

Silicon enhances growth independent of silica deposition in a low-silica rice mutant, *lsi1*

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Abstract To examine whether silica bodies are essential for silicon-enhanced growth of rice seedlings, we investigated the response of rice, *Oryza sativa* L., to silicon treatment. Silicic acid treatment markedly enhanced the SPAD (soil plant analytical development) values of leaf blades and the growth and development of leaves and lateral roots in cvs. Hinohikari and Oochikara, and a low-silicon mutant, *lsi1*. Combination of ethanol–benzene displacement and staining with crystal violet lactone enabled more detailed histochemical analysis to visualize silica bodies in the epidermis under bright-field microscopy. Supply of silicon induced the development of motor cells and silica bodies in epidermal cells in Hinohikari and Oochikara but not or marginal in *lsi1*. X-ray analytical microscopy detected silicon specifically in the leaf sheath, the outermost part of the stem, and the leaf blade midrib, suggesting that silicon is distributed

to tissues involved in maintaining rigidity of the plant to prevent lodging, rather than being passively deposited in growing tissues. Silicon supplied at high dose accumulated in all rice seedlings and enhanced growth and SPAD values with or without silica body formation. Silicon accumulated in the cell wall may play an important physiological role different from that played by the silica deposited in the motor cell and silica bodies.

Keywords Cultivar · EDX · Electron microscopy · Histochemical analysis · *lsi1* · Motor cell · *Oryza sativa* · Rice · Silica body · X-ray analytical microscope

Abbreviations

eV Electron voltage
Si Silicic acid
SPAD Soil and plant analysis development

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Introduction

Silicon (Si) is the second most abundant element after oxygen in the Earth's crust. Silicon exists ubiquitously in soil, sharing 50% to 70% of soil dry weight as SiO_2 form. Various plant species take up Si in the soil via their roots and accumulate Si in specific tissues. Among plant species, rice (*Oryza sativa* L.) contains silicic acid at about 10% of above-ground dry weight (Epstein 1999; Ma and Takahashi 2002). Rice accumulates Si as silica opal and silica bodies in the shoots, and the roots of rice absorb several times more Si than common nutritional minerals such as N, P, and K (Okuda and Takahashi 1962; Takahashi et al. 1990). Because Si is considered to have a marginal effect on the photosynthetic rate or photosynthesis apparatus by itself, it has long been questioned how Si is involved in the metabolism and growth of plants; see reviews by Epstein (1999) and Raven (2003).

Despite numerous studies of Si application to improve lodging resistance and resistance to pathogens and insects of rice (Yoshida et al. 1959; Takahashi and Hino 1978), there has been little evidence of the mechanisms involved in the assimilation and transport of silicon or of the direct biochemical process modulated by silicon in rice and other plants. Recent molecular and genetic studies in silicon-uptake-deficient rice mutants have revealed that silicon is transported into the plant apoplast and symplast via specific transporters. Ma et al. (2006) identified two transporter genes (*Lsi1* and *Lsi2*) responsible for high Si accumulation capacity in rice. Examination of the deduced sequence of the amino acids encoded by *Lsi1* indicated that *Lsi1* encodes an aquaporin-like protein and that *Lsi1* protein functions in the passive influx of uncharged silicic acid, whereas *Lsi2* functions in the H^+ -driven active efflux of silicic acid anions. Specific tissue distribution of *Lsi1* and *Lsi2* appeared to be the root exodermis and endodermis, where the Casparian bands are located. Interestingly, *Lsi1* are preferentially distributed on the distal side and *Lsi2* are on the proximal sides of root cells, respectively (Ma et al. 2006, 2007a, b). Si is transported via *Lsi1* and *Lsi2* from the root epidermis into the root steles and then moved to the shoot by transpirational water flow via the xylem. Silicon accumulated in the xylem forms monosilicic acid (Ma and Takahashi 2002; Casey et al. 2003; Mitani et al. 2005). Finally, Si is accumulated in the cell wall

and intracellular spaces of motor cells, forming silica-cuticle double layers, called "plant opal", and silica bodies composed of $[\text{SiO}_x(\text{OH})_{4-2x}]_n$ (Ma and Takahashi 2002; Prychid et al. 2004).

Despite the abundance of studies of the effects of Si fertilizers and electron-microscopic observations of silica depositions within plants (Takahashi et al. 1990; Tsuda et al. 2000; Agarie et al. 1998a, b), few of the morphological analysis of silica deposition in rice tissues have used histochemical staining and conventional microscopic techniques available to field researchers. Although there are a few reports using X-ray scanning analytical microscopy with which described silica deposition detected in a dicot plant such as *Arabidopsis halleri* (Zhao et al. 2001; Hokura et al. 2006), X-ray scanning microscopy have been broadly applied for analysis of silica bodies and other elements in rice tissues (Kaufman et al. 1972; Soni et al. 1972; Dikeman et al. 1981; Takahashi et al. 2006). However, there are few studies with X-ray scanning microscopy comparing accumulation of silicon and other elements using a silica deficient mutant. Here, we characterized the effect of silicon on silica body formation, nutritional element content, and lateral root generation by using the mutant (*lsi1*) in comparison with two wild-type rices, cvs. Hinohikari and Oochikara. We optimized a technique for silica body-specific staining to visualize silica deposits in rice tissue by bright field microscopy and a technique for determining the in situ content of elements, including silica, by X-ray scanning microscopy. We also briefly discuss the effects of the physiological water status of rice on silica accumulation.

Materials and methods

Plant materials and growth conditions common to all treatments

Two cultivars of Japonica rice (*Oryza sativa* L., cvs. Hinohikari and Oochikara) and a mutant of Oochikara background, *lsi1*, were used. Oochikara and the *lsi1* mutant were donated by Okayama University.

Eighty seedlings of each cultivar and the mutant were cultured hydroponically in growth chamber settled in a glass house under sunlight (The averages of light intensity at day time were ranging between 800 and $1,200 \mu\text{mol m}^{-2}\text{s}^{-1}$) at 25°C and with 70% humidity

for 14 days, with one seedling per cloth-covered plastic pot (10 cm diameter×8 cm depth, with a 5-mm-diameter hole in the bottom). Each pot was inserted into a plastic pot (13 cm diameter×12 cm depth) filled with nutrient solution containing half-strength Kimura B complete nutrient solution (48.2 mg L⁻¹ (NH₄)₂SO₄, 65.9 mg L⁻¹ MgSO₄, 18.5 mg L⁻¹ KNO₃, 24.8 mg L⁻¹ KH₂PO₄, 15.9 mg L⁻¹ K₂SO₄, 59.9 mg L⁻¹ Ca(NO₃)₂, and 5 mg L⁻¹ Fe₂O₃) adjusted to pH 5.5 to 6.0. To test if short- and long time incubation of rice seedlings in silicic acid supplement medium show result in difference of silica deposit accumulation between Hinohikari and Oochikara in addition of comparison between Oochikara and *lsi1* mutant. Preliminary experiments were performed on time courses of incubation time. So, 24 h and 14 days were used as an optimum condition to show the difference of silica deposits between Hinohikari and Oochikara. Control rice seedlings were kept with incubation in the same condition.

In a paddy field at Kaizuka area of Kyushu University, rice plants of cv. Hinohikari were grown for 2 months to observe a typical silica bodies in leaf tissue at the maximum tillering stage.

Silicic acid treatment

The following treatments were performed: 13-day-old seedlings (each 80 seeds of cvs. Hinohikari and Oochikara and the *lsi1* mutant) were grown for the duration of the experiment in nutrient solution containing no silicic acid (control); 13-day old seedlings (each 80 seeds of cvs. Hinohikari and Oochikara and the *lsi1* mutant) were grown at 25°C for 24 h in nutrient solution to which silicic acid had been added (1-day silicic acid treatment); and seedlings (each 80 seeds of cvs. Hinohikari and Oochikara and the *lsi1* mutant) were grown for 14 days from germination in nutrient solution to which silicic acid had been added (14-day silicic acid treatment). The concentration of silicic acid in the treatment solution was adjusted to 100 ppm and pH 5.5 to 6.0 by using Na–metasilicic acid, continuously.

Measurement of length and SPAD value of leaves

The lengths of fully expanded leaves of 14-day-old rice plants (each 80 seeds of cvs. Hinohikari and Oochikara, and the *lsi1* mutant) in the control and silicic acid treatment groups were subjected to SPAD

analysis. The first leaves from 25 plants randomly selected from 80 seedlings in each group of conditions and cultivars/mutant were selected and measured to calculate average leaf length. The chlorophyll contents of the leaves of 14-day-old rice plants in the control and 14-day silicic acid treatment groups were estimated with a SPAD-502 chlorophyll meter (Minolta, Japan). The chlorophyll content, calculated from average SPAD (soil and plant analysis development) values, was measured in two fully expanded leaves in each of 25 plants in each group by measuring the ratio of light transmittance at a wavelength of 650 nm to that at 940 nm.

Histochemical analysis of rice leaf blade at vegetative growth stage by silica body staining

Pieces of leaf 30 mm long were cut off at the leaf base, center, and tip, and pieces of root tissue 30 mm long were cut. The samples were fixed in FAA solution (formamide, 80% ethanol, 100% acetic acid, 90:5:5) for 24 h. Fixed samples were transferred and incubated at 25°C sequentially for 24 h each time by sequential dehydration treatments with 80%, 90%, and 100% ethanol. The dehydrated samples were transferred to, and incubated in, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% benzene (in ethanol) for 24 h each time to exchange the solvent with benzene in accordance with the methods of Kaufman et al. (1985) and Morikawa and Saigusa (2004), with minor modifications. Benzene-equilibrated samples were stained in 0.1% crystal violet lactone solution (in benzene) to visualize the silica bodies. The samples were observed under a bright-field microscope (Eclipse 80i, Nikon Co., Japan).

Analysis of Si, P, S, K, Na, and Ca in rice leaf blades by X-ray analytical microscopy

Pieces 20 mm long were cut off at the leaf base, center, and tip and freeze-dried. The freeze-dried samples were mounted on carbon stubs with carbon tape and then coated with carbon. The resultant samples were subjected to an energy-dispersive X-ray spectrometer (Horiba XGT-5000 X-ray analytical microscopy). The analytical parameters were as follows: a tube voltage of 50 keV, tube current of 1.0 mA, X-ray radiation diameter of 100 mm, sample distance of 1 mm, room temperature, atmospheric pressure (100 kPa) and the collection time for spectrum of 300 sec. The software

installed in XGT-5000 was used for a quantitative analysis of elemental intensity of fluorescence on multi-points and a mapping analysis of the X-Y scanning. Relative intensities of elements in samples was calculated with the intensity of Rh characteristic spectrum (Rh-L, 2.7 keV) with X-ray tube (Rh target) as a standard of 100%. X-ray fluorescence maps, profiles of concentrations along transect lines, and X-ray fluorescence spectra were recorded on randomly chosen 10 spots on the cut piece of leaf blade to detect the spectra and energy of X-rays characteristic of each element (Si at 1,900 eV, P at 2,000 eV, S at 2,300 eV, K at 3,300 eV, Na at 1,000 eV, Ca at 3,600 eV, and Mg at 1,200 eV). Leaf pieces prepared from 4 independent rice plant treated with the same condition were subjected to EDX analysis. The photo counts in each 10 eV range along the energy spectrum were used for comparison of composition of elements in samples. To normalize photo counts of signals for each elements, the photo counts at 2,700 eV indicating a specific peak of Rh specific spectrum by XGT-5000 in the condition indicated were gained with 1894, 1628 and 1995 in leaves of Hinohikari, Oochikara and *lsi1* which were grown in silicic acid solution and 1593, 1651 and 1419 in leaves of Hinohikari, Oochikara and *lsi1* which were grown in control solution in Fig. 6. The content and relative intensity of each element in the tissues were estimated from the spectrum type and intensity (Yokoyama et al. 2002; Miah and Chino 1999), with signals of rhodium (Rh) as an internal standard.

Results

Effects of silicic acid treatment on growth and SPAD values

To examine the effects of silicon supply on the growth indices of rice, the length and SPAD

values of fully expanded leaves of control rice plants or plants treated for 14 days with silicic acid were determined (Table 1). The average leaf-lengths of cvs. Hinohikari and Oochikara and the *lsi1* mutant under 14-day silicic acid treatment increased by 68%, 89%, and 34%, respectively (Table 1, Fig. 1d–f), compared with those in the controls (Table 1, Fig. 1a–c). The SPAD values in cvs. Hinohikari and Oochikara and the *lsi1* mutant after 14 days of silicic acid treatment increased by 540%, 245%, and 337%, compared with those in the controls (Table 1). The length and SPAD values of the leaves indicated that supply of silicon enhanced the growth and chlorophyll content of Hinohikari, Oochikara and *lsi1* mutant and that the effect of silicon differs in degree between in cultivars and the mutant. Furthermore, all rice plants grown in silicon-supplemented nutrient solution showed high germination percentages and normal growth (data not shown). On the other hand, rice plants in the control groups often showed silicon-deficient disease-like phenotypes as previously described (Dobermann and Fairhurst 2000).

To examine the effect of silicon treatment on root morphogenesis and therefore root silicon uptake capacity, roots of cvs. Hinohikari and Oochikara and the *lsi1* mutant were harvested after treatment of 13-day-old seedlings with silicic acid treatment for 1 day. The root shape of each plant was observed after crystal violet lactone staining. There were no visible silica bodies in the roots of any of the control rice plants (Fig. 2a–c) or the plants treated for 1 day (Fig. 2d–f). The most marked elongation of the lateral roots with silicic acid treatment was observed in cv. Oochikara, which had no lateral root formation in the controls (Fig. 2b). Lateral roots were present in both the control and silicic acid treated plants of cv. Hinohikari and *lsi1* (Fig. 2a, c).

Table 1 Leaf lengths and SPAD values of leaf blades or rice plants in 14-day silicic acid treatment

Treatment	Leaf length (cm)			SPAD value		
	Hinohikari	Oochikara	<i>lsi1</i>	Hinohikari	Oochikara	<i>lsi1</i>
Control	4.91±0.46	3.74±0.37	7.57±0.59	2.97	5.30	5.74
Silicic acid	8.26±0.48*	7.05±0.76	10.15±1.21*	19.03	18.29	25.10

Means of biological replicates ($n=25$) are shown. *Significant different from control at ($p<0.05$)

Fig. 1 Effects of Si-treatment on growth of cvs. Hinohikari, Oochikara and *lsi1* mutant. Growth and SPAD values of wild type rice cvs. Hinohikari (a, d), Oochikara (b, e) and *lsi1* mutant (c, f) were observed after control nutrient treatment of rice seedlings (a, b, c) and 14-day silicic acid treatment of rice seedlings (d, e, f) as described in materials and methods

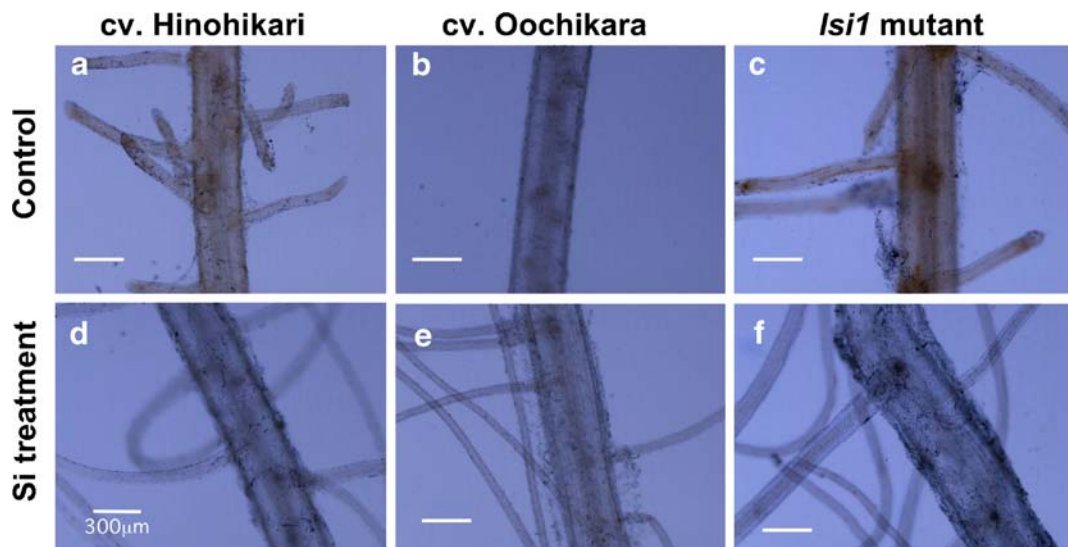
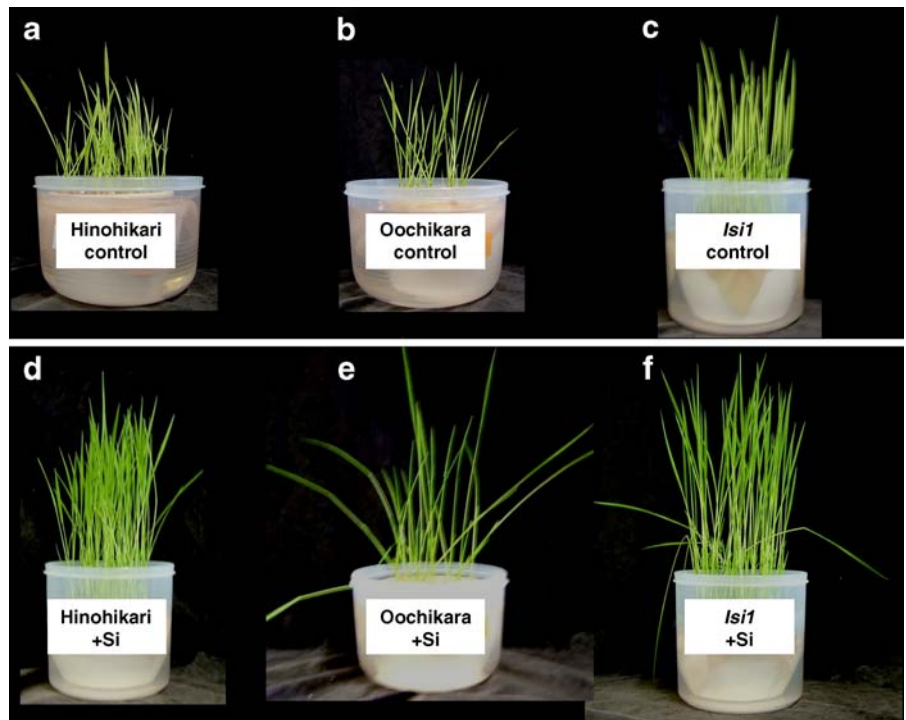


Fig. 2 Effects of Si-treatment on roots of cvs. Hinohikari, Oochikara and *lsi1* mutant. Root system and lateral roots of wild type rice cvs. Hinohikari (a, d), Oochikara (b, e) and *lsi1* mutant (c, f) were observed by light microscopy after control nutrient treatment of rice seedlings (a, b, c) and 14-day silicic

acid treatment of rice seedlings (d, e, f) as described in materials and methods. Four specimens of root samples were observed for each set of experiments with rice cultivars or *lsi1* mutant

Effects of silicic acid treatment on silicon contents of leaves in cvs. Hinohikari and Oochikara and the *lsi1* mutant

In the field, silica bodies were observed in the motor cells of the leaf blades of cv. Hinohikari at the maximum tillering stage (Fig. 3a), and silica opal was also observed in the blades (Fig. 3b). In enlarged images of silica cells located along the vascular bundles of cv. Hinohikari treated with silicic acid for 14 days, accumulation of silica was observed as clear X shapes (Fig. 3c).

To evaluate silicon-uptake performance in these two cultivars and the *lsi1* mutant, accumulation of silica in the rice blades was analyzed by observation of silica bodies in control rice plants after crystal violet lactone staining (Fig. 4a, b, c) and in 13-day-old rice plants treated for 1 day with silicic acid (Fig. 4d, e, f). In the leaf fragment samples of cv. Hinohikari treated with silicic acid for 1 day, substantial numbers of silica bodies were accumulated in the motor cells and silica cells along the vascular bundles (Fig. 4d); they were absent in the controls

Fig. 3 Crystal violet lactone staining of motor cell and silica body in leaf blade of cv. Hinohikari. A, motor cell and silica bodies in cross-section of leaf blade; B, rice opals in motor cells of leaf blade; C, a line of X-shaped silica bodies in leaf sheath. Rice opals in motor cells and silica bodies in epidermis cells were stained with crystal violet lactone using leaf blade of cv. Hinohikari in paddy field at the maximum tillering stage as described in materials and methods. Motor cells and X-shaped silica bodies are indicated by lines and arrow heads, respectively

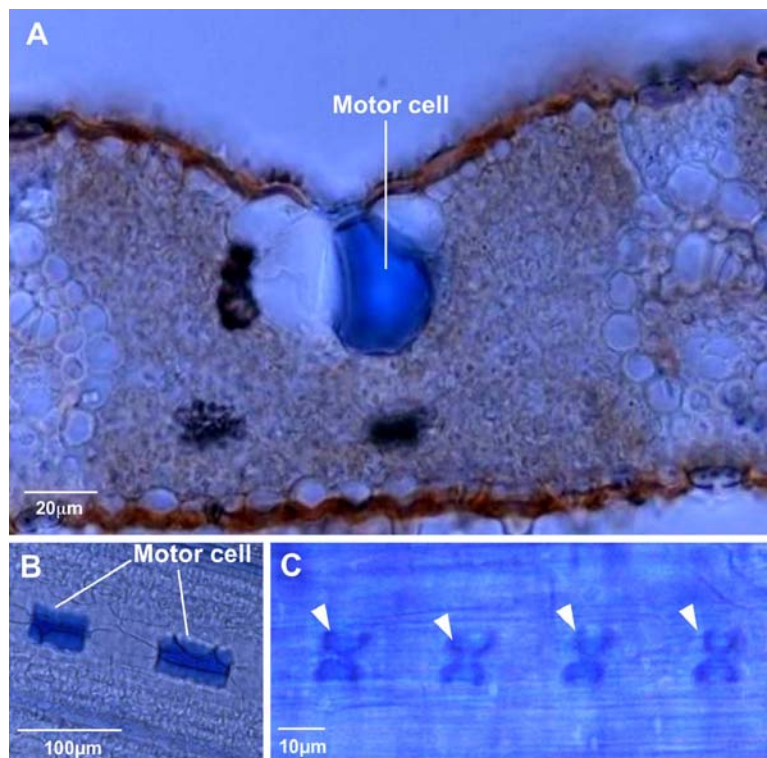
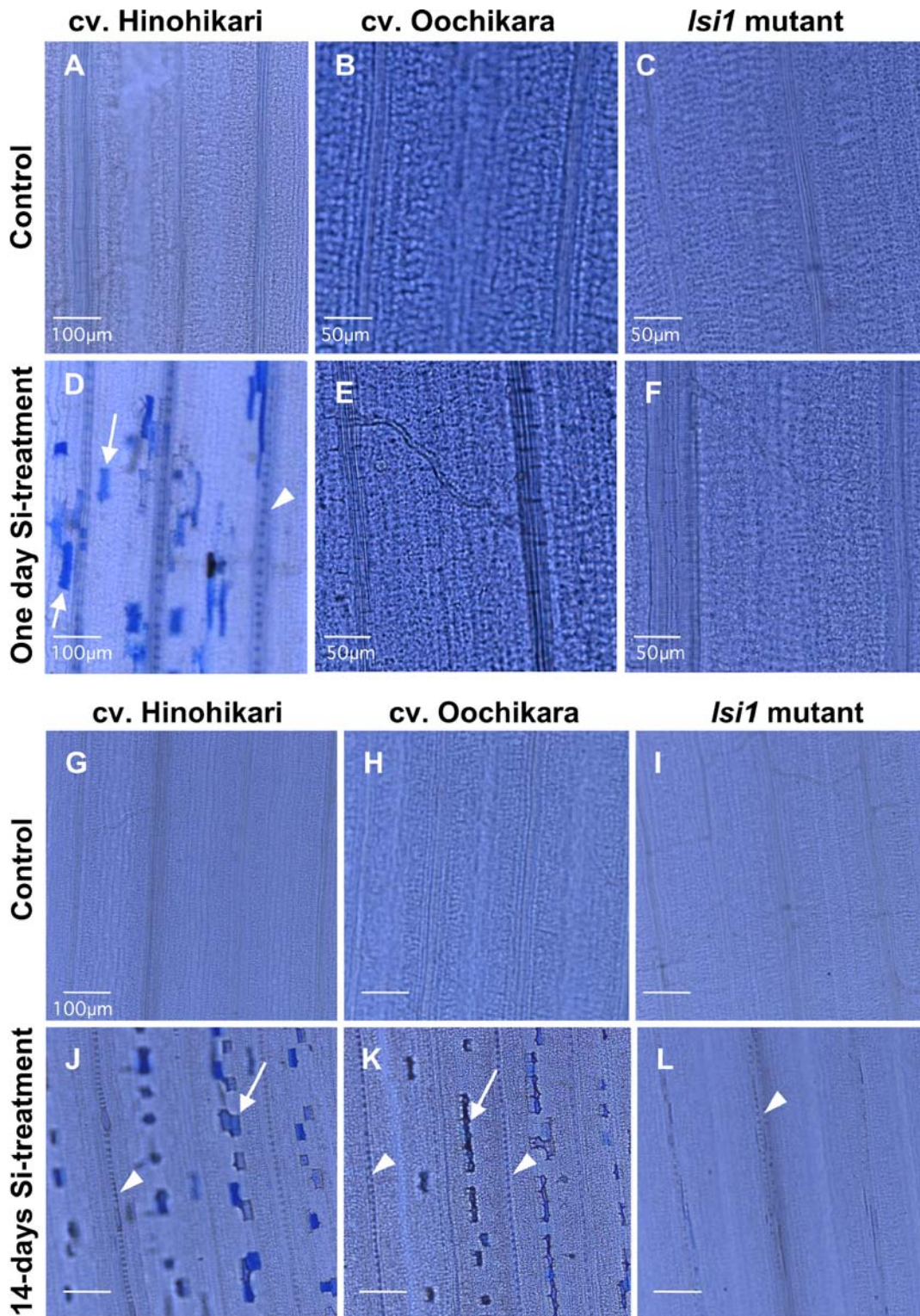


Fig. 4 Effects of Si-treatment on accumulation of silica in leaf blades of cvs. Hinohikari, Oochikara and *lsi1* mutant. Motor cells and silica bodies in leaf blades of cvs. Hinohikari (a, d, g, j), Oochikara (b, e, h, k), *lsi1* mutant (c, f, i, l) were stained with crystal violet lactone. Rice plants (a, b, c, g, h, i) were incubated in control nutrient solution for 14 days (Control). Thirteen-day old rice seedlings (c, d, e) were subjected to one-day silicic acid treatment (1-day Si-treatment). Rice seedlings (j, k, l) were continuously subjected to 14-day silicic acid treatment after seeding (14-day Si-treatment). Rice opals in motor cell and a line of X-shaped silica bodies in epidermis cells were indicated with arrow and arrow head, respectively. Six specimens of root samples were observed for each set of experiments with rice cultivars or *lsi1* mutant

(Fig. 4a). In cv. Oochikara and the *lsi1* mutant, no silica bodies were observed in the control (Fig. 4b, c) or treated (Fig. 4e, f) epidermal cells.

We next observed the accumulation of silicon in the leaf blades of rice plants subjected to 14 days of silicic acid treatment after germination. Substantial numbers of silica bodies accumulated in the motor cells and silica cells along the vascular bundles of cv. Hinohikari after treatment (Fig. 4j), but not in the controls (Fig. 4g). In cv. Oochikara, only small,



deformed silica bodies were observed in the motor cells and silica cells along the vascular bundles of treated plants (Fig. 4k), but not controls (Fig. 4h). In the *lsi1* mutant, small numbers of silica bodies accumulated in the silica cells, but not in the motor cells, along the vascular bundles (Fig. 4l). No silica bodies were observed in the epidermal cells of the controls (Fig. 4i).

Analysis of elements in rice leaves by X-ray analytical microscopy

To measure the content of Si and other elements in the leaf tissues of each rice cultivar and *lsi1* mutant, 14-day-old seedlings were analyzed by X-ray analytical microscopy. Examination of the Si-specific fluorescence spectra of characteristic X-rays showed that the energy-dispersal signals of Si (Fig. 5b, e, h) were clearly distinguishable from those of K (Fig. 5c, f, i) and bright-field microscopic images are given for comparison (Fig. 5a, d, g). Interestingly, Si signals were detected specifically on the leaf sheath of the above-ground part of the branching shoot after 14 days of silicic acid treatment (Fig. 5b), whereas K signals were distributed over all the tissues, including the surface and inside of the shoot and roots (Fig. 5c). Furthermore, the density of Si signals was higher in the midrib than in other leaf areas (Fig. 5e), whereas K signals were detected broadly over the leaf area, except in the midrib (Fig. 5f). In the shoot cross-section, Si signals were observed mainly on the outside of the leaf sheath, not in the marginal areas or the inside of the stem (Fig. 5h), whereas K signals were evenly distributed over the whole stem section (Fig. 5i). In the energy dispersal spectrum after 14 days of silicic acid treatment, a single and specific peak at about 1,900 eV was attributed to Si (Fig. 5j). The Si content of cv. Hinohikari rice plants subjected to 14 days of silicic acid treatment was substantially higher (Fig. 6d) than that of the controls (Fig. 6a). The relative intensities indicating the Si contents of the leaves of control and treated plants were 11% and 52%, respectively (Fig. 6a, d). In cv. Oochikara, the relative intensities were 12% (control) and 55% (treated plants). In contrast, the relative intensity in the treated *lsi1* mutant was 23% (Fig. 6f), only slightly higher than that in the control (10%; Fig. 6c). Thus the *lsi1* mutant had a lower ability to take up Si, even in the form of silicic acid in the nutrient solution.

For P content, indicated at 2,000 eV in the spectrum, the relative signal intensities in the control plants and 14-day silicic acid-treated plants of cv. Hinohikari were 20% and 10%, respectively (Fig. 6a, d). In cv. Oochikara, the relative P signal intensity in treated plants was 10%—the same as in the controls (Fig. 6b, e). In the *lsi1* mutant, the relative signal intensity in treated plants was 11%—lower than the 17% in the controls (Fig. 6c, f).

The relative signal intensity for the S content of cv. Hinohikari control plants was 42%, whereas that in 14-day silicic acid-treated plants was 15% (Fig. 6a, b). In cv. Oochikara, the relative signal intensity for S content in treated plants was 20%—lower than the 30% in the controls (Fig. 6c, f). In the *lsi1* mutant, the relative signal intensity for S content in treated plants was 15%, whereas it was 29% in the controls (Fig. 6c, f).

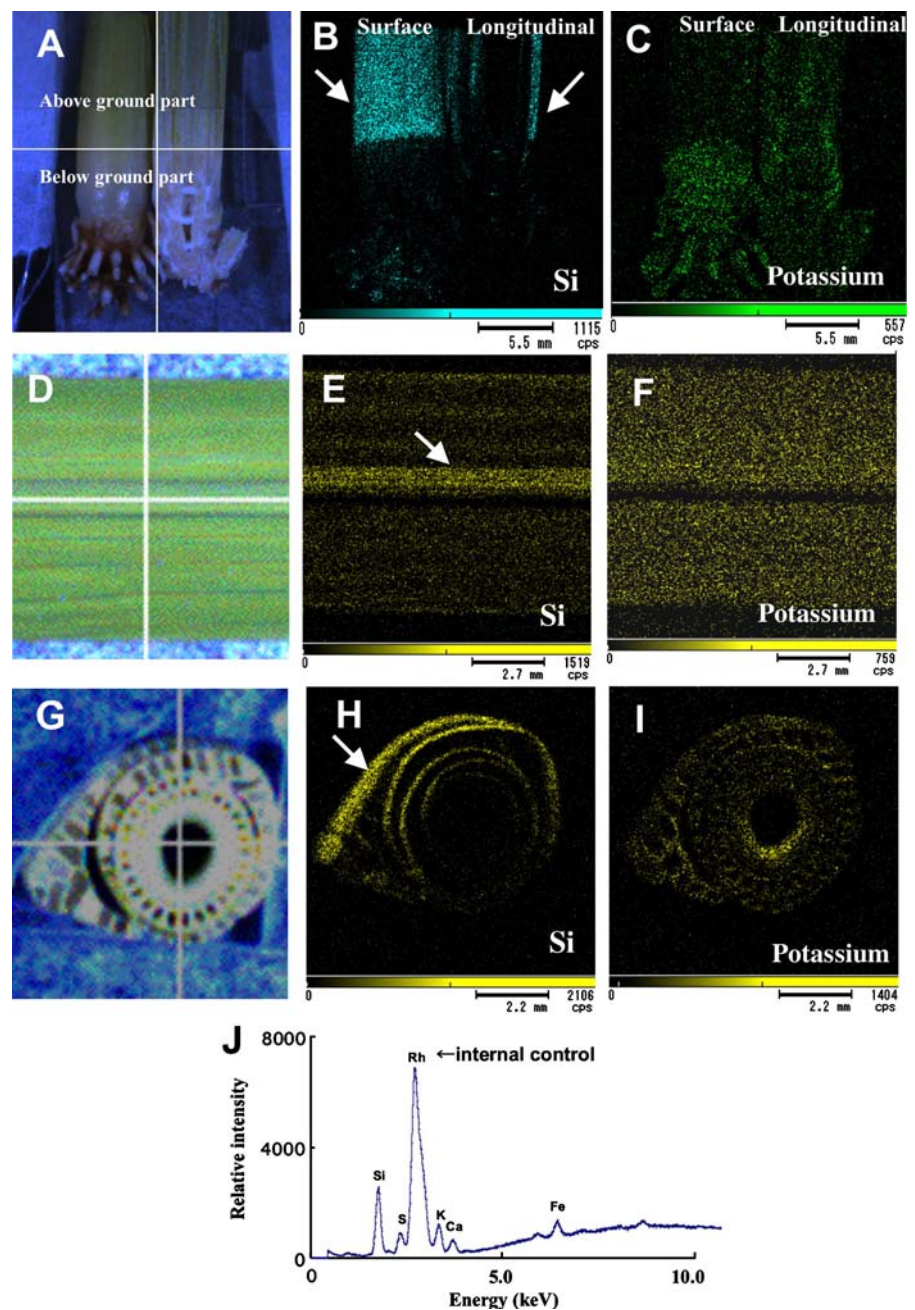
The signals indicating K content of the two cultivars and *lsi1* after 14 days of silicic acid treatment tended to be lower than, or equal to, those in the controls. In cv. Hinohikari, the relative intensity in treated plants was 30%—lower than the 61% in the controls (Fig. 6a, d). In cv. Oochikara, the relative intensities in the controls and treated plants were 40% (Fig. 6c, f). In the *lsi1* mutant, the relative intensity in treated plants was 43%—lower than the 62% in the controls (Fig. 6c, f).

Discussion

lsi1 mutant

The low-silicon rice mutant *lsi1* was isolated by screening tolerance to germanium, a toxic analog of silicic acid, as a selection parameter. This mutant has a very low silicon uptake capacity: one-tenth that of cv. Oochikara (Ma et al. 2002). A single nucleotide substitution in the influx transporter gene *Lsi1* results in loss of function of the *Lsi1* gene (Ma et al. 2006). When the *lsi1* mutant is cultivated in the field, yield is markedly decreased, possibly because of low silicon accumulation (Ma et al. 2006). Our results revealed markedly greater Si accumulation in the motor cells and silica cells of cv. Hinohikari than in those of cv. Oochikara when the rice plants were incubated in silicic acid treatment for 1 day (Fig. 4d, e). In contrast, accumulation of silicon was detected in both cvs.

Fig. 5 Distribution of Si in stem and root of cv. Hinohikari and EDX analysis by fluorescence X-ray analytical microscopy. **a, d, g:** bright microscopic observation of surface, **b, e, h:** 2-Distribution of Si signals, **c, f, i:** 2-D distribution of potassium signals. **a, b, c:** longitudinal section (*right*) and surface (*left*) of stem (Above ground part) and roots (below ground part) of cv. Hinohikari; **d, e, f:** a piece of fully expanded leaf blade, **g, h, i:** cross section of stem with leaf sheath. **j:** a typical diagram of fluorescence X-ray analysis spectrum indicates compositions of Si, P, S, K, Ca and Fe in stem and root of cv. Hinohikari. Horizontal scales are eV. Accumulation of silica is indicated with arrow. Three specimens of shoot and leaf were subjected to EDX analysis to observe the distribution of each element



Hinohikari and Oochikara when the plants were grown with silicic acid treatment for 14 days (Fig. 4j, k). The observation that, in the *lsi1* mutant, the controls (Fig. 4c), plants treated for 1 day (Fig. 4f), and plants treated for 14 days (Fig. 4i) all failed to accumulate Si (or accumulated only small amounts, in the case of the 14-day treatment) is consistent with a previous report that the *lsi1* mutant is deficient in Si uptake (Ma et al. 2002). The difference in silicon uptake between cvs.

Hinohikari and Oochikara in the 1-day silicic acid treatment suggests that cv. Hinohikari has a greater Si-uptake capacity than cv. Oochikara, possibly resulting from the action of factors other than the *Lsi1* gene. This lower Si-uptake capacity of Oochikara suits the use of this cultivar as fodder, because digestion by ruminants is faster for rice straw with a low silicon content (Agbagla-Dohnani et al. 2003). In the 14-day silicic acid treatment, substantial amounts of Si were

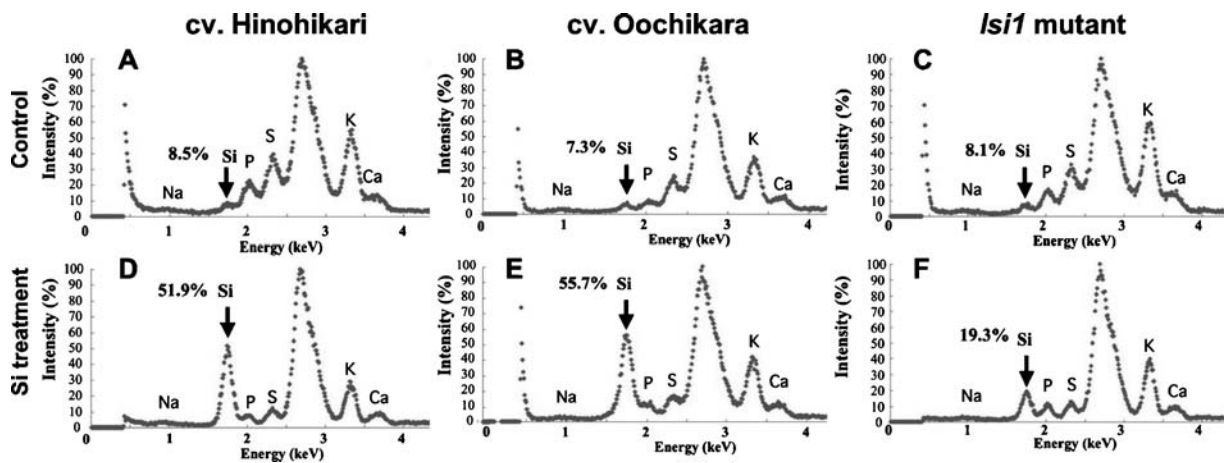


Fig. 6 Effects of Si-treatment on compositions of Si, P, S, K, Ca and Fe in leaf blades of cvs. Hinohikari, Oochikara and *lsi1* mutant. Compositions of each element in leaf blades were analyzed with X-ray analytical microscopy using rice seedlings in control nutrient solution (control) and one-days silicic acid treatment (+ Si). **a, d**, cv. Hinohikari; **b, e**, cv. Oochikara; **C, F**, *lsi1* mutant. **a, b, c**: the rice plants were incubated in control

nutrient solution for 14 days. **d, e, f**: The rice plants were subjected to control nutrient solution and one-day silicic acid treatment after incubation in control nutrient solution for 13 days, described in materials and methods. The plots in each panels shows percentage ratio of the photo counts per cent at 2,700 eV according to Rh specific spectrum as an internal standard

accumulated in both the silica cells and motor cells of cvs. Hinohikari (Fig. 4j) and Oochikara (Fig. 4k), whereas in 1-day Si treatment silica bodies were detected in cv. Hinohikari (Fig. 4d) but not in cv. Oochikara (Fig. 4e), suggesting that the difference in silicon accumulation between 1-day- and 14-day silicic acid treatments in cv. Oochikara resulted from lower silicon uptake capacity than in cv. Hinohikari. The relatively lower capacity of silicon uptake in cv. Oochikara compared with that of Hinohikari is possibly involved in genotypic difference between the two cultivars, such as the difference in expression levels of silicon transporter genes (Ma et al. 2007a, b).

Effect of Si treatment on development of rice plants

Examination of growth indices indicated that silicic acid treatment of rice seedlings accelerated elongation of the fully expanded leaves to 134% to 189% of the lengths in the controls (Table 1). This agrees with the previous findings that silicic acid treatment for 21 days caused a 1.3-fold increase in plant height (Okuda and Takahashi 1962). Okuda and Takahashi (1962) also reported that silicon absorbed from the root increases leaf area index; the resultant increase in photosynthetic production by the rice plant can then supply metabolites supporting a high growth rate and increased yield. Therefore, it is conceivable that the difference in plant

height between silicic acid treated plants (Fig. 2d–f) and controls (Fig. 2a–c) was caused by silicon stimulated increase of photosynthesis. The increase in SPAD of all rice plants under silicic acid treatment also indicated that silicon is required for normal development of the photosynthetic apparatus and for chlorophyll synthesis in the leaf and stem, which is involved in growth and yield. Silicon application appeared to suppress senescence of rice leaves (Agarie et al. 1998a, b) and to increase antioxidant enzyme activity in cucumber leaves (Zhu et al. 2004), whereas silicon is not required for activities of metabolic enzymes involving photosynthesis cascade. Therefore, it is possible that silicon has a role on protection of chlorophyll from oxidative damage and/or senescence leading to the silicon-stimulated increases of SPAD value and growth. Because it is well known that SPAD value is enhanced by nitrogen supply, it is possible that silicic acid treatment stimulates nitrogen transport on root or amino acid metabolism in leaf. Tamai and Ma (2008) reported that there was no significant difference in tiller number or SPAD value between the *lsi1* mutant and the wild type at all growth stages when rice plants were grown in the field. Here, we demonstrated essentially the same results of growth (Table 1) and SPAD values of leaves of the two wild rice cvs. Hinohikari (Fig. 1d) and Oochikara (Fig. 1e) and the *lsi1* mutant (Fig. 1f), which were grown in Si-treatment. Our observation

that supplementation with silicon caused a remarkable increase in SPAD value in all the tested rice samples indicates that silica treatment significantly benefits development of the photosynthetic apparatus, even though deposits of silica did not accumulate in the leaf tissues of the *lsi1* mutant when it was treated for 1 or 14 days with silicic acid (Fig. 4f, l). Rice has at least two genes, *Lsi1* and *Lsi6*, of aquaporin-related silicon transporter in its genome (Ma and Yamaji 2006). Expression of *Lsi6* in root can account for the residual capacity of silicon uptake of the *lsi1* mutant. Furthermore, when rice plants were incubated in 1 mM silicic acid solution, silicon concentration of symplastic solution of root did not differ between wild type and *lsi1* mutant rice plant, while silicon concentration of xylem sap in *lsi1* mutant is less than 20% of that in wild type rice (Ma et al. 2004). Therefore, it can be assumed that SPAD values and growth of rice shoot are affected by silicon concentration in external solution and/or cortical cells of root rather than those in xylem sap and/or leaf tissues. In the case, silicon-induced signal regulating photosynthesis systems may be transduced from root to shoot by an unknown mechanism. Furthermore, observation of the rice roots indicated that the 14-day silicic acid treatment stimulated the development of lateral roots (Fig. 2b, e), leading to accelerated uptake of nutrients, including silicon, by the roots. Watanabe et al. (2004) reported that up-regulation of a zinc finger protein and down-regulation of metallothionein-like protein, chlorophyll a/b binding protein and carbonic anhydrase in microarray and quantitative RT-PCR with rice plants were observed in response to increase in silicon concentration in the growth medium. Among the rice genes regulated by silicon nutrition, specific genes functioning in root morphology and in the function of silicon-sensing molecules might be involved in the lateral root formation observed in cv. Oochikara under silicic acid treatment (Fig. 2e). The silicon uptake capacity of the RH2, a mutant lacking root hairs, is almost the same as that of wild-type rice, but the silicon uptake capacity of RM109, a mutant lacking lateral roots, is one-third that of wild-type rice (Ma et al. 2001), suggesting that the lateral roots play an important role in absorbing silicon.

Accumulation of silica bodies

Crystal violet lactone staining was an effective method of visualizing various shapes of silica opals and silica

bodies deposited on the walls of the epidermal cells of leaf blades and stems, forming X-shaped silica cuticles along the vascular bundles (Fig. 3c). Successful staining reactions for observing silica bodies in plant tissues have been developed; they involve the use of methyl red, silver amine chromate, and crystal violet lactone (Dayanandan et al. 1983; Kaufman et al. 1985). In accordance with the reactivity of the silanol groups on the surfaces of the silica bodies, the crystal violet lactone dye stains the silica bodies exclusively, clearly allowing their shapes to be observed. Calcium carbonate granules in the plant tissues also interact with crystal violet lactone, but calcium carbonate can be easily removed by fixation and successive treatments. Silicic acid treatment of rice plants for 14 days enhanced the chlorophyll content, growth (Fig. 1) and biomass (Table 1) of the leaf blades and stems, suggesting that silicic acid treatment increased the photosynthetic rate, thus enhancing lateral root formation (Fig. 2). These pleiotropic effects of silicic acid treatment may help to prevent lodging and to improve the light-receiving capacity (Takahashi 1995). Uptake of silicon therefore has important roles in enhancing rice plant growth and changing leaf (Idris et al. 1975; Ando et al. 2002) and root (Ma et al. 2001) morphology to enhance nutrient uptake capacity. Silicate fertilizer appears to be effective in improving rice productivity by increasing photosynthesis with more erect canopy and thereby improving light interception parameters (Epstein 1999; Ma et al. 2001).

Specific distribution of Si in the leaf sheath and midrib in lodging resistance

Lodging resistance of rice depends on morphological features such as the leaf sheath and midrib and biochemical components such as starch, cellulose, lignin, and silica. Rigidity of the stem and leaf blade is important to prevent lodging, so silicon appeared to play a major role in increasing the mechanical strength and rigidity of the cell wall (Agarie et al. 1998a, b; Epstein 1999). Despite many studies of the localization and structure of silica deposition within the rice plant (Takahashi 1987; Kim et al. 2002; Ueno and Agarie 2005), it was unclear whether tissue specific accumulation of silicon is involved in enhancing the strength of the rice cell wall. Here, X-ray fluorescence microscopy revealed that Si was specifically localized in the leaf sheath and on the

outermost parts of the shoot, but not in the inside area of the stem (Fig. 5b). It is reasonable that Si is distributed and accumulated selectively in these outer parts of the stem, which would seem important to resist bending, whereas the Si content is very low in the inner stem tissue, which has little effect on stem strength. The thickness of the midrib of the leaf blade is greater than that of the rest of the leaf, and this helps increase the resistance of the leaf and shoot to bending. *Drooping leaf (dl)* mutations cause defects in midrib formation in leaves, resulting in reduced rigidity and drooping leaf phenotypes, indicating that the midrib is essential for leaf strength (Nagasawa et al. 2003). The observation that substantial amounts of Si accumulated in the midrib of the leaf blade (Fig. 5e) and in the leaf sheath and the outermost part of the stem (Fig. 5b, h) prompts us to hypothesize that silicon is effectively distributed to specific tissues (such as the leaf sheath and midrib) that are involved in rigidity of the rice plant, rather than being passively deposited in any growing tissues. Interestingly, substantial amounts of Si were detected in the leaf blade of the *lsi1* mutant under Si treatment (Fig. 6f), even though few silica bodies were observed under the same treatment (Fig. 4l). Substantial amounts of silicon in rice tissues occur in cell wall complexes with calcium, hemicelluloses, pectins, and lignins, thus increasing the rigidity of the plant body (Inanaga and Okasaka 1995). These results suggest that the *lsi1* mutant still has the capacity to store silicon, possibly in the cell wall, but not as silica deposits. Furthermore, supply of silicon to two wild-type cultivars and the *lsi1* mutant caused an increase in growth and silicon content, suggesting that silicon accumulated in the cell wall can form complexation with hemicellulose, pectin and/or lignin, which is not stained with crystal violet lactone. Based on these observation, it is conceivable that the silicon complexation as an unstained form by crystal violet lactone plays an important physiological role that differs from that played by silica opal, a typical silica deposit.

Effect of Si treatment on uptake of N, P, S, Na, and K

Silicon and other elements, including S, P, Ca, Na, and K, in the leaf tissues were also quantified by X-ray analytical microscopy. The concentrations of N, P, and K in the *lsi1* mutant are similar to those in wild-type rice (Tamai and Ma 2008). According to

studies by Ma et al. (2001), plants classified as Si accumulators contain more than 10 mg Si g⁻¹ dry weight and have a molar ratio of Si to Ca of more than one. When cvs. Hinohikari and Oochikara were subjected to 14-day silicic acid treatment, the signal intensities of Si in all rice plants by X-ray analytical microscopy were higher than those of Ca, indicating that cvs. Hinohikari and Oochikara are typical silicon accumulators. Furthermore, the signal intensity of Si in the *lsi1* mutant under 14-day silicic acid treatment appeared higher than that of Ca, suggesting that the *lsi1* mutant has the potential to absorb substantial amounts of silicon and can still be defined as a silicon accumulator (Fig. 6f).

Measurement of S and K by X-ray analytical microscopy indicated that silicic acid treatment caused an apparent reduction in uptake of sulfate as a major S source in the cultivars and *lsi1* mutant and that silicic acid treatment caused an apparent reduction in uptake of K in Hinohikari, but not in Oochikara and *lsi1* mutant. These data suggest that there was a negative relationship between K or S uptake and 14-day silicic acid treatment in cv. Hinohikari and the mutant, but not in Oochikara. It can be assumed that a competitive effect of silicic acid suppresses transport systems of sulfate and K in the roots (Fig. 6a-f). It is possible that the large beneficial effect of Si in enhancing total mineral uptake capacity by generating lateral root development overcame the competitive effect of silicic acid against the uptake of other nutrients.

Silicic acid treatment resulted in a decrease, in P uptake in Hinohikari and *lsi1* mutant but lack of change in Oochikara, suggesting that Si treatment on Oochikara had little effect on the P content. Those observations are consistent with previous observations (Takahashi 1987; Ma and Takahashi 1990). Inhibition of P absorption by excess silicic acid treatment has been found in various silicon accumulator crops (Takahashi 1987). In the case of rice, silicic acid treatment enhances translocation of phosphate assimilated from the roots in soils with low P concentration, whereas it suppresses excessive uptake of phosphate in soils with relatively high phosphate concentration (Ma and Takahashi 1990). Details of the relationship between phosphate and silicic acid nutrient uptake systems are still unclear, and further research is expected to clarify the silicon-modulated mechanism contributing to phosphate uptake.

Physiological function of silica bodies in rice

Motor cells filled with silica function in bending the leaf to prevent water loss from mesophyll cells in response to drought stress (Dingkuhn et al. 1989). Accumulation of silica in the *lsi1* mutant was less than one half of that in cvs. Hinohikari and Oochikara (Fig. 6d–f). Silica bodies in the epidermal cells and silica opals in the motor cells were observed in the leaf blades of cvs. Hinohikari (Fig. 5j) and Oochikara (Fig. 5k) after 14 days of silicic acid treatment, whereas there was little silica in the epidermal cells of the *lsi1* mutant after the same treatment (Fig. 4l). This finding agrees with the results of previous studies (Ma et al. 2001, 2006; Yamaji and Ma 2007). It is possible that silica compounds localized in the epidermal cells affect the cell walls of guard cells, thus altering physiological water processes under drought conditions (Agarie et al. 1998a, b). Low silicon levels cause excessive transpiration of the spikelets, leading to increased sterility (Tamai and Ma 2008). Moist conditions in the husk must be maintained for normal development of the spikelets. The density of stomata in the husk is lower than that in the leaf blade, and water in the husk is lost by transpiration only through the cuticle layers (Yoshida 1965; Ma and Takahashi 2002). Silica bodies in the cuticle of the husk may be involved in silicon-dependent resistance to water deficit by stopping excessive transpiration (Yoshida 1965; Ma and Takahashi 2002). Different effects of silica fertilizer on drought tolerance have been observed between rice (Goto et al. 2003), sorghum (Lux et al. 2002, 2003; Hattori et al. 2003a, b) and maize (Gao et al. 2004).

Conclusion

Under X-ray fluorescence analytical microscopy, the tissues of the leaf sheath, the outermost part of the basal stem, and the midrib of the leaf blade appeared to function in silicon storage, in addition to the silica bodies and motor cells in the leaf blade. Silicon was distributed in tissues functioning to increase the rigidity of the plant body but not in the central region of the stem, which is not important to plant body strength. Si therefore accumulates specifically in tissues involved in rigidity and is not passively deposited in growing organs or old leaves. In addition, the observation that supply of silicon to the *lsi1* mutant resulted in increased growth

and accumulation of silicon with the formation of only a few silica bodies suggests that complexation of silica with hemicelluloses, pectins, and lignins in the cell wall plays an important role in increasing rigidity and/or growth that is separate from the function of silica body formation. It is conceivable that synthesis of cell walls containing silicon in the leaf sheath and midrib is regulated spatially and temporally, such as in response to changes in developmental stage and/or environmental stresses, to maximize the efficiency of silicon, which competes with the uptake of various minerals from external solution. However, these processes for sensing silicon concentration in solution, such as silicon-induced signaling and/or silicon-regulated transcriptional regulation, remain to be understood.

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