorophyll and tissue damage. Since the levels of nitric oxide reached a peak after UV-B+SNPs treatment, a protective role via the modulation of NO levels was proposed by the authors.

Silica nanoparticles also conferred protection via mitigation of oxidative stress in pea seedlings treated with Cr(VI): the activities of enzymes such as superoxide dismutase, ascorbate peroxidase increased significantly in the presence of SNPs, while catalase, glutathione reductase and dehydroascorbate reductase were less inhibited by Cr(VI) in the presence of SNPs (Tripathi et al., 2015b).

Silica nanoparticles (12 nm) were also found to improve germination in a known Si-excluder, tomato: at a concentration of 8 g/L, SNPs improved seedling germination, as well as fresh and dry weight by 116.6 and 117.5% respectively (Siddiqui and Al-Whaibi, 2014).

Nanostructured SiO_2 (TMS) was shown to be valuable in larch seedling production, because, when applied to the roots of 1-year-old seedlings via soaking for 6 h, it promoted lateral root growth, main root length and chlorophyll content (Bao-shan et al., 2004).

The effect of SNPs was, however, shown to be dependent on the plant species, as in Bt-transgenic cotton they significantly decreased plant growth (Le et al., 2014). SNPs toxicity may be linked to pH and nutrient adsorption problems. Indeed, in thale cress, SNPs phytotoxicity was triggered when the pH of the medium was not adjusted or silanol groups were not removed from the surface (Slomberg and Schoenfisch, 2012). The alkaline pH (pH 8 ca.) makes nutrients less available for uptake, while the negatively charged SNPs tend to adsorb nutrients.

Si AND FIBER CROPS

Fiber crops like textile hemp (*Cannabis sativa* L.) are natural resources which provide long and strong cellulosic fibers (a.k.a. bast fibers) used in both the textile and biocomposite sectors (Guerriero et al., 2014; Andre et al., 2016; Guerriero et al., 2016b). Given the positive effects of Si on plants, its use for fiber crop growth may provide an enhanced biomass yield and, therefore, an increased production of bast fibers. The association of SiO_2 with the fiber cell walls may provide new properties, notably and increased durability. In this respect, it should be noted that hemp woody fibers, which contain SiO_2 and therefore bind well with lime, are already used to manufacture a lightweight concrete-like material used in eco-construction and known as hempcrete. The few studies available on the specific Si impact on fiber crops confirm protection against abiotic stresses. In ramie [*Boehmeria nivea* (L.) Gaud.], the application of Si ameliorated Cd toxicity via stimulating the activities of antioxidant enzymes (Tang et al., 2015). Bakry et al. (2015) and Shedeed et al. (2016) reported that foliar application of Si improved the nutrient status of flax and increased straw and oil yield/plant.

Silicon accumulation in fiber crops is genetically controlled, as demonstrated for bamboos by Collin et al. (2012). Exogenous Si did not reduce Cu absorption by bamboos growing on contaminated solution, but reduced toxicity symptoms (Collin et al., 2013, 2014). Si also improved the growth of cotton exposed to Cd but, in this case, Si reduced Cd uptake and

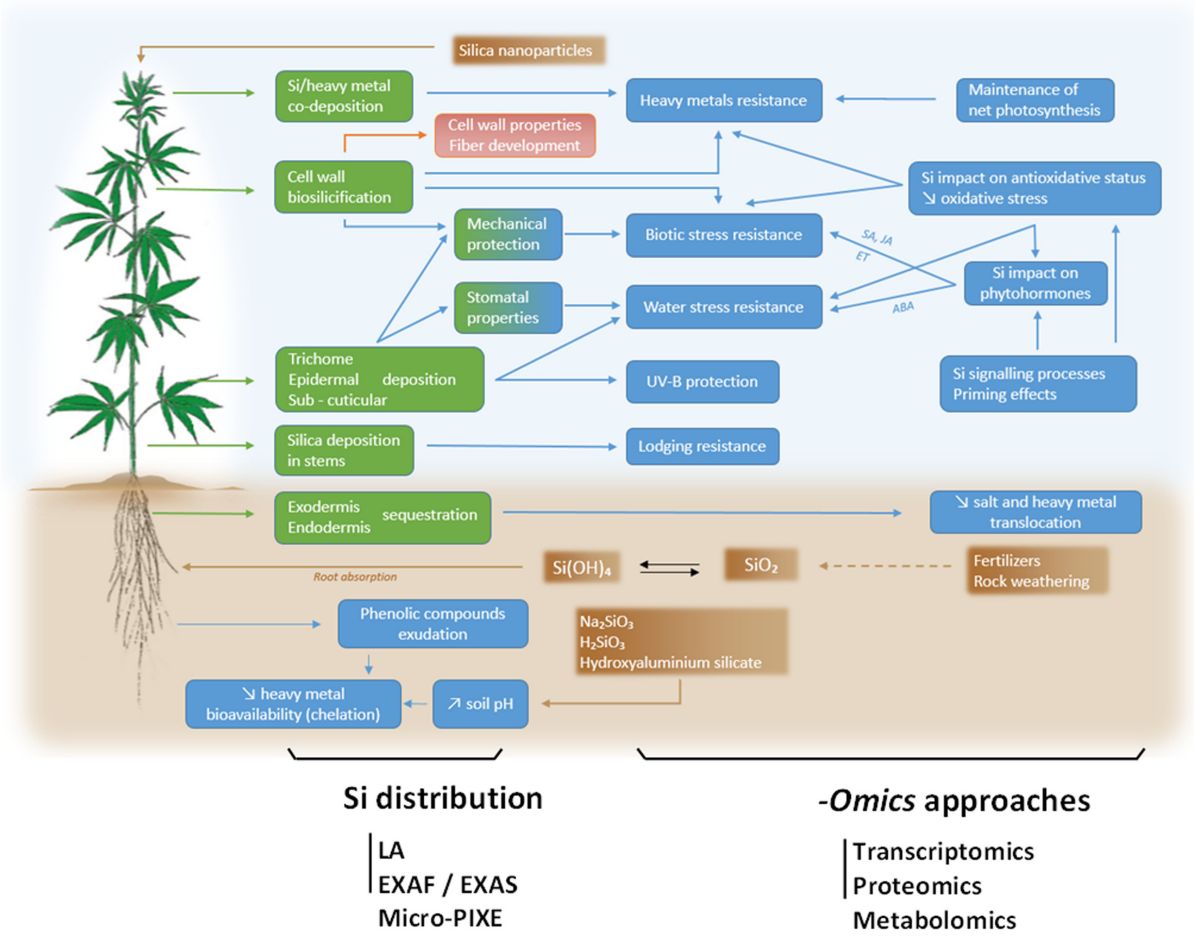

FIGURE 1 | Global overview of Si impact on hemp (*Cannabis sativa* L.), here depicted as a model plant in light of its economic importance as a source of bast fibers. Speciation of Si in soil and application of SiO_2 nanoparticles are indicated in brown boxes and possible sites of Si deposit in the plant are indicated in green boxes. Resulting consequences of Si accumulation in terms of stress resistance and underlying physiological processes are indicated in blue boxes. For further details, please refer to the text. A deep understanding of processes involved in Si absorption, translocation and physiological consequences require holistic *-omics* approaches including transcriptomics, proteomics and metabolomics tools. The precise Si distribution may be assessed by laser ablation (LA), extended X-ray fine structure (EXAFS), X-ray absorption near edge structure (XANES) and micro particle-induced X ray emission (micro-PIXE).

mitigated the adverse effect of this heavy metal by improving plant growth, biomass and photosynthetic parameters in stressed plants (Farooq et al., 2013).

Data concerning the direct influence of Si on fiber development itself are crucially lacking. Some old studies, however, provided indirect evidences that Si may assume important functions in this respect. Khan and Roy (1964) reported that soil application of silicate improved the size of the commercial fiber jute by increasing cell elongation and fineness. According to Boylston (1988), the Si concentration is high during the elongation phase of cotton fiber development but decreases as the fiber matures. The ratio of the amount of Si per mass of fiber peaks at the time when secondary wall initiation occurs (Boylston et al., 1990). Si is known to interact with cell walls (see Introduction), although the mechanisms underlying the final incorporation of polymerized Si into the cell wall remain elusive. Kido et al. (2015) recently demonstrated that the interaction of mixed linkage glucan (1;3, 1;4)-β-D-glucan with Si may have obvious mechanical consequences.

Si beneficial influence on natural fiber properties is confirmed by the use of Si-containing compounds during industrial processing of harvested fibers. Natural fibers are gaining attention in engineering composite industry. However, cell wall polymers often bear hydrophilic hydroxyl groups able to form new hydrogen bonds with water molecules, which hinder hydroxyl group to react with the polar matrix of the composites (Mwaikambo and Ansell, 2002). Silane is an inorganic compound (SiH_4) commonly used to improve tensile strength and thermal stability of natural fibers (Abdelmouleh et al., 2004) which may be due to the emergence of Si-O-C and Si-O-Si links on the cellulose surface (Lu et al., 2013). Other Si treatment, including siloxane and nano Si dioxide are also used for similar purposes (Kabir et al., 2012; Siengchin and Dangtungee, 2014; Orue et al., 2016). It may thus be hypothesized that Si treatment *in vivo* during

fiber development (and not only *in vitro* on harvested mature fibers) may lead to several promising application. This exciting goal, however, requires a multidisciplinary approach to gain a better understanding of Si influence on the modalities of fiber development (**Figure 1**).

CONCLUSION AND FUTURE PERSPECTIVES

Silicon is an abundant element on Earth and its positive effects on plants make it important in agriculture. The study of the Si-plant binomium has still much to teach us and this is particularly the case for e.g., the cell wall-related mechanisms underlying its prophylactic role under stress. The plant cell wall takes active part in the response to (a)biotic stresses by establishing a signaling cascade toward the cell interior (Hamann, 2015) and by undergoing a remodeling (Tenhaken, 2014). It is therefore clear that part of the beneficial effects of Si on plants is linked to direct/indirect effects on the cell wall.

In the future, research activities centered on specific aspects of the interaction Si-plants will be important to devise agricultural strategies aimed at improving crop yield.

AUTHOR CONTRIBUTIONS

GG conceived the idea of writing the paper. ML, J-FH, SL, and GG collected the literature data and wrote the manuscript.

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A Model of Silicon Dynamics in Rice: An Analysis of the Investment Efficiency of Si Transporters

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Silicon is the second most abundant element in soils and is beneficial for plant growth. Although, the localizations and polarities of rice Si transporters have been elucidated, the mechanisms that control the expression of Si transporter genes and the functional reasons for controlling expression are not well-understood. We developed a new model that simulates the dynamics of Si in the whole plant in rice by considering Si transport in the roots, distribution at the nodes, and signaling substances controlling transporter gene expression. To investigate the functional reason for the diurnal variation of the expression level, we compared investment efficiencies (the amount of Si accumulated in the upper leaf divided by the total expression level of Si transporter genes) at different model settings. The model reproduced the gradual decrease and diurnal variation of the expression level of the transporter genes observed by previous experimental studies. The results of simulation experiments showed that a considerable reduction in the expression of Si transporter genes during the night increases investment efficiency. Our study suggests that rice has a system that maximizes the investment efficiency of Si uptake.

Keywords: silicon, mathematical model, silicon transporter, rice, silicon transport

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INTRODUCTION

Once taken up by roots, mineral elements are transported to upper part with transpiration stream, followed by distributing to different organs and tissues. Understanding of the mechanisms that control the dynamics of both water and mineral elements is an important issue in plant science. Over the past decade, many transporters for uptake of mineral elements have been identified, including those in rice for N, P, K, Mg, B, Mn, Zn, Fe, and Si (Sasaki et al., 2016). These transporters are located in the plasma membrane. Moreover, some these transporters show polar localization. For example, Si influx transporter Lsi1 is localized to the distal side of the root exodermis and endodermis (Ma et al., 2006), while Si efflux transporter Lsi2 is localized to the proximal side of the same cells (Ma et al., 2007).

An important challenge is to develop a mathematical model that can simulate the dynamics of both water and mineral elements in the whole plant to quantitatively understand the complex mineral element transporting systems. Transporter expression depends on mineral concentrations

in tissues (Sasaki et al., 2016). To quantify the dynamics of mineral element transport, it is necessary to consider multiple factors simultaneously including transporter activities in roots and shoots, the dynamics of xylem sap and phloem sap, and the expression levels of the transporter genes.

For water flow in plants, the models using an analogy with an electric circuit (Landsberg and Fowkes, 1978) is one of the most historical models. In these models, water potential (potential energy of water per unit volume relative to pure water) is treated like voltage in a circuit. Water flows according to the difference in water potentials. Because the flow of water in the phloem is strongly related to sucrose concentration, some models treat water and sucrose dynamics simultaneously (Daudet et al., 2002; Lacointe and Minchin, 2008; Lobet et al., 2014; Seki et al., 2015). For the transportation of substances in plant, several different concepts are used to model transport of mineral elements and organic pollutants from roots. In the compartment model, the plants are divided into several compartments (such as root and leaf), and substances are assumed to be transported among the compartments according to transition rates that are determined as parameter values (Fryer and Collins, 2003; Fantke et al., 2013; Trapp, 2014). However, these macro-scale models do not consider not only water flow dynamics but also such micro-scale characteristics as the location and activity of the transporters. On the other hand, in models that simulate the dynamics of substances at the micro-scale (Grieneisen et al., 2007, 2012; Sakurai et al., 2015; Yamaji et al., 2015; Foster and Miklavcic, 2016), substance flow is simulated by diffusion and convection in which the location and polarity of the transporters are considered. However, these models simulate dynamics at the cell or tissue level but not in the whole plant level.

The purpose of this study is to develop a new mathematical model that simulate silicon (Si) transport in whole plant in rice. Si is abundant in soils and is beneficial for plant growth (Ma and Takahashi, 2002). Si deposited in plant tissues enhances tolerance to abiotic and biotic stresses via alleviation of water stress, improvement of light interception characteristics by keeping the leaf blade erect, and an increase in resistance to diseases, pests, and lodging (Epstein, 1994; Savant et al., 1997; Ma and Takahashi, 2002). In rice roots, the passive transporter Lsi1 (OsLsi1) and the active transporter Lsi2 (OsLsi2) are involved in Si uptake (Ma et al., 2006, 2007). Both are located in the plasma membranes of the exodermal and endodermal cells, where Casparian strips are located. Lsi1 transports Si along the Si gradient between the plasma membrane, whereas Lsi2 transports Si from the symplast to the apoplast, and this transport is coupled with proton antiport (Ma et al., 2011). In rice nodes, Lsi2, Lsi3 (OsLsi3, active), and Lsi6 (OsLsi6, passive) transport Si from enlarged vascular bundles (EVB) to diffuse vascular bundles (DVB) for preferential distribution of Si to the grains (Yamaji and Ma, 2014; Yamaji et al., 2015). Dehydration stress decreases the expression of Lsi1 and Lsi2 via abscisic acid (ABA) in root (Yamaji and Ma, 2007). The expression of these transporter genes show a diurnal variation (Yamaji and Ma, 2007). More recently, it was reported that the expression of Lsi1 and Lsi2 genes is controlled by Si accumulation in the shoots, not in the roots (Mitani-Ueno et al., 2016). However, the mathematical models that simulate Si dynamics in whole plant have not been constructed yet.

In recent mathematical models of Si transport in both the root and node of rice (Sakurai et al., 2015; Yamaji et al., 2015), Si is assumed to be transported via diffusion and convection. In a previous study (Sakurai et al., 2015), we modeled the dynamics of Si transport from external solution to the cortex and stele of the root using a diffusion equation in which Si diffuses along the gradient of Si concentrations on a two-dimensional grid ($4 \times 4\,\mu$m). Using this micro-scale diffusion model, we could explain the dynamics of the micro-scale Si transport and could account for the characteristics of the spatial location of the transporters. The parameters of the models estimated using statistical computation methods, and the simulation output match the empirical data well. However, it is difficult to extend these models to whole-plant simulation.

In this study, we propose a new mathematical model that simulates Si transport in the whole plant using empirical data and knowledge from previous modeling studies. Using the model, we examined the factors and mechanisms affecting the expression of Si transporter genes. We assumed three possible signaling mechanisms that control the expression of Si transporter genes in this model: accumulation control, shortage control, and water stress control. Under accumulation control, expression is reduced by excess Si concentration in leaf cells. Under shortage control, expression is increased by low Si concentration in leaf cells. Under water stress control, expression responds to water stress (indicated by transpiration rate in this model). We compared the expression levels simulated by the models with those observed. Finally, using the model, we investigated the reason for the diurnal change of transporter gene expression levels in rice from the point of view of investment efficiency.

MODEL AND DATA

Model Structure

To consider the dynamics of water, sucrose, starch, Si, and the signals that control the expression level of Si transporter genes at the whole-plant level, we divided the whole plant into multiple points and connected them like in an electric circuit (**Figure 1**). In an electric circuit, a point of two or more elements is referred to as a "node," but in biology the junction region of leaves and branches to the stem is also referred to as a "node;" therefore, we call a point in a circuit a "hydraulic node" (**Figure 1**).

Water Flow

The flow of water and sucrose in the xylem and phloem was calculated following the model proposed by Daudet et al. (2002). In this model, the axial water flow between hydraulic nodes conforms to the Ohm's law:

$$J_{\mathrm{W}(i,j)\mathrm{X}} = -\frac{\Psi_{\mathrm{X}(j)} - \Psi_{\mathrm{X}(i)}}{r_{\mathrm{X}(i,j)}} \tag{1}$$

where $J_{\mathrm{W}(i,j)\mathrm{X}}$ is axial water flow in the xylem from the hydraulic node i to j, $\Psi_{\mathrm{X}(i)}$ and $\Psi_{\mathrm{X}(j)}$ is xylem water potential at the hydraulic node i and j, and $r_{\mathrm{X}(i,j)}$ is xylem flow resistance (Daudet

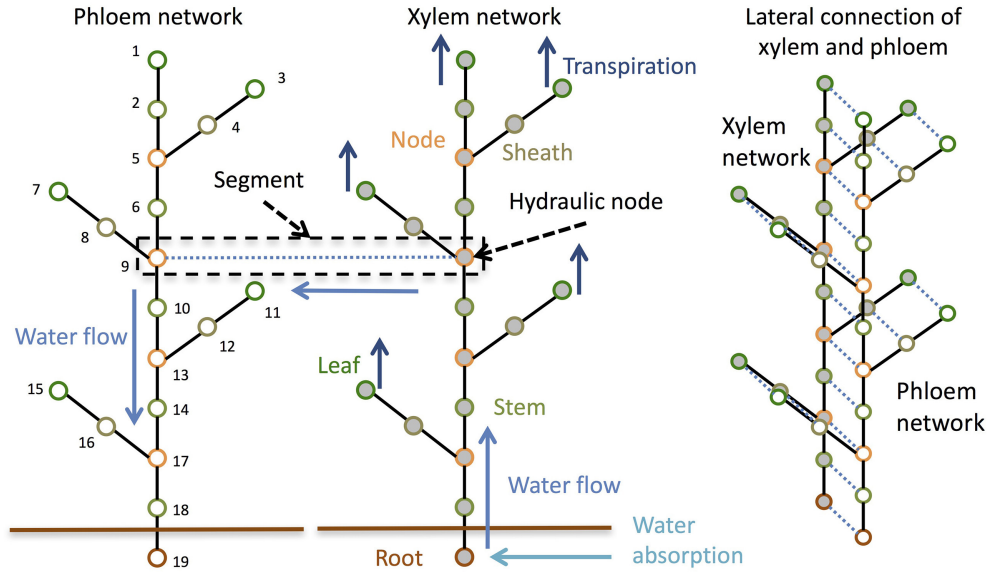

FIGURE 1 | Schematic diagram of the model structure. The model is composed of a phloem network and a xylem network. The two networks are combined at each hydraulic node.

et al., 2002). Water moves from one area to another in accordance with the gradient of water potential in the xylem (Cosgrove, 2010). Daudet et al. (2002) suggested a formula to describe the flow between the phloem and xylem and within the phloem. The lateral water flow between the xylem and phloem ($J_{W(i)Lat}$) is described as:

$$J_{W(i)Lat} = -\frac{\Psi_{P(i)} - \Psi_{X(i)}}{r_{Lat(i)}} \quad (2)$$

where $\Psi_{X(i)}$ is water potential in the xylem, $\Psi_{P(i)}$ is water potential in the phloem, and $r_{Lat(i)}$ is the sum of the apoplastic pathway resistance between the xylem and phloem (Daudet et al., 2002). Axial water flow in the phloem [$J_{W(i,j)P}$] is described as:

$$J_{W(i,j)P} = -\frac{P_{P(j)} - P_{P(i)}}{r_{P(i,j)}} \quad (3)$$

where $P_{P(i)}$ and $P_{P(j)}$ is hydraulic (mainly turgor) pressure in the phloem at the hydraulic node i and j and $r_{P(i,j)}$ is phloem flow resistance. Because gravity can be ignored when calculating water flow on a small scale, the following equation holds (Daudet et al., 2002):

$$P_{P(i)} = \Psi_{P(i)} - \Pi_i \quad (4)$$

where $\Psi_{P(i)}$ is water potential in the phloem and Π_i is osmotic potential. The latter can be described as:

$$\Pi_i = -R \cdot T_i \cdot C_{S(i)} \quad (5)$$

where R is the universal gas constant, T_i is absolute temperature, and $C_{S(i)}$ is sucrose concentration. The axial phloem solute flow [$J_{S(i,j)}$] is described as:

$$\begin{cases} J_{S(i,j)} = J_{W(i,j)P} \cdot C_{S(i)}, & \text{when } J_{W(i,j)P} > 0 \\ J_{S(i,j)} = J_{W(i,j)P} \cdot C_{S(j)}, & \text{when } J_{W(i,j)P} < 0 \end{cases} \quad (6)$$

where $J_{W(i,j)P}$ is the axial water flow in the phloem. Because the purpose of the model developed in this study was to estimate the dynamics of mineral transport rather than sucrose flow, we ignored lateral sucrose flow for simplicity.

The flow of water should be conserved at any hydraulic node in the xylem and phloem. Therefore, the following equation should hold:

$$\sum J_W = 0 \quad (7)$$

where J_W is the water flow from the target hydraulic node to the connected nodes. As this equation should hold at any node, we can estimate water potential at each hydraulic node at any time point by solving simultaneous equations under an appropriate boundary condition.

To calculate osmotic potential, we need sucrose concentration. In this model, we simply input the photosynthetic and transpiration rates as the boundary condition. Following photosynthesis, starch is synthesized in the leaf. The starch is dehydrated to sucrose and gradually loaded into the phloem, which is conveyed by the flow in the phloem, which follows the hydraulic pressure. The models of the dynamics of starch and sucrose in leaves and other tissues, which are similar to that of Daudet et al. (2002), are explained in Supplementary Information.

Si Transport

To understand the dynamics of Si in the whole shoot, we developed a simple two-compartment model that emulates the

transport of Si in root. If we assume the compartmentation of the root cortex between the external solution (soil) and root stele, then the flow of Si from external solution (soil) to the cortex can be described as:

$$J_{\mathrm{M(o:c)}} = \alpha \cdot tr_{\mathrm{exo}} \cdot C_{\mathrm{M:out}} - p_{\mathrm{cm}} \cdot (C_{\mathrm{M:cor}} - C_{\mathrm{M:out}}) \quad (8)$$

where $J_{\mathrm{M(o:c)}}$ is the flow of Si from external solution (soil) to the cortex, α regulates the expression level of the transporter (from 0 to 1), tr_{exo} is the transportation capability at the maximum expression level in exodermal cells, $C_{\mathrm{M:out}}$ and $C_{\mathrm{M:cor}}$ are Si concentrations in external solution and the cortex, respectively, and p_{cm} is the permeability of the cell membrane (Sakurai et al., 2015). Si is transported by endodermal transporters from the cortex to the stele; this flow can be described as:

$$J_{\mathrm{M(c:s)}} = \alpha \cdot tr_{\mathrm{end}} \cdot C_{\mathrm{M:cor}} - p_{\mathrm{cm}} \cdot \left(C_{\mathrm{M}(nr)} - C_{\mathrm{M:cor}}\right) \quad (9)$$

where $J_{\mathrm{M(c:s)}}$ is the flow of Si from the cortex to the stele, tr_{end} is the transportation capability at the maximum expression level in the endodermis, $C_{\mathrm{M}(nr)}$ is the Si concentration in the stele (i.e., in the hydraulic node of the root), and *nr* is the sequential number of the hydraulic node of the root.

Note that both tr_{exo} (Equation 8) and tr_{end} (Equation 9) include the activity of both Lsi1 and Lsi2. The change of Si concentration can be described as:

$$\frac{dC_{\mathrm{M:cor}}}{dt} = \frac{J_{\mathrm{M(o:c)}} - J_{\mathrm{M(c:s)}}}{V_{\mathrm{cor}}} \quad (10)$$

$$\frac{dC_{\mathrm{M}(nr)}}{dt} = \frac{J_{\mathrm{M(c:s)}} - J_{\mathrm{M}(nr,nr-1)}}{V_{\mathrm{st}(i)}} \quad (11)$$

where V_{cor} is the tissue volume of the cortex (assumed to be 1 ml for simplicity), $J_{\mathrm{M}(nr,nr-1)}$ is the flow of Si from the root to the hydraulic node above the root, and $V_{\mathrm{st}(i)}$ is the tissue volume of the sieve tube.

We assumed that Si absorbed in the root is transported with the flow of water only in the xylem. Therefore, the following equation holds for any node:

$$J_{\mathrm{M}(i,j)} = C_{\mathrm{M}(i)} \cdot J_{\mathrm{W}(i,j)\mathrm{X}} \quad (12)$$

where $J_{\mathrm{M}(i,j)}$ is the axial Si flow in the xylem and $C_{\mathrm{M}(i)}$ is Si concentration. The model assumes only transpiration as the force driving the water in the xylem; it does not consider the case when $J_{\mathrm{W}(i,j)\mathrm{X}}$ is negative.

Using a diffusion equation for Si transport between EVB and DVB, we previously revealed the importance of the apoplastic barrier at the bundle sheath cells and suggested that transporters generate large differences in Si concentration between DVB and EVB to enable rice to transfer sufficient Si upward (Yamaji et al., 2015). Here, to model the dynamics of Si in the whole shoot, we simplified the model of Si distribution at the node as follows:

$$J_{\mathrm{M}(i,j)} = J_{\mathrm{W}(i,j)} \cdot C_{\mathrm{M}(i)\mathrm{DVB}} \quad (13)$$

$$J_{\mathrm{M}(i,k)} = J_{\mathrm{W}(i,k)} \cdot C_{\mathrm{M}(i)\mathrm{EVB}} \quad (14)$$

$$C_{\mathrm{M}(i)\mathrm{DVB}} = \rho_i \cdot C_{\mathrm{M}(i)\mathrm{EVB}} \quad (15)$$

$$C_{\mathrm{M}(i)\mathrm{EVB}} = \frac{J_{\mathrm{W}(i,k)} + J_{\mathrm{W}(i,j)}}{\rho_i \cdot J_{\mathrm{W}(i,k)} + J_{\mathrm{W}(i,j)}} \cdot C_{\mathrm{M}(i)} \quad (16)$$

where $J_{\mathrm{M}(i,j)}$ and $J_{\mathrm{M}(i,k)}$ are Si flow, $J_{\mathrm{W}(i,j)}$ and $J_{\mathrm{W}(i,k)}$ are water flow, $C_{\mathrm{M}}(i)_{\mathrm{DVB}}$ and $C_{\mathrm{M}(i)\mathrm{EVB}}$ are Si concentrations in DVB and EVB, respectively, and ρ_i determines how Si concentration increases in DVB. We assume that hydraulic node *j* is connected to hydraulic node *i* via DVB and that *k* is connected to *i* via EVB (see also Supplementary Figure 1). Increasing the concentration of Si in DVB can distribute a large amount of Si to upper developing tissues, to which DVB connects (Yamaji et al., 2015). This is the function of ρ_i.

We assumed that Si is unloaded in each tissue according to the following equation:

$$J_{\mathrm{M}(i)\mathrm{unload}} = k_{\mathrm{M:unload}} \cdot C_{\mathrm{M}(i)} \cdot V_{\mathrm{con}(i)} \quad (17)$$

where $J_{\mathrm{M}(i)\mathrm{unload}}$ is Si flow from the xylem to tissue cells, $k_{\mathrm{M:unload}}$ is a parameter, and $V_{\mathrm{con}(i)}$ is the tissue volume of the conduit. The Si concentration in tissue cells, $C_{\mathrm{M}(i)\mathrm{cyt}}$, is calculated as:

$$\frac{dC_{\mathrm{M}(i)\mathrm{cyt}}}{dt} = \frac{J_{\mathrm{M}(i)\mathrm{unload}}}{V_{\mathrm{cyt}(i)}} \quad (18)$$

where $V_{\mathrm{cyt}(i)}$ is the volume of the cytoplasm in the tissue.

Transport of a Signaling Substances

Lsi2 expression is decreased by high Si accumulation in the shoot through an unknown signal from shoots to roots (Yamaji and Ma, 2011), and the expression of Lsi1 and Lsi2 is decreased by dehydration stress (Yamaji and Ma, 2007, 2011); the response to dehydration stress is more rapid than the response to Si accumulation. In this study, we used the following models to investigate the dynamics of the unknown signal. We considered the following three types of models for generation of the signal:

$$\text{Accumulation control}: GR = slp_{\mathrm{c}} \cdot C_{\mathrm{M}(i)\mathrm{cyt}} \quad (19)$$

$$\text{Shortage control}: GR = slp_{\mathrm{r}} \cdot (1 - C_{\mathrm{M}(i)\mathrm{cyt}}) \quad (20)$$

$$\text{Water stress control}: GR = slp_{\mathrm{J}} \cdot Trans \quad (21)$$

where *GR* is the rate of signal generation, slp_{c}, slp_{J}, and slp_{r} are parameters that determine the generation rate of signal in response to the target factor, $C_{\mathrm{M}(i)\mathrm{cyt}}$ is Si concentration in leaf cells, and *Trans* is transpiration rate in leaf. For simplicity, we assumed that the signal is generated only in the leaf. Moreover, because the signal is an unknown substance, we did not define its units. Equation (19) represents that the signal is generated according to Si concentration in leaf cells. Equation (20) represents that the signal is generated according to the transpiration rate in leaf. In this situation, we assume that the water stress is in proportion to the transpiration rate. This assumption may be rough approximation because the water stress in a plant would be affected by several factors such as soil moisture, water absorption history, and transpiration rate. However, we adopt this simple assumption because estimating exact water stress is beyond the purpose of this study. Equation (21) represents that the signal is generated according to the shortage of Si in leaf cells. In this situation, the signal transmits the information about shortage of Si. On the other hand, in

Equations (19), (20), the signal transmits the information about excess of Si. The signal decays according to decay rate *dec*. That is,

$$\frac{dC_{R(i)}}{dt} = -dec \cdot C_{R(i)} + GR \tag{22}$$

where $C_{R(i)}$ is signal concentration. We assumed that the generated signal is transferred via the phloem water flow. When the signal reaches the root, the expression level of the Si transporter is regulated according to the signal concentration as follows:

$$\alpha = \max(0, -C_{R(nr,\, t-\chi)} + 1) \tag{23}$$

$$\alpha = \min(1, C_{R(nr,\, t-\chi)}) \tag{24}$$

where α is a factor that regulates transporter expression level, $C_{R(nr,t)}$ is signal concentration at time t (hour), and χ is delay of time. It is set to 0 for Accumulation control assumption and Shortage control assumptions but set to 5 for Water stress control situation because the previous study suggests that there is time lag until the decrease of the expression level by dehydration stress (Yamaji and Ma, 2011). Equation (23) is used for Accumulation control and Water stress control assumptions. Equation (24) is used for Shortage control assumption.

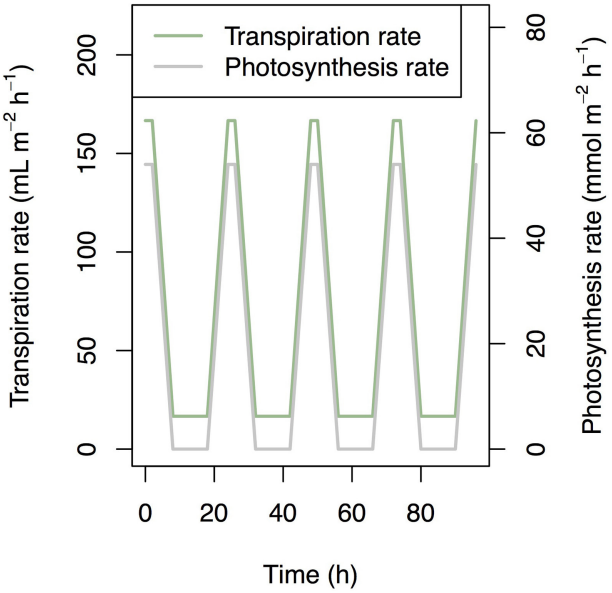

FIGURE 2 | Time series of transpiration rate and photosynthesis rate that was used as a boundary condition in simulation.

Simulation Settings

We set the values of transpiration rate, photosynthesis rate, and temperature as input data. We set the standard transpiration rate to 0.4 ml cm^{-2} day^{-1} (Kuwagata et al., 2012) and standard photosynthesis rate to 0.0015 mol cm^{-2} s^{-1}. We assumed (i) 10-h night, (ii) 2-h peaks of photosynthesis and transpiration (**Figure 2**), and (iii) a transpiration rate during darkness of 10% of the standard transpiration rate. We also assumed a simplified rice plant structure: (i) the root represented by one segment; (ii) five leaves; (iii) identical internode length; (iv) identical sheath length; and (vi) constant biomass during simulation. The other structural settings are described in Supplementary Table 1. The simulation period was 4 days.

Parameters for Transporters in Roots

To estimate the values of tr_{exo}, tr_{end}, and p_{cm}, we used time-series Si concentrations in xylem sap of 1-month-old seedlings exposed to 1.0 mM Si solution and measured every 5 min (Sakurai et al., 2015). We estimated parameter distribution using Markov chain Monte Carlo methods with the following likelihood function:

$$L(\theta|Data) = \prod \frac{1}{\sqrt{2\pi}\,\sigma_t} \exp\left(-\frac{(C_{M(nr),t}(\theta) - C'_{M(nr),\,t})^2}{2\sigma_t^2}\right) \tag{25}$$

where L is the likelihood of parameter set θ with the observed data set *Data*, σ_t is the standard deviation of the error distribution at time t, $C_{M(nr),t}(\theta)$ is the Si concentration in xylem sap at time t estimated with the parameter set θ, and $C'_{M(nr),t}$ is the observed Si concentration. We assumed that σ_t is equivalent to the standard deviation of the observed data. The initial setting for $C_{M:cor}$ (Equation 8) was 0.0 mM. The observed time-series data for xylem sap and estimated data are shown in Supplementary Figure 2. The model adequately estimated the observed Si concentrations.

Estimation of Parameters Relevant to Respiration

We estimated sucrose dynamics under several parameter settings. The estimated parameter values were 0.1, 0.15, and 0.2 for k4 and 2.0e-5, 4.0e-5, 6.0e-5, 8.0e-5, 10.0e-5, 12.0e-5, 14.0e-5, 16.0e-5, 18.0e-5, and 20.0e-5 for k1 (see Supplementary Information for k1 and k4). The parameter set where sucrose has the stable cyclic dynamics (k1 = 8.0e-5 and k4 = 0.1) was used for the simulation experiment (Supplementary Figure 3).

Simulation of Si Dynamics

To estimate Si dynamics, we used Equations (19), (20), or (21). In each case, we simulated Si dynamics with several parameter sets for *slp* and *dec*. For the other parameters and input data, we used the same values in all equations. The definitions of variables are described in Supplementary Table 2. The parameter values used for the simulation are described in Supplementary Tables 2, 3.

Investment Efficiency

To evaluate investment efficiency, we defined it as:

$$IE = \frac{\int_{t=stri}^{t=endi} J_{M(1,t)unload}\, dt}{\int_{t=stri}^{t=endi} \alpha_t\, dt} \tag{26}$$

where IE is investment efficiency, t is time, *stri* is the starting time of the calculation, *endi* is the ending time of the calculation, $J_{M(i,t)unload}$ is Si flow from the xylem to the cells of top leaf at time t, and α_t is a factor that regulates transporter expression

level at time t. This equation means that the investment efficiency is the amount of Si accumulated in top leaf divided by the total expression level of Si transporter genes.

Simulation under Natural Environment

Finally, we simulated Si dynamics under the natural environmental condition. In the above simulation setting, the artificial pattern of transpiration rate and photosynthesis rate were used (**Figure 2**). To confirm the result that is found in the above simulation experiment, we simulated the Si dynamics with the input data that was measured in the field experiment. The data were observed at the paddy site using the eddy covariance method during the growing season in 2004. The observation site was located at Mase, Tsukuba City, Ibaraki prefecture, Japan. In this observation, not only LE (Latent heat flux) and NEE (Net Ecosystem CO_2 Exchange) but also LAI (Leaf Area Index) were measured. We used LE and NEE for the estimation of transpiration and photosynthesis rates, respectively, assuming that the effects of evaporation from the water surface and heterotrophic respiration from the soil on the observed fluxes were negligible in our analysis. We used the data during day 33 and 35 after transplanting.

RESULTS

Si Dynamics and Expression of Si Transporter Genes under Accumulation Control

Using Equation (19), we simulated the model with several parameter sets (0.05, 0.1, and 0.2 for *dec* and 0.005, 0.01, and 0.02 for slp_c). During the day, Si concentration in the xylem of the top leaf rapidly increased and then decreased (**Figure 3A**) according to changes in leaf transpiration rate (**Figure 2**). At night, Si concentration reached zero, and then it increased again according to the increase in transpiration rate. Although Si concentration did not reach zero in the lower leaf (Supplementary Figure 4), a similar pattern was observed. Though the pattern was similar at all parameter settings, Si concentrations during the day were low in the models with low *dec* values (slow decay of signaling substance) and high slp_c values (rapid generation of the signaling substance). Si concentration in leaf cells gradually increased, and the differences among parameter sets became apparent after 24 h (**Figure 3B**). Si concentration in leaf cells was the lowest at $dec = 0.05$, $slp_c = 0.02$, and was only about half of that at $dec = 0.2$, $slp_c = 0.005$. The pattern was the same in the lowest leaf (Supplementary Figure 5). The signal level in phloem sap of the top leaf (**Figure 3C**) and roots (**Figure 4**) gradually increased with time and reached local maxima at dawn for the top leaf and at dusk for the roots. The signal level was the lowest at $dec = 0.2$, $slp_c = 0.005$, and was about one tenth of that at $dec = 0.05$, $slp_c = 0.02$) for the roots. The expression level of the transporter genes in roots gradually decreased with time (**Figure 5**). Two parameter sets ($dec = 0.05$, $slp_c = 0.01$; $dec = 0.1$, $slp_c = 0.02$) fit best the observed expression levels (mean mRNA levels) of Lsi1 measured using real-time RT-PCR by Yamaji and Ma (2011). Local maxima were reached during the day with all parameter sets.

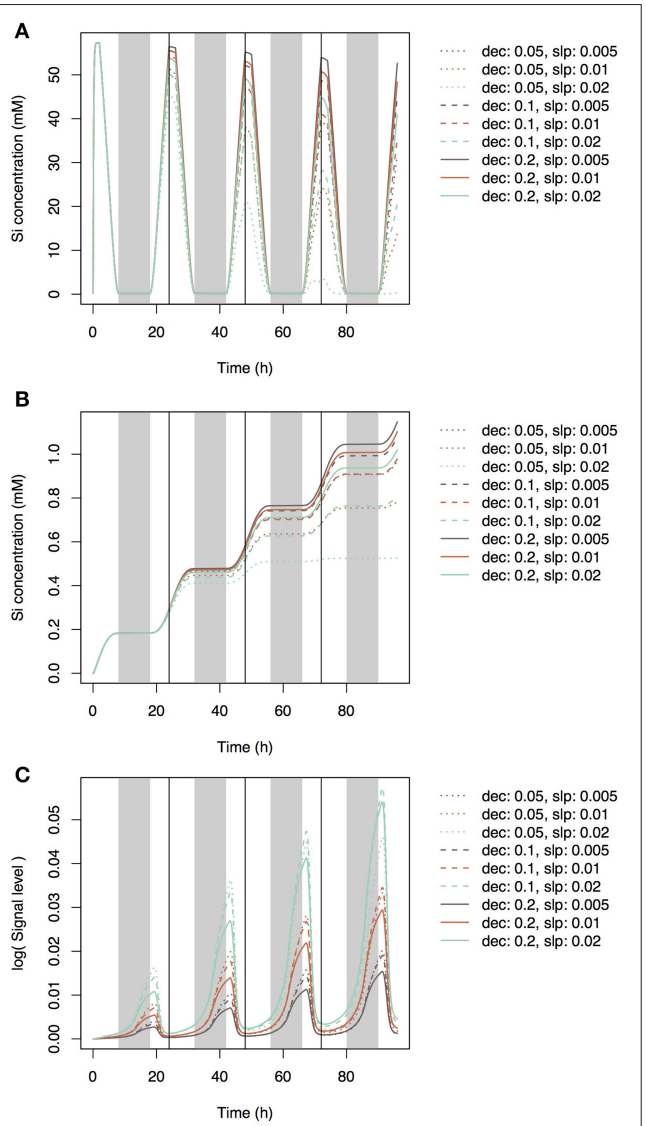

FIGURE 3 | Si concentration in xylem sap **(A)** and leaf cells **(B)** of the top leaf and the signal level in xylem sap **(C)** of the top leaf simulated with multiple parameter sets under the accumulation control assumption. Parameter *dec* is the decay rate of the signaling substance. Parameter *slp* is the generation rate of the signaling substance and corresponds to slp_c in Supplementary Table 2.

Expression of Si Transporter Genes under Shortage Control

Using Equation (20) and the same parameter sets, we simulated the expression level of the transporter genes in roots (**Figure 6**). As with accumulation control, the expression level gradually decreased with time, but local maxima were reached at dusk and local minima were reached at dawn with all parameter sets.

Si Dynamics under Water Stress Control

Using Equation (21) and the same parameter sets, we simulated the dynamics of Si concentration in the xylem (Supplementary Figure 6A) and in top-leaf cells (Supplementary Figure 6B).

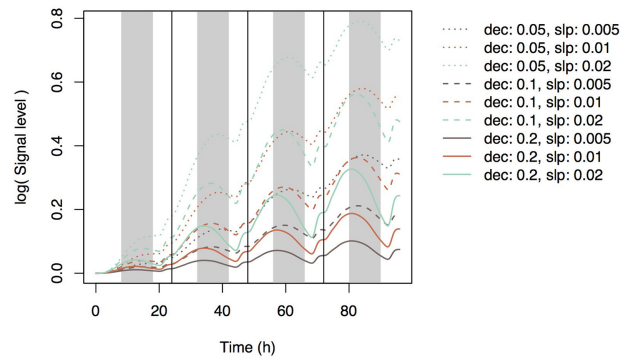

FIGURE 4 | Signal level in xylem sap of the root simulated with multiple parameter sets. Parameter *dec* is the decay rate of the signaling substance. Parameter *slp* is the generation rate of the signaling substance and corresponds to slp_C in Supplementary Table 2.

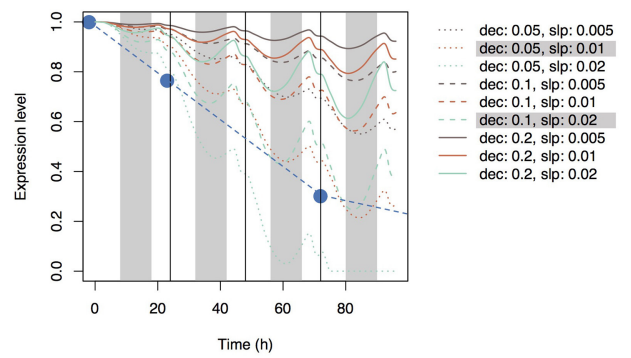

FIGURE 5 | Time series of the expression levels of Si transporter genes simulated with multiple parameter sets under the accumulation control assumption. Parameter *dec* is the decay rate of the signaling substance. Parameter *slp* is the generation rate of the signaling substance and corresponds to slp_C in Supplementary Table 2. Blue circles indicate the observed values of the mean expression level of *Lsi*1 (Yamaji and Ma, 2011).

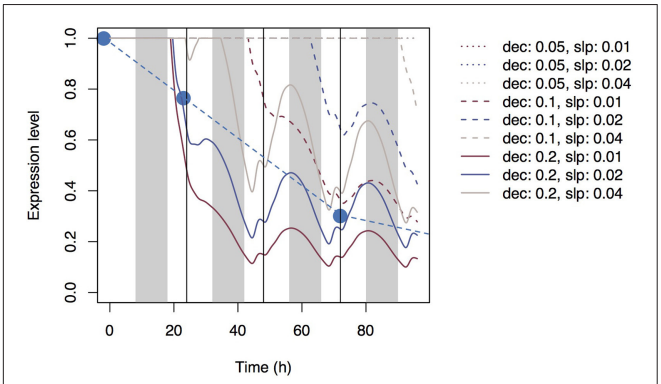

FIGURE 6 | Time series of the expression levels of Si transporter genes simulated with multiple parameter sets under the shortage control assumption. Parameter *dec* is the decay rate of the signaling substance. Parameter *slp* is the generation rate of the signaling substance and corresponds to slp_r in Supplementary Table 2. Blue circles indicate the observed values of the mean expression level of *Lsi*1 (Yamaji and Ma, 2011).

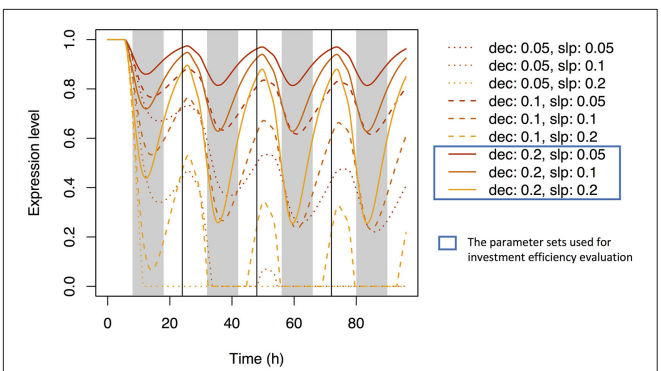

FIGURE 7 | Time series of the expression level of Si transporter genes simulated with multiple parameter sets under the water stress control assumption. Parameter *dec* is the decay rate of the signaling substance. Parameter *slp* is the generation rate of the signaling substance and corresponds to slp_J in Supplementary Table 2.

The patterns were similar to those under accumulation control. However, the pattern of the signal level in xylem sap differed: the local maxima were reached at dusk, but the signal level did not increase with time (Supplementary Figure 6C). A diurnal expression pattern in roots was found with some parameter sets (**Figure 7**); local maxima were reached during the day and local minima at night. This pattern is similar to that observed by Yamaji and Ma (2007) for the *Lsi1* expression level in the root.

Investment Efficiency

We investigated the reason why the expression level of the transporter genes shows diurnal variation in the point of view of investment efficiency. We used $dec = 0.2$ with $slp_J = 0.05$ (low sensitivity of Si transporter expression to water stress), 0.1 (intermediate), or 0.2 (high) under the water stress control assumption (see **Figure 7**). Under "constant" setting (which means that the expression level of the transporter does not change in response to water stress), the investment during the night was 71.4% of that during the day. The nighttime investment decreased with increasing sensitivity (**Figure 8A**). As the results, the investment efficiency increased with increasing sensitivity: it was 5.8% at low sensitivity, 13.0% at intermediate sensitivity, and 34.9% at high sensitivity (**Figure 8B**).

Simulation under Natural Environment

Patterns of photosynthesis and transpiration rates were similar between the artificial input data and empirical data (compare **Figures 2**, **9A**). As the results, the simulated patterns of the expression levels of the transporter genes were similar between them (compare **Figures 7**, **9B**), with local maxima during the day and local minima during the night. The investment efficiency increased by 4.2% at low sensitivity, 10.3% at intermediate sensitivity, and 27.4% at high sensitivity in comparison with that at "constant" setting.

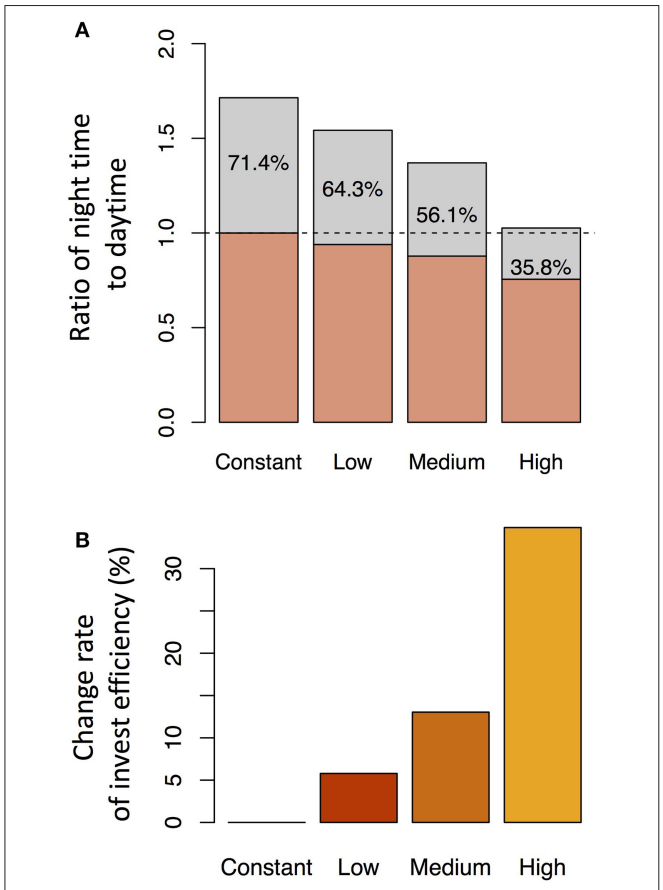

FIGURE 8 | Ratio of the investment during the night to that during the day for each parameter set **(A)** and the change rate of the investment efficiency relative to that under the constant expression of the transporter genes **(B)**. The parameter sets were constant (no sensitivity of Si transporter expression to water stress; $dec = 0.0$, $slp_J = 0.0$), low sensitivity ($dec = 0.2$, $slp_J = 0.05$), intermediate sensitivity ($dec = 0.2$, $slp_J = 0.1$), and high sensitivity ($dec = 0.2$, $slp_J = 0.2$) under the water stress control assumption (see **Figure 7**).

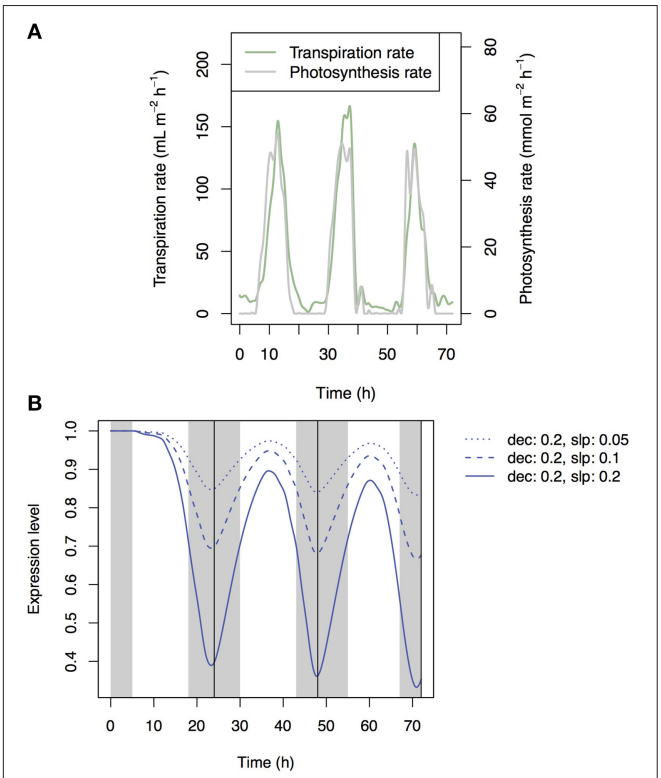

FIGURE 9 | Time-series of measured transpiration and photosynthesis rates **(A)** and of the transporter expression level at low, intermediate, and high sensitivity simulated under the water stress control assumption **(B)**.

DISCUSSION

In this study, we proposed a new mathematical model that simulate the Si dynamics in whole plant in rice and investigated the possible mechanisms underlying diurnal variation of the expression level of the transporter genes. To simulate the dynamics of mineral nutrients in rice, we have to simulate not only water flows in the xylem and phloem but also the transport and distribution of mineral nutrients via transporters. Models have been developed that simulate water flows in the xylem and phloem (Daudet et al., 2002; Lacointe and Minchin, 2008; Lobet et al., 2014; Seki et al., 2015) and transport of mineral nutrients from roots or mineral distribution at nodes (Sakurai et al., 2015; Yamaji et al., 2015). However, no study has been conducted on modeling the dynamics of mineral nutrients by considering both water flow and transporter expression level.

The conceptual characteristics of the model proposed here are as follows: (1) it can simulate the dynamics of a mineral nutrient in a whole rice plant while considering plant morphology (multiple leaves, nodes, and stems); (2) the model can simulate mineral transport from roots and its distribution at nodes; and (3) the model can simulate the control of the expression level of the transporter genes in roots. This concept can also be applied to other mineral nutrients and crops if the experimental data on the absorption and distribution of the target mineral nutrients can be obtained.

In the present study, we assumed that three mechanisms control transporter expression levels. The first mechanism is accumulation control, in which a signaling substance is generated in response to Si concentration in leaf cells and is then transported to roots through phloem sap flow. The model based on this mechanism reproduces the experimental data to some extent: the *Lsi1* expression level gradually decreases after root exposure to Si solution of sufficient concentration (**Figure 5**). The best parameter sets that agree with the empirical data were $dec = 0.05$, $slp_c = 0.01$ and $dec = 0.1$, $slp_c = 0.02$ (**Figure 5**). For all parameter sets, the estimated Si concentration in xylem sap was nearly zero during the night because a low transpiration rate at night decreases the differences between water potentials of hydraulic nodes. Interestingly, the concentration of the signaling substance in the leaf xylem showed diurnal variation (**Figure 3C**) despite the steady increase in the generation rate of the signaling substance in response to the Si concentration in leaf cells (**Figure 3B**). This phenomenon may be related to the dynamics of phloem sap. At dawn, the difference in the

potential between hydraulic nodes in phloem became small (Supplementary Figure 7) because of the depletion of starch in leaves by this time. Therefore, the flow of phloem sap from top to bottom decreased and the signaling substance remained in the leaves. In the root, the concentration of the signaling substance decreased at dawn (**Figure 4**). The diurnal variation in the transport rate of the signaling substance generated the diurnal variation in the expression level of the transporter genes (**Figure 5**).

Under the assumption of shortage control, the expression level of the transporter genes gradually decreased, as under the assumption of accumulation control, but local minima were reached at dawn (**Figure 6**). The downward convex curve of the expression level under shortage control may be attributable to the mechanisms that determine how fast the expression is suppressed. Under accumulation control, the decrease in the suppression rate depends mainly on the rate of generation of the signaling substance in leaves, whereas under shortage control, it depends mainly on the rate of decay of the signaling substance. The convex curve of the expression level under shortage control appears to fit the data reported by Yamaji and Ma (2011). Therefore, shortage control may be a more likely mechanism for the control of Si dynamics than accumulation control from the aspect of the shape of the curve (but see below).

Simulation under the assumption of water stress control shows diurnal variation of the expression level of the transporter genes (**Figure 7**). Local minima were reached around midnight. All parameter sets produced similar expression cycles, but the amplitudes differed depending on *slp* parameter values. The parameter set of $dec = 0.2$, $slp = 0.2$ fit well the experimental data of Yamaji and Ma (2007), which show a decrease in the *Lsi1* expression level at midnight to one-third of that at daytime.

Why does rice have a control system that generates the diurnal pattern of the expression level of the transporter genes? To answer this question, we compared the investment efficiency at different parameter values. A decrease in the transporter expression level during the night decreased the relative investment into the expression of the transporter genes (**Figure 8A**). As a result, the investment efficiency was highest in the simulation that had the largest amplitude of the diurnal variation of expression (**Figure 8B**) because of the difference in the transpiration rates between day and night. During the day, the transpiration rate is high, xylem sap flow is large, and Si absorbed in the root is efficiently transported to the upper tissues. During the night, the transpiration rate is low, xylem sap flow is small, and Si is not efficiently transported. In other words, when a conveyor belt is moving rapidly, many pieces of baggage can be loaded, but loading many pieces of baggage on a slow conveyor belt is not a good strategy. Interestingly, the increasing rate of the Si concentrations in the tissue cells of the roots during the night were higher than during the day (Supplementary Figure 8). It is because "many pieces of baggage" fell from the slow conveyor. As the results, the average Si concentration of all hydraulic nodes constantly increased even during the night to some extent (Supplementary Figure 9). This result is consistent with the previous experimental studies in which the rate of Si uptake did not slow down during the night (Ma and Takahashi, 2002).

We also investigated the investment efficiencies of accumulation control and shortage control. Under accumulation control, the investment efficiencies do not change greatly among parameter sets (Supplementary Figure 10A). Under shortage control, on the other hand, they were greatly decreased at all settings (Supplementary Figure 10B), perhaps because the level of expression of the transporters decreased during daytime rather than nighttime. Therefore, accumulation control may be preferable for rice from the aspect of investment efficiency. Although, it was reported that dehydration stress decreases the expression of Lsi1 and Lsi2 via ABA in root (Yamaji and Ma, 2007), the mechanism that gradually decreases the expression of transporter genes is not well-understood. Evaluating whether accumulation control or shortage control is the actual mechanism in rice is a task for future study.

The transpiration rate during the night used for the present simulation setting (10% of the daytime transpiration rate) may be large from the actual night-time transpiration rate. However, if the actual transpiration rate during the night is lower than 10% (e.g., Nakano et al., 2010), the conclusion discussed above would not change. It is because lower transpiration rate during the night should slow the xylem sap flow.

To confirm the result, we simulated the model with field data and found a similar diurnal pattern of transporter expression (**Figure 9**). The investment efficiency was highest when the model was simulated with the most sensitive (large-amplitude) parameters ($dec = 0.2$, $slp = 0.2$).

A previous study suggested that the localization and polarity of transporters observed in rice roots provide highest investment efficiency among all possible patterns evaluated (Sakurai et al., 2015). The present study suggests that rice maximizes the investment efficiency in terms of not only the spatial pattern but also the temporal pattern. A gradual decrease in the expression level of Si transporter genes in response to Si concentration in leaf cells might be the mechanism that increases investment efficiency. In rice, many positive effects of Si have been reported with no detectable negative effects of excess Si intake (Ma and Takahashi, 2002). However, the control of the transporter expression level in response to Si concentration in shoot should improve the efficiency of resource allocation.

In the current model, the processes of Si transport in roots and distribution in nodes are simplified. Including more detailed processes will be needed if the aim is to focus on the dynamics of Si at finer scales, such as the dynamics inside and outside of the cell membrane or the localization and polarity of transporters. However, as the current model was designed to describe the dynamics of Si at the whole-plant scale, its degree of simplicity is appropriate. Moreover, the photosynthate dynamics modeled in this study would be a general pattern of plants and does not include characteristic partitioning processes of carbohydrates found in grasses. Grasses store carbohydrates in mainly stem tissue when carbohydrates from the source is greater than whole plant demand (Slewinski, 2012). However, the non-structural carbohydrates in the stem is mainly expended during reproductive growth period (Slewinski, 2012) and leaves

would be the main source of carbohydrates during daytime and nighttime in rice (Eom et al., 2012). Therefore, it could be assumed that the downward transport of sucrose is predominant during nighttime at least in the early vegetative period. This was the case of our simulation in the present study. If the model is applied for the Si dynamics during the reproductive period, it might have to be modified so as to include the effect of the storage.

In the current study, the model structure and values of resistance may be oversimplified. The purpose of this study was to propose a new model to investigate qualitatively why rice controls the expression of Si transporter genes. For quantitative understanding of mineral transport, more realistic structure and resistance of water flow values should be reflected in the model, which would be require a large amount of additional experimental data.

CONCLUSION

We developed a new model that simulates the dynamics of Si in a whole rice plant by considering Si transport in the roots, its distribution at the nodes, and the control of the expression level of Si transporter genes by a signaling substances. The model reproduced a gradual decrease and diurnal variation of the expression level of the transporter genes observed by Yamaji and Ma (2007, 2011). Our modeling suggests that a considerable reduction in the expression level of Si transporter genes during the night increases investment efficiency (the amount of Si accumulated in top leaf divided by the total expression level of Si transporter genes). Our study suggests that rice has a system that maximizes the investment efficiency for Si uptake in terms of not only the spatial pattern (Sakurai et al., 2015) but also the temporal pattern.

AUTHOR CONTRIBUTIONS

GS, NY, NM, MY, and JFM designed the study. GS performed the simulations. KO measured field data. All authors contributed to drafting the paper.

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SUPPLEMENTARY MATERIAL

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An ABC Transporter Is Involved in the Silicon-Induced Formation of Casparian Bands in the Exodermis of Rice

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Silicon (Si) promotes the formation of Casparian bands (CB) in rice and reduces radial oxygen loss (ROL). Further transcriptomic approaches revealed several candidate genes involved in the Si-induced formation of CB such as ATP binding cassette (ABC) transporter, Class III peroxidases, ligases and transferases. Investigation of these genes by means of overexpression (OE) and knockout (KO) mutants revealed the contribution of the ABC transporter (*OsABCG25*) to CB formation in the exodermis, which was also reflected in the expression of other *OsABCG25* in the Si-promoted formation of CB genes related to the phenylpropanoid pathway, such as phenylalanine-ammonia-lyase, diacylglycerol O-acyltransferase and 4-coumarate-CoA ligase. Differential CB development in mutants and Si supply also affected the barrier function of the exodermis. OE of the ABC transporter and Si supply reduced the ROL from roots and Fe uptake. No effect on ROL and Fe uptake could be observed for the KO mutant. The presented research confirms the impact of the *OsABCG25* in the Si-promoted formation of CB and its barrier functions.

Keywords: radial oxygen loss, Fe uptake, phenylpropanoid metabolism, CaMV 35s enhancer trap lines, LRR, bypass flow

INTRODUCTION

Silicon (Si) is not an essential element, but has several beneficial effects on plant growth. It is one of the most abundant elements in the soil surface, with a soil solution concentration of 2.5–20 mg $Si^{*}L^{-1}$ silicic acid (Bogdan and Schenk, 2008; Marxen et al., 2016). Plants differ in their ability to accumulate Si and rice is known to be a strong accumulator, containing Si in even higher concentrations than nitrogen, potassium or calcium (Epstein, 1994). One of the Si effects is the formation of mechanical barriers in leaves and roots (Cai et al., 2009). Rice is cultivated under flooded (anaerobic) and unflooded (aerobic) conditions. Under submerged conditions adventitious rice roots develop aerenchyma by the lysis of cell walls in the cortex to ensure oxygen supply to the root tips from the shoot (Nishiuchi et al., 2012). However, oxygen diffuses from the aerenchyma into the rhizosphere which is hampered by the Casparian band (CB) in the exodermis. The CB development starts about 5–8 cm behind the root tip in the anticlinal cell walls and is mostly completed at a 12–13 cm distance from the root tip, reducing the radial oxygen loss (ROL) from roots (Steudle, 2000; Vaculík et al., 2009; Fleck et al., 2011). The CBs in the exodermis are also

thought to reduce the inflow of freely available ions from the soil solution into the cortex (Yeo et al., 1999; Gong et al., 2006; Faiyue et al., 2010). The CB development in the exodermis is stimulated by the Si supply in rice and also in other species, such as *Zea mais*, *Allium cepa*, *Tradescantia virginiana*, and *Guizotia abyssinica,* restricting the ROL to the first 5 cm behind the root tip in rice (Fleck et al., 2015). Furthermore, it was shown that Si supply reduces the Fe concentration in shoot matter (Ma and Takahashi, 1990; Dufey et al., 2013). It is hypothesized that Si-stimulated CB formation reduces the Fe flow into the cortex, where it is bound to Deoxymugineic acid and taken up via yellow stripe- like transporters (Yehuda et al., 1996; Nozoye et al., 2011).

The CBs occur in the endodermis of all species and in the exodermis of most species, including rice, maize and onion, but not in *A. thaliana* (Schreiber et al., 1999; Ma and Peterson, 2003; Naseer et al., 2012; Fleck et al., 2015). The main components are lignin and suberin (Schreiber et al., 2005b; Naseer et al., 2012). Suberin is a biopolymer consisting of aliphatic components (ω-hydroxy acids, α, ω-dicarboxylic acids, fatty acids, alcohols) and aromatic components (ferulic acid; Franke et al., 2005). The CB formation in the endodermis of *A. thaliana* starts with the deposition of lignin hampering the flux from the cortex into the stele. In the second step, suberin is deposited (Naseer et al., 2012). The CB in the exodermis of rice is formed by simultaneous incorporation of lignin and suberin into anticlinal cell walls (Kotula et al., 2009; Fleck et al., 2015). These CB compounds are provided by the phenylpropanoid pathway and it has been shown that some of the genes, like phenylalanine-ammonia-lyase (PAL), 4-coumarate-CoA ligase (4CL), glycerol-3-phosphate acyltransferase (AT), diacylglycerol O-acyltransferase (DGOAT), ATP binding cassette (ABC) transporter and class III peroxidases (POD) involved in this secondary metabolism pathway are upregulated through Si in roots (Fleck et al., 2011). Additionally, expression of a leucine-rich repeat (LRR) family protein and an ABC transporter (*OsABCG25*; LOC_Os10g30610) was enhanced. The transporter *OsABCG25* was suggested to be involved in the transport of monolignols or suberin monomers in the Si-induced development of CB in the exodermis. The PAL desaminates phenylalanine to cinnamic acid, which is metabolized via several steps to precursors of lignin and suberin (Zhong et al., 1998; Eckardt, 2002). The 4CL catalyzes the formation of monolignols from coumaroyl-CoA, feruloyl-CoA, or sinapoyl-CoA. Suberin consists of aliphatic and aromatic components where the aliphatic components are provided from fatty acids by POD. The aliphatic and aromatic components, are bound to glycerol by AT, such as DGOAT to suberin monomers. Both monolignols and suberin monomers are most probably transported by ABC transporters into the apoplast (Landgraf et al., 2014; Shiono et al., 2014). The function of these genes in Si-stimulated CB development was studied in overexpression (OE) and knockout (KO) mutants. We observed the involvement of the *OsABCG25* in CB formation and, additionally, investigated the expression of genes related to lignin and suberin metabolism. Furthermore, the barrier function of exodermal CB with regarding ROL from roots and Fe uptake were investigated in these mutants.

MATERIALS AND METHODS

Selection of Rice Mutant Lines

We selected 24 rice mutant lines carrying a T-DNA insertion that contains multimerized cauliflower mosaic virus (CaMV) 35S enhancers leading to an OE of nearby genes (Jeong et al., 2002; Chern et al., 2007). As selection criteria, the T-DNA insertion of the mutant lines should be located within 10,000 bp upstream or downstream of one of eight candidate gene for suberin or lignin synthesis and must not interrupt the sequence of a non-target gene. In contrast to the other lines, the line *1B-14436* carried an insertion in the exon sequence of a candidate gene, resulting in an interrupted transcription of the gene *OsABCG25* (LOC_Os10g30610; Genebank ID: ABB47708.1; Uniprot ID: B9G5Y5). The positions of the T-DNA relative to the target gene were calculated using the GenomeBrowser of the OryGenesDB database[1] (Droc et al., 2006). The mutant lines selected were ordered from the Pohang University of Science and Technology (Postech; Pohang, Republic of Korea; Jeon et al., 2000) and from the Taiwan Rice Insertional Mutants Database (TRIM; Taiwan). **Table 1** summarizes the mutant lines, the target gene identifiers, the position of the insert relative to the start codon of the target gene, and the supplier of the seeds. Out of these 24 rice mutant lines a total of ten homozygous lines were obtained.

[1]http://orygenesdb.cirad.fr/

TABLE 1 | Mutant lines, target genes, position of the insert relative to the gene and supplier of the seeds.

Mutant line	Target gene	Position relative to start codon of gene	Supplier
1B-04415	LOC_Os01g67540	245 upstream	Postech
3A-14487	LOC_Os01g67540	759 upstream	Postech
2D-41110	LOC_Os02g41680	5270 downstream	Postech
M0060856	LOC_Os02g41680	59 downstream	TRIM
4A-50856	LOC_Os05g20100	161 downstream	Postech
5A-00450	LOC_Os06g16350	3745 upstream	Postech
5A-00464	LOC_Os06g16350	3820 upstream	Postech
3A-01911	LOC_Os06g16350	10457 upstream	Postech
M0038578	LOC_Os06g22080	3644 downstream	TRIM
3D-01082	LOC_Os06g22080	9232 downstream	Postech
3A-01215	LOC_Os08g02110	392 downstream	Postech
3A-02897	LOC_Os08g02110	420 upstream	Postech
3A-08589	LOC_Os08g02110	9509 upstream	Postech
3A-06124	LOC_Os10g30610	1466 upstream	Postech
3A-16329	LOC_Os10g30610	3131 upstream	Postech
3A-16331	LOC_Os10g30610	3874 upstream	Postech
3A-02127	LOC_Os10g30610	4537 upstream	Postech
3A-60593	LOC_Os10g30610	4139 upstream	Postech
2D-00893	LOC_Os10g30610	5277 downstream	Postech
M0033740	LOC_Os11g14050	7665 upstream	TRIM
M0058854	LOC_Os11g14050	3592 downstream	TRIM
2A-20141	LOC_Os11g14050	1810 downstream	Postech
M0066685	LOC_Os11g14050	12599 downstream	TRIM
1B-14436	LOC_Os10g30610	Exon	Postech

Plant Material, Growth Conditions and Harvest, T1 Seeds

Rice (*Oryza sativa* L.) seeds of the insertion lines (**Table 1**) were delivered as T1 seeds containing a mixture of wild type (WT), heterozygous and homozygous mutant plants. Seeds were germinated in tap water for several days for seed propagation and the seedlings were then transferred to 10-L pots containing soil from the local campus and grown submerged in a greenhouse with average temperatures around 28°C and a minimum of 220 μmol M^{-2} s^{-1} light intensity until maturity. After a few weeks, the genotype of the plants was determined using DNA from the leaves and only homozygous mutant plants were further cultivated further. Whole plants were individually enwrapped in plastic sleeves at the time of flowering to prevent crosspollination with other plants.

Genotyping

The DNA extraction was performed using a crude leaf extract from a NaOH-Tris-extraction method for genotyping (Collard et al., 2007). An amount of 200 mg of a leaf were harvested, transferred to a 2.0-ml tube containing one steel ball and 100 µl of 0.5 M NaOH. The leaf was homogenized in a swing mill for 1 min at 30 Hz and then 900 µl of 0.1 M Tris was added. Samples were centrifuged for 3 min at 13000 × *g*, the supernatant was transferred to a fresh 1.5-ml tube and stored at −20°C.

An amount of 1 µl of the DNA extracted was used in 25 µl PCR reaction mix containing 2.5 µl 10x reaction buffer, 3.6 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas, St. Leon-Rot, Germany), 0.75 U *Taq*-DNA-polymerase (DNA cloning service, Hamburg, Germany), 0.25 µM forward primer and 0.25 µM reverse primer. Two PCR runs with different primer combinations were used for each insertion line. A primer pair targeting at genomic regions flanking the insert was used (W-primer pair) in the first PCR, and an insert-specific primer targeting at a sequence near the border of the T-DNA was used together with one primer of the first PCR (I-primer pair) in the second PCR. The PCR products were electrophoretically separated on a 1% agarose gel. The WT plants showed a band with the W-primer pair, homozygous mutant plants were identified by a band with the I-primer pair, while heterozygous plants were characterized by bands with both primer pairs. The PCR runs included both negative controls with water instead of DNA and positive controls with DNA from WT plants. The genotyping primers used for each line are summarized in Supplementary Tables S1, S2.

Growth Conditions and Harvest, T2 Seeds

The T2 seeds of homozygous mutant plants and corresponding WT plants were germinated in tap water for several days and then placed between two layers of filter paper standing in tap water for 7 days. Seedlings were transferred to nutrient solution in 5-L pots containing 0.43 mM NH_4NO_3, 0.32 mM NaH_2PO_4, 0.51 mM K_2SO_4, 1 mM $Ca(NO_3)_2$, 1.6 mM $MgSO_4$, 1.82 µM $MnSO_4$, 0.03 µM $(NH_4)_6Mo_7O_{24}$, 9 H_3BO_3, 0.6 µM $ZnSO_4$, 0.15 µM $CuSO_4$, and 35.81 µM Fe^{EDDHA}. The pH value was adjusted to 6.0 by the addition of 10% H_2SO_4 and 1 M KOH and the nutrient solution was renewed weekly for the first 2 weeks. After 2 weeks, the nutrient solution was changed twice a week until harvest. Plants were cultivated in a climate chamber (photoperiod 14/10 h light/dark; temperature, 25/20°C day/night; 75% relative humidity and a light intensity of 220 µmol m^{-2} s^{-1}).

The WT seeds and T2 seeds of homozygous mutant plants of the lines *1B-14436* (KO) and *3A-16329* (OE) were germinated and cultivated in nutrient solution as described above, but with two Si treatments, to determine the effect of Si supply. Si was applied as silica gel and Si concentrations were 3 or 30 mg L^{-1} resulting in Si concentrations of 0.1/0.1 and 60/3 mg*g^{-1} shoot / root DM, respectively (Supplementary Figure S1).

The homozygous genotype of the plants was confirmed during the cultivation using DNA from the leaves. After 28 days in nutrient solution, root zones 4–6 cm behind the root tip were harvested and either stored in 70% ethanol at 4°C for subsequent histochemical determination of CB or transferred immediately to liquid nitrogen and stored at −80°C for transcript analysis. Shoot and root were separated, dried at 60°C for 4 days and weighed.

Histochemical Examination of Roots

Freehand cross sections of adventitious roots fixed in 70% EtOH were stained with 0.1% (w/v) berberine hemisulfate for 60 min, washed three times with distilled water and counterstained with 0.5% (w/v) aniline blue for a further 30 min for detection of CB (Brundrett et al., 1988). Stained sections were mounted in 0.1% (w/v) $FeCl_3$ in 50% (v/v) glycerine and examined using an Axioskop fluorescence microscope (Zeiss, Jena, Germany) with UV illumination and excitation filter G 365, chromatic beam splitter FT 395 and barrier filter LP 420. Pictures were taken with the AxioCam MRc (Zeiss) and picture recording software (AxioVision Ac, Version 4.4, Zeiss). Suberin exhibited a blue-white color under UV light. The development of CB in the anticlinal exodermal cell walls was determined and allocated to one of four stages: 0% (stage I), 0–25% (II), 25–50% (III) and 50–100% (IV) development of CB in the anticlinal cell wall of the exodermis.

Five roots without lateral roots were taken from each of the four replicates for cross-sectioning and 20 cells each from five cross sections were used for microscopic examination, therefore, the degree of development of CB was based on 400 cell walls per treatment.

Transcript Analysis

Frozen root material was ground under liquid nitrogen and total RNA was isolated using TRIsure® Reagent (Bioline, Luckenwalde, Germany), following the manufacturer's instructions. The RNA quality was determined electrophoretically by 2% non-denaturating agarose gel and fluoretically using a Nanophotometer (Implen, Munich, Germany). The total RNA (1 µg) and random hexamer primers were used to synthesize first-strand cDNA using the Revert Aid™ H Minus Kit (Fermentas, St. Leon-Rot, Germany), following the manufacturer's instructions for GC-rich templates.

In the qRT-PCR experiments, 100 ng cDNA was used as a template in 25 µl reaction mix containing 2.5 µl 10x buffer,

3.6 mM $MgCl_2$, 0.2 mM dNTPs mix (Fermentas, St. Leon-Rot, Germany), 0.25 µl 1:1000 diluted SYBR-Green (Invitrogen, Carlsbad, CA, USA), 0.75 U HotStart-Taq-DNA-Polymerase (DNA cloning service, Hamburg, Germany), and 0.25 µM forward and 0.25 µM reverse primers. The qRT-PCR runs were performed in the CFX96 cycler (Bio-Rad, München, Germany), using an initial 95°C-step for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s, and a final melting curve procedure with a stepwise increment of 1°C ranging from 60 to 95°C.

The eukaryotic elongation factor 1-alpha (eF1-α) was used as an endogenous control due to its stable transcript abundance in rice (Jain, 2009). A list of primer sequences used can be found in Supplementary Table S3. Three technical and three biological replicates were used for each target in qRT-PCR. The relative quantity was calculated using the R-Macro "qpcrmix" (Steibel et al., 2009), based on the $2^{-\Delta\Delta CT}$ method.

Cell Wall Isolation and Preparation for Suberin Analysis

Root zones 4–6 cm behind the root tip were harvested and the root surface was scanned using WinRHIZO software (Regent Instruments Inc., Quebec, QC, Canada). The cell wall isolation and preparation was performed as described in detail by Schreiber et al. (1994). Briefly, root zones were washed with H_2O_{dest} and then incubated at room temperature for 4 days in 1 ml enzyme solution (0.1 M citric acid monohydrate, 1% pectinase (v/v), 1% cellulase (v/v), 0.1% NaN_3), which was renewed daily. After enzymatic digestion, the non-degradable outer part of the root comprising the exodermal cell wall fraction and the sclerenchyma was separated from the tissue containing the stele by using two forceps under a binocular. The exodermal cell wall material was incubated in enzyme solution for another 2 days to remove any residual cortex material. Subsequently, the isolates were washed with H_2O_{dest} and incubated in borate buffer (0.01 M sodium borate, pH 9) for 2 days.

Dried isolated cell wall material was extracted for 5 days with a 1:1 mixture of chloroform and methanol, which was changed daily. After the extraction, the isolated samples were dried for 2 h in the desiccator over silica gel. The dry weight was determined just prior to the suberin analysis of isolated samples.

Suberin Analysis

The dried sample isolates were incubated for 16 h in 1 N methanolic boron trifluoride ($MeOH/BF_3$; Fluka/Sigma-Aldrich, St. Louis, MO, USA) at 70°C for transesterification. Saturated NaOH was added to stop the transesterification reaction and to advance the following phase separation. Dotriacontan (C_{32} alkane, 10.025 mg/ 50 ml) was added to each sample as an internal standard. The soluble hydrophobic components were extracted by adding chloroform. The chloroform phase was transferred to a new vial and extraction was repeated three times. The extract was dried with water-free Na_2SO_4 and the volume was reduced to 50 µl by evaporation under N_2 flow.

Samples were derivatized in 20 µl BSTFA (N,N-bis(trimethylsilyl)-trifluoracetamide; Machery-Nagel, Düren, Germany) and 20 µl dry pyridine (GC-grade, Merck, Darmstadt, Germany) for 40 min at 70°C. Pyridine catalyzed the derivatization reaction and BSTFA masked free hydroxyl- and carboxyl-groups forming the corresponding trimethylsilyl derivatives (Schreiber et al., 2005b). Samples were analyzed by gas chromatography (GC; Type: 6890N, Agilent Technologies, Santa Clara, CA, USA) and mass spectroscopy (MS: Type: 5973N, Agilent Technologies, Santa Clara, CA, USA). The GC and MS analyses were performed as described previously in detail in Zeier and Schreiber (1997). The quantification of the monomers was performed using a gas chromatograph combined with a flame ionization detector. Four replicates of each rice line were used.

Visualization of Radial Oxygen Loss

In order to visualize the ROL, adventitious roots of plants were grown for 28 days in nutrient solution with and without Si supply, as described above. An adventitious root was placed between two acryl glass plates (16 cm × 6 cm; 0.5 cm apart) which were sealed with Plasticine (Pelikan, Hannover, Germany) and the rest of the root system remained in nutrient solution. The space between the plates with the root was filled with 38°C warm semisolid agar medium containing FeS by use of a pipette and the top was sealed with paraffin wax. The medium was prepared by adding 0.8% agar to iron-free nutrient solution and subsequent heating to solubilize the agar. The solution was amended with 1.4 g $FeSO_4 \times 7\ H_2O\ L^{-1}$ and 0.32 g $Na_2S\ L^{-1}$, whereupon a black FeS precipitation developed (Trolldenier, 1988). Finally, the solution was buffered by the addition of 0.5 g $CaCO_3\ L^{-1}$ and adjusted to pH 6.0. The acryl glass plates, held together by clamps, and the plant were fixed using a tripod. The plates were covered with aluminum foil and scans of the plates were taken by a flatbed scanner (Expression 1600, Seiko Epson K.K., Suwa, Nagano, Japan). 12 h after embedding in agar, six roots were investigated for each treatment. The area of ROL was determined using the Fiji imaging software (Schindelin et al., 2012), transforming the picture into a binary image and analyzing particles bigger than 50 pixels.

Chemical Analysis

In order to determine the Si concentration in the shoot and root, 200 mg dried and ground plant matter was digested in 3 ml 65% HNO_3, 2 ml H_2O and 2 ml 30% H_2O_2 in a microwave for 12 min at 190°C, then diluted with 20 ml 10% NaOH, neutralized with HNO_3 (Haysom and Ostatek-Boczynski, 2006) and filled up to a final volume of 100 ml.

In order to determine the Fe, Cu, Mn and K, 50 mg of dried and ground shoot matter was digested in 2 ml 65% HNO_3, 2 ml H_2O, and 0.5 ml 30% H_2O_2 in a microwave for 25 min at 190°C and then diluted with distilled water to 25 ml.

The Si, Fe, Cu, Mn, and K in the plant extracts and nutrient solution were determined by ICP-MS (7500c Agilent Technologies, Santa Clara, CA, USA).

Statistical Analysis

All treatments were replicated four times unless stated otherwise and the mean of the treatments were compared using *t*-test,

Tukey or Bonferroni test using Sigma Plot (Systat Software Inc., San Jose, CA, USA). A cumulative link mixed model was calculated with $p < 0.05$ using the package ordinal in R Software (R Development Core Team, 2011) for comparison of the developmental stages of CB. The statistical qRT-PCR analysis was performed using the R-Macro of Steibel et al. (2009).

RESULTS

A total of ten homozygous rice lines were investigated to observe the function of Si enhanced genes involved in the lignin and suberin synthesis (**Table 2**). Only one KO (1B-14436) and one OE (3A-16329) line showed a significant reduction or increase of the transcript level compared to WT for the ABC transporter *OsABCG25* (LOC_Os10g30610).

Results given in **Figure 1** confirm down- and upregulation of this gene in the respective mutant at low and high Si supply. The expression in OE plants was increased by a factor of four at both Si levels, whereas the transcript level in KO plants was reduced to 1/6. High Si supply enhanced the gene expression by a factor of 2 in WT and OE plants, but did not in KO plants.

However, there was no pronounced effect of differential gene expression in mutants on suberin fractions in outer cell layers (OPR). The Total content of ω-hydroxy fatty acids and 2-hydroxy fatty acids, and fractions of C24 acids, C24 alcohols, C24 diacids, ω-hydroxy fatty acids and 2-hydroxy fatty acids were not significantly affected (Supplementary Figures S2, S3). The content of aromatic compounds, both coumaric acid and ferulic acid, in the OPR was decreased in the KO mutants, but the OE mutants did not differ clearly from the WT plants (Supplementary Figure S4).

The downregulation of one gene involved in CB development should result in an altered regulation of other genes known to be involved in CB development from previous studies (Fleck et al., 2011). The KO of *OsABCG25* resulted in a downregulation of PAL (LOC_Os02g41680) and LRR (LOC_Os11g14050) by 30% (**Figure 2**). The OE resulted in an upregulation of DGOAT (LOC_Os06g22080), PAL and LRR by a factor of 1.5 to 2. Additionally, 4CL (LOC_Os01g67540) was slightly downregulated. Moreover, POD1 and 2 (LOC_Os08g02110 and LOC_Os06g16350) and AT (LOC_Os05g20100) were not regulated.

Furthermore, microscopic evaluation of the exodermis development in berberin-aniline stained hand cuttings of the zone 4–6 cm behind the root tip showed different patterns of CB formation (**Figure 3**). High Si supply enhanced in WT and the mutants the CB development which was reflected in a decreased number of cell walls without CB and a higher number of cell walls of Stage III or even fully developed CB. Furthermore, the CB formation in the OE mutant compared to the WT was significantly enhanced in both Si levels. However, the KO mutant was not different from the WT.

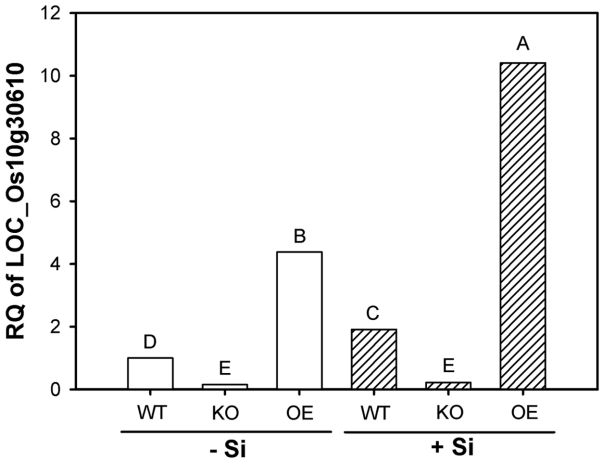

FIGURE 1 | Transcription of the gene LOC_Os10g30610 (OsABCG25) in root zone 4–6 cm of wild type (WT), knockout (KO) line 1B-14436 and overexpression (OE) line 3A-16329 (OE) plants grown in nutrient solution with low (–Si, 3 mg Si L^{-1}) and high (+Si, 30 mg L^{-1}) Si supply. Relative quantity (RQ) of WT –Si = 1. Different letters indicate significant differences between treatments at $p < 0.01$.

TABLE 2 | Homozygous rice insertion lines and target genes related to suberin and lignin synthesis, gene loci and changes in target gene transcription relative to wild type (WT) plants.

Insertion line	Target gene	Gene Locus	Transcription of target gene in insertion line relative to WT
2D-41110	Phenylalanine ammonia-lyase	LOC_Os02g41680	1.44 n.s.
3A-14487	4-coumarate-CoA ligase-like 6	LOC_Os01g67540	0.53 n.s.
3A-01911	Class III peroxidase	LOC_Os06g16350	1.83 n.s.
3A-08589	Class III peroxidase	LOC_Os08g02110	1.05 n.s.
1B-14436 (KO)	ABC transporter OsABCG25	LOC_Os10g30610	0.21***
3A-06124	ABC transporter OsABCG25	LOC_Os10g30610	1.05 n.s.
3A-16329 (OE)	ABC transporter OSABCG25	LOC_Os10g30610	5.01***
3A-02127	ABC transporter	LOC_Os10g30610	1.7 n.s.
3A-60593	ABC transporter	LOC_Os10g30610	1.36 n.s.
M0066685	Leucine-rich repeat receptor kinase	LOC_Os11g14050	1.78 n.s.

*n.s, not significant ($p > 0.05$); ***significant with p-Value < 0.05.*

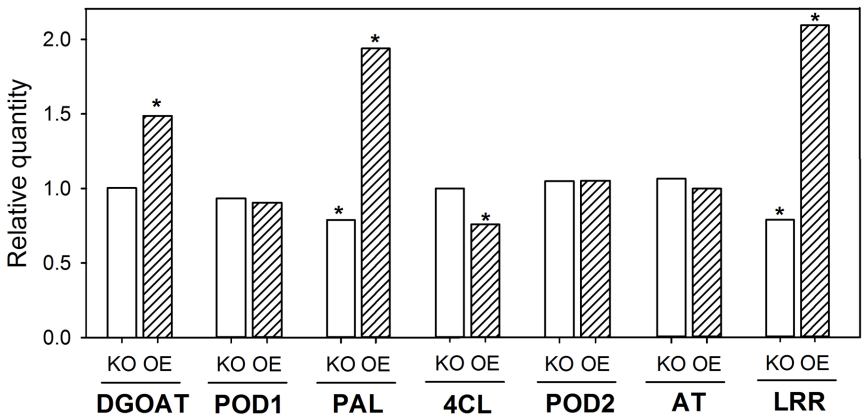

FIGURE 2 | Transcription of Casparian band (CB)-related genes in root zone 4–6 cm of WT, KO line *1B-14436* and OE line *3A-16329*. The OE plants were grown in nutrient solution with low (–Si, 3 mg Si L^{-1}) Si supply and KO grew in high (+Si, 30 mg L^{-1}) Si supply. The RQ of corresponding WT (–Si /OE; +Si/KO) = 1. Abbreviations of the genes are: 4CL, 4-coumarate ligase (LOC_Os01g67540); AT, glycerol-3-phosphate acyltransferase (LOC_Os05g20100); DGOAT, diacylglycerol O-acyltransferase (LOC_Os06g22080); LRR, leucine-rich repeat family protein (LOC_Os11g14050); PAL, phenylalanine-ammonia-lyase (LOC_Os02g41680); POD1 and 2, peroxidase (LOC_Os08g02110 and LOC_Os06g16350). A *indicates significant differences between the WT and mutant plant at $p < 0.01$.

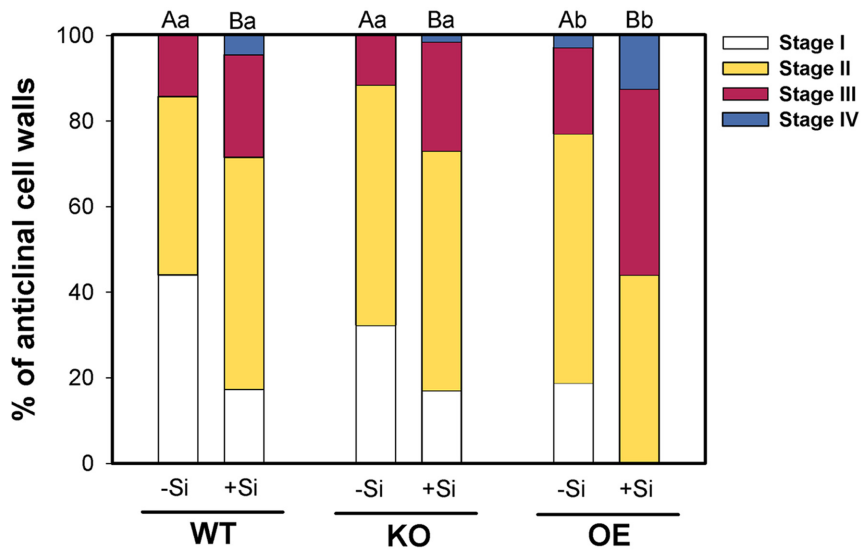

FIGURE 3 | Development of CB in the exodermis of WT plants and the mutant lines *1B-14436* (KO) and *3A-16329* (OE) as affected by Si supply (concentration are given in the legend of Figure 1). The root zone investigated was 4–6 cm behind the root tip. The CB formation was classified in four stages: I:0, II: 0–25, III: 25–50, IV: >50% of the length of the anticlinal cell wall with CB $n = 4$. Different capital and small letters indicate significant differences between Si treatments within a genotype and for genotypes within the same Si level, respectively; cumulative link mixed models with $p < 0.05$.

These findings agreed well with the observations on ROL from rice roots (**Figure 4**). Rice roots were embedded in iron-sulfur agar to visualize the ROL as a clear zone along the root. The KO line was investigated under +Si conditions, since a fully developed ROL can be already expected for WT roots under −Si conditions. By contrast, ROL was investigated for the OE line under −Si, since the ROL of WT roots under +Si is already heavily restricted to the 5 cm behind the root tip (Fleck et al., 2011).

The results confirmed previous reports (Fleck et al., 2011, 2015) that Si reduces the ROL from WT roots. The clear zone in −Si plants, extended along the whole root length and even precipitation of Fe^{3+} hydroxide/oxide was visible at the root surface. The area of ROL was reduced by half under high Si and restricted to the first 5 cm behind the root tip. The OE line had a clearly reduced ROL compared to WT, whereas the KO mutant behaved like the WT.

Silicon application was reported to decrease the Fe concentration in rice shoots (Ma and Takahashi, 1990; Dufey et al., 2013). We speculated that this effect might be related to the CB-formation and analyzed the Fe concentration in shoot matter (**Figure 5**). Si application reduced Fe concentration in

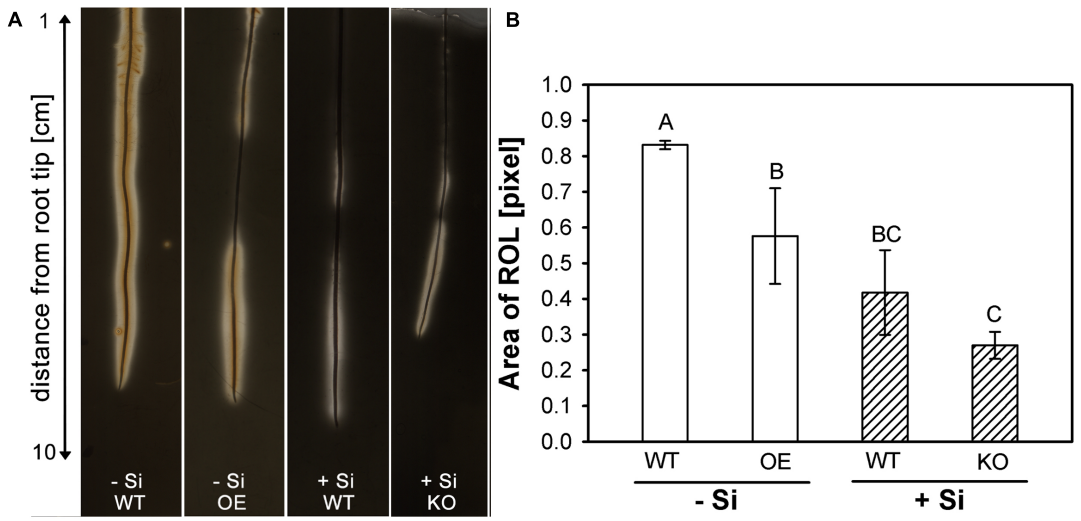

FIGURE 4 | (A) Visualization of radial oxygen loss (ROL) from rice roots using a FeS-agar. The WT plants were grown with low (–Si) and high (+Si) Si supply (conc. are given in the legend of **Figure 1**), whereas the insertion line 1B-14436 (KO) was cultivated only in + Si and mutant 3A-16329 (OE) only in –Si; **(B)** Area of ROL of roots of WT plants and the mutation lines *1B-14436* (KO) and *3A-16329* (OE). Different letters indicate a significant difference between treatments; Tukey test with $p < 0.05$.

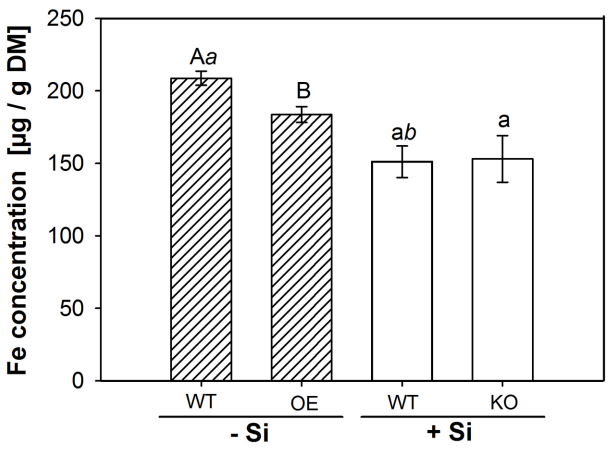

FIGURE 5 | Iron content in shoot dry matter. The WT plants were grown with low (–Si) and high (+Si) Si supply (conc. are given in the legend of **Figure 1**), whereas the insertion line 1B-14436 (KO) was cultivated only in +Si and mutant 3A-16329 (OE) only in –Si. Different capital, small and italic letters indicate a significant difference between treatments; students *t*-test with $p < 0.05$.

WT shoots by 20%. A significant reduction compared to the WT under −Si was also observed in the OE mutant, whereas the KO line was not different to the WT under +Si.

DISCUSSION

Gene Expression

The KO line and one OE line out of nine homozygous lines showed a significant differential expression of the gene of interest indicating a very low efficiency of the CaMV 35s promoter enhancer-trap transgenic lines (**Table 2**). The CaMV 35s was described to be less active in monocots, roots and rhizodermal cells (Battraw and Hall, 1990; McElroy et al., 1990). Obviously, CaMV 35s enhancer-trap transgenic lines were not suitable to investigate gene function in rice roots. Additionally, we observed only heterozygous progenies for some CaMV 35s enhancer trap-lines (data not shown). The reasons could be lethality of homozygous plants and/or apomixis (Bicknell and Koltunow, 2004).

The KO and OE of the ABC transporter *OsABCG25* (LOC_Os10g30610) resulted in down- and upregulation of some genes involved in the lignin and suberin metabolism, as summarized in **Figure 6** (**Figures 1**, **2**). The PAL (LOC_Os02g41680), catalyzing the first step of the phenylpropanoid pathway, and the LRR receptor-like kinase (LOC_Os11g14050) were downregulated in KO, but upregulated in OE. Furthermore, the 4CL (LOC_Os01g67540) in OE was downregulated and an upregulation of DGOAT (LOC_Os06g22080) was observed. The 4CL metabolizes p-coumaric acid and ferulic acid to monolignols (Gross and Zenk, 1974). DGOAT is most probably involved in the esterification of glycerol compounds into aliphatic suberin precursors in roots (Cases et al., 1998; Lu et al., 2003), to which ferulic acid is transferred by aliphatic suberin feruloyl transferase (Molina et al., 2009) to generate suberin monomers. These observations suggest that the formation of monolignols in the OE mutant was reduced, whereas the pathway to suberin monomers was enhanced, leading to the speculation that suberin monomers are the substrate of the OE ABC transporter *OsABCG25* (**Figure 6**). This transporter has no sequence similarity to *ABCG1*, a suberin transporter which has already been described from *Arabidopsis thaliana* (*ABCG1*, Landgraf et al., 2014). Furthermore, the ABC

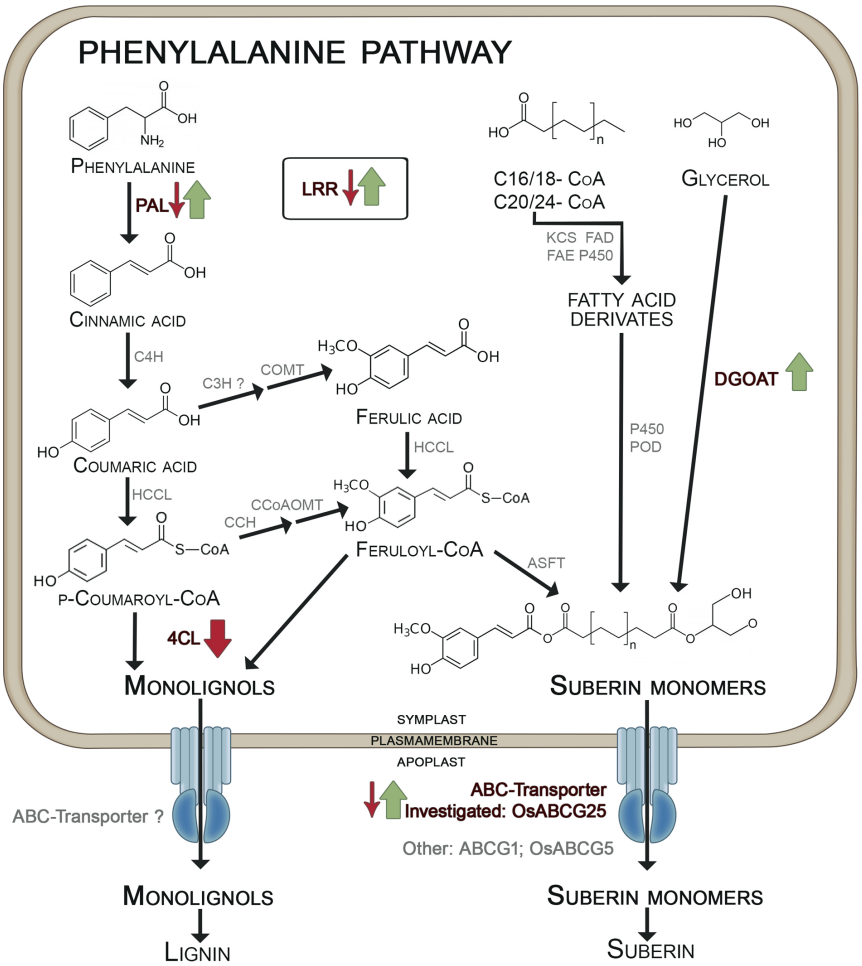

FIGURE 6 | Differential expression of genes involved in exodermal CB development in mutants of OsABCG25. KO mutant: small arrows; OE mutant: big arrows. Phenylalanine-ammonia-lyase (PAL) catalyzes the formation of cinnamic acid which is metabolized by cinnamate-4-hydroxylase (C4H) to coumaric acid. Ferulic acid is built by coumaroyl-CoA-3-hydroxylase (C3H) and caffeic acid O-methyltransferase (COMT). Hydroxycinnamate-CoA-ligase (HCCL) catalyzes the formation of coumaryl-CoA and feruloyl-CoA which are converted to monolignols by 4-coumarate ligase (4CL) (Zhong et al., 1998; Eckardt, 2002). From coumaryl-CoA also feruloyl-CoA is generated by p-coumaroyl CoA 3-Hydroxylase (CCH) and caffeoyl-CoA O-methyltransferase (CcoAOMT) which is bound to suberin monomers by aliphatic suberin feruloyl transferase (ASFT). The aliphatic components of suberin monomers originate from fatty acid derivates and glycerol, which is incorporated by diacylglycerol O-acyltransferase (DGOT) (Franke et al., 2005; Franke and Schreiber, 2007; Li-Beisson et al., 2013). Both, monolignols and suberin monomers are transported by ABC-transporter from the symplast into the apoplast. We suggested that the investigated OsABCG25 transports suberin monomers. Other suberin transporters are ABCG1 (Landgraf et al., 2014) and OsABCG5 (Shiono et al., 2014). Further proteins are: CCH, p-coumaroyl CoA 3-hydroxylase; FAD, fatty acid desaturase; FAE, fatty acid elongase; KCS, β-ketoacyl-Coa synthase; LRR, leucine-rich repeat family protein; P450, cytochrome P450 monooxygenase; POD, peroxidase. Arrows indicate significant regulation in KO/OE mutants vs. +WT/−WT (p-value > 0.01), green stands for upregulation, red stands for downregulation. The scheme based on Eckardt (2002) and Franke and Schreiber (2007).

transporter *OsABCG5* (LOC_Os03g17350) shows 72% sequence similarity on a protein level to the ABC transporter in this study. *OsABCG5* is necessary for the CB formation in the exodermis (Shiono et al., 2014), confirming the need of the ABC-transporter for suberin- transport and subsequent CB development.

The LRRs are widely described to be involved in many functions, such as signal transduction, sensing, pathogen-response (Shiu and Bleecker, 2001; Shiu et al., 2004) or even development of root exodermal cells (Huang et al., 2012). It was shown that another LRR, the *Docs1*, belonging to the LRR RLK group in the LRR II subfamily, is necessary for the formation of sclerenchyma and exodermis in young lateral roots of rice. The LRR in this study belongs to the same structure group and subfamily with an LRR-domain, protein-kinase and transmembrane domain similar to *Docs1*. The LRR was upregulated through Si and by OE of the ABC-transporter. The LRR was downregulated in KO. This indicates a role of LRR in CB development.

Suberization, Oxidation Power, and Fe Uptake

The suberin analysis in this work showed the typical pattern of rice root aliphatic components (Schreiber et al., 2005a,b).

The OPR contained the typical abundant ω-hydroxy acids for rice in C16, 28 and 30. C24 diacids also appeared as expected, but were not different between genotypes (Supplementary Figures S2, S3). However, the total aromatic suberin amount, both coumaric acid and ferulic acid, was lower in KO (Supplementary Figure S4). This may indicate a general disturbance of cell wall metabolism in OPR.

The supply with Si enhanced the development of the CB (**Figure 3**), as was previously described. It was hypothesized that Si crosslinks phenols with cell walls or induces precipitation of the phenols leading to an enhanced formation of CB (Fleck et al., 2011, 2015). Furthermore, it was shown that Si promotes the deposition of aliphatic and aromatic compounds synchronously in the exodermis as investigated by serial cuttings stained with Berberine-Aniline-Blue and Fluorol Yellow 088 (Fleck et al., 2015). Contrarily, *Arabidopsis thaliana*, a species without CB as an exodermal diffusion barrier, first develops lignin monolayers and later suberin lamellae in the endodermal CB (Naseer et al., 2012). Thus, in the early stages of CB development, CB in the exodermis consists of both suberin and lignin, while it is lignin in the endodermis.

A significant enhanced development of the CB could also be observed in the OE mutant, but no difference compared to the WT occurred in the KO mutant (**Figure 3**). These findings agree well with the fact that the OE of the ABC transporter stimulated the expression of PAL and DGOAT, supplying metabolites for the formation of suberin (**Figure 2**). The CB development in the KO plant was not different from the WT, suggesting that the function of the knocked-out transporter may be substituted by other ABC transporters.

The different CB in Si-supplied WT and in mutants was also reflected in the area of the ROL (**Figure 4**). The enhanced CB development in Si-supplied WT plants and in the OE mutant resulted in a clearly decreased ROL. The WT plants grown in −Si solution had an oxidation zone along the whole root length, whereas the oxidation zone in WT/+Si and OE/−Si was limited to the first 5 cm from the root tip and to the zone 7–10 cm, where lateral root development starts. The oxidation zone was unaffected under +Si in the KO mutant; this fits with the observation that CB development was not affected. Oxygen-transport in rice is provided by aerenchyma from the upper plant organs to the root tip (Nishiuchi et al., 2012). Exodermal CB functions as a diffusion barrier reducing the ROL (Kotula et al., 2009; Fleck et al., 2011). Differential CB development depending on Si supply and mutant was also paralleled by variations of the Fe concentration in shoot matter, which was reduced in WT plants by Si supply and in the OE mutant, but was unaffected in the KO mutant (**Figure 5**). This agrees with previous findings that Si supply in the nutrient solution reduces the concentration of Fe, Mn and other nutrients by 20–50% in leaf DM (Ma and Takahashi, 1990; Dufey et al., 2013). Si, supplied as monosilic acid, can bind with Cu^{2+}, Mn^{2+} or Fe^{2+} (Stevic et al., 2016). Such binding to monosilic acid may also occur with Fe^{EDDHA}, as was supplied in this research, and may decrease the bioavailability in rice, a strategy II species. Iron uptake in strategy II species grown in nutrient solution is assumed to take place through binding of Fe^{3+} to deoxymugineic acid in the apoplast of roots (Takagi, 1976; Bienfait et al., 1985) and subsequent uptake by yellow stripe-like transporters (Koike et al., 2004; Inoue et al., 2009; Lee et al., 2009). The flux of Fe from the nutrient solution into the apoplast may be impaired by the Si-enhanced development of the exodermis. This conclusion is supported by the observation in the OE mutant that the increased development of exodermal CB resulted in a decreased Fe concentration in shoot matter (**Figure 6**), which is in line with the consideration of the exodermal CB as a diffusion barrier controlling the ion flow into the apoplast (Faiyue et al., 2010). Exodermal CB hampers the apoplastic pathway of water and Na uptake (Yeo et al., 1987; Steudle, 2000) and it was shown that Si supply reduces the apoplastic Na transport across the root of rice (Yeo et al., 1999; Gong et al., 2006; Krishnamurthy et al., 2009). The presented research confirms the function of OsABCG25 and indicates the involvement of genes related to the phenylpropanoid pathway, such as PAL, DGOAT and 4CL in the Si-promoted formation of CB. The OE of the ABC transporter as well as silicic acid supply enhanced the CB formation in the exodermis leading to a decrease of both, ROL and Fe uptake. This supports the view that the exodermis acts as diffusion barrier controlling fluxes in and out of roots.

AUTHOR CONTRIBUTIONS

MH, MS, and AF designed the research; MH, AF, EB, and NN performed the experiments; LS provided reagents and helpful discussions and MH, MS, and AF wrote the manuscript.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00671/full#supplementary-material

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Silicification in Grasses: Variation between Different Cell Types

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Plants take up silicon as mono-silicic acid, which is released to soil by the weathering of silicate minerals. Silicic acid can be taken up by plant roots passively or actively, and later it is deposited in its polymerized form as amorphous hydrated silica. Major silica depositions in grasses occur in root endodermis, leaf epidermal cells, and outer epidermal cells of inflorescence bracts. Debates are rife about the mechanism of silica deposition, and two contrasting scenarios are often proposed to explain it. According to the passive mode of silicification, silica deposition is a result of silicic acid condensation due to dehydration, such as during transpirational loss of water from the aboveground organs. In general, silicification and transpiration are positively correlated, and continued silicification is sometimes observed after cell and tissue maturity. The other mode of silicification proposes the involvement of some biological factors, and is based on observations that silicification is not necessarily coupled with transpiration. Here, we review evidence for both mechanisms of silicification, and propose that the deposition mechanism is specific to the cell type. Considering all the cell types together, our conclusion is that grass silica deposition can be divided into three modes: spontaneous cell wall silicification, directed cell wall silicification, and directed paramural silicification in silica cells.

Keywords: cell wall, grasses, inflorescence bracts, root endodermis, silica cells, silicification mechanism, transpiration, trichomes

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INTRODUCTION

Silicon is a ubiquitous soil element that along with oxygen forms 50–70% of soil mass (Ma and Yamaji, 2006). Plant roots absorb silicon as mono-silicic acid [$Si(OH)_4$], a solute that is released to soil by the weathering of siliceous minerals. Near most soil pH, silicic acid is an uncharged molecule with pKa 9.8. Its concentration in soil solutions usually varies between 0.1 to 0.6 mM, but may range anywhere between 0.01 to 2.0 mM (Haynes, 2014). Silicon affects plants' physiology in many beneficial ways, imparting tolerance against biotic stresses and alleviating adverse effects of abiotic stresses (Liang et al., 2015). Although the benefits of silicon in agriculture are known for a long time, no general mechanism of action was defined.

Silicon content across plants varies between about 0.1% to more than 10% on dry weight basis (Epstein, 1994). Plants belonging to fern family Equisetaceae, and monocots belonging to families Poaceae, Cyperaceae, and Commelinaceae have relatively large silicon content of about 4% (Hodson et al., 2005; Currie and Perry, 2007). Among them, Poaceae (the grass family) is the agriculturally most important family, with rice, wheat, and barley constituting the basis for human nutrition worldwide. In grasses, the root uptake of silicic acid (herein referred to as Si) is mediated by the cooperative action of an aquaporin-like channel Low silicon 1 (Lsi1) and a proton

antiporter, Lsi2. Afterwards, Si moves with the transpiration stream and is unloaded from the xylem in the leaves by another aquaporin-like channel, Lsi6 (reviewed by Ma et al., 2011; Ma and Yamaji, 2015). In addition, so far unknown Si transporters might be involved in directing further Si transfer within the leaf tissues and concentrating it in target locations. The solute terminally polymerizes with concomitant loss of water molecules, forming hydrated silica ($SiO_2 \cdot nH_2O$). Plant silicification occurs in cell walls, cell lumens, and intercellular spaces. While most of the mineral is found in the shoot, some Si polymerizes in the roots (Sangster, 1978).

Two contrasting hypotheses are often proposed to explain silica deposition. The first is based on a passive mode of silicification, relying on the spatial correlation between silica deposition and organ transpiration (Yoshida et al., 1962; Sangster and Parry, 1971; Rosen and Weiner, 1994; Euliss et al., 2005). In this case, specific cell wall components and cuticular structures may additionally affect the location of bio-silicification (reviewed by Exley, 2015; Guerriero et al., 2016). This hypothesis infers that silica deposition in plants is a spontaneous process resulting from auto-condensation of Si molecules as the sap undergoes dehydration (Yoshida et al., 1962). The second hypothesis suggests that the formation of plant silica structures is catalyzed by biological entities (Kaufman et al., 1969; Hayward and Parry, 1973; Sangster et al., 1983; Parry et al., 1984; Kumar et al., 2017). Some authors suggest that silica deposition cannot be explained solely by any one of the two hypotheses, and both the mechanisms may be involved simultaneously (Hayward and Parry, 1975; Sangster et al., 2001; Motomura et al., 2004; Markovich et al., 2015). A review of literature is thus pertinent to better understand this biomineralization process.

SITES OF SILICIFICATION IN GRASSES

Silica is deposited in all the organs of grasses. The most intensely silicified tissues are usually root endodermis, leaf epidermis, and abaxial epidermis of inflorescence bracts (**Figure 1**). In most cases, silica impregnates the cell walls, directly laid down onto the cell wall matrix (Bauer et al., 2011; He et al., 2013; Hodson, 2016). The composition of the silicifying matrix may vary between species and cell types, thus influencing silicification pattern (reviewed by Guerriero et al., 2016; Hodson, 2016). In particular, grasses have a unique hemicellulose composition, containing glucuronoarabinoxylan and mixed-linkage glucans, instead of the xyloglucan in non-commelinid monocots and dicots. Furthermore, grass cell walls contain more phenylpropanoids and less pectin compared with dicots (Guerriero et al., 2016). Silica is often proposed to crosslink the cell wall polymers, adding to their compressive strength (Currie and Perry, 2009; He et al., 2013; Kido et al., 2015), similar to the role of lignin in lignified walls (Salmén, 2015). In addition, structural trade-off between silica, lignin and cellulose was observed in rice (Suzuki et al., 2012; Yamamoto et al., 2012) and in a number of wetland species (Schoelynck et al., 2010). As the metabolic costs of silica deposition were estimated to be 20-fold lesser than that of lignification (Raven, 1983), silicification can present preferable solution for improving mechanical properties of plant tissues. However, silica seems not to provide water repelling properties comparable to lignin and its utilization thus require some degree of regulation (Soukup et al., 2017).

Silicification in Inflorescence Bracts

Translocation of Si in plants is driven mostly by transpiration (Sangster and Parry, 1971), and coordinated by specific distribution of Si transporters. Silicic acid is selectively transported to the panicle of rice during its maturation (Yamaji et al., 2015), possibly due to its increasing sink strength (Detmann et al., 2013). In panicles, Si is concentrated and deposited in the inflorescence bracts (Hodson and Sangster, 1988), which serve as a tough protecting shield to the developing caryopses. Silica deposition is restricted to the epicarp hairs (Bennett and Parry, 1981; Parry et al., 1984) and the outer wall of aleurone layer (Hodson and Parry, 1982), whereas it does not accumulate in the caryopsis endosperm (Jones et al., 1963). Bract silicification also provides a safe disposing location for the mineral, which would polymerize anyway, as the transpired water evaporates.

Silicification of the Glume Prickle Hairs, Papillae, and Long Cells

The abaxial epidermis of the *Phalaris canariensis* glume consists of stomatal complexes, long cells, prickle hairs, papillae, marco-hairs and silica-cork cell pairs (Hodson et al., 1985). **Figure 1B** shows a scanning electron micrograph of a *Triticum aestivum* (wheat) glume, exhibiting stomata, prickle hairs, papillae, and macro-hairs. In this section we will discuss silicification in glume prickle hairs, papillae and long cells, whereas the silicification of silica cell will be discussed in a separate section. Presence of stomatal complexes on the abaxial epidermis indicates substantial transpiration after their emergence. Nevertheless, the outer tangential cell wall of papillae and prickle hair tips are already silicified at emergence (Sangster et al., 1983; Hodson et al., 1985). The flag leaf sheath encloses the inflorescence before emergence and limits its transpiration, raising the possibility that the cell wall is conducive for spontaneous silica deposition (Nissan et al., 2015). Callose [β-(1→3)-D-polyglucose] induces silica deposition in undersaturated silicic acid solution (Law and Exley, 2011), and it is possible that similarly, other polysaccharides might play such role in cell wall silicification. Cross-sections of mature prickle hairs and papillae show that the lumen of these cells is also filled with silica, without the in-growth of cell wall into the cell lumen to template the silicification (Hodson et al., 1985; Hodson, 2016). Lumen silicification in dead cells suggests that it might be driven either by passive transpiration via silica granule formation (Bennett, 1982), or templated by organic matrix that is accessible to Si only after cell death (Sangster, 1970). However, the fact that lumen silicification continues long after cell death rather supports the transpiration driven passive mode of silicification. Thus, it might be possible that silica is deposited in two stages in these cells, starting at the tip and the outer wall induced by wall materials, followed by a spontaneous precipitation inside the lumen driven by the degradation of the protoplast and evapo-transpiration (Motomura et al., 2006; Markovich et al., 2015).

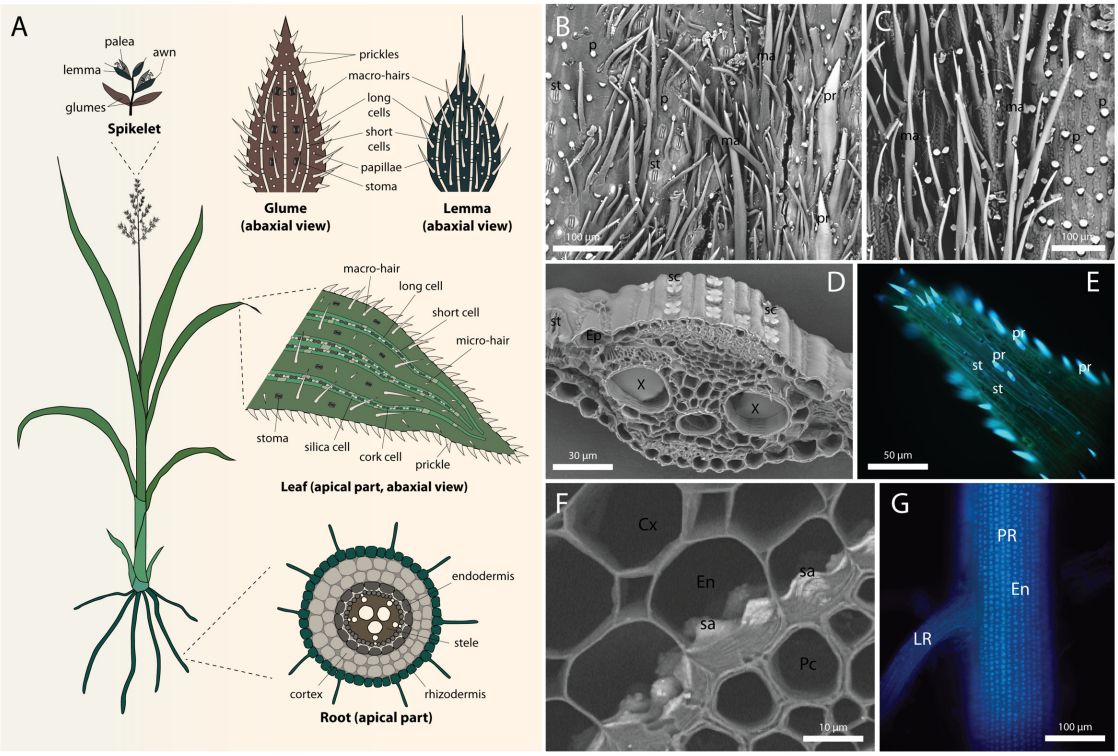

FIGURE 1 | Silica deposition in grasses. (A) Diagram showing a full view of a generalized grass, and typical silicification patterns in the inflorescence (top), leaf epidermis (middle), and root cross-section (bottom). White represent silicified cells. **(B)** Scanning electron micrograph (SEM) of the abaxial epidermis of glume in *Triticum aestivum* L. **(C)** SEM of the abaxial epidermis of lemma in *T. aestivum*. **(D)** SEM of *Sorghum bicolor* (L.) Moench leaf cross-section showing silica cells in the epidermis. **(E)** Fluorescence micrograph of prickles at the leaf tip in *S. bicolor* visualized by alkali-induced fluorescence (Soukup et al., 2014). **(F)** SEM of *S. bicolor* root cross-section showing silica aggregates anchored in the inner tangential cell walls of endodermis. **(G)** Alkali-induced fluorescence micrograph of *S. bicolor* primary root showing extensive distribution of silica aggregates in the endodermis. Root cortex was mechanically removed to expose the inner tangential cell walls. Cx, cortex; En, endodermis; Ep, epidermis; LR, lateral root; ma, macro-hair; p, papilla; Pc, pericycle; PR, primary root; pr, prickle cell; sa, silica aggregate; sc, silica cell; st, stoma. SEMs were collected at the back scattered electron mode, rendering silicon atoms brighter than carbon atoms.

In long cells, the outer wall thickens about 1 week after glume emergence. Silica deposition seems to initiate 2 weeks after the glume emergence, in parallel to cell death and collapse of the long cells and parenchymatic cells (Hodson et al., 1985). Thus, long cell silicification seems to depend on water evaporation.

The Outer Epidermal Cells and Macro-Hairs in Lemma

The outer epidermis of the lemma of *Phalaris canariensis* lacks stomata, suggesting low transpiration rates (Tambussi et al., 2007). Silicification, however, occurs in macro-hairs and typical rectangular epidermal cells (Hodson et al., 1984), a trait common to many grasses. Before panicle emergence, both the macro-hairs and outer epidermal cells have a large vacuole. The presence of Si was not detected at this stage. **Figure 1C** shows a scanning electron micrograph of a *T. aestivum* glume, exhibiting papillae, and macro-hairs, but no stomata.

Macro-hairs are unicellular trichomes, often with lengths greater than 1 mm on *Phalaris canariensis* lemma (Perry et al., 1984a). Macro-hairs also start to silicify after panicle emergence. Silica deposition initiates at the hair tip (Perry et al., 1984b). During the week following emergence, wall thickening proceeds to the base, and the outer layer of the wall is silicified. The nanometric morphology of the deposited silica is governed by the newly laid-down polysaccharides. Sheet-like structures form during the deposition of arabinosylated xylan and cellulose, globular particles deposit simultaneously with mixed linkage β-(1→3, 1→4)-D-glucan, and fibrous silica forms after the cell wall thickening stops. Apparently, the polysaccharides provide chemical environments necessary to stabilize the deposited silica (Perry et al., 1987). By 2 weeks after emergence, silicification of the wall material continues in concentric rings (Hodson et al., 1984; Perry et al., 1984a). The cytoplasmic content of macro-hairs breaks down leaving behind an empty lumen (Hodson et al., 1984).

Deposition of silica at the outer epidermal cells is also templated by their cell wall. At emergence, the inner tangential cell walls thicken to occupy most of the cell volume, leaving only little space for active cytoplasm. In the week following emergence, cytoplasm degrades and silicification initiates in the cell wall region close to the pre-existing cytoplasm. Within 2 weeks, the whole cell wall is impregnated with silica (Hodson et al., 1984). We conclude that in macro-hairs and outer epidermal cells of the

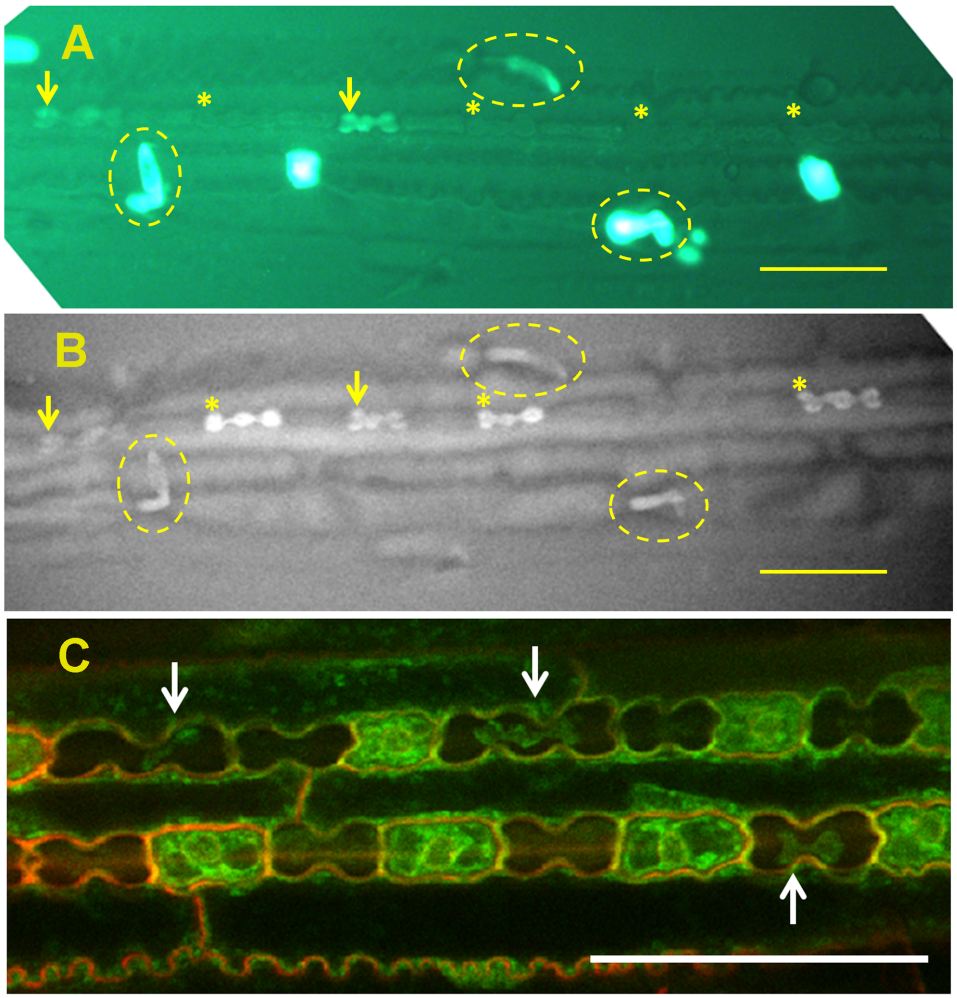

FIGURE 2 | Silica deposition in the epidermis of sorghum leaf. (A) Viability assay of epidermal peel showing viable cells' cytoplasm green. Viable silica cells are indicated with arrows whereas dead silica cells are indicated with asterisks. Micro-hairs are shown with broken ovals. **(B)** Back-scattered electron micrograph of the same field of view, showing high signal intensity emanating from viable silica cells (arrow) and micro-hairs (broken oval). Dead silica cells are already silicified (asterisks), although one dead non-silicified silica cell can also be seen [compare **(A,B)**]. **(C)** Silica cells displaying shrunken but viable cytoplasm (arrows) indicating extra-membranous silica deposition. All scale bars represent 50 μm. Images adapted from Kumar et al., (2017) with permission from the John Wiley and Sons publications. Copyrights of the image rest with the original authors and publisher.

lemma, silicification templated by the cell wall coincides with the onset of spikelet transpiration.

Leaf Silicification

Among all plant organs, leaves usually exhibit highest transpiration volumes. The evaporation of water promotes xylem sap condensation and contributes to the formation of solute sediments, including silica. However, even though most of the water evaporates from mesophyll cells and passes through stomata to the atmosphere, silicification of the guard cells occurs at slow pace, advances with age, and never reaches all of the cells (**Figures 1D,E**), (Motomura et al., 2004). Among other epidermal cell types, long cells accumulate silica in their walls as soon as the leaf starts to transpire (Sakai and Sanford, 1984). Silicification in the mesophyll and bulliform cell walls is rather characteristic to mature, sometimes senescent leaves (Sangster, 1970; Dinsdale et al., 1979). Silicification is probably spontaneous in the cell wall of these cells, resulting from Si auto-polymerization. In case of lumen silicification in bulliform cells, granules of silica are observed (Motomura et al., 2004).

Silicification of Leaf Micro-Hairs

Micro-hairs are bicellular trichomes having a basal and a cap cell. Basal cells of micro-hairs in sugarcane are the first cell type to silicify, even before leaf exposure to the outside environment (Sakai and Sanford, 1984). In sorghum, silicification of the basal cell initiates in viable cells (**Figure 2**), probably at the cell wall. The cells probably die later on, and their lumen passively fills up with silica (Motomura et al., 2000). In bamboo (*Sasa veitchii*), cap cells accumulate significant amounts of silica only after leaf opening. The number of silicified micro-hairs increases

with age (Motomura et al., 2004), suggesting a dependency on transpiration. Silicification in micro-hairs seems thus to share similar mechanism to prickle hairs and papillae in the inflorescence bracts.

Silica Deposition in Silica Cells

In grasses, silica cells are found as stretches of silica-cork cell pairs in the epidermis of leaves (Sangster, 1970), stem internodes (Kaufman et al., 1969) and abaxial epidermis of glumes (Hodson et al., 1985). Silica cells are among the first type of cells to be silicified in a tissue, sometimes even before the tissue is exposed to the atmosphere (Kaufman et al., 1969; Sangster, 1970; Motomura et al., 2006; Kumar et al., 2017). The silica deposition occurs over hours (Blackman, 1969; Kaufman et al., 1969), suggesting that the process is metabolically controlled. In rice, the walls of silica cells lignify before silica deposition (Zhang et al., 2013). Prior to silica deposition, silica cells are metabolically very active with large nucleus and high numbers of ribosomes and mitochondria. Silica cells are well connected to their neighboring cork cells, but not to the neighboring long cells (Lawton, 1980). Silica cells are viable at the time of silica deposition. The mineralization process initiates in the extra-membranous space and proceeds centripetally (**Figure 2**). The forming mineral is limited by the cell wall on one side and by the membrane on the other side. The shrunk cytoplasm maintains its viability and the deposited silica does not interfere with cell-to-cell diffusion (Kumar et al., 2017). Silica deposition occurs also in leaf segments with very limited transpiration flow (Sangster and Parry, 1971; Kumar et al., 2017). The deposition is reduced in leaves treated with a metabolic inhibitor 2,4-dinitrophenol (Sangster and Parry, 1971) and does not occur in dead silica cells present in live leaves (Markovich et al., 2015; Kumar et al., 2017). These findings further indicate on a metabolic process controlling the silicification.

Organic matter with N/C ratio indicative of amino acids is continuously distributed in wheat silica cells (Alexandre et al., 2015), suggesting possible role of proteins in the templating process, similar to diatoms (Kröger et al., 1999). Depending upon the phytolith extraction process, some of nitrogen detected may result from the use of nitric acid in the extraction process or represent acid hydrolyzed proteins occluded within the silica structure (Watling et al., 2011), even without their direct participation in silicification.

Silicification in Roots

Roots are the first organs exposed to silicic acid, allowing its uptake and controlling the extent of Si supply to the entire plant. In most cases, the deposition of silica in roots cannot rely on evaporation of water for concentrating silicic acid. A passive mode of silicification in roots thus assumes that Si condensation occurs as water is absorbed by the symplasm, leaving behind concentrated silicic acid solution in the apoplasm (Exley, 2015). Such separation may occur at the Casparian bands, where the passive diffusion of Si and water is blocked (Sakurai et al., 2015). Indeed, in the aerial parts of adventitious roots of *Phalaris canariensis*, silica deposition occurs in the epidermis and outer cortical layer, but not in the endodermis (Hodson, 1986). This observation suggests the involvement of transpiration in silica deposition in these aerial roots. However, transpiration-dependent model does not conform to silicification at the endodermal inner tangential walls, which are located centripetally to the Casparian bands (Sangster, 1978). Depending on the grass species, silica impregnates the endodermal inner tangential and radial cell walls (Parry and Soni, 1972; Hodson and Sangster, 1989; Lux et al., 2003a), or forms discrete aggregates anchored in the inner tangential wall (Sangster and Parry, 1976a; Parry and Kelso, 1977; Sangster, 1977) (**Figures 1F,G**).

The active uptake of Si that bypasses the Casparian bands occurs at the apical part of roots. Afterward, Si is transferred with the transpiration stream basipetally through the central cylinder (Lux et al., 2003b; Soukup et al., 2017). While most of the Si is transported to the shoot, some Si binds to the root endodermis with high affinity (Markovich et al., 2015). Endodermal silicification is usually associated with the thickening of inner tangential walls and the deposition of polyphenols suberin and lignin in the mature parts of the roots (Parry and Kelso, 1975; Sangster and Parry, 1976a). Since Si is taken up actively in the root apex, the mature endodermis is supplied with Si by its centrifugal flow from the central cylinder. This model was evidenced in sorghum, where the aerial parts of adventitious roots silicified even before reaching the growth medium (Sangster and Parry, 1976b). Accordingly, silica deposition was detected in the basal parts of roots grown with Si supply provided to their apices only (Lux et al., 2003b), even if the cortical tissues between those regions were removed (Soukup et al., 2017).

In sorghum, silica aggregation initiates in non-lignified sites of the inner tangential cell walls, possibly templated by arabinoxylan–ferulic acid complexes. The aggregation sites are established even in the absence of Si, indicating that the formation of silica aggregates is at least partially controlled by the structure and composition of the endodermal cell walls (Soukup et al., 2017). The aggregates seem to swell the silicifying wall and protrude from the endodermal inner tangential wall toward the cell lumen (**Figure 1F**).

In older roots, the deposition of silica can extend also to other root regions, e.g., to the outer tangential walls of endodermis or intercellular spaces of cortex. Such deposition is probably a result of Si condensation due to water uptake by the symplasm, water evaporation, or it can be induced by increasing ionic strength of the apoplasm or pH changes. With the increasing age of the roots, stele, sclerenchyma and conductive tissues may also silicify (Parry and Kelso, 1975; Montgomery and Parry, 1979; Hodson and Sangster, 1989).

CONCLUSION

Transpiration plays a major role in moving Si throughout the plant. Water evaporation and water uptake by the symplasm can efficiently condense Si and lead to silica precipitation. However, uncontrolled and spontaneous silica deposition may be harmful for the functioning of the plant. The evolution of mechanisms for a safe disposal of Si was thus essential. Based on the matrix that templates the silicification and the participation of transpiration in this process, we identified three types of silica deposition in

grasses that describe silicification in most of the cell types. (1) Passive cell wall silicification: This type is distinctive to mature and/or intensely transpiring organs, where the condensation of Si is driven by dehydration. A continuous supply of Si infiltrates the non-silicified cell walls and its deposition occurs without being metabolically controlled by the cells. (2) Controlled cell wall silicification: Silica is deposited directly on the cell wall matrix, even before the organ is exposed to the atmosphere/transpiration. Silicification is possibly templated by the cell wall polymers inducing the silicic acid polymerization. In some cases, the cell protoplast dies, allowing spontaneous silica deposition driven by transpiration in the cell lumen, without further organic template. (3) Silica cells are a special case, where the mineral is deposited on the external side of a functional plasma membrane, possibly in a volume that contains materials that enhance silica deposition, independent of transpiration.

Thus we saw, a plant as a whole does not follow one silicification mechanism but the observed mechanism is specific to the cell-type chosen for study. Sometimes, a cell type follows two stage silicification: the early stage being cell wall silicification followed by granular silica deposition in the dead lumen. Thus, silicification in grasses is not an entirely active or passive process, and its mechanism is cell-type specific.

AUTHOR CONTRIBUTIONS

SK prepared the initial draft. All authors commented, added and revised the manuscript and approved for publication.

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Expression of Genes for Si Uptake, Accumulation, and Correlation of Si with Other Elements in Ionome of Maize Kernel

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The mineral composition of cells, tissues, and organs is decisive for the functioning of the organisms, and is at the same time an indicator for understanding of physiological processes. We measured the composition of the ionome in the different tissues of maize kernels by element microanalysis, with special emphasis on silicon (Si). We therefore also measured the expression levels of the Si transporter genes *ZmLsi1*, *ZmLsi2* and *ZmLsi6*, responsible for Si uptake and accumulation. Two weeks after pollination *ZmLsi1* and *ZmLsi6* genes were expressed, and expression continued until the final developmental stage of the kernels, while *ZmLsi2* was not expressed. These results suggest that exclusively ZmLsi1 and ZmLsi6 are responsible for Si transport in various stages of kernel development. Expression level of *ZmLsi* genes was consistent with Si accumulation within kernel tissues. Silicon was mainly accumulated in pericarp and embryo proper and the lowest Si content was detected in soft endosperm and the scutellum. Correlation linkages between the distribution of Si and some other elements (macroelements Mg, P, S, N, P, and Ca and microelements Cl, Zn, and Fe) were found. The relation of Si with Mg was detected in all kernel tissues. The Si linkage with other elements was rather specific and found only in certain kernel tissues of maize. These relations may have effect on nutrient uptake and accumulation.

Keywords: ionome, maize kernel, PCA analysis, silicon (Si), Si correlation with elements, *ZmLsi* transporter genes

INTRODUCTION

The composition of minerals in a cell, tissue, or the whole organism is referred to as the ionome. The ionome consists of essential and also non-essential elements, thus it represents the inorganic component of plant organism (Salt et al., 2008). Recently, increased attention has been paid to the study of the plant ionome, especially in cereals (Lombi et al., 2009; Baxter et al., 2013, 2014; Baxter, 2015; Bokor et al., 2015; Asaro et al., 2016; Shakoor et al., 2016) or other plant species (White et al., 2012; Parent et al., 2013; Pongrac et al., 2013b). Plant ionome, element profiles, distribution, and

correlation among elements in various tissues may depend on environmental stimuli. For example, element tissue composition can vary greatly upon various abiotic stress conditions such as metal toxicity as it was found out in maize root and shoot (Bokor et al., 2015). In those plants, the root ionome was much more affected than the shoot ionome due to Zn exposure, and, surprisingly, also by Si application, however with lower intensity. Other metals can considerably cause alterations of the element profile in plants, as it was, for example, revealed in sensitive population of *Silene paradoxa* under Cu exposure (Pignattelli et al., 2013). Similarly, the altered distribution of elements in response to the influence of non-essential toxic element Cd in two isolates of *Salix caprea* was reported by Vaculík et al. (2012). Phylogenetic aspect is also important, because shoot ionome varies at ordinal level in angiosperms (Broadley et al., 2004). Understanding of relationships between elements is crucial to unravel the regulation of ionome. These relationships can exist regardless of the tissue, species, or environment; however, many associations will vary with a combination of these factors (Baxter, 2009). For the experiments with element tissue distribution, seeds may represent an ideal object, because the ionome of mature seeds represents a developmental end-point, showing plant life history with a combination of genetic program and interactions of environment (Baxter et al., 2014). Analysis of buckwheat seeds showed different mineral concentration and accumulation in various seed tissues, including pericarp, endosperm, cotyledons and testa with aleurone layer. Most of the measured elements were preferentially localized in cotyledons of the seed, although Ca was predominant in pericarp tissue. In cotyledons, the co-localisation of Mg and P indicated a possible binding of Mg to phytic acid (Pongrac et al., 2013b). In cereals, higher mineral accumulation is characteristic for embryo and external tissues of the grain, whereas the starchy endosperm is less enriched by minerals (Mikuš et al., 1992, 1995; Lombi et al., 2011). Different distribution of various micro- and macronutrients varied remarkably also in different tissues of rice grain, including husk, bran, endosperm, and embryo (Lombi et al., 2009). Taken together, mineral elements are not concentrated uniformly along the seed or grain, not even in a cob carrying variable amount of kernels in maize. Different accumulation of Na, S, Ca, Fe, Cu, Zn, and Sr resulted in unequal concentration of these elements in the base, middle and the tip of the corn cob in maize (Baxter et al., 2014). Concentration and accumulation of elements is the result of many physiological processes, including root uptake, remobilisation and translocation within the plant, and subsequently deposition and storage in the seed (Shakoor et al., 2016).

Silicon, as a beneficial element for plants, has been studied frequently, mainly in experiments dealing with its relation to abiotic and biotic stress. To this date, an essential function of Si has not been proven in higher plants, however, the list of beneficial effects is still increasing (for details see reviews: Liang et al., 2007, 2015; Meharg and Meharg, 2015; Líška et al., 2017). As an example, the importance of Si in increased crop yield became evident; Detmann et al. (2012) showed that higher crop yield of rice resulted from increased weight of grains after Si application. Silicon accumulation ranges from 0.1 to 10% of dry weight in plants and in many species, especially in monocots, Si uptake is an active process (Epstein, 1994; Ma et al., 2006; Currie and Perry, 2007). In maize, *ZmLsi1*, *ZmLsi2,* and *ZmLsi6* genes are responsible for direct Si transport within the plants (Mitani et al., 2009a,b). The information revealing Si concentration in various harvested portions of crops, mainly in grains, is limited. Some known data show averaged Si concentration of 2.6 g kg^{-1} in corn kernel and 23 g kg^{-1} in rice panicles, including grain and the husk (Tubana et al., 2016). In southern India, the critical limit of 12 g kg^{-1} Si was established for rice grain; below this concentration there is the likelihood of a crop significantly responding to Si fertilization (Narayanaswamy and Prakash, 2009). Ma et al. (2003) observed a large variation in grain Si concentration of barley grain ranging from 0 to 3.8 g Si kg^{-1} of dry weight; silicon was mostly localized in the hull (more than 80% of total Si). In their study, various barley varieties grown in the same field were tested, and authors suggested that variation in grain Si concentration is controlled genetically. Genotypic variation in Si concentration of grain may be associated with variation in the ability of root to take up Si, in differences in translocation and accumulation of Si, and in other factors (Ma et al., 2003).

In human physiology, silicon is an essential element, mainly important in bone formation and crucial in connective tissues. However, its bioavailability from solid foods is limited. Especially, Si bioavailability from phytolits in plant-base foods is very low (Jugdaohsingh et al., 2002 and references therein). Silicon deprivation affects collagen at different stages in bone development, collagen-forming enzymes, or collagen deposition in other tissues would have implications that Si is important for both wound healing and bone formation (Seaborn and Nielsen, 2002).

In the present study, we investigated molecular mechanisms of Si transport in maize kernel. We focused on two principal questions: which *ZmLsi* genes are expressed and where is Si accumulated in the kernel. Another aspect of this work concerns the possible correlation of micro- and macroelement with Si in various kernel tissues, including pericarp, soft endosperm, scutellum, and embryo proper. The possible role of a specific *ZmLsi* expression and possible physiological explanation for the existence of multilateral correlation patterns among Si and other elements in maize kernel tissues are discussed.

MATERIALS AND METHODS

Plant Material and Cultivation

Maize (*Zea mays*, hybrid Novania) kernels were obtained from plants cultivated in soil under natural conditions. The plants were grown in sandy-loam soil in the yard of the Department of Plant Physiology, Comenius University in Bratislava and watered regularly from April till October 2015. No insecticides or additional foliar spraying neither addition of nutrients in the form of fertilizers were applied. The concentration of bioavailable Si from the soil (113 $\pm$ 15 mg kg^{-1}) was analyzed according to Rodrigues et al. (2003) with modification. After extraction by 0.5 M acetic acid, Si was measured by

ICP-MS in place of colorimetric determination using blue silicomolybdous acid procedure as used in the original procedure. The concentration of Si in other plant organs was determined by AAS (Supplementary Table S1) as described previously by Bokor et al. (2014).

Male and female flowers were protected in a bag to prevent uncontrolled pollination. During flowering, pollination was performed manually. During maize kernel development, we selected three developmental stages of kernels for analyses: 2 weeks after pollination (the 1st developmental stage), 1 month after pollination (the 2nd developmental stage), and mature dry kernel (the 3rd developmental stage).

Real-Time PCR Analysis

RNA of the kernel from the 1st developmental stage was extracted from pericarp and the rest of the kernel, because it was possible to extirpate the embryo at this stage. At the 2nd developmental stage, RNA was extracted from pericarp, embryo, and the whole kernel. At the 3rd developmental stage, RNA was extracted from the pericarp and embryo. The Real-time PCR analysis was performed according to our previous work (Bokor et al., 2015).

Briefly, total RNA was extracted and DNase I treated using Spectrum Plant Total RNA kit (Sigma–Aldrich). In addition, RNA extraction of the kernel in the 3rd developmental stage was done after 24 h imbibition in sterile conditions. Synthesis of cDNA was performed by ImProm-II Reverse Transcription System (Promega) using Oligo(dT)15 primers and after that, cDNA was purified by DNA Clean & Concentrator^{TM}-5 (ZymoReseach). *ZmLsi1*, *ZmLsi2*, *ZmLsi6,* and *beta actin* genes were amplified by Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific) using Light Cycler II 480 (Roche). The relative changes in gene expression were estimated according to Pfaffl method including amplification efficiency of selected genes (Pfaffl, 2001). After the collection, the samples were stored in −80°C before analysis. All samples for PCR experiments were analyzed in three biological and technical replicates.

Scanning Electron Microscopy (SEM) Coupled with X-ray Microanalysis

Longitudinally sectioned and air-dried mature maize kernels (the 3rd developmental stage) were coated with carbon and fixed on aluminum stubs covered with a carbon sticker. Surface conductivity was increased by carbon coating resulting in a uniform thickness of the carbon layer of approximately 60 nm. Kernel tissues (**Figure 1**) and the distribution of 11 elements (N, Zn, Ca, K, P, S, Si, Mg, Cl, Cu, Fe) were observed and analyzed with the Jeol JSM-IT300 scanning electron microscope (SEM) equipped with an energy dispersive X-ray (EDX) analyser.

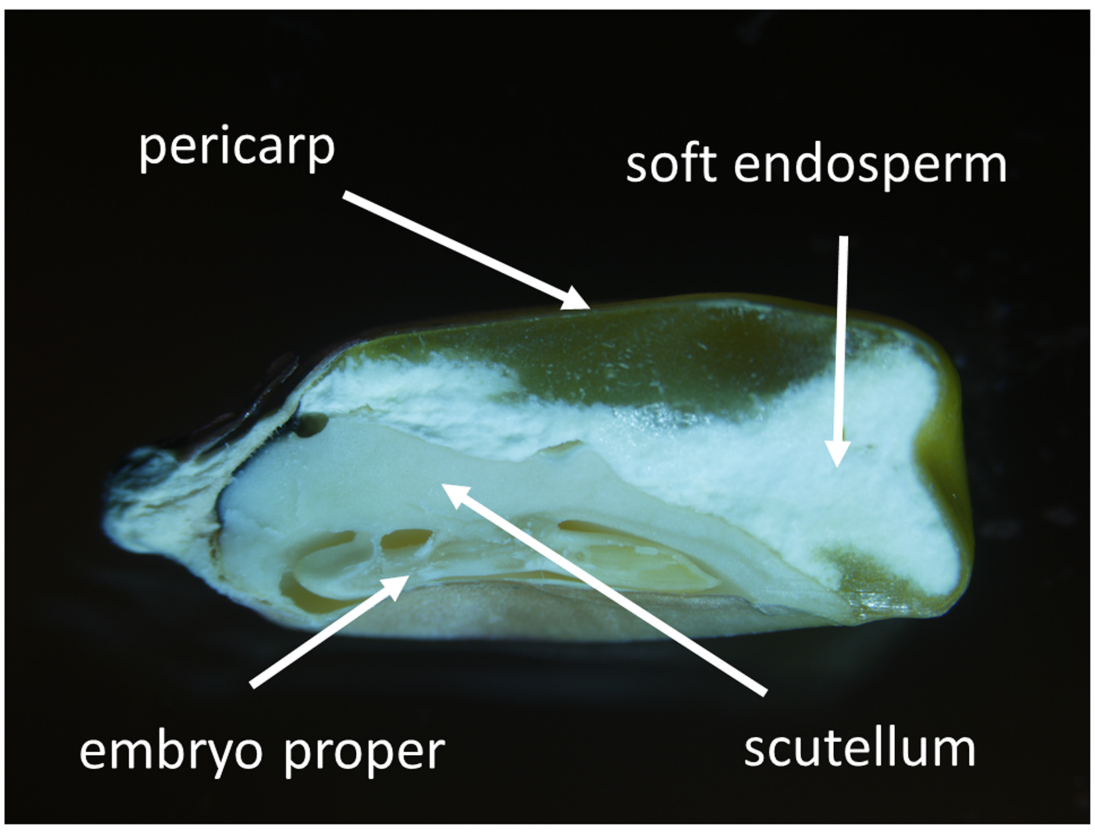

FIGURE 1 | Longitudinal section of mature maize kernel (the 3rd developmental stage). Kernel tissues including pericarp, soft endosperm, scutellum, and embryo proper are indicated.

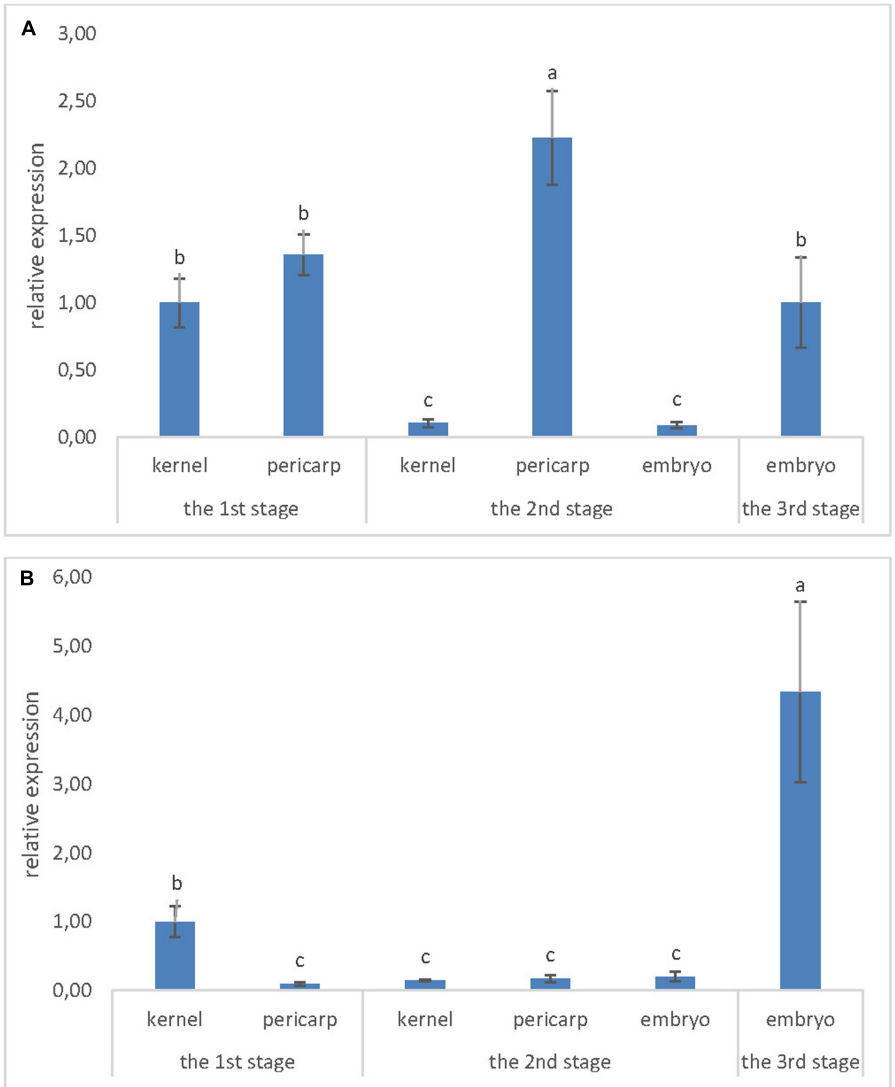

FIGURE 2 | Expression level of **(A)** *ZmLsi1* and **(B)** *ZmLsi6* genes determined by real-time PCR in whole kernel or kernel tissues pericarp and embryo. The x-axis represents various developmental stages of maize kernel and related kernel tissues. Values are means ± SE. Different letters indicate significant differences at 0.05 level.

Initial mapping showed relatively low differences in element distribution within kernel tissues (**Supplementary Figure S1**), therefore for relative quantification of element distribution all samples were observed at 1000x magnification and 20 kV accelerating voltage, working distance was 11.0 mm. Data were collected for 50 live seconds. As uneven surfaces may heavily distort EDX measurements (Goldstein, 2003), only flat parts of the samples were selected for analysis. For EDX measurements, three biological and four technical replicates were performed.

In total, four different mature maize kernels were investigated by measuring four different spots on each of four maize kernel areas (pericarp, soft endosperm, embryo proper, and scutellum). This indicates that data for statistical processing were obtained from 16 individual measurements of each maize kernel zone. Raw data were processed with the TEAM Enhanced, Version 4.3 (EDAX-Ametek, United States) software and all the values are expressed as weight % of total analyzed elements.

Statistical Analysis

Statgraphics Centurion (version 15.2.05) software was used for statistical evaluation. The statistical significance of the means was considered at 0.05 probability level. Analysis of variance (ANOVA) and least significant difference (LSD) were performed on all experimental data sets. Additionally, standard principal component analysis (PCA) and multivariate regression analysis were applied to the study of ionome using Microsoft Excel 2013 and R version 3.0.2 software.

The data from EDX measurements were used for the study of ionome. Ionome analysis was first performed by PCA. The original data matrix consisted of 11 element variables (Ca, Cl,

Cu, Fe, K, Mg, N, P, S, Si, and Zn) measured in 63 observations differentiated among four different tissue categories (embryo proper 16, soft endosperm 16, scutellum 15, pericarp 16). Principal components analysis was performed by singular value decomposition of the centered and scaled data matrix using the function "prcomp" in R version 3.0.2. Variable loadings revealed the correlation structure between original variables and extracted components. Multivariate regression analysis was performed by ordinary least squares estimation procedure "lm" in R version 3.0.2. Structural category embryo proper and Si element were assigned as references, to which all remaining categories and elements, as well as their two-way interaction terms were compared. The resulting model was statistically significant (F-statistic 420.9 on 43 and 649 degrees of freedom, p-value 0.00) with adjusted R-squared at the level 0.96.

Additional insights to standard statistical procedures was found in the analysis of network representation of ionome with focus on Si. Pair-wise conditional probabilities were constructed from mutual above-average element contents detected in corresponding tissue category. This approach is inspired by the network of relatedness between products of national economies, so called "product space" (Hidalgo et al., 2007), identifying conceptual analogy between international trade linking geographical territories with biological transport of elements between different tissues. Each observation was assigned above- or below-average element content, based on relative share from total sums of all element variables. Conditional probabilities corresponded to the empirical frequencies of mutual source and target elements above-average divided by the frequency of source element above-average. An oriented weighted network is created in each tissue. This method enables insights in a complexity of relationships between elements, a formation of tissue-specific clusters of elements, a hierarchy arising between the central and the peripheral roles in maize kernel ionome. For purpose of visualization we generalized full network in the fragment above threshold probability of 0.50 to focus our attention on Si and its relation to other elements in different maize kernel tissues.

RESULTS

Gene Expression Level

Expression of *ZmLsi* genes was detected in maize kernel at the 1st developmental stage 2 weeks after pollination (**Figure 2**). In this developmental stage, *ZmLsi1* and *ZmLsi6* genes were expressed; but the *ZmLsi2* gene was not. The same pattern of gene expression was observed also in older kernels. In the 2nd stage, only *ZmLsi1* and *ZmLsi6* were expressed in pericarp, embryo and also in the whole kernel containing endosperm tissue (**Figure 2**). In the 3rd developmental stage, pericarp tissue was senescent, thus total RNA was not possible to extract. Expression of *ZmLsi1* and *ZmLsi6* genes was found in the embryo; however, also at this stage *ZmLsi2* was not expressed. Analysis of *ZmLsi* genes corresponds with Si accumulation in adult kernel (the 3rd developmental stage). EDX analysis showed the highest Si accumulation in embryo proper and pericarp. However, very low Si content was

TABLE 1 | Macro- and microelement content expressed as weight % of total analyzed elements in mature maize kernel (the 3rd developmental stage).

	Mg	Si	P	S	Cl	K	Ca	Fe	Cu	Zn	N
Embryo proper	0,312 ± 0,022 a	0,078 ± 0,011 a	0,489 ± 0,087 a	0,148 ± 0,019 b	0,117 ± 0,013 a	0,382 ± 0,064 b	0,071 ± 0,009 b	0,081 ± 0,009 b	0,056 ± 0,009 c	0,044 ± 0,009 b	7,54 ± 0,3 a
Scutellum	0,059 ± 0,019 c	0,033 ± 0,012 b	0,092 ± 0,023 b	0,083 ± 0,015 c	0,131 ± 0,022 a	0,822 ± 0,098 a	0,191 ± 0,057 a	0,157 ± 0,032 a	0,152 ± 0,025 a	0,135 ± 0,024 a	4,20 ± 0,26 c
Soft endosperm	0,006 ± 0,003 d	0,030 ± 0,006 b	0,059 ± 0,009 b	0,202 ± 0,017 a	0,101 ± 0,016 a	0,150 ± 0,014 c	0,079 ± 0,01 b	0,094 ± 0,012 b	0,121 ± 0,017 ab	0,109 ± 0,020 a	5,65 ± 0,16 b
Pericarp	0,141 ± 0,019 b	0,103 ± 0,012 a	0,149 ± 0,013 b	0,230 ± 0,02 a	0,120 ± 0,016 a	0,225 ± 0,061 bc	0,066 ± 0,005 b	0,069 ± 0,006 b	0,077 ± 0,01 bc	0,061 ± 0,006 b	5,78 ± 0,29 b

Values are means ± standard error. Different letters in column indicate significant differences between the treatments at 0.05 level.

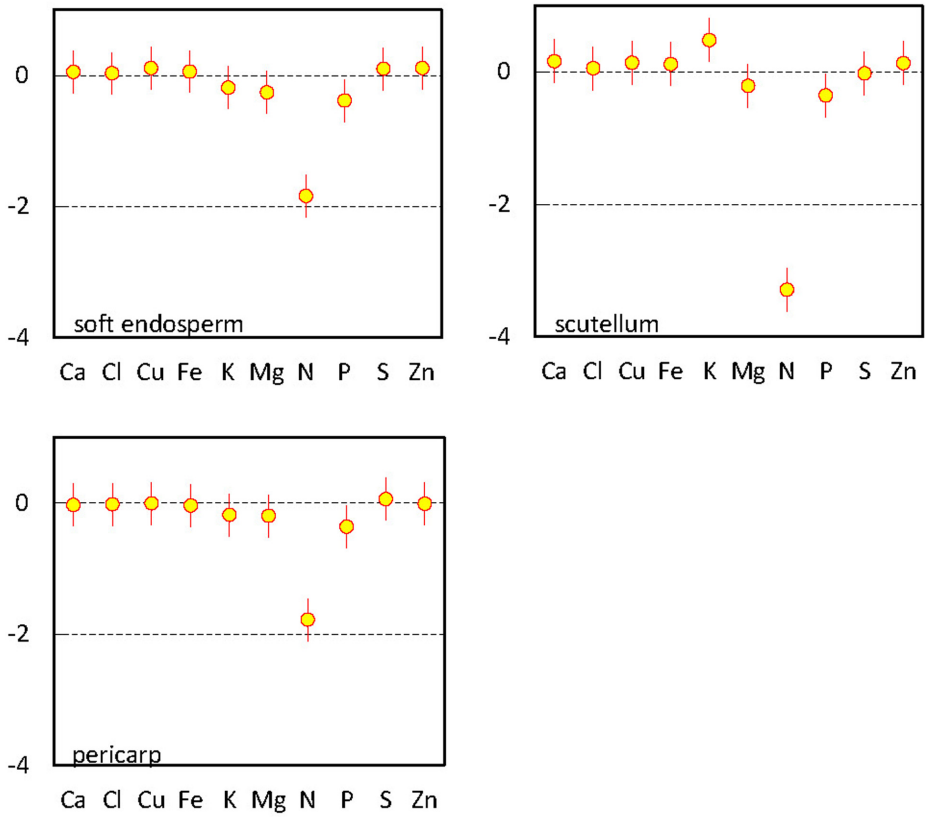

FIGURE 3 | Multivariate regression analysis of elements in mature maize kernel (the 3rd developmental stage). Parameter estimates for interaction terms between tissue variables for soft endosperm, scutellum, and pericarp and remaining elements compared with reference variable (Si) in embryo proper, including 95% confidence interval bars.

also detected in soft endosperm and scutellum of the embryo (**Table 1**).

Ionome Analysis

Energy dispersive X-ray measurements showed significantly higher concentration of the macroelements Mg, P, and N in the embryo proper in comparison to all other kernel tissues (**Table 1**); while the microelements Zn and Cu showed the lowest content found in the embryo proper. In the scutellum, microelements Zn, Cu, Fe and macroelements Ca and K were significantly higher when compared to the other kernel tissues. Additionally, Ca did not significantly differ between embryo proper, soft endosperm, and pericarp. Soft endosperm tissue was characteristic for very low Mg, P, K, and N content, although some elements (S, Zn, and Cu) were higher, when compared mainly with the embryo proper. In the pericarp, the concentration of microelements Fe, Cu, and Zn did not differ from values found in embryo proper. Pericarp and soft endosperm contained the highest amount of kernel sulfur. Nitrogen accumulation reached similar values between scutellum and pericarp. Preferentially, N was accumulated in the embryo, while the lowest content was found in soft endosperm.

Multivariate regression analysis revealed characteristic ionome changes in soft endosperm, scutellum, and pericarp tissues using comparison with Si and embryo proper as a reference category (**Figure 3**). Only three elements have significantly different content than Si detected in embryo proper, namely macronutrients K, P, and N. Considering differences between embryo proper and other tissues, content of N and P was significantly below the content of Si in embryo proper in all three remaining categories. Potassium is significantly elevated only in scutellum tissue in comparison to embryo proper (**Figure 3**). Remaining elements in the scutellum, soft endosperm and pericarp showed statistically indistinguishable contents in comparison to reference element in embryo proper in the presence of N; its content varies at a substantially higher order of magnitude and shadows variation among other elements.

In order to introduce additional aspect to ionome analysis, we used standard PCA, capable to determine relationships between specific tissues (pericarp, soft endosperm, scutellum, and embryo proper) and varying element composition in maize kernel (**Figure 4**). The values of mineral content were set as source variables and used for PCA. This method revealed three significant principal components (PC); PC1, PC2, and PC3 which explain 69.6% of total variance in maize kernel ionome. The PC1 (*SD*: 2.13) accounts for 41.2%, PC2 (*SD*: 1.33) accounts for 16.1% and PC3 (*SD*: 1.16) accounts for 12.3% of total variance. Projection of eigenvectors in plane defined by PCs reveals complex correlation structure in ionome. Acute angle

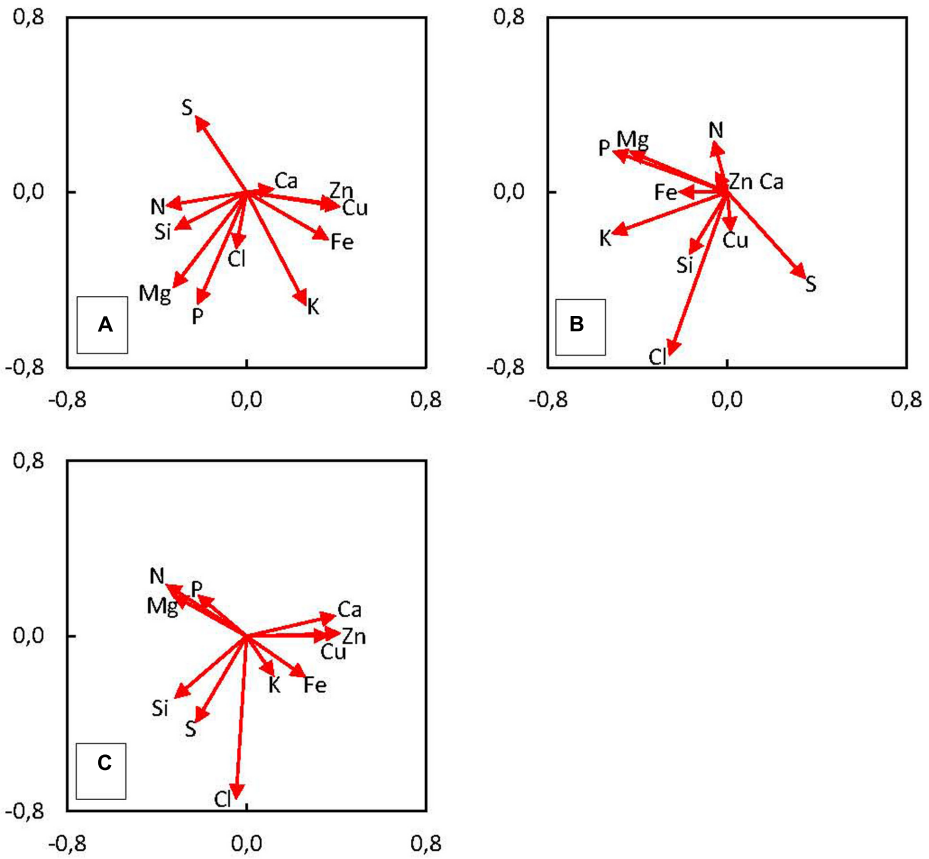

FIGURE 4 | Principal components analysis (PCA) shows the correlation of measured variables (content of elements in mature kernel tissues – the 3rd developmental stage) to principal components **(A)** PC1 and PC2, **(B)** PC2 and PC3, **(C)** PC1 and PC3. The length of arrows (eigenvectors) represents the strength of correlation with the PCs. The angle between eigenvectors represents the correlation among elements.

between eigenvectors of certain elements represents a positive correlation, obtuse angle shows negative correlation and 90° angle refers to mutually uncorrelated elements. Thus, clustering of certain elements displays elemental relationships effectively.

We have found positive correlation of Cu, Zn, Fe, and K and negative correlation of N, Si, Mg, S, and P with the PC1 (**Figure 4A**). The PC2 is positively correlated only with S, negatively correlated with K, P, Mg, Cl, and Fe (**Figure 4B**). Finally, PC3 is positively correlated with N, Mg, and P and negatively correlated with Cl, S, and Si (**Figure 4C**). In the case of Si clustering with other elements, correlation was found between Si and N, Mg, and P (**Figure 4A**), the other correlation represents Si and Cl together with Cu (**Figure 4B**) and the last cluster consists of Si and S and Cl (**Figure 4C**). Projection of observations in plane defined by PCs suggests the existence of underlying regularity, which is clearly tissue related, as location of four categories mostly remains well-defined in these plots, especially in **Figure 5A**, which is a plane defined by two most significant PCs. Hence, this demonstrates different accumulation of elements in various kernel tissues. Higher scores of PC1 correspond with observations categorized in soft endosperm and scutellum, lower scores of PC1 correspond with observations categorized in embryo proper and pericarp (**Figure 5A**). Concerning PC2 as a dimension orthogonal with the former one, higher scores of PC2 correspond with observations categorized in soft endosperm and pericarp, lower scores of PC2 correspond with observations categorized in embryo proper and scutellum (**Figure 5B**). Observations are clustered in four quadrants of the scatterplot PC1 vs. PC2. Third dimension is not offering any similar clustering of observations, therefore the interpretation seems to be less tissue related and more hidden in complex multilateral relationships linking together groups of elements by specific functional routes (**Figure 5C**).

Generalized network representation of ionome in kernel tissues is a novel analytical tool, proposed in order to reveal the structure of multilateral relationships within ionome equilibrium, which can be visualized similar to correlation wheels between pairs of elements. However, unlike standard correlation, pair-wise probability of mutual above average content is not necessarily reciprocal, nor even bidirectional (notice different size of arrow on a link between two elements). Thus, this method reveals varying probability patterns between different tissues corresponding with different functional relationships among elements. For example, in soft endosperm, the most evident

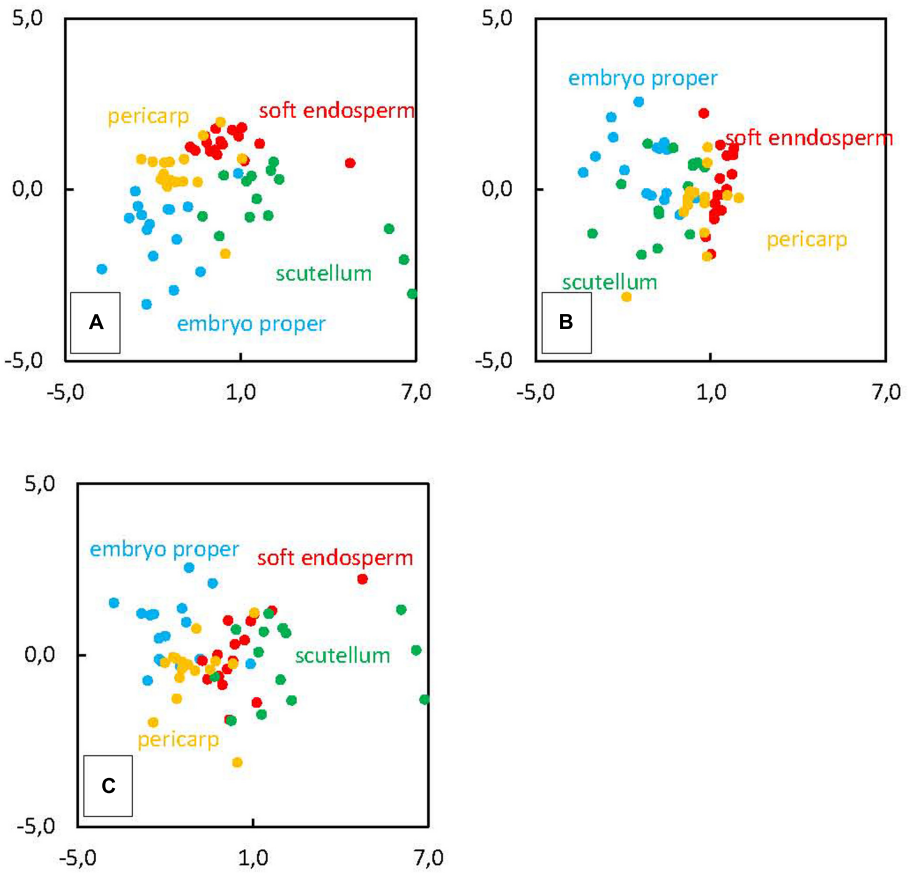

FIGURE 5 | Principal components analysis of element distribution in mature kernel tissues (the 3rd developmental stage) pericarp, soft endosperm, scutellum and embryo proper. Original observations are projected into PCs score of **(A)** PC1 and PC2, **(B)** PC2 and PC3, **(C)** PC1 and PC3.

reciprocal probability is detected between Si and Cl. It means, that if Si has above average content, there is relative high certainty that also Cl has above average content (**Figure 6**), and vice versa. However, the other connected elements, Si and S are detected with above-average content with less certainty in soft endosperm, because the correlation is weak (**Figure 6**). In scutellum, main correlations consist of Si and Mg, P, and S (**Figure 6**). In general, tissue of embryo proper contains weaker network of Si-elements relationships in comparison to scutellum, soft endosperm, and pericarp. Here, the most evident relation represents Si and N, the rest of Si relations are much weaker. In pericarp, Si is strongly correlated with P and Mg (**Figure 6**). The other correlation in pericarp represents weak Si correlation with Cl, Ca, Zn, and Cu.

The relation between Si and Mg is found in every kernel tissue, however this correlation is unidirectional only in soft endosperm (**Figure 6**). Interestingly, Si and P interaction is found only in soft endosperm, scutellum and pericarp tissue and is missing in embryo proper, where it is below threshold. In general, the most abundant relation exists between Si and Mg, P, and Cl. These findings are also supported by PCA, where these relations are clustered with Si, although Si and Cu correlation found in PCA is showed by network representation only in pericarp (weak relation, graphically not visible). Considering all kernel tissues, Si was in relation with macroelements consisting of N, P, S, Ca, and Mg and microelements including Fe, Zn, and Cl.

DISCUSSION

The novelty of this work represents the evidence of *ZmLsi* genes expression in developing maize kernel. It is surprising, that *ZmLsi1* and *ZmLsi6* expression occurred in maize kernel (especially embryo and pericarp), while the *ZmLsi2* gene was not expressed during kernel development at all. It is known that Lsi1 and Lsi6 transporters (NIP subfamily of aquaporins) are not energetically dependent in comparison to different property of Lsi2 transporter that needs energy to maintain its transport function (Ma et al., 2007; Zangi and Filella, 2012). The relation of Lsi1 and Lsi6 transporters to aquaporins may explain the importance of water transport during the life cycle of kernel: during early stages of its development and growth, water is very important to the point, when kernel starts dehydration process and dormancy. After imbibition of mature kernel, again, water uptake is essential for germination. Thus, the expression of aquaporins may be essential in these processes and this may be the reason for increased *ZmLsi1* and *ZmLsi6* expression. Since

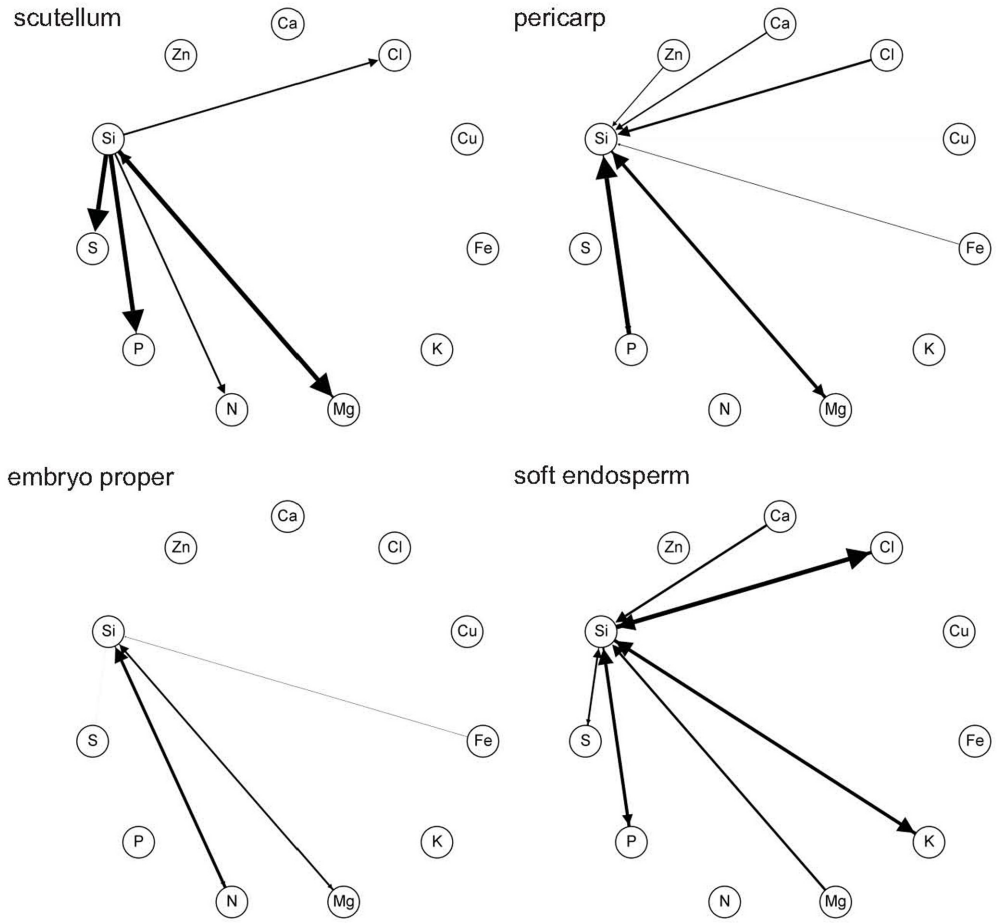

FIGURE 6 | Network representation using pair-wise probabilities of mutual above-average element content in tissues: pericarp, soft endosperm, scutellum, and embryo proper of mature maize kernel (the 3rd developmental stage). This figure shows all probabilities above threshold 0.50.

these transporters are capable of Si transport, Si is accumulated in kernel tissues, mainly in embryo and pericarp. As embryo is the most important part of the maize kernel and will later develop into the new organism, the expression of both *Lsi* genes in this specific region also suggests the importance of Si for optimal growth and development of maize already from early stage of plant development, i.e., embryogenesis. Additionally, increased Si accumulation in pericarp may also indicate protective function against various mechanical and biotic stresses.

Statistical analyses including PCA and network representation of elements uncovered specific correlations and/or relations of Si with other micro- and macroelements in maize kernel. Relationship of Si with Mg was found in all kernel tissues. This interaction is based on promoted uptake of Mg and also Ca by Si presence (Islam and Saha, 1969). Overally, Si related with N, P, S, Ca, Mg, K and also Cl, Fe, and Zn in the kernel. Very high positive correlations were found between Si and Mg, K, and S in reproductive organs of crops (Monti et al., 2008). Synergic effect of Si between N, P, and Ca is responsible for increased concentration of these macroelements in plants, after Si treatment (Mali and Aery, 2008a). In wheat, lower addition of Si increased concentration of macroelements K and Ca related with improved growth under stress conditions, mainly drought stress (Mali and Aery, 2008b). However, in rice plants, Si caused decrease in uptake of N, K, and Fe (Islam and Saha, 1969). Anyway, these opposing data are consistent with our results, because, e.g., in scutellum, if Si is increased, there is high probability that also N is increased (thus, Si promotes N uptake, because of arrow direction from Si to N). However, in embryo proper, if Si is increased it means that N is increased with lower probability than in reciprocal relation (if N is increased there is a high probability that Si is increased; thicker arrow direction from N to Si). The cations Ca, Mg, and K share similar chemical properties that is also responsible for interaction between them (Fageria, 2001), thus this may be the reason why Si is related with these elements. In case of microelements, Si application increases Zn and Fe content in seeds of rice (Mehrabanjoubani et al., 2015), although did not influence the concentration and tissue distribution of Zn in cucumber (Bityutskii et al., 2014). However, in maize roots and shoots, Si caused decrease in Zn concentration indicating antagonistic effect of Si (Bokor et al., 2014). Negative correlation of Cl and Si was found in crops by Monti et al. (2008).

Thus, our findings support the idea, that not only ionome, but also relation of Si with other nutrients is specific, and, that Si has impact on nutrient uptake and accumulation within kernel tissues. The predominant computed synergic effect (based on pair-wise probability of mutual above average content) of Si with other nutrients may indicate its positive role in kernel tissues to promote nutrient accumulation important in seed metabolism and physiology.

In maize kernel tissues, measurements of micro- and macroelement accumulation showed interesting results. Correlation between elements in grain or seed may point to common molecular mechanisms of uptake and metabolism of these elements, or it may represent common adaptation to environment (Vreugdenhil et al., 2004). The elements Mg and P, clustered in PCA were mostly accumulated in the embryo proper. Co-localisation of Mg and P in cotyledons of the embryo was supported also by a high correlation coefficient of these elements in the pseudocereal buckwheat (Pongrac et al., 2013b). Since phytic acid is the main storage molecule of P and strongly binds Mg (and also other elements), this clearly suggests, that this correlation represents an association of Mg – P within phytic acid (Mikuš et al., 1992, 1995; Veiga et al., 2006; Persson et al., 2009; Kumar et al., 2010; Pongrac et al., 2013b). A cluster of Zn, Cu, and Fe is also detected by PCA and network of elements (however, not in soft endosperm), which proposes a shared mechanism of accumulation. Such clustering was recently found in sorghum grain and also in wheat, where the similarity between the distribution of Cu and Zn was shown (Lombi et al., 2011; Shakoor et al., 2016).

Sulfur is an element specifically included in storage proteins that may be located in cotyledons as well as in testa and aleurone of buckwheat (Pongrac et al., 2013b). Thus, N and S assimilation is coordinated (Zhao et al., 1997). We found also correlation in PCA, however, not so strong. In wheat, S can be strongly partitioned in the subaleurone tissue (Pongrac et al., 2013a). In rice grains, S occurred together with Zn and was accumulated not only in the aleurone/pericarp tissues but also in the endosperm (Lombi et al., 2009). This is similar to our results, where both Zn and S content was increased in the soft endosperm tissue in comparison to the embryo proper. Thus, complexes of Zn with –SH groups of phytochelatines and metallotioneins are indicated, although this role in cereal grains is still not fully understood (Lombi et al., 2009). In our study, we did not observe cluster Zn – S in PCA analysis, thus more investigation is needed.

Nitrogen is localized preferentially in the embryo proper of the kernel. Increased content of N may be linked to increased protein synthesis. Scutellum and pericarp contain less N than embryo proper, and soft endosperm shows the lowest content of N. Nitrogen supplementation is key component in protein synthesis and its supplementation increases total protein content in grain (Triboi et al., 2000). Positive correlation of N and Si was also supported by PCA. Silicon improves N use efficiency and enhances vegetative and generative biomass production in wheat plants grown under various Si treatments. However, grain yield nitrogen use efficiency was positively affected only by higher doses of Si (Neu et al., 2017). The highest concentration of proteins, which are metabolically active is found in embryo and less in aleurone layer. In endosperm, protein concentration is generally low and most of them represent storage proteins (Lasztity, 1995).

Mineral elements Zn, Cu, Fe, Ca, and K were higher in the scutellum in comparison to other tissues, however, Zn and Cu did not differ significantly from soft endosperm. In buckwheat, Zn, Cu, Fe, and K were preferentially localized in cotyledons of the embryo and Ca was abundant in the pericarp (Pongrac et al., 2013b). In rice grain, Zn and Cu content increased in the embryo and Fe was mainly localized in external parts of the grain (Lombi et al., 2011). The maize pericarp did not show increased micro- and macroelement accumulation in comparison to the rest of kernel tissues, except of S and Si.

The PCA analyses showed, that the elemental composition is specific for the different tissues of the kernel; it further demonstrated a regularity of mineral content in each kernel tissue. Therefore, it could be concluded that each tissue is represented by its own ionome. In a case of Si, we found several correlations of Si and other elements (the macroelements Mg, N, P, Ca, K, and S and the microelements Cl, Fe and Zn in network representation and PCA) that are also tissue specific, except of Si and Mg relation present in all tissues of maize kernel. Considering the fact that many beneficial roles of Si so far are not understood, research about its correlation with other elements in the various tissues becomes especially important. However, additional investigation is necessary to find out the background and role of this correlation in the maize kernel ionome.

CONCLUSION

We found that high accumulation of Si was detected in the embryo proper and the pericarp what is supported by expression of *ZmLsi* genes in these tissues. During the developmental stages of kernel, only *ZmLsi1* and *ZmLsi6* genes were expressed, but*ZmLsi2* gene not at all. In general, the analysis of the kernel ionome showed that element correlations may be tissue specific and that each investigated tissue (pericarp, soft endosperm, embryo proper, and scutellum) is characteristic by its own ionome with more likely different level of element distribution regulation. The analyses of Si linkage to other elements showed mainly synergic effect (based on probability of above-average content of Si and other element in network representation model) on nutrient accumulation in the kernel tissues. Silicon, as important component of the maize ionome, mainly related with Mg non-specifically in all tissues. The other macroelements P, S, N, K, and Ca related with Si in at least two kernel tissues showing higher degree of tissue specificity. Similarly, Si linkage to microelements Cl, Fe, and Zn was rather tissue specific. These results show that nutrient accumulation in maize kernel may be affected by silicon.

AUTHOR CONTRIBUTIONS

BB designed the research, performed real-time PCR and wrote the article; SO performed statistical analysis including

PCA, multivariate regression and network representation; MV performed EDX analyses and contributed to the design of research and figure preparation; SB considerably contributed to the real-time PCR experiments and analysis; MW and IL enabled EDX analyses; JT and AL supervised the project; and all authors discussed the results and commented on the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01063/full#supplementary-material

FIGURE S1 | Informative map of element distribution within kernel tissues investigated in this study using SEM coupled with EDX-microelement analysis. Red frame denote the zone of maize kernel that was analyzed and distribution of each investigated element (11 in total) is marked by different color. Bar = 500 μm.

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