Effects of Silicon on Tolerance to Water Deficit and Heat Stress in Rice Plants (*Oryza sativa* L.), Monitored by Electrolyte Leakage

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Abstract : To evaluate the positive effects of silicon on the stress tolerance of rice plants, we measured the electrolyte leakage (El) from leaf tissue caused by desiccation with polyethylene glycol (PEG) and by high temperature to estimate the integrity of cell membranes. The El caused by 30% and 40% solutions of PEG decreased with the increase in the level of Si in leaves. In leaves of plants grown with 100 ppm SiO_2, the level of polysaccharides in cell walls, which is one of the factors related to tolerance to desiccation, was 1.6-fold higher than that in leaves of plants grown without Si. Ultrastructural observations of leaves revealed that polymerized Si accumulated in the walls of epidermal cells but not in those of the mesophyll cells, which are probably the main sites of El. These findings suggested that silicon in rice leaves is involved in the water relations of cells, such as mechanical properties and water permeability and plays a role in preventing El through the synthesis and functions of cell walls. The El caused by high temperature (42.5°C) was also lower in the leaves grown with Si than in the leaves grown without Si, suggesting the involvement of silicon in the thermal stability of lipids in cell membranes. These results suggested that silicon prevents the structural and functional deterioration of cell membranes when rice plants are exposed to environmental stress.

Key words : Electrolyte leakage, *Oryza sativa* L., Silicon, Stress tolerance.

Silicon (Si) has beneficial effects on the growth and development of rice plants (*Oryza sativa* L.) (Agarie et al., 1992 ; Elawad and Green jr, 1979 ; Lewin and Reimann, 1969 ; Ma et al., 1989), but, the physiological role of silicon is not fully understood.

The effects of silicon on the growth of several higher plants become clear under stressful conditions, such as drought (Emadian and Newton, 1989 ; Raven, 1983 ; Tanaka and Park, 1966 ; Yoshida et al., 1959), high and low temperatures (Okamoto, 1969ab), high salt (Ahmad et al., 1992 ; Bradbury and Ahmad, 1990 ; Matoh et al., 1986), supercooling (Larcher et al., 1991), and toxic levels of Al (Li et al., 1989) and Mn (Horiguchi, 1988 ; Horst and Marschner, 1978 ; Vlamis and Williams, 1967). Although the growth of Si-deficient plants is considerably stunted under these conditions, the growth of Si-treated plants is close to normal. Thus, silicon clearly mitigates the harmful effects of stress.

Cell membranes have been proposed as a primary critical target of environmental stress (Liebermann et al., 1958 ; McKersie and Tomes, 1980 ; McKersie et al., 1982 ; Siminovitch et al., 1964). Indeed, many physiological symptoms that are caused by such stress are essentially associated with damage to cell membranes. The fact that Si-treated plants acquire tolerance to these conditions suggests that silicon plays a significant role in maintenance of the integrity of cell membranes. However, to our knowledge, the involvement of silicon in the rigidity of cells has not been studied.

Leakage of cytoplasmic solutes from plant tissues after exposure to stress has often been used as an indicator of membrane lesions. The degree of leakage tendency has been shown to be correlated with the extent of damage due to stress, such as chilling (Leopold, 1980 ; Lieberman et al., 1958 ; Tanczos, 1977), freezing (Dexter et al., 1932 ; Sobczyk et al., 1980), high temperature (Martineau et al., 1979), drought (Dlugokecka and Kacperska-Palacz, 1978 ; Blum and Ebercon, 1981), Al-toxic conditions (Chen et al., 1991) and pollution (Zwiazek and Shay, 1988). In the present study we measured the electrolyte leakage (El) from leaf tissues after exposure of rice plants to osmotic and high-temperature stresses in an attempt to estimate membrane integrity. Possible roles of silicon in the maintenance of normal growth of rice plants under stress are discussed in relation to the integrity of cell membranes.

Material and Methods

1. Plant material

Seeds of rice (*Oryza sativa* L., cv. Koshihikari) were

Abbreviation : El, Electrolyte leakage.

sown in plastic petri dishes that contained distilled water. Husks were removed from the seeds to prevent contamination by SiO_2, which is deposited in the husks. After two weeks, seedlings were transplanted to mats that were floating on the surface of water in culture tanks (480 L), which had been set up in a greenhouse on the campus of Kyushu University, Fukuoka, Japan. The rice plants were cultured as described elsewhere (Agarie et al., 1993) using Yoshida's standard nutrient solution (Yoshida et al, 1976) that contained 20, 40, and 100 ppm SiO_2, and the nutrient solution without SiO_2. To prevent the nutrient solutions from contamination by SiO_2, which is contained in tap-water, deionized water was used. The culture solutions were renewed once a week, and the pH of solutions was adjusted to between 5.0 and 5.5 with 1 M NaOH and 1 M HCl. Leaves of plants grown in the nutrient solution with 100 ppm SiO_2 (+Si) and without SiO_2 (−Si) were used for all experiments with the exception that leaves of plants that had been grown in solutions with various concentrations of SiO_2 were used for measurements of levels of SiO_2 and El. After 30 to 40 days of treatment with SiO_2, when the twelfth leaf on the main culms emerged, samples were taken from the uppermost fully expanded leaves on the main culm and primary tillers. In order to examine the effect of water stress on El, plants were transferred, after 30 days of treatment with SiO_2, to pots (4-liter pots) that were filled with the nutrient solutions with 100 ppm SiO_2 and without SiO_2, that contained mannitol (final concentration, 0.2 M, equivalent to−0.68 MPa of water potential) and were cultured for 20 h.

2. Determination of levels of Si in leaves

Levels of SiO_2 in leaves and in culture solutions were determined by gravimetric procedures and a colorimetric method with molybdenum blue (Yoshida et al., 1976), respectively. Four replicate plants were prepared for each measurement.

3. Electrolyte leakage (El) from leaves of plants treated with polyethylene glycol (PEG) and high temperature.

El from leaf tissues induced by treatment with PEG and high temperature were measured as described elsewhere (Agarie et al., 1995). Samples of leaves were cut into squares of 1 cm^2. Twenty or thirty pieces were put into a 100-mL flask and washed gently with three changes of deionized distilled water to remove adhering electrolytes from the surface. Then, the leaf pieces were submerged in 30 mL of 30% or 40% solutions of PEG (PEG 600) for 24 h at 10°C in darkness. After this treatment, the pieces were quickly washed three times with deionized distilled water. Thirty mL of deionized distilled water were then added and samples were again stored for 24 h at 10°C in darkness. Subsequently, the flask was shaken well at 25°C, and the initial electrical conductivity of the distilled water in the flask (Ei) was measured with an electric conductivity meter (CM-

30ET ; TOA, Tokyo, Japan). After the measurement, the leaf tissues were killed by autoclaving for 15 min to release all ions from the tissues. The flask was shaken well at 25°C, and total electrical conductivity (Et) was measured. El was calculated as :

$$El = [1 − (1 − Ei/Et)/(1 − Ci/Ct)] \cdot 100$$

where, Ei is the initial electrical conductivity, Et is the total electrical conductivity, Ci is the initial electrical conductivity of non-treated control, and Ct is the total electrical conductivity of non-treated control. In general, El occurred in the leaves treated with PEG, but not in the leaves soaked in the solution of 10°C without PEG, suggesting that low temperature has no effect on El. Hence, Ci/Ct values were always very small.

Measurement and calculation of El that was induced by high temperature treatment were performed as described for the treatment with PEG with the exception that ten pieces of leaves were incubated in 30 mL of deionized water at 42.5°C instead of in a solution of PEG.

4. Measurement of the osmotic potential (Ψ_π) of leaves

Discs (6 mm in diameter) were cut from the uppermost fully expanded leaves and immediately put in the sample chamber of a thermocouple psychrometer (Wescor C-52 ; Wescor Inc., Logan, UT, USA). The water potential of the leaf discs was measured psychrometically after an equilibration period of 2 h with a microvoltmeter (Wescor HR33 ; Wescor Inc.). Then the leaf discs were removed from chambers to 1.5-mL plastic tubes. The samples were frozen in a freezer (−80 °C) and then thawed at room temperature. They were returned again to the chambers for measurement of Ψ_π. Ψ_π was measured psychrometically, after an equilibration period of 30 min, with the microvoltmeter.

5. Quantitation of total carbohydrate (TC) and polysaccharide in cell walls (PCW)

TC in leaves was extracted as described by Motomura (1990). The leaf samples were dried for at least two days in a dryer (80°C). The dried samples (0.1 g) were pulverized with a mortar and pestle and hydrolyzed in 10 mL of 0.7 M HCl for 2.5 h. The solution was shaken gently with ion-exchange resin (Amberlite IR-120B ; Organo, Tokyo, Japan) for 10min. After centrifugation at 3000 rpm for 10min at 25°C, the total amount of sugar in the supernatant was determined as described by Nelson (1944). TC was expressed in terms of the dry weight of the leaf samples.

PCW was extracted from leaves as described by Motomura (1990). The pulverized sample (0.1 g) was incubated in 80% EtOH for 10min at 80°C. After centrifugation at 3000 rpm for 10min at 25°C, the pellet was suspended in 0.1 M KOH and boiled for 30min. The solution was neutralized with 1 M CH_3COOH, and treated with amyloglucosidase (600 unit mL^{-1}) for 55min at 60°C. After centrifugation at 3000rpm for 10min at 25°C, the pellet was suspended in 0.5 M HCl and boiled

for 2.5 h. The total sugar in the solution was determined as described by Nelson (1944).

6. Ultrastructural observations

Ultrastructural observations of leaves were made using sections prepared from samples that had been fixed with glutaraldehyde and osmium tetroxide and embedded in Spurr's resin, as described by Ueno (1995). In order to examine the accumulation of silica, sections on nickel grids that had been coated with Formvar were treated with a 2.5% (v/v) solution of hydrogen fluoride for 30min at room temperature. After several washings with distilled water, the sections were stained with uranyl acetate and lead citrate.

Results

1. Effects of silicon on electrolyte leakage

To investigate the effects of silicon on the membrane integrity in water stress conditions, we measured the El from leaf tissues with different levels of Si after exposure
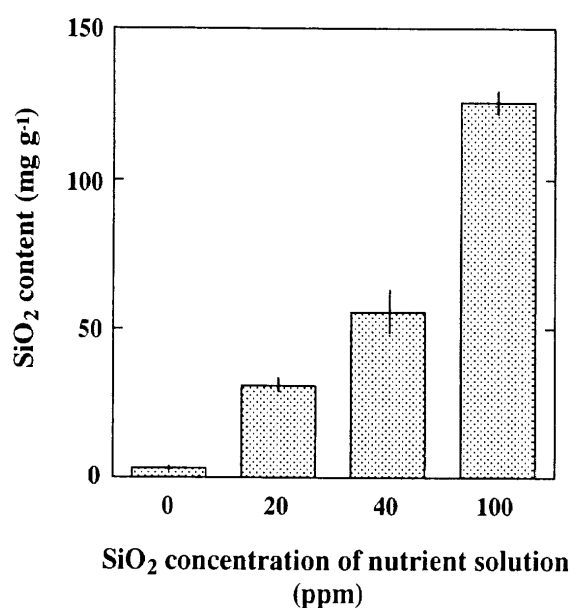
of rice plants to osmotic stress. When rice plants were grown in the nutrient solutions that contained 20, 40 and 100 ppm SiO_2, and without Si, the level of SiO_2 per gram dry weight of leaves increased in parallel with the increase in the level of Si in the nutrient solution (Fig. 1). The leaves of the treated plants were examined for El (Fig. 2). The degree of El decreased with the increase in the level of Si in leaves upon treatment with 30% and 40% solutions of PEG. The treatment with a 40% solution of PEG caused greater El than that with a 30% solution of PEG. Irrespective of the PEG treatments, a significant difference (P<0.05) was found in the El between leaves of plants grown with 100 ppm SiO_2 (+ Si) and without SiO_2 (−Si).

The water stress induced by the addition of mannitol to the nutrient solutions before the treatment with PEG caused an increase in the El of both +Si and −Si leaves, but the degree of increase with water stress was slightly

SiO₂ concentration of nutrient solution (ppm)

Fig. 1. Levels of SiO_2 per gram dry weight in leaves of rice plants that had been grown in nutrient solutions with different concentrations of SiO_2. Data represent the means and SE of results from four replicates.

Table 1. Effects of silicon on leakage of the electrolytes from leaves, as affected by water stress.

Si	Control (%)	Stress (%)
+	29.1±2.9*	38.4±1.8**
−	40.3±3.9	47.2±1.5

The leaf samples were collected from plants that had been grown in nutrient solutions with 100 ppm SiO_2 (+Si) and without SiO_2 (−Si). Electrolyte leakage (El) was measured after treatment with 30% solutions of PEG for 24 h. Data represent the means ± SE of results from four replicates. One and two asterisks indicate statistical significance in differences of the El between +Si and −Si leaves at the 5% and 1% levels, respectively.
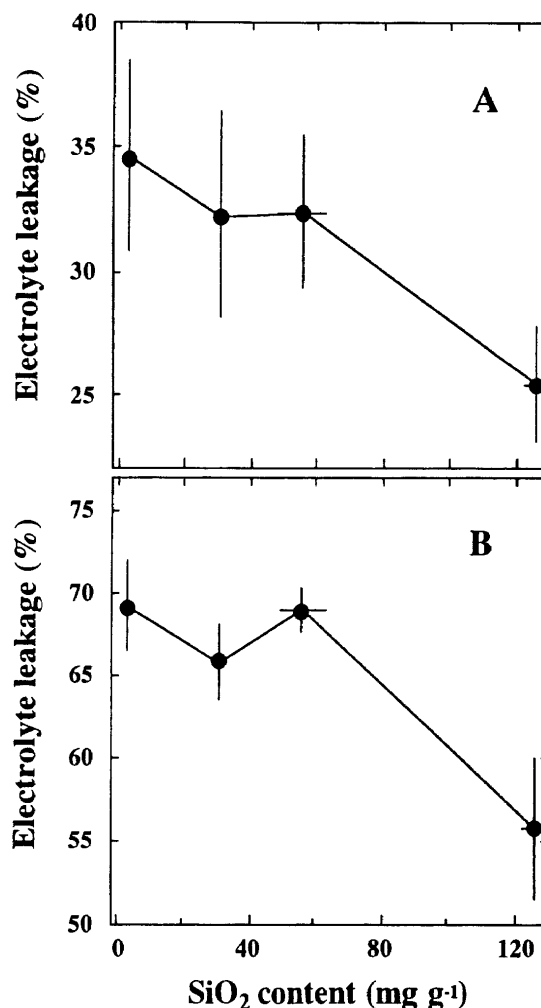

SiO₂ content (mg g⁻¹)

Fig. 2. Electrolyte leakage from leaves with different levels of Si. The leaf samples were collected from plants that had been grown in nutrient solutions with 20, 40 and 100 ppm SiO_2 and without SiO_2. Leakage (El) was measured after treatment with 30% (A) and 40% (B) solutions of PEG for 24 h. Data represent the means and SE of results from four replicates. A significant difference (P<0.05) was found in the El between leaves of plants grown with 100 ppm SiO_2 and without SiO_2 in both PEG treatments.

higher in the former (Table 1). The El from unstressed $-$Si leaves ($40.3\pm3.9\%$) was similar to that from stressed $+$Si leaves ($38.4\pm1.8\%$).

To determine the mechanisms by which PEG causes El leaf tissues, we measured the osmotic potential (osmotic concentration of cell sap, Ψ_π) of leaf tissues. Figure 3 shows the relationships between Ψ_π and El for $+$Si and $-$Si leaves. The Ψ_π in both $+$Si and $-$Si leaves was between -1.2 and -1.9 MPa, indicating that silicon had no effect on the solute concentration of the cell sap. However, there was a tendency for El to decline with decreasing Ψ_π. Moreover, a very significant correlation ($P<0.05$; $r=0.77$) was detected between Ψ_π and El in $+$Si leaves but not in $-$Si leaves.

To compare the properties of cell membranes, we examined the effects of high temperature ($42.5°C$) on El from $+$Si and $-$Si leaves (Fig. 4). In $-$Si leaves, El clearly increased with the passage of time after the high temperature treatment, whereas the El from $+$Si leaves increased only slowly. The El from $-$Si leaves was about 2.5 times that from $+$Si leaves 24 h after the treatment.

2. Effects of silicon on the cell walls

We investigated the level of polysaccharide in cell walls (PCW), which is considered to be one of the factors involved in the water relations of cells (Table 2). Although the level of total carbohydrate (TC) was similar in $+$Si and $-$Si leaves, the level of PCW was about 1.6-fold higher in $+$Si leaves than in $-$Si leaves. As a result, the ratio of PCW to TC was higher in $+$Si leaves than in $-$Si leaves.

The internal structural features of $+$Si and $-$Si leaves were also investigated under an electron microscope

(Fig. 5). There were no clear differences in the arrangement and structure of mesophyll cells or in the thickness and structure of their cell walls between $+$Si and $-$Si leaves (data not shown). However, there was clear difference in the structure of the cell walls of epidermal cells between the two types of leaves. Accumulation of electron-dense particles was observed in the cell walls of $+$Si leaves (Fig. 5A) but not in those of $-$Si leaves (Fig. 5B). The electron-dense particles accumulated at higher levels in the outer walls and in the outer half of the radial walls than in the inner tangential walls and the inner half of the radial walls, forming dense bands of particles. When sections of $+$Si leaves were treated with hydrogen fluoride, the particles disappeared (Fig. 5C). In the sections of $-$Si leaves, treatment with hydrogen fluoride had little effect on the structure of cell walls (Fig. 5D). This indicated that the electron-dense particles were composed of polymerized silica gel (SiO_2 nH_2O). There was no significant difference in the thickness of cell walls of epidermal cells between $+$Si and $-$Si leaves.

Fig. 4. Effects of high temperature on electrolyte leakage from leaves of plants that had been grown in nutrient solutions with 100 ppm SiO_2 (●) and without SiO_2 (○). Measurements were made with the uppermost fully expanded leaves after heating in water at $42.5°C$. Data represent the means and SE of results from four replicates.
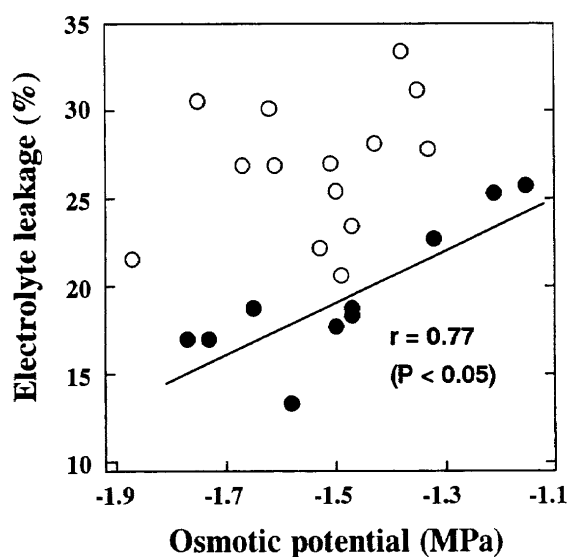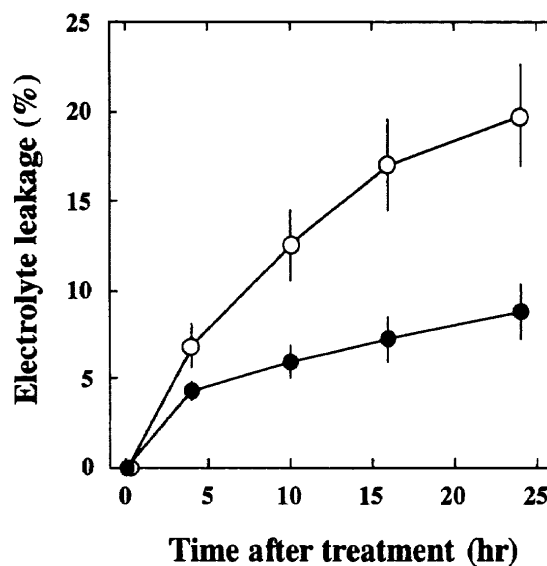

Fig. 3. Relationships between osmotic potential and electrolyte leakage from leaves of plants that had been grown in nutrient solutions with 100 ppm SiO_2 (●) and without SiO_2 (○). Measurements were made with the uppermost fully expanded leaves. Leakage was measured after treatment with a 30% solution of PEG for 24 h. A coefficient of correlation was calculated for the data from $+$Si leaves. r, correlation coefficient. P, level of significance.

Table 2. Effects of silicon on the levels of total carbohydrate (TC) and polysaccharide in cell walls (PCW) of leaves.

Si	TC (mg g^{-1})	PCW (mg g^{-1})	PCW/TC (%)
+	134.6 ± 7.0	64.7 ± 1.7***	48.3 ± 1.5***
−	135.1 ± 5.4	41.0 ± 3.5	30.5 ± 3.0

Data represent the means$\pm$SE of results from four replicates. Three asterisks indicate statistical significance at the 0.1% level.
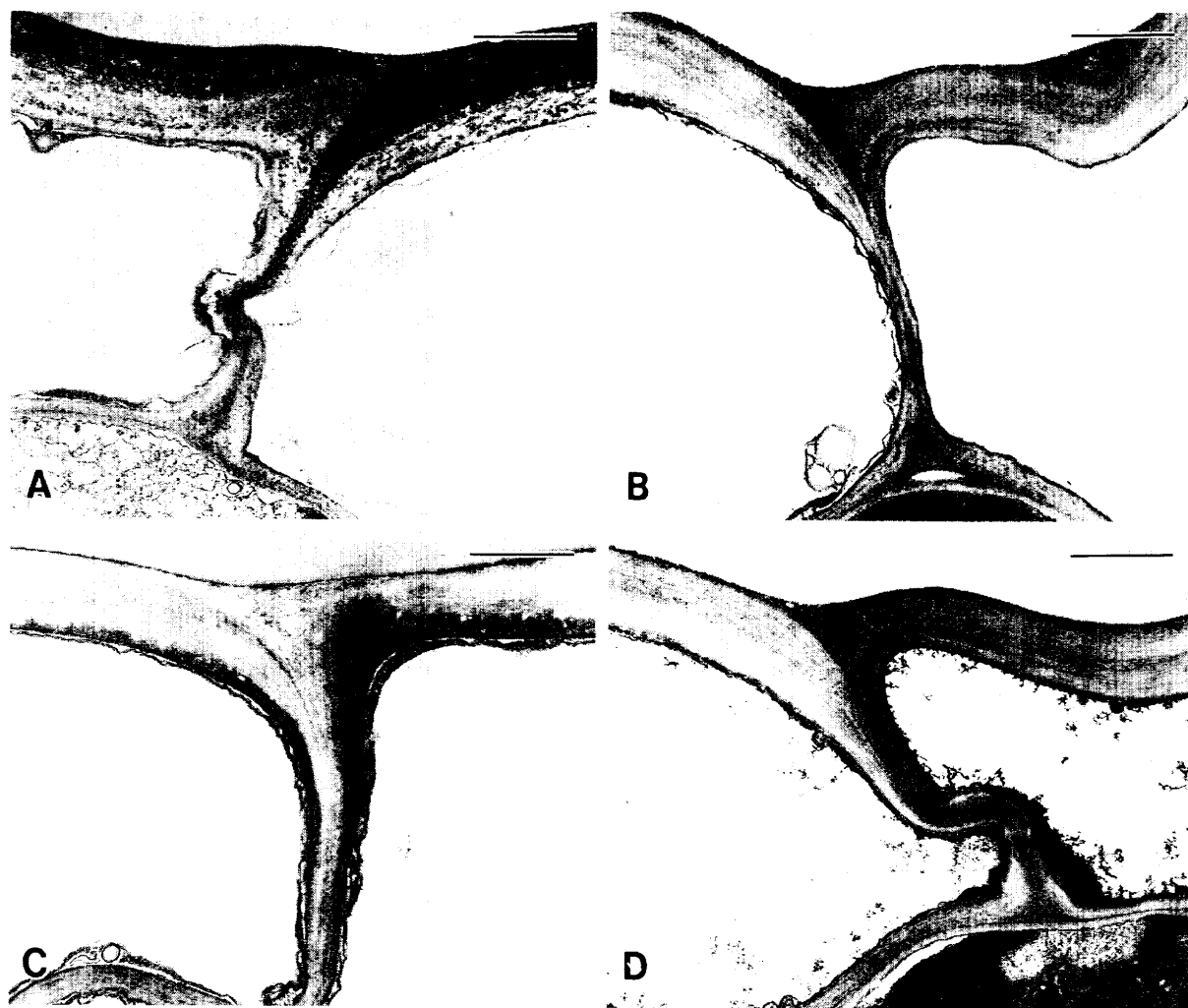
Fig. 5. Fine structure of cell walls of leaves of plants that had been grown in nutrient solutions with 100 ppm SiO_2
(A) and without SiO_2 (B). Scale bars = 1 μm. Note the electron-dense particles that accumulated in the walls of
epidermal cells in +Si leaves (A). The particles disappeared after treatment with hydrogen fluoride of +Si leaves
(C). Epidermal cells of −Si leaves that had been treated with hydrogen fluoride are also shown as a control (D).

Discussion

1. Silicon maintains the integrity of cell membranes

In general, a water deficit causes a profound change in the structure and functions of cell membranes. When cells are subjected to water stress, the lipids of cell membranes change from the liquid-crystalline phase to the gel phase, and finally the permeability of cell membranes increases (Crowe et al., 1987 ; Ferrari-Iliou et al., 1984 ; Maroti et al., 1984 ; Senaratna et al., 1984). Treatment with PEG causes desiccation of leaf tissues. Thus, destructive events associated with desiccation may occur, together with El, in the cell membranes. The water relations of leaves are regulated by complex mechanisms, and many factors responsible for the resistance to desiccation are involved in the regulation of El. Therefore, the El induced by PEG should reflect the secondary effects of the factors that are responsible for the resistance to desiccation, as well as the properties of lipids in cell membranes. In the present study, we measured Ψ_π and PCW in leaves as two factors that are related to the

resistance to desiccation induced by PEG.

The osmotic potential of the 30% and 40% PEG solutions was −2.48 MPa and −3.16 MPa, respectively. When the gradient of water potential between the tissue and the solution of PEG is small, damage to cell membranes is not necessarily severe because the water loss from the tissue is minimal. Indeed, in several higher plants, such as maize and sorghum, Ψ_π is well correlated with El (Premachandra et al., 1989, 1992). A similar relationship was observed in the present study of rice leaves : leaves with a low Ψ_π had limited El. By contrast, Ψ_π was similar in +Si and −Si leaves, while El was always greater from −Si leaves than from +Si leaves over the entire range of values of Ψ_π (Fig. 3). These results suggest that, although Ψ_π could be one of the factors responsible for the regulation of El, Ψ_π does not explain the lower El from +Si leaves, as compared with −Si leaves.

The properties of cell walls, such as rigidity and water permeability, are involved in the water relations of cells and, hence, they are important factors in the regulation of El. From anatomical observations of rice leaves,

Yoshida and Kitagishi (1962) postulated that the accumulation of silica leads to thickening of cell walls, and in particular of the walls of epidermal cells, and that such modification of cell walls also affects the water relations in leaf tissue. In the present study, however, we observed little difference in the thickness of the cell walls between +Si and −Si leaves (Fig. 5), an indication that deposited silica might not participate in the thickening of cell walls in rice leaves.

It seems likely that the EI occurs mainly from the mesophyll rather than through the surface of epidermis. Ultrastructural observations revealed that polymerized SiO$_2$ was predominant in the epidermal cells, and in particular in the outer cell walls of these cells (Fig. 5). The present study showed that +Si leaves contained higher levels of PCW than −Si leaves (Table 2), suggesting the involvement of silicon in the biosynthesis of components of cell walls. Our results suggest that non-polymerized silicon, which is located in the cell walls of mesophyll cells, is responsible for the reduced EI via improvements in the mechanical properties of cell walls and consequent effects on the permeability of the cell walls to water. Silicon reportedly affects the levels and properties of the constituents of cell walls, such as pectic acid, polyuronides, proteins (Schwarz, 1973), galactose (Engle, 1953) polyphenols and lignin (Parry and Kelso, 1975 ; Raven, 1983). Some of the cell wall-bound silicon is present as an ester-like derivative of silicic acid, acting as a bridge in the structural organization of polyuronides (Schwarz, 1973). The crosslinking of silicon with components of cell walls and the interactions of silicon with the biosynthesis and deposition of lignin suggest that silicon affect the mechanical properties of the cell walls, such as their rigidity (Adatia and Besford, 1986 ; Yoshida and Kitagishi, 1962), architecture, resilience (Schwarz, 1973) and elasticity (Emadian and Newton, 1989). An X-ray microanalysis of leaf sections is required to validate this hypothesis.

We also examined the effects of high temperature on EI from leaves (Fig. 4). In this way, the properties of cell membranes could be evaluated without any effects of factors that are related to desiccation tolerance. In the present study, the EI from −Si leaves was always greater than that from +Si leaves. Moreover, the EI from −Si leaves increased with the passage of time after heat treatment, whereas the EI from +Si leaves increased more slowly (Fig. 4). These findings suggest that silicon is also involved in the thermal stability in cell membranes. High temperature influences membrane proteins and lipids that compose cell membranes. Temperature-induced changes in membrane lipids are key factors in the regulation of heat resistance of plant (Somerville and Browse, 1991), but, to our knowledge, the involvement of membrane protein with EI in relation to high temperature resistance of plant has not been studied. In general, EI caused by high temperature is maximum around the transition temperature at which fluid-phase and solid-phase lipids coexist in cell membranes (Blok et al.,1976).

Therefore, the extent of EI caused by the high-temperature treatment presumably reflects differences in the properties of leaves, in terms of responses of lipids to temperature. In diatoms, silicon is considered to be an essential element, and is involved in lipid metabolism (Taguchi et al., 1987). It remains to be investigated whether or not silicon plays a specific role in lipid metabolism in higher plants.

2. Physiological importance of the increased integrity of cell membranes

Transpiration from rice plants significantly increases under Si-deficiency. We have previously confirmed this phenomenon by measurements of gas exchange rates of rice leaves (Agarie et al., 1992, 1993). We also found that, in Si-deficient rice plants, the higher rate of transpiration accounts for a dramatic decline in leaf water potential when plants are subjected to water stress (Agarie et al., 1998). Taking the results of the present study together with the above observations, we suggest that water stress is easily caused in Si-deficient plants due to excessive transpiration (Agarie et al., 1998) and that the cell membranes of the plants are inclined to be easily damaged by water stress. In the experiment that involved pre-treatment with water stress (Table 1), the extent of EI from -Si leaves that had not been subjected to water stress was similar to that of EI from water-stressed +Si leaves. This supports the idea that the cell membranes of Si-deficient plants have already been injured under environmental conditions in the absence of water stress, as a result of excessive transpiration and the inferior quality of their cell membranes.

As mentioned above, the earliest symptoms of injury caused by environmental stress are the loss of function or the destruction of the structure of cell membranes. Severe injury, which cannot be repaired, hasten the aging progress (McKersie et al., 1988 ; Sacher, 1957). Wilting and necrosis, which are often observed as typical symptoms in aged leaves of Si-deficient plants, are conspicuous under conditions of high temperature with low humidity (Lewin and Reimann, 1969). This observation supports the validity of the hypothesis that stress hastens the aging process.

We found previously that the increase in rates of growth and dry-matter production of rice plants upon application of silicon is attributable to prevention of retardation of the rate of photosynthesis and of the destruction of chlorophyll in aged leaves (Agarie et al., 1992, 1993). The present study demonstrates that silicon prevents the structural and functional deterioration of cell membranes when rice plants are exposed to environmental stress. The increased stability of cell membranes caused by silicon might also contribute to prevention of the aging process in leaves. These effects of silicon on the physiological activities of leaves may contribute not only to the maintenance of photosynthetic capacity, but also to the capacity for growth of rice plants under various types of stress.

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**In Japanese with English abstract.
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