Analysis of the Mechanism of Secondary Cell Wall Synthesis Controlled by Silicon in Rice (*Oryza sativa* L.)

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Abbreviations

Ara: arabinose

CAD: cinnamyl alcohol dehydrogenase

CCR: Cinnamoyl CoA Reductase

cDNA: complementary deoxyribonucleic acid

CesA: cellulose synthase catalytic subunit A

Fuc: fucose

Gal: galactose

GalA: galacturonic acid

Glc: glucose

GlcA: glucuronic acid

Lsi: low silicon rice

Man: mannose

Os: *Oryza sativa*

PAL: phenylalanine ammonialyase

Rha: rhamnose

RNA: ribonucleic acid

rpm: round per minute

RT-PCR: reverse transcription polymerase chain reaction

SD: standard deviation

Si: silicon

TFA: trifluoroacetic acid

TMS: trimethyl silylated

UDP: uridine diphosphate

UTR: untranslated region

UV: ultraviolet

Xyl: xylose

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 and physical strength of leaf blade became weak. Sugar amount in the cellulosic fraction and lignin amount 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. In addition, some *OsSWN*s, which is NAC type transcriptional factors involved in synthesis of secondary cell wall, was up-regulated under –Si condition. These results suggest that an increase in secondary cell wall components occurs in rice leaves to compensate for Si deficiency and this process was regulated by NAC type transcriptional factors.

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 (Epstein 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 has 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) (Epstein 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 and Ma 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 2009). 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 biosynthesis (Rogers and Campbell 2004), and cinnamyl alcohol dehydrogenase (CAD) catalyzes the last step of monolignol biosynthesis (Rogers and Campbell 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℃ and then transferred to halfstrength 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 without Si (- Si condition), and grown at 30℃ under continuous light of 250 l mol 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 interval (Fig. 1 c), 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

Four weeks 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 ºC 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 gasliquid 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 ºC 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 ºC 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) and ISOGEN (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg 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’ -GTCCATCCTTCTCCCA-3’ and 5’-CTGCTCAATCTCCTCG-3’ for *OsSWN2* (Os04g15920); 5’ -CTTGCATGCAGTTCATTGCT-3’ and 5’-TGGCTGCCAGATCAAAACAAG-3’ for *OsSWN3* (Os08g0103900); 5’ -AAGGAGCCCAGTTACT-3’ and 5’-TCAAATCACACCGCAATGTT-3’ for *OsSWN6* (Os04g15920); 5’ -GGAAGAAGGGTGGGTGGTAT-3’ and 5’-GGGCGCAATATTGATGATCT-3’ for *OsSWN7* (Os04g15920); 5’ -GACTCTGGTGATGGTGTCAGC-3’ and 5’-GGCRGGAAGAGGACCTCAGG-3’ for *ACTIN* (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) calcofluor 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 (Leica Microsystems, Wetzlar, Germany) 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. 1 a, b). To evaluate this phenomenon quantitatively, the leaf blades were subjected to mechanical testing using a creep meter (Fig. 1 c). 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. 1 d) The load when the moving distance was 1.5 mm was 1.3-fold higher in the - Si condition compared to the +Si condition (Fig. 1 e). In contrast, the load and moving distance when leaf blade was broken was 1.7- and 1.5-fold higher in the - Si condition compared to the +Si condition (Fig. 1 f, g).

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. 2 a, 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. 2 c, 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. 2 c). In leaf sheaths, the sugar content in the hemicellulosic fraction under - Si condition was 1.5-fold higher than that under +Si condition (Fig. 2 d). 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. 2 e, f). Lignin content under- Si condition was about two-fold 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 between two treatments in terms of monosaccharaides (Fig. 3), but in the pectic fraction tended in –Si condition to have more galactose and less galacturonic acid.

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 calcofluor white staining for cellulose and phloroglucinol staining for lignin (Fig. 4). There was increased localization of cellulose under - Si condition in short cells in the

adaxial epidermis (Fig. 4 b, d, arrow) and in the cell layer just beneath the abaxial epidermis (Fig. 4 b, f, arrowheads). The phloroglucinol staining pattern showed no significant change between +Si condition and -Si condition (Fig. 4 g, 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). I 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 (Vanholme et al. 2008; Wang et al. 2010; Hirano et al. 2012; Kawasaki et al. 2006). Among the 12 CAD genes in rice genome (Tobias and Chow 2005), it is known that *OsCAD2* works as a major CAD gene in rice culms and OsCAD7 mutation exhibited late heading time, semidwarf and flexible culm phenotype (Zhang et al. 2006; Li et al. 2009). In my 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* (suppressors of NH1-mediated lesion formation) 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 my 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.

Effect of mechanical stress on secondary cell wall synthesis

To reveal the regulatory mechanisms, we focused on the drooped leaf blade in Si deficient condition, because it was considerable that drooped leaf blade was exposed to mechanical stress and that the mechanical stress may be switch of the promotion of secondary cell wall synthesis. To clarify this hypothesis, load test and hold test was performed. The load test was attaching the weight of aluminum foil to droop leaf blade of +Si condition (Fig. 6a) and the hold test was supporting the leaf blade of –Si condition to prevent drooping (Fig. 6b). Plants grown in these conditions for one week and lignin content were measured. In load test, lignin content was slightly higher in –load condition than +load condition (Fig. 6c). In hold test, there was no significant change in lignin content (Fig. 6d). These results suggest that mechanical stress may not affect on secondary cell wall synthesis.

Expression of genes to transcriptional factors involved in cellulose and lignin synthesis

To reveal the regulatory mechanisms, we focused on NAC type transcriptional factors that involved in secondary cell wall synthesis. Secondary wall NAC (SWN) is involved in synthesis of secondary cell wall, and there are seven *SWN*s in rice genome (Hirano *et al.,* 2013, Zhong *et al.*, 2011). So, the expression level of these *SWN*s in +Si condition and –Si condition was analyzed.

In a result, the expression level of *OsSWN3* was higher in –Si condition than +Si condition (Fig. 7a). And then, the expression level of *OsSWN2*, *6*, *7* was also higher in –Si condition than +Si condition (Fig. 7b). These results suggest that secondary cell wall synthesis under Si deficient condition was controlled by *SWN*s. To reveal the regulation mechanisms more in detail, time-dependent changes of plant response when Si was deficient was analyzeda. Tested Si condition was two patterns; constant +Si condition (+Si/+Si), +Si to -Si condition (+Si/-Si). As a result, lignin content in leaf blade was higher in +Si/-Si condition than +Si/+Si condition (Fig. 8a). Moreover, in +Si/-Si condition, the increase of lignin content from 0 to 7 days after Si condition change was about 94% of lignin content at day 0, although the increase was about 50% both in +Si/+Si condition (Fig. 8a). The expression level of NAC type transcriptional factors was analyzed. In a result, expression level of *OsSWN3* was increased in one day after Si condition was shifted and higher in +Si/-Si condition on 7 day after Si condition was shifted (Fig. 8b).

Discussion

In the mechanical test, leaf blades growing under -Si condition were deformed plastically and showed high durability to fracture (Fig. 1 c, 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.

It is known that secondary cell wall synthesis regulated by NAC type and MYB type transcriptional factors in many plants, including rice (Hirano *et al.,* 2013, Zhong *et al.*, 2011). NAC act as master switch of xylem differentiation and secondary cell wall synthesis, while MYB act downstream of NAC and directly bind promoter region to regulate the expression of genes that codes enzymes of secondary cell wall synthesis (Hirano *et al.,* 2013, Zhong *et al.*, 2011). In this study, it was clarified that some *OsSWN*s were up-regulated in –Si condition and expression level was changed in one week (Fig. 7, 8b). These results suggest that secondary cell wall synthesis under Si deficient condition was regulated by NAC type transcriptional factor. Although expression level of MYB type transcriptional factors was not analyzed in this study, it is considerable that MYB works downstream of NAC and up-regulate the genes that code enzyme of secondary cell wall synthesis, such as *PAL*, *CAD* and *CCR*. Further, these results suggest that expression level of some *OsSWN*s was affected by Si condition, and it is considerable that there are any factors that sense Si density of environment and signal to downstream genes. So I considered that mechanical stress was one of factor that sense Si density, I perform the load test and hold test to clarify the effect of mechanical stress on secondary cell wall (Fig. 6a, b). But, in a result, there were no significant changes in lignin content both

load test and hold test (Fig. 6c, d). Therefore, mechanical stress may not be the factor that sense Si density and any other factor may act as sensor of Si density.

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). The reason 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 suggests 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, histochemical staining showed the change of cellulose localization. In comparison with cellulose staining, the pattern of lignin staining did not change under -Si condition (Fig. 4 g, 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 2004).

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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Figures

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 load when moving distance was 1.5 mm (n = 5). **f** Average load when leaf blade was broken (n = 5). **g** Average moving distance when leaf blade was broken (n = 5). Black and white bars indicate -Si and +Si conditions, respectively.

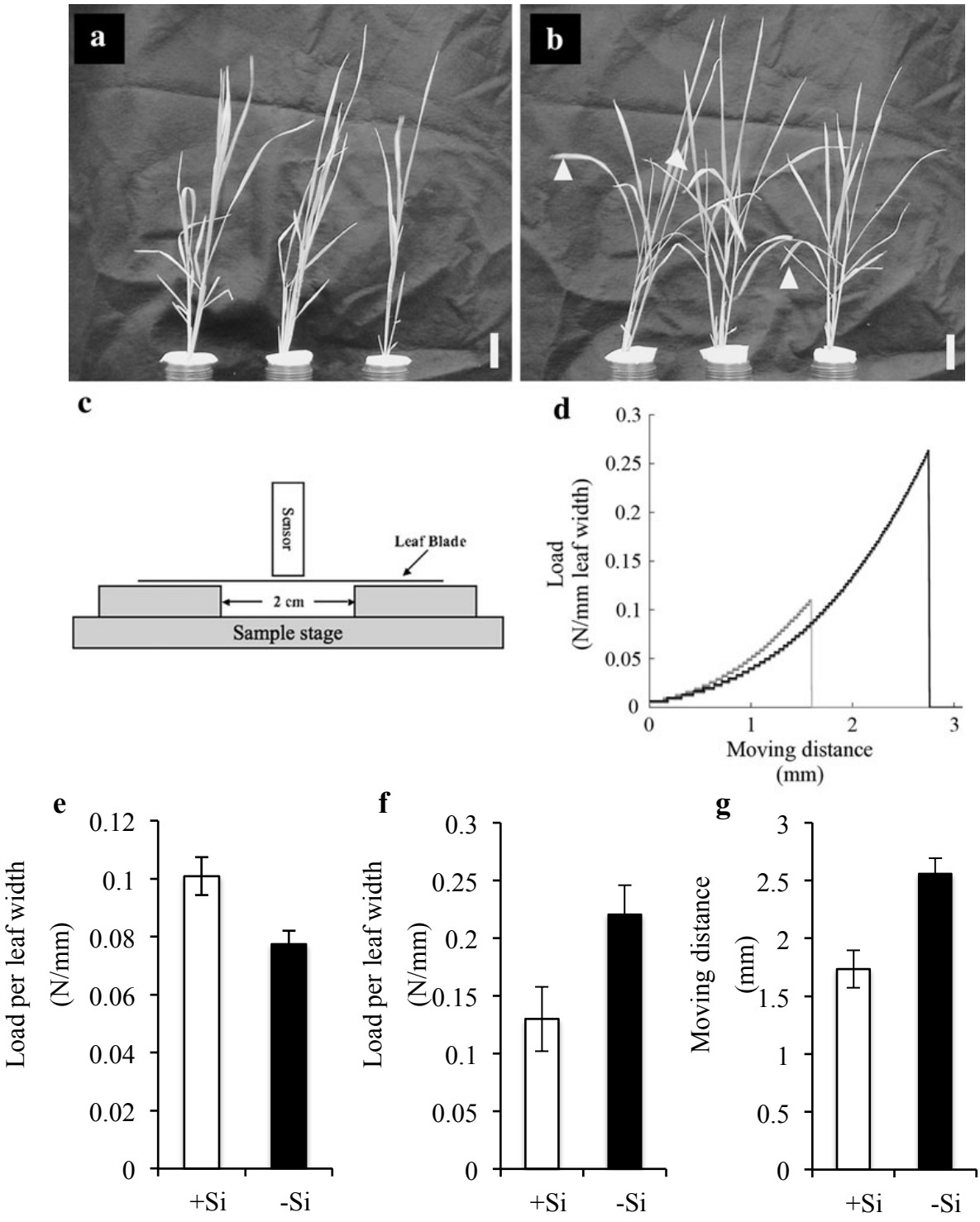

a
b
c
Sensor
Leaf Blade
2 cm
Sample stage
d
Load
(N/mm leaf width)
Moving distance
(mm)
e
Load per leaf width
(N/mm)
+Si
-Si
f
Load per leaf width
(N/mm)
+Si
-Si
g
Moving distance
(mm)
+Si
-Si

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

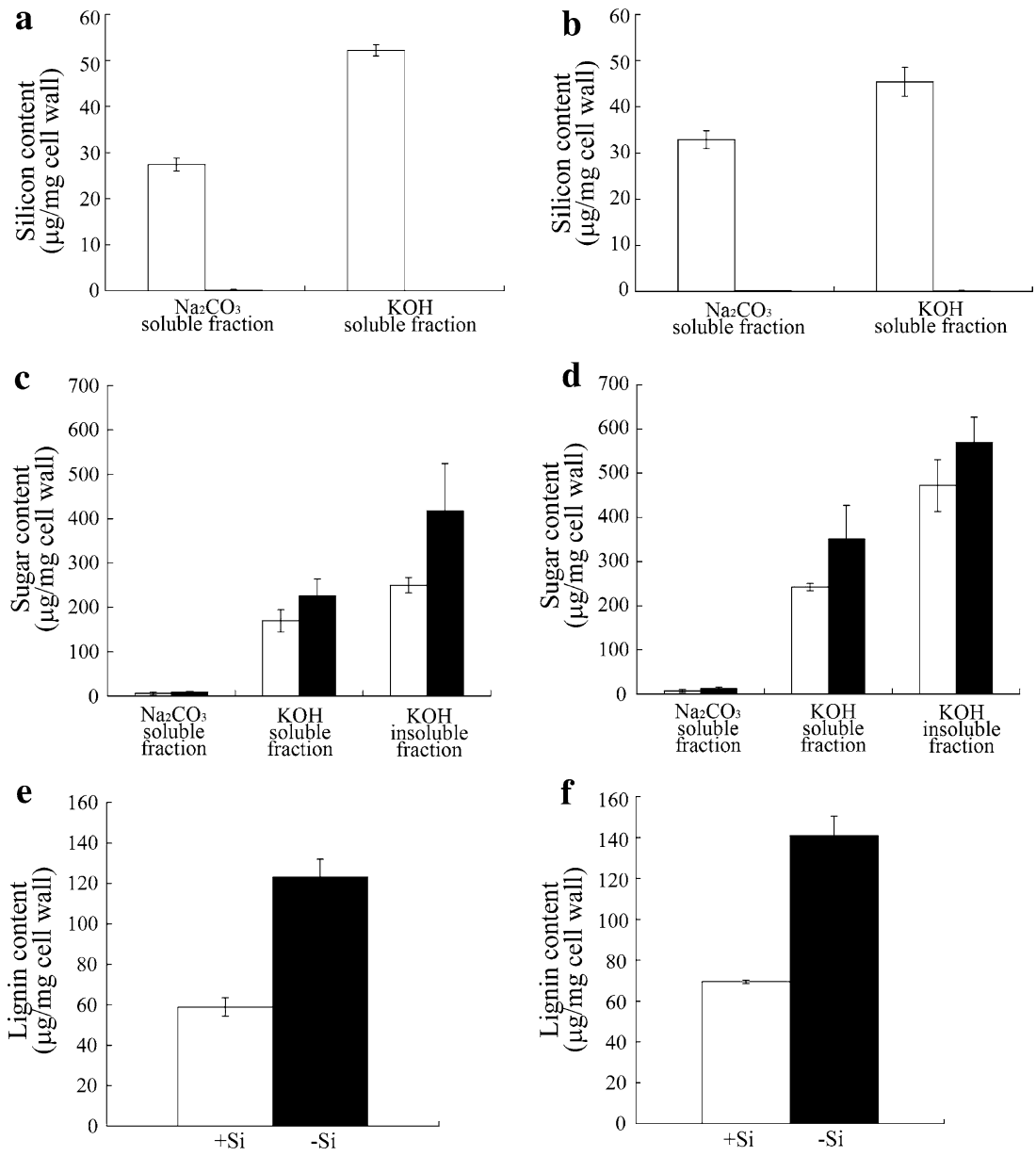

a
Silicon content (μg/mg cell wall)
60
50
40
30
20
10
0
Na2CO3 soluble fraction
KOH soluble fraction
b
Silicon content (μg/mg cell wall)
60
50
40
30
20
10
0
Na2CO3 soluble fraction
KOH soluble fraction
c
Sugar content (μg/mg cell wall)
700
600
500
400
300
200
100
0
Na2CO3 soluble fraction
KOH soluble fraction
KOH insoluble fraction
d
Sugar content (μg/mg cell wall)
700
600
500
400
300
200
100
0
Na2CO3 soluble fraction
KOH soluble fraction
KOH insoluble fraction
e
Lignin content (μg/mg cell wall)
160
140
120
100
80
60
40
20
0
+Si
-Si
f
Lignin content (μg/mg cell wall)
160
140
120
100
80
60
40
20
0
+Si
-Si

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.

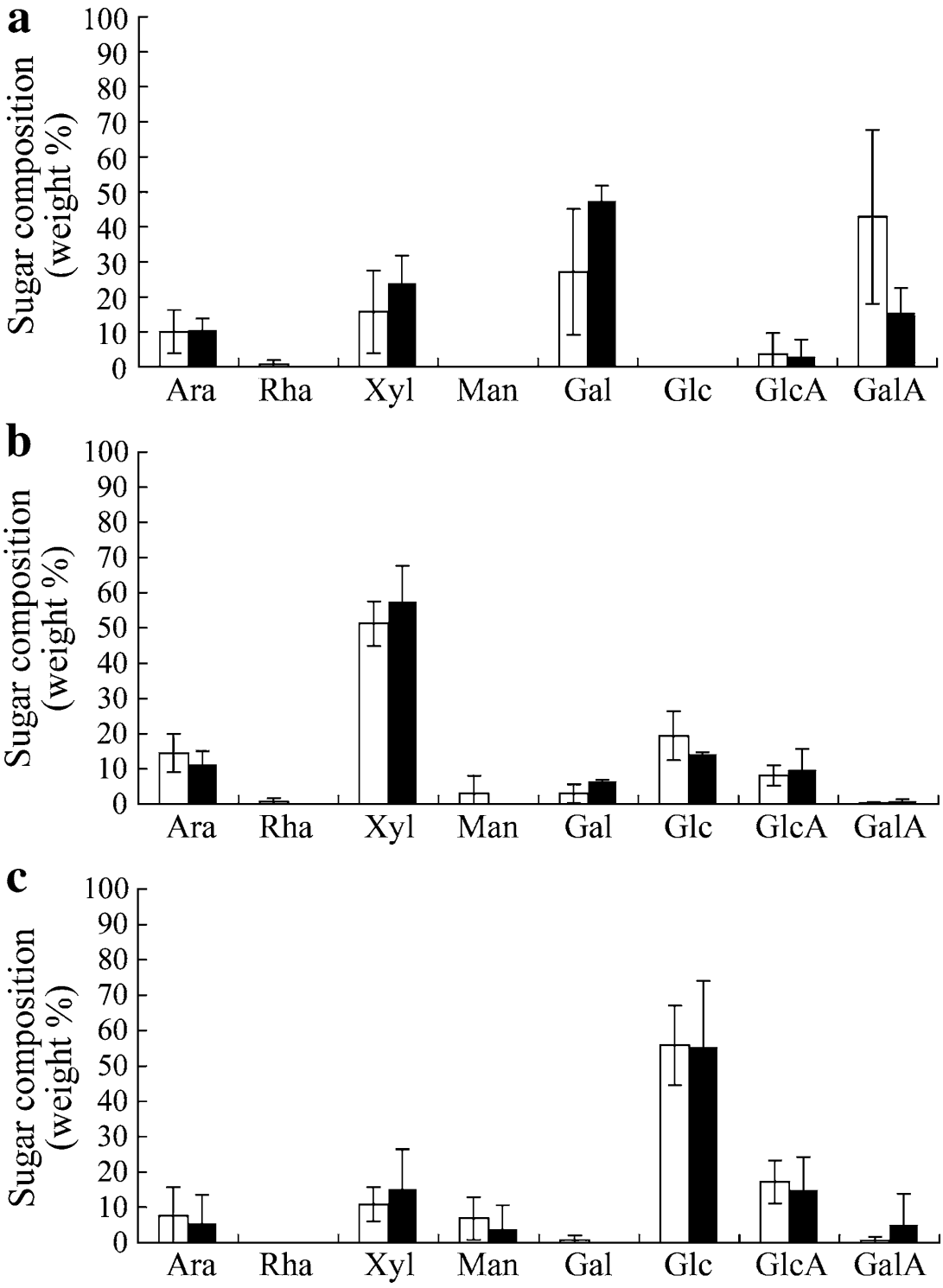

a
b
c
Sugar composition (weight %)
100
90
80
70
60
50
40
30
20
10
0
Ara
Rha
Xyl
Man
Gal
Glc
GlcA
GalA

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 μ m

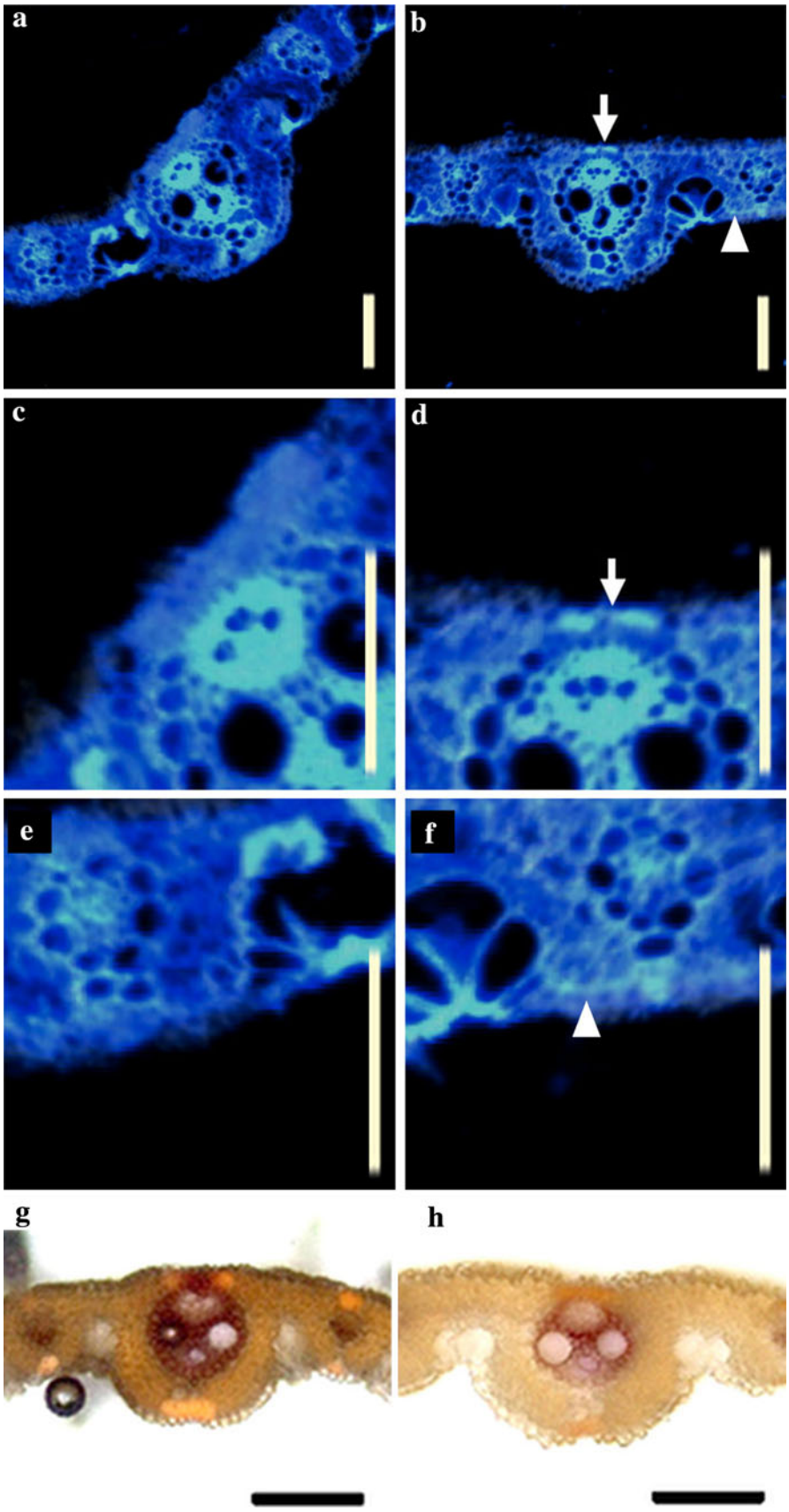

a
b
c
d
e
f
g
h

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

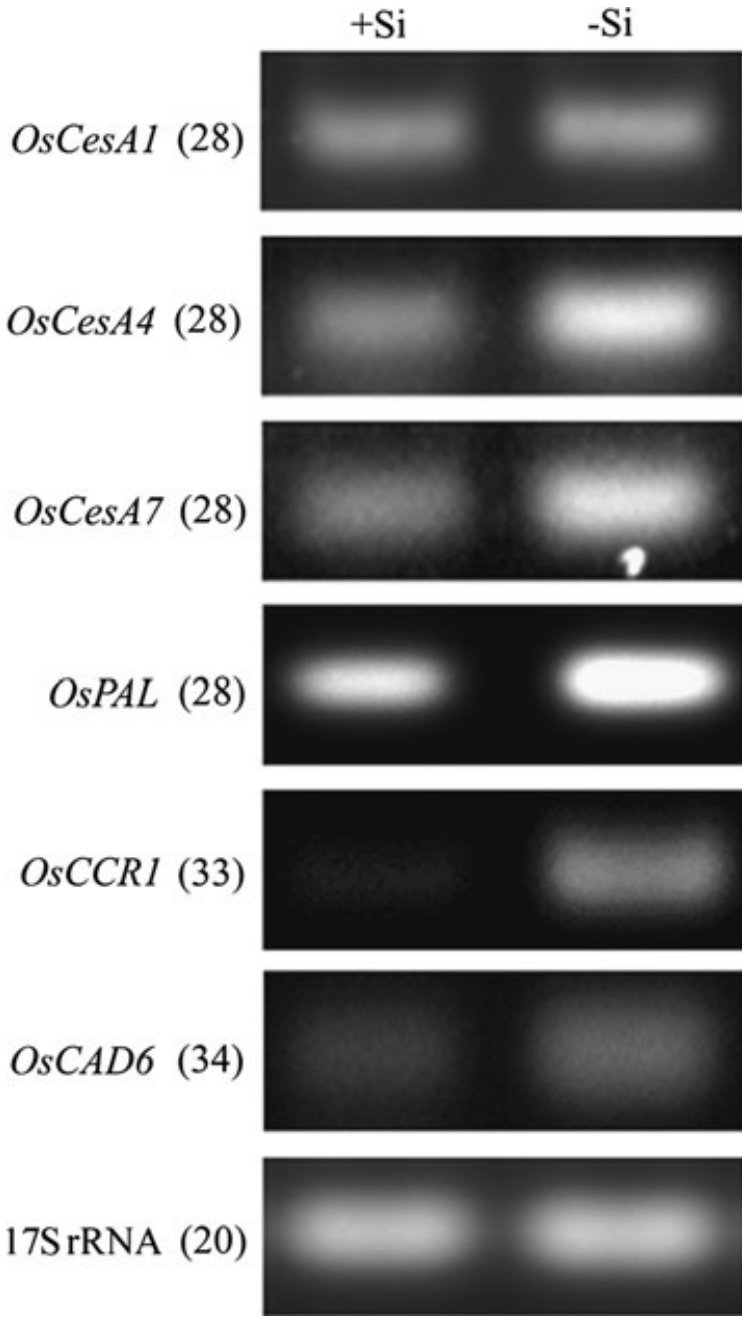

+Si
-Si
OsCesA1 (28)
OsCesA4 (28)
OsCesA7 (28)
OsPAL (28)
OsCCR1 (33)
OsCAD6 (34)
17S rRNA (20)

Fig. 6 Load test in +Si condition and Hold test in –Si condition. Plants were grown hydroponically for 3 weeks under +Si or –Si conditions, and then transferred load test (**a**) and hold test (**b**). Lignin content in the cell wall of the leaf blades in load test (**c**) and hold test (d) (n = 3). Bars=5 cm

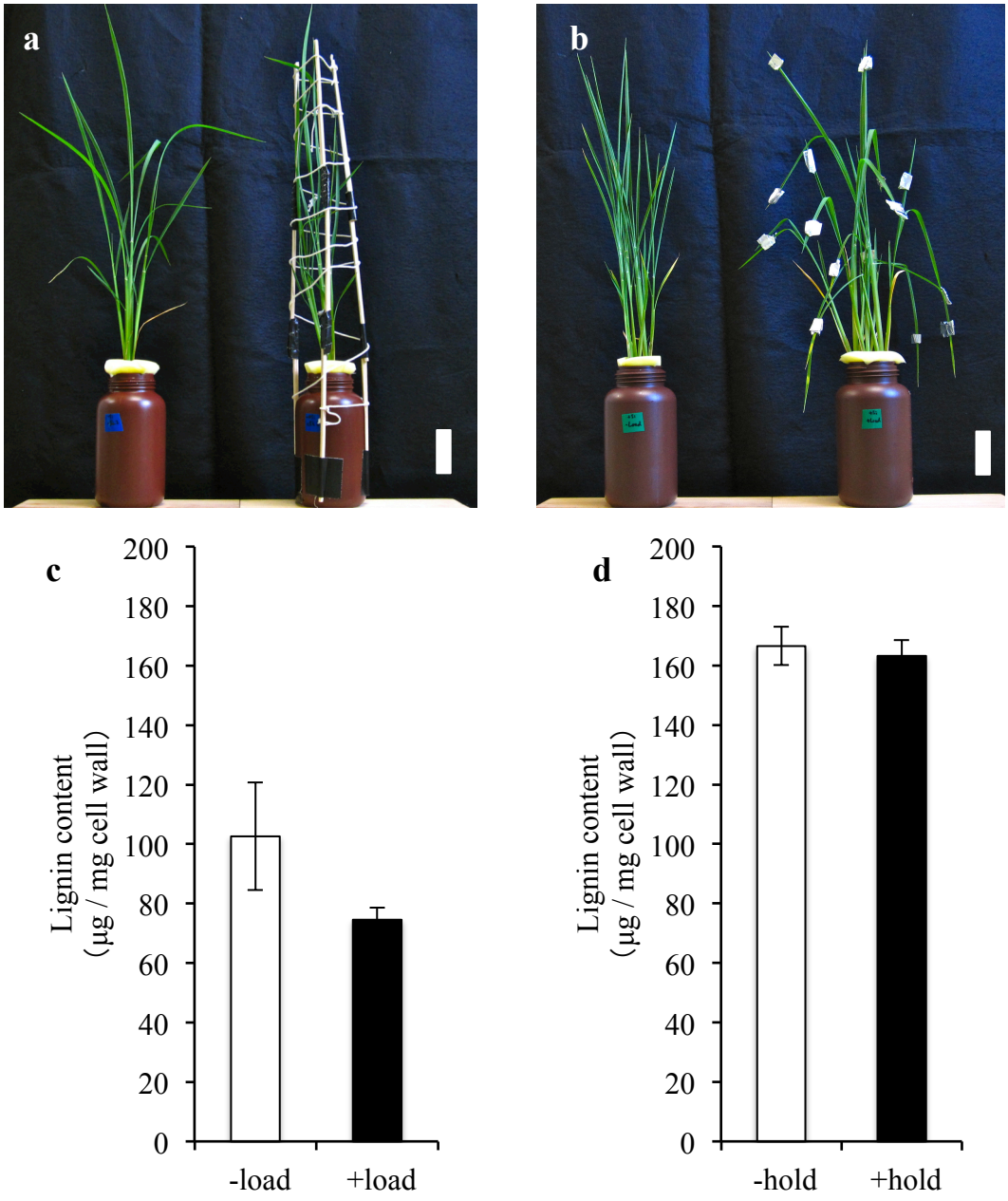
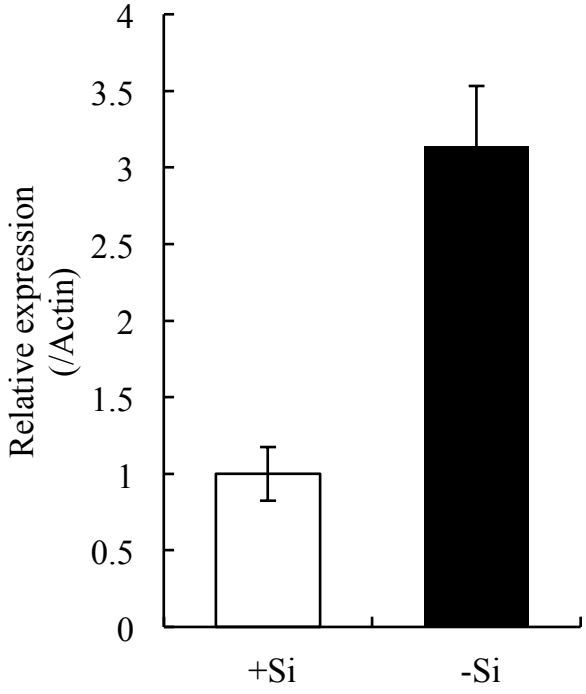

a
b
c
d
Lignin content
(μg / mg cell wall)
200
180
160
140
120
100
80
60
40
20
0
-load
+load
-hold
+hold

Fig. 7 Expression of *OsSWN*s in leaf blades of rice grown under +Si/-Si conditions for 4 weeks. a Expression level of *OsSWN3* measured by qRT-PCR (n = 3). Black and white bars indicate -Si and +Si conditions, respectively. **b** Expression level of *OsSWN2*, *OsSWN6*, *OsSWN7* measured by RT-PCR. Numbers in brackets indicate the PCR cycle number.


Relative expression
(/Actin)
4
3.5
3
2.5
2
1.5
1
0.5
0
+Si
-Si

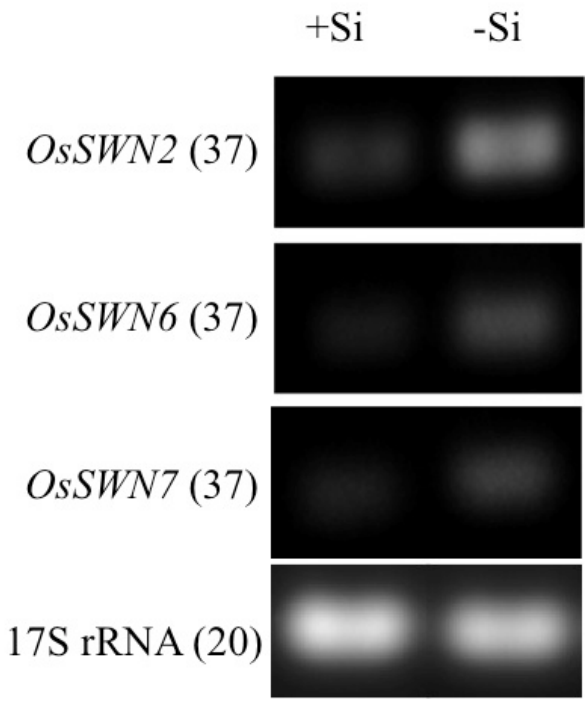
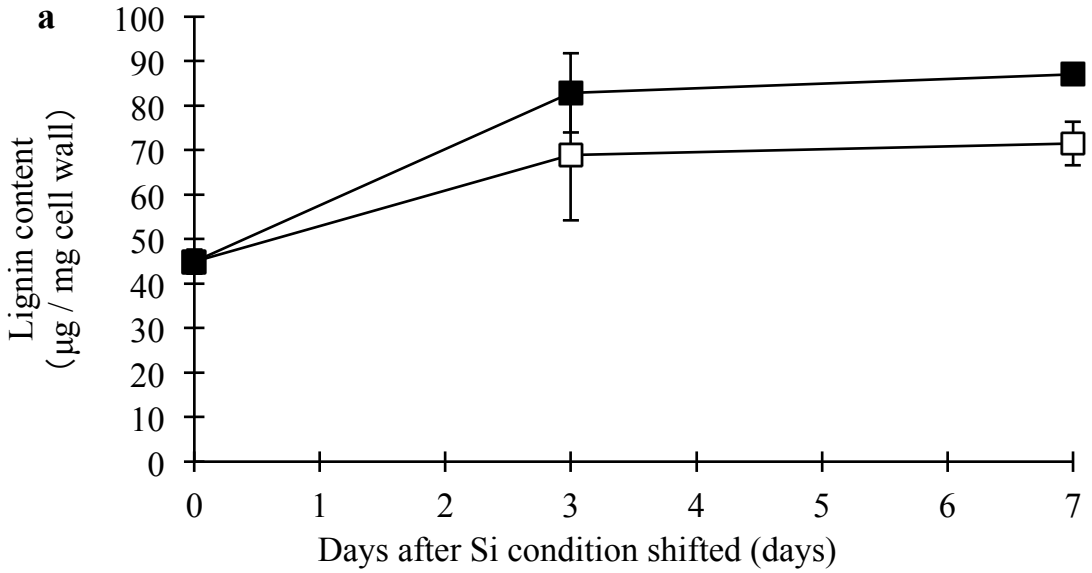
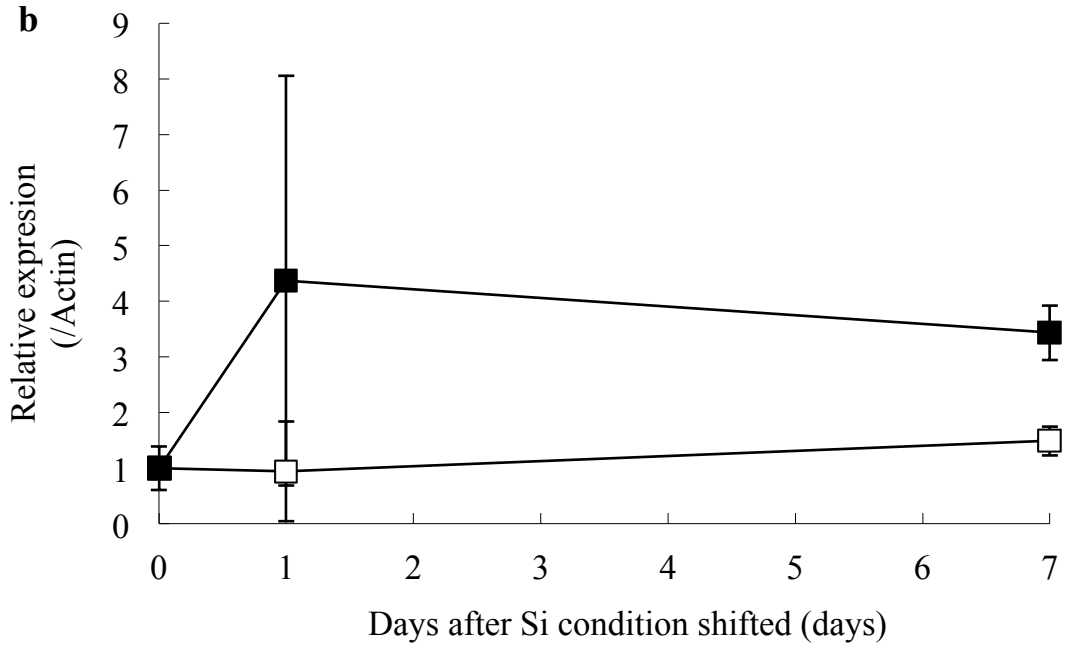

+Si
-Si
OsSWN2 (37)
OsSWN6 (37)
OsSWN7 (37)
17S rRNA (20)

Fig. 8 Time-dependent Analysis When Silicon Condition Was Shifted.

a Lignin content of leaf blade in +Si/-Si and +Si/+Si condition (n = 3).

b Expression level of *OsSWN3* +Si/-Si and +Si/+Si condition (n = 3). Black and white symbol indicate +Si/-Si and +Si/+Si conditions, respectively


a
Lignin content
(μg / mg cell wall)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
Days after Si condition shifted (days)


b
Relative expresion
(/Actin)
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
Days after Si condition shifted (days)

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