Title

Effect of silicon deficiency on secondary cell wall synthesis in rice leaf

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Abstract

Rice (*Oryza sativa* L.) is a typical Si-accumulating plant and is able to accumulate Si up to >10% of shoot dry weight. The cell wall has been reported to become thicker

under Si-deficient condition. To clarify the relationship between Si accumulation and cell wall components, the physical properties of, and macromolecular components and Si content in, the pectic, hemicellulosic, and cellulosic fractions prepared from rice seedlings grown in hydroponics with or without 1.5 mM silicic acid were analyzed. In the absence of Si (the –Si condition), leaf blades drooped, but physical properties were enhanced. Sugar content in the cellulosic fraction and lignin content in the total cell wall increased under –Si condition. After histochemical staining, there was an increase in cellulose deposition in short cells and the cell layer just beneath the epidermis in the –Si condition, but no significant change in the pattern of lignin deposition. Expression of the genes involved in secondary cell wall synthesis, *OsCesA4*, *OsCesA7*, *OsPAL*, *OsCCR1* and *OsCAD6* was up-regulated under –Si condition, but expression of *OsCesA1*, involved in primary cell wall synthesis, did not increase. These results suggest that an increase in secondary cell wall components occurs in rice leaves to compensate for Si deficiency.

Keywords

Cellulose, Lignin, Rice, Secondary cell wall synthesis, Silicon

Introduction

Silicon (Si) is the second most abundant element in soil, after oxygen. Silicon dioxide comprises 50–70% of soil mass, and all plants rooting in soil contain some Si in their tissues (Epstain 1999). Today, Si is still not recognized as an essential element for plant growth, but the benefits of this element to growth, development, rigidity of plant body, yield, and disease resistance have been observed in a wide variety of plant species (Ma 2004).

Plants differ greatly in their ability to accumulate Si, and levels of Si in plants range from 0.1% to 10% (dry weight) (Epstain 1999; Ma and Takahashi 2002; Richmond and Sussman 2003). Rice (*Oryza sativa* L.) is a typical Si-accumulating plant, and Si is accumulated up to 10% of shoot dry weight, which is several-fold higher than in other Gramineae, such as maize or barley (Ma and Takahashi 2002). Recently, molecular mechanisms of Si uptake have been revealed. Lsi1 and Lsi2 are the influx and efflux transporters for silicic acid, respectively (Ma et al. 2006, 2007a). After

transmembrane transport via Lsi1 and Lsi2 into the root stele, Si is translocated to the shoot by transpiration flow through the xylem. Re-uptake of Si from the xylem is performed by Lsi6, which is an influx transporter for silicic acid and mainly localized in the xylem parenchyma cells of the leaf blades and sheaths (Yamaji et al. 2009).

Previously, the effects of Si on the responses of plants to disease and drought and on agricultural traits were studied. Si polymerizes in motor cell and cuticle layers of the shoot, and the polymerized Si acts as a physical barrier to disease or drought (Ma and Yamaji 2006). However, the relationship between Si and other cell wall components, including polysaccharides and lignin, has not been well investigated. As an interaction between cell wall components and Si, an increase in cell wall thickness was observed in Si-deficient plants (Kim et al. 2002), and the existence of a higher molecular weight silicon complex was reported in rice cell wall with enzymatic degradation (Ishii and Matsunaga 2008). Therefore, a relationship between Si and cell wall macromolecules, as well as a compensatory role of Si for cell wall organic components, has been suggested.

Plant cell walls can be classified into two types: as general characteristics, the

primary cell wall is synthesized in developing cells and is flexible and extensible, while the secondary cell wall is synthesized after cell development and is rigid. Secondary cell wall is mainly composed of cellulose, hemicellulose and lignin. Cellulose synthase A (CesA) catalyzes polymerization of UDP-glucose to synthesize cellulose microfibrils (Holland et al. 2000; Wang et al. 2010), phenylalanine ammonia lyase (PAL) catalyzes the first step of the lignin synthesis pathway (Korth et al. 2001; Sewalt et al. 1997), the cinnamoyl-CoA reductase (CCR) enzyme catalyzes the conversion of cinnamoyl-CoAs to cinnamaldehydes in lignin byosynthesis (Rogers and Campbel, 2004), and cinnamyl alcohol dehydrogenase (CAD) catalyzes the last step of monolignol biosynthesis (Rogers and Campbel, 2004). Therefore, it is widely accepted that normal plant growth is dependent on the strict regulation of genes at specific times and in specific tissues.

The aim of this study was to reveal changes in cell wall organic components induced by Si deficiency in rice. The expression of genes involved in the synthesis of cell wall components was also investigated under Si-deficient condition.

Materials and Methods

Plant material and growth conditions

Seeds of rice (*Oryza sativa* L. cv Nipponbare) were soaked in water overnight at 30°C and then transferred to half-strength Kimura B solution. On day 7, seedlings were transferred to a 3-L plastic pot containing half-strength Kimura B solution with 1.5 mM Si (+Si condition) or no Si (-Si condition), and grown at 30°C under continuous light of 250 μmol m^{-2} s^{-1}. Si stock solution was prepared by passing potassium silicate solution through cation exchange resin (Amberlite IR-120B, H^+ form, Organo, Tokyo, Japan) according to Ma et al. (2007b).

Analysis of physical properties of leaf blades with a creep meter

Rice leaf blades grown for 1 week after transfer to +Si/-Si conditions were used for creep tests. Both sides of a cut leaf blade were attached with scotch tape to the edges of separate stages at 2-cm intervals (Fig. 1c), the stages were positioned to load the middle of the sample onto the sensor, and then the change in load and the distance moved were monitored with a creep meter (Rheoner II Creep Meter, REZ-33005B, Yamaden, Tokyo, Japan).

Extraction and analysis of cell wall polysaccharides

4 week after transfer to +Si/-Si conditions, leaf blades and sheaths were frozen in liquid nitrogen and ground with a Tissue Lyser II (Qiagen, Tokyo, Japan) at 30 Hz for 2 min; the resulting powder was washed in 80% ethanol. The extraction and fractionation of cell wall polysaccharides were performed according to the protocol in Selvendran and O'Neill, 2006. The supernatant was removed after centrifugation for 5 min at 17,400g. The pellet was washed three times with water, three times with methanol: chloroform (MC = 1:1), and three times with acetone. A drop of phenol:acetic acid:water (PAW = 2:1:1) was added to the pellet and mixed. Two drops of MC were then added to the sample, which was then washed with acetone. This process was repeated three times, and the sample was then dried at room temperature for over 1 h. Starch was removed by digestion with amylase (2 unit/ml, Wako, Osaka, Japan) in 50 mM acetate buffer at 37°C for 3 h. After this reaction, the samples were centrifuged and the residues washed three times with water, 80% ethanol, MC, and acetone. After washing, the samples were air-dried for over 12 h and used as cell wall material. The

cell wall material was treated with 50 mM Na_2CO_3 containing 20 mM $NaBH_4$ at room temperature for 3 h, and centrifuged for 5 min at 17,400g. The supernatant was used as the Na_2CO_3-soluble (pectic) fraction. The pellet was treated with 4 M KOH containing 20 mM $NaBH_4$ at room temperature for 2 h and centrifuged for 5 min at 17,400g. The supernatant was used as the KOH-soluble (hemicellulosic) fraction, and the pellet was used as the KOH-insoluble (cellulosic) residue. Each fraction and residue was neutralized with glacial acetic acid. The pellets were hydrolyzed with 72% H_2SO_4 at room temperature for 2 h and then diluted to 4% H_2SO_4 and boiled for 1 h. The H_2SO_4 solutions were neutralized with $Ba(OH)_2$. Sugars in each fraction and in the residue were treated with methanol–hydrogen chloride and the resulting methyl glycosides were trimethyl silylated and analyzed using gas-liquid chromatography (GC-14, Simadzu, Kyoto, Japan). The sugar content in each fraction and residue was determined by the phenol sulfuric acid method (Dubois et al. 1956).

Measurement of silicon content

The silicon concentration in each fraction and in the residue was determined using

the colorimetric molybdenum blue method. To 2.7 ml H_2O, a 0.2-ml sample was added, followed by 1.5 ml 0.2 N HCl, 0.2 ml 10% $(NH_4)_6Mo_7O_2$, 0.2 ml 20% tartaric acid, and 0.2 ml reducing agent. The reducing agent was prepared by dissolving 1 g Na_2SO_3, 0.5 g 1-amino-2-naphthol-4-sulfonic acid, and 30 g $NaHSO_3$ in 200 ml water. After 1 h, the absorbance was measured at 600 nm with a spectrophotometer (Jasco, Tokyo. Japan).

Measurement of lignin content

The measurement of lignin content followed the method for high-throughput determination of thioglycolic acid lignin from rice (Suzuki et al. 2009). The cell wall was dried in vacuo and weighed, 1 ml 3 N HCl and 0.1 ml thioglycolic acid (Nacalai Tesque, Kyoto) were added, and the mixture was then heated at 80°C for 3 h. After centrifugation at 17,400g for 10 min at room temperature, the supernatant was removed and the pellet vortexed for 30 s in 1 ml distilled water. After centrifugation at 17,400g for 10 min at room temperature, the supernatant was discarded, and the pellet was resuspended in 1 ml 1 N NaOH and then shaken vertically at 80 rpm for 16 h. The samples were centrifuged at 17,400g for 10 min at room temperature, and the

supernatant (1 ml) transferred to fresh 1.5-ml tubes and acidified with 0.2 ml concentrated HCl. After chilling the tubes at 4°C for 4 h, they were centrifuged at 17,400g for 10 min at room temperature. The supernatant was removed and the pellet dissolved in 1 N NaOH. Absorbance was measured at 280 nm with a spectrophotometer (Jasco).

Expression of genes involved in the synthesis of cell wall components

Total RNA was extracted from leaf blades using an RNeasy plant extraction mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 mg total RNA using an oligo(dT)18 primer and the random hexamer, RevarTra Ace qPCR RT kit (Toyobo, Osaka, Japan). PCR was performed in a 20-μl reaction volume containing 2 ml 1:5 diluted cDNA, 200 nM each gene-specific primer, and Ex Taq (Takara Bio, Otsu, Japan). The primers used for RT-PCR were followings. 5'- CCTTGGGGCAATGCGGTGTG-3' and 5'-ACCCCTCAAACAAATGACTA-3' for *OsCesA1* (Os05g08370); 5'-CTAATGCGACGAAGACGATG-3' and 5'-GATTTAACGGTGCCCTCTCA-3' for

OsCesA4 (Os01g54620); 5’-TCCATCTTCTCCCTCGTCTG-3’ and 5’-GAATCATCCATCCGGTCATC-3’ for *OsCesA7* (Os10g32980); 5’-ACCGCTTCGTGTATCTTCAG-3’ and 5’-AAGGATGGAATCGAGTAGCA-3’ for *OsPAL* (Os02g41630); 5’-CTCATCCGTGGCTACCACGTC-3’ and 5’-GGGTAGGACTTCTTGGTGCC-3’ for *OsCCR1* (Os02g56460); 5’-CCAACAGTCAGGAACAGCAA-3’ and 5’-ACATCCCGCAGTACTTCACC-3’ for *OsCAD6* (Os04g15920); 5’-GCAAATTACCCAATCCTGAC-3’ and 5’-CTATTGGAGCTGGAATTACC-3’ for 17S rRNA.

Histochemical staining of leaf blades

Rice leaf blades were fixed in 4% (w/v) paraformaldehyde, 0.05 M phosphate buffer, and 0.25% glutaraldehyde, and then embedded in paraffin. Sections, sliced to 15-μm thick, were incubated in PBS containing 0.01% (w/v) calcoflour white for 5 min for cellulose staining. For lignin staining, hand-cut sections of rice leaf blades were incubated in 1% (w/v) phloroglucinol in 20% (w/v) HCl. The sections were observed with a microscope (DMRB, Leuca, Bellevue, WA, USA) under UV and white light.

Results

Effects of silicon on leaf growth and properties

The posture of rice plants grown for 4 weeks after transfer to +Si/-Si conditions differed, and under –Si condition mature leaf blades bowed outward (Fig. 1a, b). To evaluate this phenomenon quantitatively, the leaf blades were subjected to mechanical testing using a creep meter (Fig. 1c). The load (N/leaf width (mm)) and distance that the sensor moved (mm) until the leaf blades broke were measured with a real-time monitoring method (Fig. 1d). Compared to the +Si condition, the load and moving distance in the -Si condition increased 1.7- and 1.5-fold, respectively (Fig. 1e).

Cell wall sugar and lignin content with or without Si

To reveal the cause of changes in leaf physical properties observed in both plant posture and the creep test, cell wall polysaccharides and lignin were analyzed. The cell wall prepared from leaf blades and sheaths was subjected to fractionation into Na_2CO_3-soluble (pectic) and KOH-soluble (hemicellulosic) fractions and

KOH-insoluble (cellulosic) residue. In both leaf blades and sheaths, Si was mainly fractionated into pectic and hemicellulose fractions (Fig. 2a, b). The amount of Si in the hemicellulose fraction was 1.9- and 1.4-fold higher than that in the pectic fraction in both the leaf blades and sheaths, respectively, and the amount in the pectic fraction of the leaf sheaths was 1.2-fold that of the leaf blades. On the other hand, Si content in the hemicellulose fraction of the leaf blade was 1.2-fold higher than that in the leaf sheaths. Under -Si condition, Si content was less than 1% of that under +Si condition in both fractions. Si content in the cellulosic residue was not measured because it was less than 1% of that in pectic and hemicellulosic fractions in rice shoots (date not shown).

Most sugars were fractionated into hemicellulosic and cellulosic fractions, and cellulosic fractions contained 1.3- to 1.9-fold more sugars than the hemicellulosic fractions (Fig. 2c, d). In leaf blades, the sugar content in the cellulosic fraction under -Si condition was 1.7-fold higher than that under +Si condition (Fig 2c). In leaf sheaths, the sugar content in the hemicellulosic fraction under -Si condition was 1.5-fold higher than that under +Si condition (Fig. 2d). In addition to these fractionated sugars, the total lignin content, one of the main components of the secondary cell wall (Boudet 2000),

was measured in the leaf blades and sheaths (Fig. 2e, f). Lignin content under -Si condition was about twofold higher than that under +Si condition in both the leaf blades and sheaths. The sugar composition of the cell wall in whole shoots did not differ significantly among fractions in terms of monosaccharides (Fig. 3), but the pectic fraction tended to have more galactose and less galacturonic acid (Fig. 3a).

Histochemical staining of the leaf blade

To reveal the effect of Si deficiency on cellulose and lignin distribution in leaf tissues, cross-sections of leaf blades were subjected to calcoflour white staining for cellulose (Fig. 4) and phloroglucinol staining for lignin. There was increased localization of cellulose under –Si condition in short cells in the adaxial epidermis (Fig. 4b, d, arrow) and in the cell layer just beneath the abaxial epidermis (Fig. 4b, f, arrowheads). The phloroglucionol staining pattern showed no significant change between +Si condition and –Si condition (Fig. 4g, h).

Expression of genes involved in cellulose and lignin synthesis

To reveal differences in cellulose and lignin synthesis under +Si/-Si conditions, expression of the genes known to be involved in their synthesis was investigated with RT-PCR (Fig. 5). We focused on *OsCesA4* and *OsCesA7* as key enzymes for cellulose synthesis and *OsPAL*, *OsCCR1* and *OsCAD* for lignin synthesis in the secondary cell wall of rice (Vamholme et al. 2008; Wang et al. 2010; Hirano et al. 2012; Kawasaki et al. 2006,). Among the 12 *CAD* genes in rice genome (Tobias et al. 2005), it is known that *OsCAD2* works as a major *CAD* gene in rice culms and *OsCAD7* mutation exhibited late heading time, semi-dwarf and flexible culm phenotype (Zhang et al. 2006; Li et al. 2009). In our experiment, *OsCAD1, 2, 3, 6* and *7* were analyzed by RT-PCR and *OsCAD6* expression was detected in leaf blades. As for *CCR*, it is known that *Snl6* mutant have reduced lignin content and OsCCR1, which works with OsRac1, is one of the main enzymes in lignin synthesis (Bart et al. 2010; Kawasaki et al. 2006). In our experimental condition, *OsCesA4*, *OsCesA7*, *OsPAL*, *OsCCR1* and *OsCAD6* were up-regulated under -Si condition, but *OsCesA1*, which is known to be involved in synthesis of the primary cell wall (Wang et al. 2010), showed no significant change between +Si/-Si conditions.

Discussion

In the mechanical test, leaf blades growing under –Si condition were deformed plastically and showed high durability to fracture (Fig. 1c, d). If the increase was observed at load factor only, it was suggested that the leaves became rigid. However, under –Si condition, drooped leaves indicated less rigidity and increases both in load and distance were observed. These suggest that rice leaves obtained higher extensibility under –Si condition. Therefore rice leaves without Si might become easy to droop without break in our experimental condition. This suggests two functional possibilities; one is that Si accumulation in the leaf blade prevents excess deformation of the cell wall. Compared to +Si condition, leaves under –Si condition bowed outward, resulting in low efficiency of photosynthesis, and this difference in posture was consistent with the suggestion that Si supports cell wall rigidity. The other possibility is that the change in cell wall components caused by Si deficiency induced a highly durable cell wall.

Increases in the sugar content in the cellulosic fraction and lignin (Fig. 2) suggest that the secondary cell wall increased under –Si condition. This increase of secondary

cell wall might induce the thickening of cell wall, which is known to occur under Si-deficient condition (Kim et al. 2002). Under –Si condition, expression of *OsCesA4*, *OsCesA7*, *OsPAL*, *OsCCR1* and *OsCAD6* increased, but *OsCesA1* was not up-regulated (Fig. 5). It is known that the former genes are involved in secondary cell wall synthesis, while *OsCesA1* is involved in primary cell wall synthesis (Tanaka et al. 2003; Vanholme et al. 2008; Wang et al. 2010). Therefore, it is also suggested that secondary cell wall synthesis was enhanced under Si-deficient condition. Generally, the secondary cell wall enhances wall rigidity. In the lignin-deficient mutant *irx4* in *Arabidopsis*, both strength and stiffness of the stems were severely reduced (Jones et al. 2001). Previously, several brittle culm (*bc*) mutants of rice were analyzed. For example, *bc1* showed brittleness in culm and flag leaf and brittleness of *bc3* and *bc6* was observed in culm and that of *bc5* was in stem node (Li et al. 2003; Hirano et al. 2010; Kotake et al. 2011; Aohara et al. 2009). These mutants showed reduced secondary cell wall. The secondary cell wall is formed inside the primary cell wall after cessation of cell growth, and develops particularly into sclerenchyma tissue and xylem elements (Reiter 2002). The developed secondary cell wall presumably provides the plant body with mechanical strength

(Carpita and Gibeaut 1993; Gibeaut and Carpita 1994). Therefore, it is suggested that the change in cell wall components induced by Si deficiency compensated for the reduced rigidity by increasing the mechanical strength of cell wall components.

Since it is known that Si enhances resistance to biotic and abiotic stress, the increase in the secondary cell wall might be a key factor that compensates for the reduction in stress resistance caused by Si deficiency. In fact, up-regulation of lignin synthesis is known to be an important factor for stress resistance (Boudet 2000; Li et al. 2011). In addition to secondary cell wall synthesis, OsPAL was reported to be involved in resistance to biotic stress via salicylic acid synthesis (Cu et al. 2000; Gayoso et al. 2010; Smit and Dubery 1997). The up-regulation of *OsPAL* expression might contribute to both lignin synthesis and biotic stress resistance in Si-deficient rice plants.

From the histochemical staining images obtained under +Si/-Si conditions, specific staining of cellulose was identified in short cells and between the first and second cell layer in -Si condition (Fig. 5). Short cells are known to accumulate Si in rice, and silicic acid is deposited as amorphous silica after polymerization (Ma and Takahashi 2002; Yamaji et al. 2008). Why polymerization of Si occurs in the short cells is unknown;

however Si deposition under +Si condition and the increase in cellulose under -Si condition in short cells suggest the involvement of short cells in leaf blade strength. Although Si accumulation between the first and second cell layer has not been reported before, similar compensation for Si with cellulose might occur in this cell layer. In addition to the increase of cellulosic sugar content in whole leaf shown by chemical analysis, the change of cellulose localization was shown by histochemical staining. In comparison with cellulose staining, the pattern of lignin staining did not change under –Si condition (Fig. 4g, h). This suggests that the increase in lignin took place in the same area in which lignin originally occurred, such as in vascular bundles. Relating to the quantitative performance, it was known that phloroglucinol-HCl method doesn't always reflect the content of lignin because phloroglucinol appears to react with the cinnamaldehyde and coniferyl end groups of lignin and this method is not so quantitative, therefore, histochemical staining of lignin didn't show the drastic change (Jensen, 1962; Wardrop, 1981).

Under –Si condition, rice becomes sensitive to several stresses, and the expression of genes involved in secondary cell wall synthesis was up-regulated, resulting in an

increase in cellulose and lignin content to compensate for reduced stress resistance. This suggests that rice might expend less energy for stress resistance by using inorganic Si instead of organic material. How plants sense Si deficiency and compensate for stress resistance will be clarified in future work.

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Figure legends

Fig. 1 Effects of Si deprivation on growth and physical properties of leaf blades in rice. Plants were grown hydroponically for 4 weeks under +Si (**a**) or –Si (**b**) conditions. Arrowheads indicate drooping leaves. Bars = 5 cm. (**c**) Schematic of the mechanical testing experiment. Physical properties of a leaf blade were measured with a creep meter, using a cut leaf blade and a moving sample stage. (**d**) Representative relationship between moving distance (mm) and load (N/leaf width (mm)) in the creep test. Points showing the highest load value indicate leaf breakage. Black and gray lines indicate -Si and +Si conditions, respectively. (**e**) Average distance and load when leaf blade broke (n = 5). Black and gray symbols indicate -Si and +Si conditions, respectively.

Fig. 2 Silicon, sugar, and lignin content in the leaf blades and sheaths of rice grown under +Si/-Si conditions for 4 weeks. Silicon (**a, b**) and sugar (**c, d**) content in Na_2CO_3-soluble, KOH-soluble fractions and KOH-insoluble residue obtained from the leaf blades (**a, c**) and sheaths (**b, d**). Lignin content in the cell wall of the leaf blades (**e**) and sheaths (**f**) (n = 3). Black and white bars indicate -Si and +Si conditions,

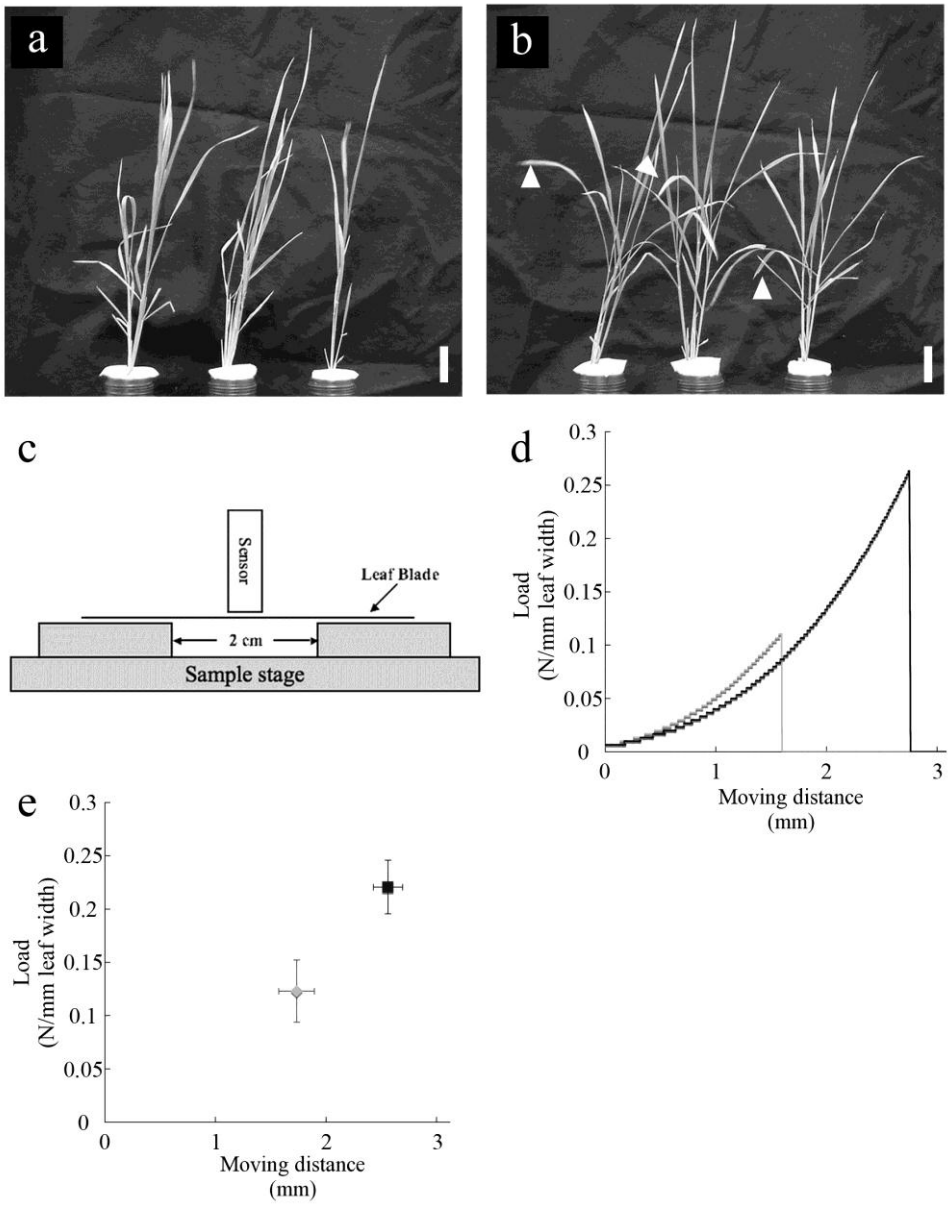
respectively.

Fig. 3 Sugar composition of cell wall fractions in rice shoots grown under +Si/-Si conditions for 4 weeks. Sugar composition of 50 mM Na_2CO_3-soluble (**a**) and 4 M KOH-soluble (**b**) fractions, and 4 M KOH-insoluble residue (**c**) obtained from rice shoots (n = 3). Black and white bars indicate -Si and +Si conditions, respectively.

Fig. 4 Histochemical staining of the leaf blade for cellulose and lignin in rice grown under +Si/-Si conditions for 4 weeks. Cross-sections of leaf blades obtained from rice grown under +Si condition (**a, c, e, g**) or –Si condition (**b, d, f, h**), and stained with calcofluor white for cellulose (**a-f**) and phloroglucinol for lignin (**g, h**). Arrows and arrowheads indicate –Si-specific cellulose accumulation observed in short cells (**b, d**) and between the first and second cell layer (**b, f**). Bars = 100 μm.

Fig. 5 Expression of *OsCesA1, OsCesA4, OsCesA7, OsPAL, OsCCR1, OsCAD6* and 17S rRNA in leaf blades of rice grown under +Si/-Si conditions for 4 weeks. Numbers

in brackets indicate the PCR cycle number.



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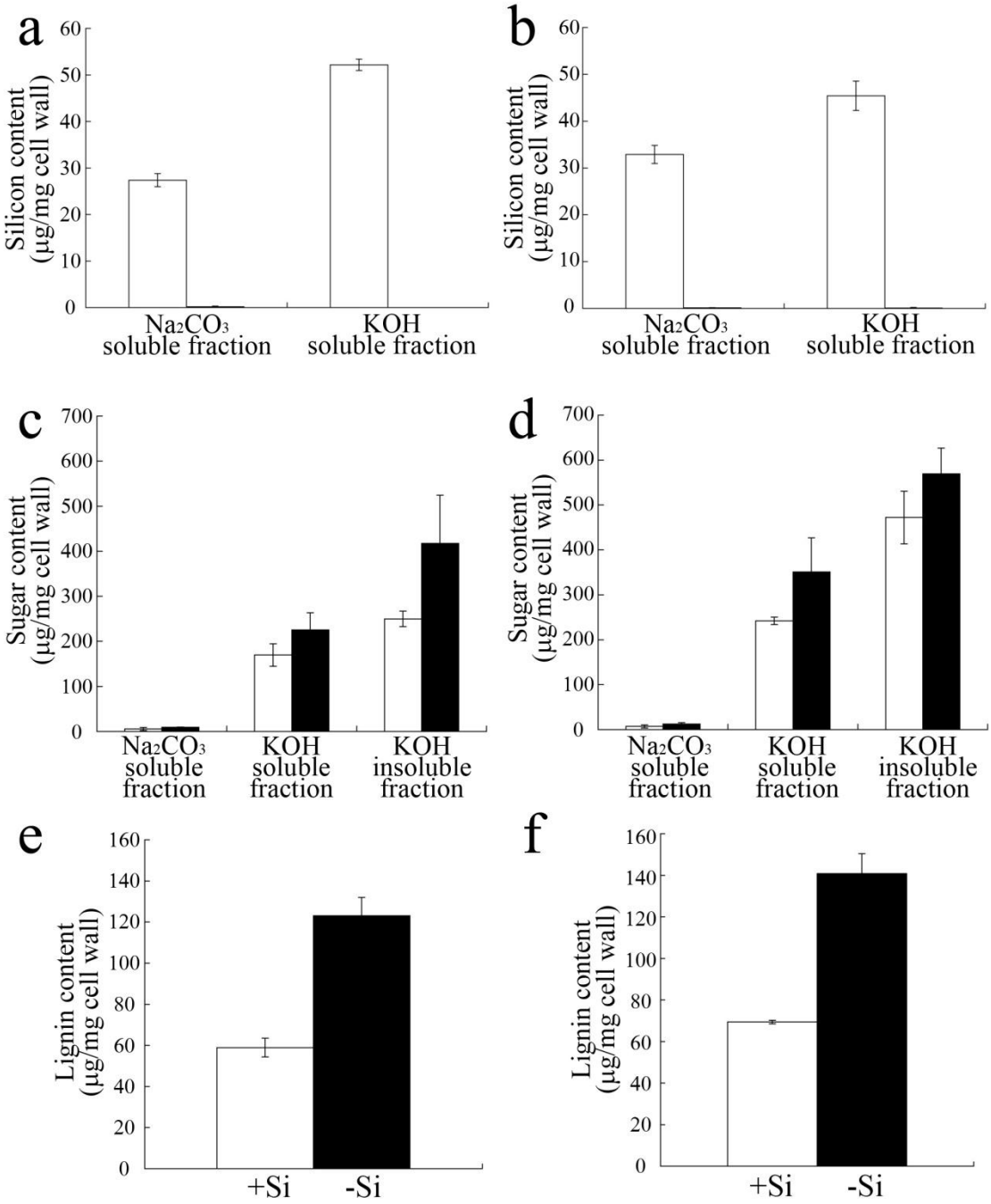

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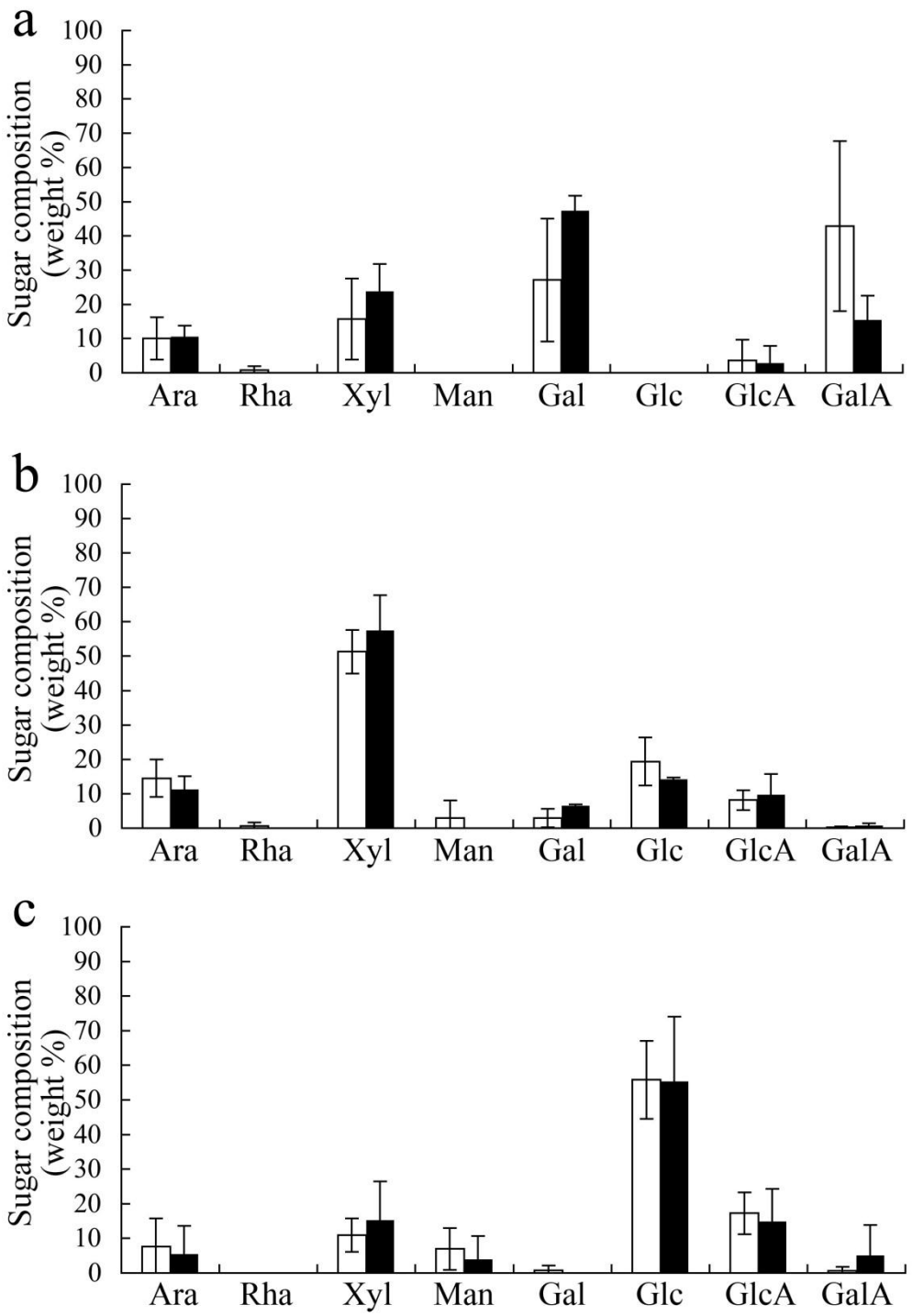

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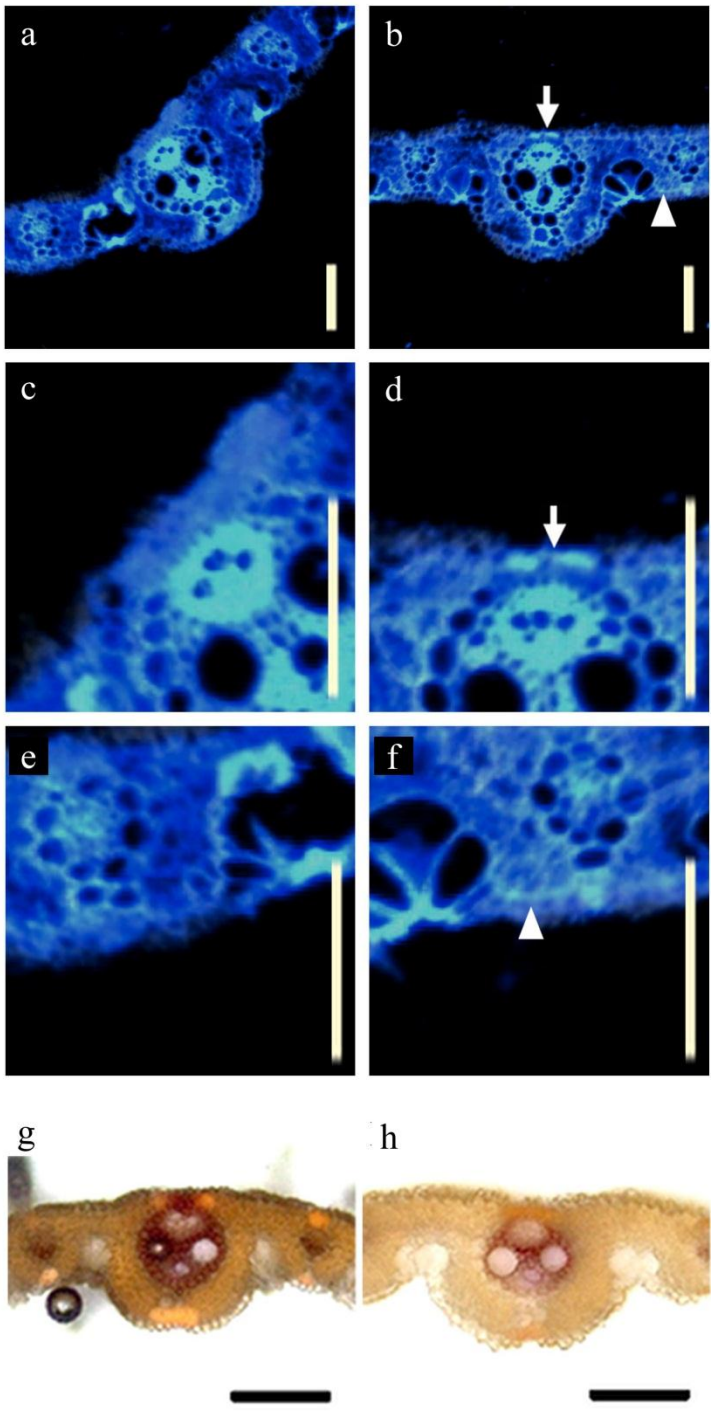

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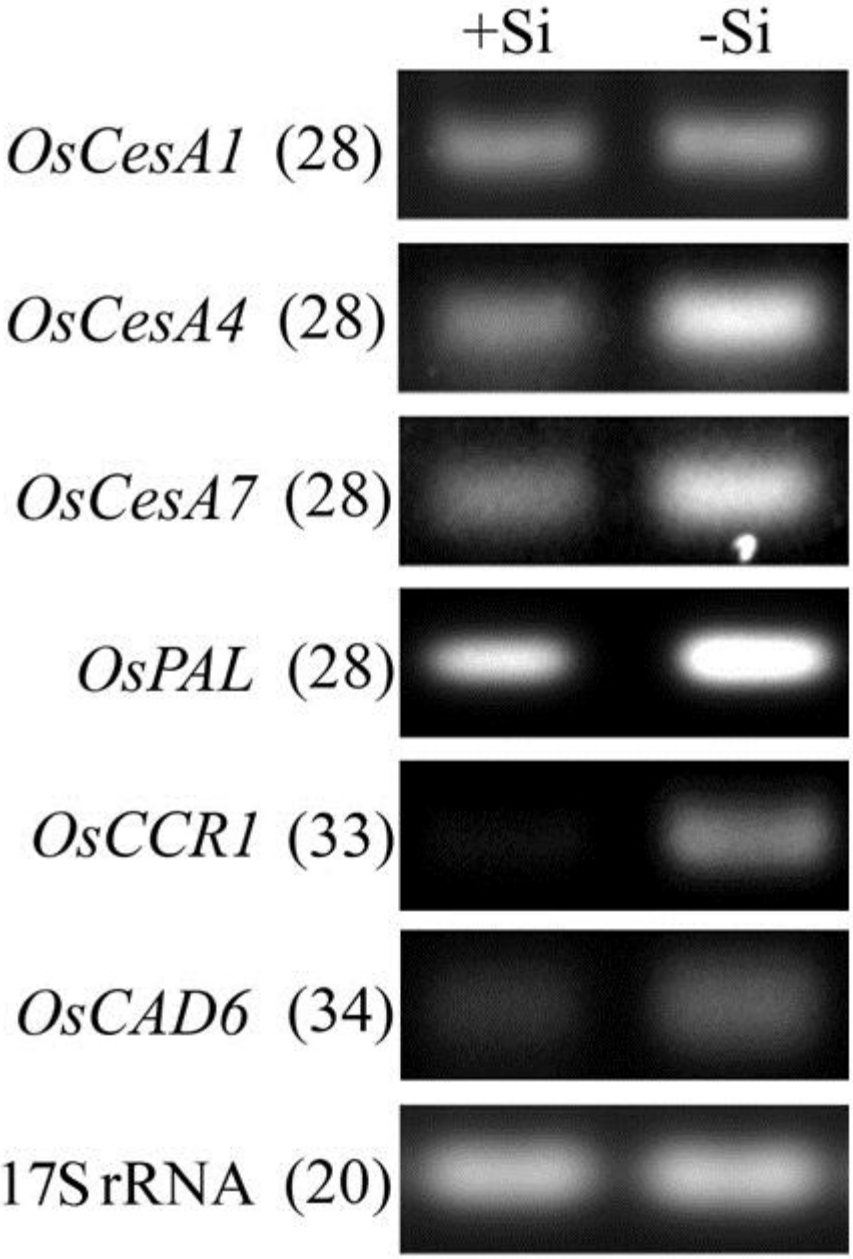

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