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Salt Tolerance Requires Cortical Microtubule Reorganization in Arabidopsis

Che Wang [1,2], Jiejie Li [1] and Ming Yuan [1,*]

[1] *State Key Laboratory of Plant Physiology and Biochemistry, Department of Plant Sciences, College of Biological Sciences, China Agricultural University, Beijing 100094, PR China*
[2] *Biological Science and Technology College, Shenyang Agricultural University, Shenyang 110161, PR China*

Although the results of some studies indicate that salt stress affects the organization of microtubules, it remains an open question whether microtubules play an active role in the plant's ability to withstand salt stress. In the present study, we showed that salt stress-induced wild-type *Arabidopsis* seedling roots display right-handed skewed growth and depolymerization of the cortical microtubules. The results of a long-term observational study showed that cortical microtubules depolymerized then reorganized themselves under salt stress. Stabilization of microtubules with paclitaxel resulted in more seedling death under salt stress, while disruption of microtubules with oryzalin or propyzamide rescued seedlings from death. Seedlings in which the cortical microtubules were reorganized did not succumb to salt stress. These results suggest that both depolymerization and reorganization of the cortical microtubules are important for the plant's ability to withstand salt stress. Depolymerizing microtubules by drugs rescues seedlings from death under salt stress. This rescue effect was abolished by removing calcium from the medium or treatment with a calcium channel inhibitor. Depolymerization of the microtubules is followed by an increase in the free cytoplasmic calcium concentration. The addition of calcium to the growth medium increased the number of seedlings in which recovery of the cortical microtubules occurred, whereas the removal of calcium decreased the number of seedlings in which recovery occurred. Therefore, depolymerization of the cortical microtubules raises intracellular calcium concentrations, while reorganization of the cortical microtubules and seedling survival may be mediated by calcium influx in salt stress.

Keywords: *Arabidopsis* — Calcium — Cortical microtubules — Salt tolerance.

Abbreviations: ABA, abscisic acid; CFP, cyan fluorescent protein; GFP, green fluorescent protein; hpt, hours post-transfer; PLD, phospholipase D; PPM, propyzamide; ROS, reactive oxygen species; SOS, salt overly sensitive; YFP, yellow fluorescent protein.

Introduction

Salt stress has serious consequences for plant growth and crop production. As a result, a great deal of research effort has been devoted to understanding the mechanism(s) of salt tolerance in plants. In saline environments, plants need to sense and transduce the stress signal(s) in order to activate response mechanism(s) to enable them to adapt to the abiotic stress. In recent years, the results of molecular biological and genetic studies have been valuable for identifying the signal transduction pathways and genes involved in the plant's response to salt stress (Serrano and Rodriguez-Navarro 2001, Zhu 2003). Zhu (2002) has proposed that plants have three adaptive responses to salt stress: (i) ion homeostasis; (ii) damage control and repair, or detoxification; and (iii) growth control.

Using molecular biological and biochemical analyses, the salt overly sensitive (SOS) signal transduction pathway, which is important for ion homeostasis in plants, has been elucidated. When plants are salt stressed, the activity of SOS1, the plasma membrane Na^+/H^+ antiporter, is induced (Shi et al. 2000, Shi et al. 2002, Qiu et al. 2003). SOS3, a calcineurin B-like calcium-binding protein, and SOS2, a serine/threonine protein kinase, control the activity of SOS1 (Qiu et al. 2002, Quintero et al. 2002). Salt stress elicits an intracellular calcium signal that is detected by SOS3. SOS3 then relays the signal to SOS2, which is probably responsible for phosphorylating SOS1 (Qiu et al. 2002). Interestingly, Shoji and colleagues reported recently that mutations in SOS1 and SOS2 suppressed both cortical microtubule disruption and helical growth of the *spiral1* mutant of *Arabidopsis thaliana*. They also reported that *sos1* and *sos2* mutants displayed abnormal responses to low doses of microtubule-interacting drugs. On the basis of these two findings, Shoji et al. (2006) concluded that cytoplasmic sodium imbalance may compromise cortical microtubule functions.

Controlling cell growth is another important mechanism that plants have developed to tolerate salt stress. For instance, DELLA proteins of *Arabidopsis*, which restrain cell proliferation and expansion, have been shown to promote survival in response to salt stress (Achard et al. 2006). Cortical microtubules play a vital role in the growth of plant cells (Smith and Oppenheimer 2005). Therefore, it is likely that cortical microtubules are involved in the plant's ability to tolerate salt stress by controlling cell growth.

*Corresponding author: E-mail, mingyuan@cau.edu.cn; Fax, +8610-62733491.

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In addition, other cell constituents that are involved in the plant's response to salt stress interact with cortical microtubules. For example, calcium is an important second messenger in the plant's responses to salt stress (Xiong et al. 2002, Chinnusamy et al. 2005), and the results of several studies have shown that cortical microtubules may be involved in regulating the activity of calcium channels (Thion et al. 1996, Thion et al. 1998). Abscisic acid (ABA), one of the several plant hormones synthesized in response to salt stress, has also been shown to affect the organization of cortical microtubules (Sakiyama and Shibaoka 1990, Shibaoka 1994). Reactive oxygen species (ROS), either as signal molecules or generated from cell components that have been damaged by salt stress in plants, have been shown to cause fragmentation of microtubules in vitro (Xu et al. 2006) and mitotic arrest in tobacco BY-2 cells (Dixit and Cyr 2003). Phospholipase D (PLD), which is involved in ABA- and ROS-mediated processes as part of the plant's response to abiotic stresses (Zhang et al. 2005), has been shown to be involved in the rearrangement of cortical microtubules (Dhonukshe et al. 2003). Furthermore, cortical microtubules have been shown to be involved in the responses to biotic stress (Takemoto and Hardham 2004) and abiotic stresses, the latter of which include osmotic stress (Balancaflor and Hasenstein 1995) and cold acclimation (Wang and Nick 2001, Abdrakhamanova et al. 2003).

Although the results of these studies suggest that cortical microtubules participate in the plant's response to various abiotic stresses, including salt stress, there is no evidence to show that they are actively involved in the plant's tolerance to salt stress. In the present study, we investigated the role of cortical microtubules in the ability of *A. thaliana* to withstand salt stress. Our experimental results showed that cortical microtubules depolymerized and underwent dynamic reorganization when salt stressed. Destabilization of cortical microtubules enhanced the ability of the plant to withstand salt stress. Seedlings in which the organization of the cortical microtubules had been restored survived when salt stressed. Salt stress-induced depolymerization of the cortical microtubules raised the free cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) in cells. The addition of calcium to salt-loaded media increased the number of seedlings in which recovery of the cortical microtubules occurred. Therefore, cortical microtubule reorganization, which itself may regulate calcium influx, is necessary for the plant's ability to withstand salt stress.

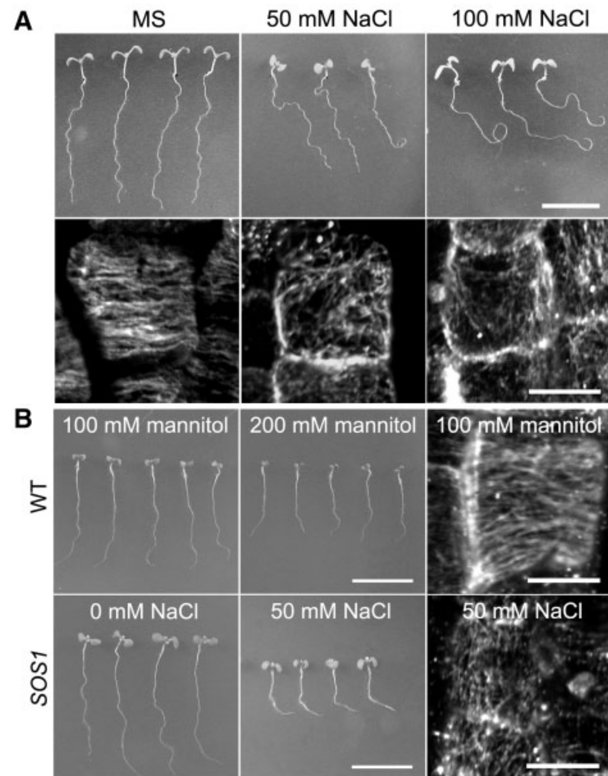

Fig. 1 *Arabidopsis* seedlings displayed skewed root growth and depolymerization of the cortical microtubules under salt stress. (A) Wild-type *Arabidopsis* seedlings were grown for 4 d in 1.5% agar MS media containing either 0, 50 or 100 mM NaCl. Bar = 1 cm for seedling images. Fluorescence images of cortical microtubules in root epidermal cells were observed at 24 h after the seedlings were transferred to MS medium that contained either 0, 50 or 100 mM NaCl. Bar = 10 μm for fluorescent images. Each experimental group involved at least 20 seedlings and each experiment was repeated three times. (B) Sodium is the major factor for inducing depolymerization of the cortical microtubules. Wild-type *Arabidopsis* seedlings were grown for 4 d in MS medium that contained either 100 or 200 mM mannitol. *Arabidopsis sos1* mutant seedlings were grown for 4 d in normal MS medium or MS medium that contained 50 mM NaCl. Cortical microtubules of root epidermal cells were observed at 24 h post-transfer using immunofluoresence microscopy. Bar = 1 cm for seedling images. Bar = 10 μm for fluorescent images. Each experimental group involved at least 20 seedlings and each experiment was repeated three times.

Results

Salt stress-induced right-handed root growth and cortical microtubule depolymerization

Root growth often exhibits a skewed pattern on inclined agar plates when the cortical microtubules are destabilized or disrupted (Thitamadee et al. 2002). We observed that right-handed skewing of root growth always occurred following the addition of varying concentrations of NaCl to the culture medium (Fig. 1A). In fact, the extent of right-handed skewing of root growth (measured as the angle of the skewed root growth against the gravity vector) increased as the concentration of NaCl increased, in a concentration-dependent manner (Table 1).

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Table 1 The skew angle of root growth following salt and microtubule-targeting drug treatments

NaCl in MS	In the absence of microtubule-targeting drugs	In the presence of microtubule-targeting drugs				
		0.1 μM paclitaxel	0.2 μM paclitaxel	0.5 μM paclitaxel	1 μM paclitaxel	0.1 μM oryzalin
0 mM	2.5 ± 3.9 (L)	30.5 ± 3.2 (L)	66.4 ± 2.4 (L)	67.8 ± 4.1 (L)	68.7 ± 3.6 (L)	6.4 ± 4.3 (R)
25 mM	12.2 ± 4.2 (R)	–	–	–	–	–
50 mM	30.1 ± 3.7 (R)	45.3 ± 1.9 (L)	64.8 ± 3.0 (L)	63.2 ± 2.8 (L)	64.4 ± 3.8 (L)	60.1 ± 3.7 (R)
75 mM	38.1 ± 3.8 (R)	–	–	–	–	–
100 mM	63.3 ± 3.4 (R)	63.6 ± 2.4 (R)	62.1 ± 2.6 (R)	53.6 ± 3.8 (R)	61.9 ± 3.1 (L)	61.4 ± 3.7 (R)
125 mM	65.7 ± 3.4 (R)	–	–	–	–	–

Data presented are means ± SE (the direction of growth). (L), left skewed; (R), right skewed. Each experimental group involved at least 20 seedlings and each experiment was repeated three times.

To investigate whether patterns of root growth are regulated by the organization/dynamics of cortical microtubules, we monitored the extent of skewing in root growth in the presence of microtubule-targeting drugs. When the seedlings were grown on medium that contained increasing concentrations of paclitaxel to stabilize the microtubules, the root growth skewed towards the left, instead of towards the right (Table 1). The direction of growth depended on the concentrations of both NaCl and paclitaxel. In fact, treatment with paclitaxel antagonized the effect of NaCl, and NaCl blunted the effects of paclitaxel (Table 1). Addition of the microtubule-disrupting drug, oryzalin, to the medium did not change the direction of NaCl-induced right-handed growth, but made the skew angle bigger (Table 1). These results show that in salt-stressed seedlings, stabilizing the cortical microtubules antagonizes right-handed growth, whereas disruption of the cortical microtubules enhances right-handed growth. Collectively, these results suggest that cortical microtubules depolymerize under conditions of salt stress.

To confirm that skewed root growth under conditions of salt stress is caused by depolymerization of the cortical microtubules, we examined the cortical microtubules in root cells using immunofluorescence microscopy. We observed that the cortical microtubules depolymerized after treatment with salt for 24 h (Fig. 1A). Treatment with 50 mM NaCl resulted in random orientation of the cortical microtubules in root cells about 100–500 μm from the root tip (Fig. 1A). In this region of the cell, the orientation of cortical microtubules was usually perpendicular to the growth axis when the seedlings were grown in normal MS medium (Fig. 1A). Increasing the concentration of NaCl to 100 mM resulted in significant fragmentation of the cortical microtubules (Fig. 1A). The extent of depolymerization of the cortical microtubules correlated well with the size of the angles of skewed root growth. These observations showed that the cortical microtubules depolymerize under conditions of salt stress at an early stage and that the extent of their depolymerization depends on the NaCl concentration.

Sodium is the major factor for the depolymerization of cortical microtubules under salt stress

To determine which factor(s) might play a role in the depolymerization of cortical microtubules in their response to salt stress, we monitored the root growth of wild-type *Arabidopsis* seedlings grown in MS medium containing either 100 or 200 mM mannitol and its *sos1* mutant grown under conditions of salt stress.

The seedlings displayed a normal pattern of root growth when grown in MS medium that contained either 100 or 200 mM mannitol (Fig. 1B). However, when grown on medium containing 50 and 100 mM NaCl, seedlings exhibited a pattern of right-skewed root growth (Fig. 1A). Using immunofluorescence microscopy, we observed that the cortical microtubules remained intact and their orientation was normal in root epidermal cells after treatment with 100 mM mannitol for 24 h (Fig. 1B), while cortical microtubules often exhibited abnormalities with 50 mM NaCl treatment (Fig. 1A). There were obvious differences in the pattern of root growth and the arrangement of the cortical microtubule following the treatments with NaCl and mannitol (Fig. 1A, B). We further counted the frequency of seedlings with microtubule abnormalities. The result indicated that about 86% of seedlings showed microtubule abnormalities in 50 mM NaCl, whereas only 6% seedlings had microtubule abnormalities in 100 mM mannitol.

SOS1 is an Na^+/H^+ antiporter that specifically transports Na^+, but not K^+ (Shi et al. 2000, Shi et al. 2002). The *sos1* mutant is hypersensitive to NaCl because of intracellular accumulation of sodium (Shi et al. 2000,

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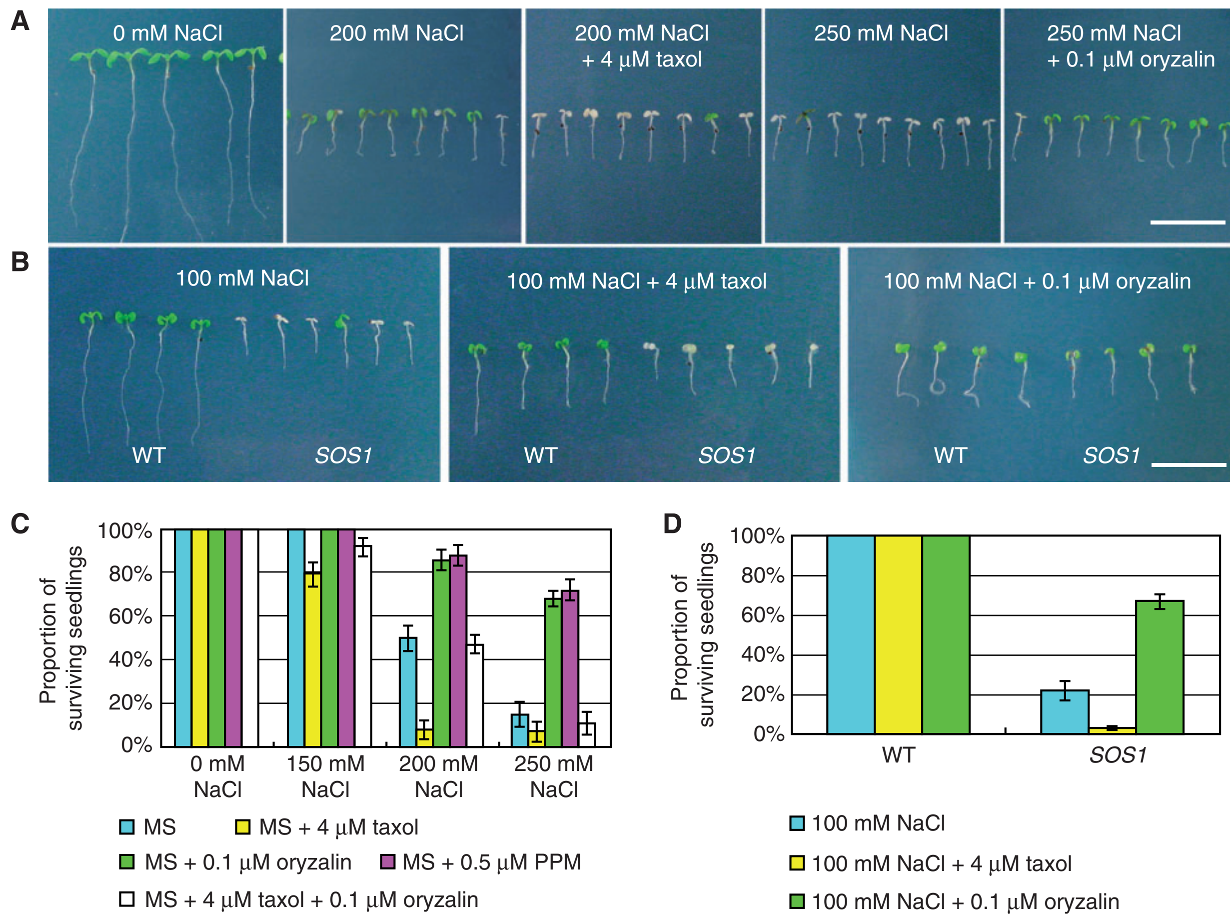

Fig. 2 Depolymerization of the cortical microtubules is important for salt tolerance of *Arabidopsis*. (A) *Arabidopsis* seedlings were grown for 4 d in MS medium that contained varying concentrations of NaCl and/or microtubule-targeting drugs. Bar = 1 cm for seedling images. (B) Seeds of wild-type *Arabidopsis* and its *sos1* mutant were grown for 4 d in MS medium containing 100 mM NaCl and/or microtubule-targeting drugs. Bar = 1 cm for seedling images. (C) The survival of *Arabidopsis* seedlings grown for 4 d in MS medium that contained either 0, 150, 200 or 250 mM NaCl and/or microtubule-targeting drugs. Data presented are means ± SE. Each experimental group involved at least 100 seedlings and each experiment was repeated three times. (D) The survival of wild-type *Arabidopsis* and its *sos1* mutant seedlings grown for 4 d on MS medium containing 100 mM NaCl and/or microtubule-targeting drugs. Data presented are means ± SE. Each experimental group involved at least 50 seedlings and each experiment was repeated three times.

Shi et al. 2002). Root growth of the *sos1* mutant seedlings was the same as that observed in the wild-type seedling when both were grown in normal MS medium. However, the *sos1* mutant seedlings that were grown in MS medium that contained 50 mM NaCl displayed a pattern of right-handed root growth with skew angles larger than those of wild-type plants grown in identical medium. In addition, the extent of depolymerization of the cortical microtubules of *sos1* mutant root cells was higher than that observed in cells from the wild-type plant (Fig. 1A, B). These results demonstrate that the intracellular accumulation of sodium may cause the cortical microtubule to depolymerize. From these observations, we propose that sodium imbalance is the major factor responsible for the depolymerization of cortical microtubules in salt-stressed plants.

Reorganization of the cortical microtubules is important for salt tolerance

The depolymerization of cortical microtubules under salt stress raises the question of whether this process is due to cell damage or a response to salt tolerance. To address this question, we measured the survival of salt-stressed *Arabidopsis* seedlings using bleaching of seedling leaves as an indicator of seedling death and observing the cortical microtubules in the presence or absence of microtubule-targeting drugs after 4 d. *Arabidopsis* seedlings grew vigorously on MS medium supplemented with 4 μM paclitaxel or 0.1 μM oryzalin (Fig. 2C). The number of surviving seedlings declined as the NaCl concentration increased (Fig. 2A). The rates of survival declined after the addition of 4 μM paclitaxel (Fig. 2A, C). In contrast,

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the rate of survival of salt-stressed seedlings increased when 0.1 μM oryzalin or 0.5 μM propyzamide (PPM) was added (Fig. 2A, C). Moreover, the addition of 0.1 μM oryzalin to the medium containing 4 μM paclitaxel resulted in a notable increase in seedling survival under salt stress (Fig. 2A, C). This result suggested that oryzalin antagonized the effect of paclitaxel on the survival of salt-stressed seedlings (Fig. 2C). Our experiments demonstrate that a drug that stabilizes the cortical microtubules reduces the ability of the *Arabidopsis* seedlings to withstand salt stress, whereas drugs that disrupt the organization of the cortical microtubules increase their ability. In addition, we tried to mimic the induction of salt tolerance by transient oryzalin treatment in the absence of salt stress. Three-day-old seedlings were treated with 0.1 μM oryzalin for 10 h before transfer to the MS medium containing 250 mM NaCl without oryzalin. The rates of survival were counted 4 d after transfer of seedlings. The results showed that pre-treatment with oryzalin resulted in an increasing survival rate of $63 \pm 3.4\%$, compared with only $22 \pm 1.9\%$ without oryzalin pre-treatment. Therefore, we conclude that the depolymerization of cortical microtubules in *Arabidopsis* is not a passive consequence of salt stress, but is a necessary component for withstanding salt stress.

To investigate this hypothesis further, we conducted additional studies using the *Arabidopsis sos1* mutant, which is hypersensitive to NaCl. The addition of 4 μM paclitaxel or 0.1 μM oryzalin to the normal MS medium had no obvious effect on seedling growth; both the wild-type and *sos1* mutant seedlings grew vigorously. However, the rate of death of *sos1* mutant seedlings grown in MS medium containing 100 mM NaCl was increased by the addition of 4 μM paclitaxel, but reduced by the addition of 0.1 μM oryzalin (Fig. 2B, D). These observations reinforce our conclusion that the depolymerization of cortical microtubules is important for the plant's ability to develop salt tolerance.

To investigate salt stress-induced dynamics and the organization of the cortical microtubules, 3-day-old *Arabidopsis* seedlings expressing green fluorescent protein (GFP)–tubulin were transferred to the 200 mM NaCl medium, and cortical microtubules were observed at various times after the treatment (Fig. 3A). Because GFP–tubulin fusion proteins are not fully incorporated into cortical microtubules in roots of *Arabidopsis* seedlings expressing GFP–tubulin (Abe and Hashimoto 2005), we observed the cortical microtubules in their cotyledon pavement cells.

After the seedlings were transferred to the salt medium, the organization of the cortical microtubules remained normal, and no significant changes were observed until 12 h post-transfer (hpt) (Fig. 3A). However, disruption of cortical microtubules was observed in some of the seedlings at 14 hpt (Fig. 3A) and in most of the seedlings at 16 hpt (Fig. 3A). The disruption of cortical microtubules persisted at 18, 20, 34 and 36 hpt (Fig. 3A). Nevertheless, cortical microtubules had recovered their normal conformation in cells of some of the seedlings at 38 hpt. This recovery continued and the conformation of the cortical microtubules was fully recovered in all cells at 40 and 42 hpt (Fig. 3A). These observations demonstrate that salt stress causes transient depolymerization of the cortical microtubules and is followed by their reorganization or restoration of their organization.

In order to establish that reorganization of the cortical microtubules occurs in surviving salt-stressed seedlings, *Arabidopsis* seedlings were grown on MS medium for 3 d and then were transferred to media that contained varying concentrations of NaCl and/or microtubule-interacting drugs. The cortical microtubules were observed at 16 and 40 hpt, and the number of seedlings with intact cortical microtubules was counted. The cortical microtubules in cotyledon pavement cells were well organized in seedlings that were not salt stressed (Fig. 3B and Table 2). Treatment with either 200 or 250 mM NaCl lowered the number of seedlings with intact cortical microtubules when observed at 16 hpt (Fig. 3B and Table 2). Treatment of these salt-stressed seedlings with 0.1 μM oryzalin did not change the number of seedlings with intact cortical microtubules (Fig. 3B and Table 2). When oryzalin was replaced with 4 μM paclitaxel, the number of seedlings with intact cortical microtubules increased (Fig. 3B and Table 2). These observations suggested that salt stress-induced depolymerization of the cortical microtubules can be inhibited by treatment with the microtubule-stabilizing drug, paclitaxel, which, in turn, results in a reduction of *Arabidopsis* seedling survival. At 40 hpt, $38 \pm 6.3\%$ (in 200 mM NaCl) and $17 \pm 2.5\%$ (in 250 mM NaCl) of salt-stressed seedlings, respectively, had intact cortical microtubules (Fig. 3B and Table 2). The addition of 0.1 μM oryzalin increased these respective numbers to 65 ± 7.5 and $50 \pm 7.5\%$ (Fig. 3B and Table 2). However, the addition of 4 μM paclitaxel reduced the number to $7 \pm 1.4\%$ in 250 mM (Table 2).

Because the treatment with 4 μM paclitaxel resulted in a much lower number of cells with intact cortical microtubules at 40 hpt than at 16 hpt, this result suggests that paclitaxel hindered cortical microtubule recovery. In contrast, the results suggest that treatment with oryzalin facilitates cortical microtubule reorganization, because after treatment with 0.1 μM oryzalin the number of cells with normal cortical microtubules at 40 hpt was greater than at 16 hpt, although the cortical microtubules initially depolymerized before reorganizing. Because the number of cells in which reorganization of the cortical microtubule occurred coincided with the number of surviving seedlings, we propose that reorganization of the cortical microtubules probably plays a role in the ability of *Arabidopsis* to withstand salt stress.

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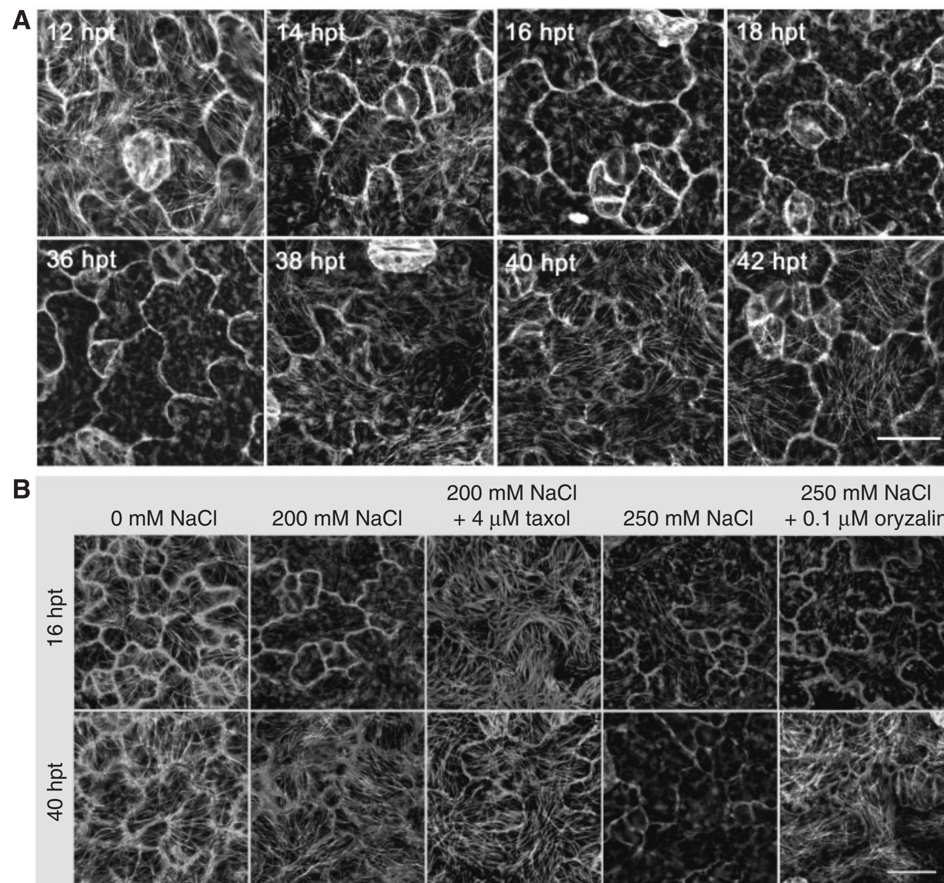

Fig. 3 Cortical microtubules initially depolymerize and then reorganize themselves when under salt stress. (A) Cortical microtubules of cotyledon pavement cells of *Arabidopsis* that expressed GFP–tubulin that were grown in MS medium containing 200 mM NaCl and were observed 12, 14, 16, 18, 34, 36, 38, 40 and 42 h post-transfer. Bar = 20 μm for fluorescent images. (B) Cortical microtubules of cotyledon pavement cells of *Arabidopsis* that expressed GFP–tubulin that were grown in MS medium containing varying concentrations of NaCl and/or microtubule-targeting drugs and were observed at 16 and 40 h post-transfer. Bar = 20 μm for fluorescent images. Each experimental group involved at least 20 seedlings and each experiment was repeated three times.

Table 2 The number of salt-stressed seedlings with intact cortical microtubules following treatment with microtubule-targeted drugs

NaCl in MS	In the absence of microtubule-targeting drugs	In the presence of microtubule-targeting drugs	
		4 μM paclitaxel	0.1 μM oryzalin
0 mM	100% (16 h); 100% (40 h)	–	–
150 mM	18 ± 6.3% (16 h); 78 ± 3.8% (40 h)	–	–
200 mM	15 ± 2.5% (16 h); 38 ± 6.3% (40 h)	100% (16 h); 50 ± 5.0% (40 h)	11 ± 3.8% (16 h); 65 ± 7.5% (40 h)
250 mM	13 ± 3.8% (16 h); 17 ± 2.5% (40 h)	57 ± 6.3% (16 h); 7 ± 1.4% (40 h)	18 ± 6.4% (16 h); 50 ± 7.5% (40 h)

The data are expressed as the percentage of *Arabidopsis* seedlings in the seedlings observed with intact cortical microtubules ± SE (hours post-treatment). Each experimental group involved at least 20 seedlings and each experiment was repeated three times.

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Table 3 Recovery of cortical microtubules is important for the survival of *Arabidopsis* seedlings under salt stress

NaCl in normal MS medium	In the absence of oryzalin	In the presence of 0.1 μM oryzalin
200 mM	100% (R); $15 \pm 2.5\%$ (D)	100% (R); $13 \pm 5.0\%$ (D)
250 mM	100% (R); $13 \pm 3.8\%$ (D)	100% (R); $15 \pm 6.6\%$ (D)

Data presented are means ± SE. (D), *Arabidopsis* seedlings with disrupted cortical microtubules; (R), *Arabidopsis* seedlings with recovered cortical microtubules. Each experimental group involved at least 20 seedlings and each experiment was repeated three times.

To investigate further whether the reorganization of the cortical microtubules is important for the survival of salt-stressed *Arabidopsis*, we observed the cortical microtubules of the *Arabidopsis* seedlings which express GFP–tubulin after various treatments at 40 hpt. Specifically, we observed two groups of cortical microtubules: cortical microtubules that remained depolymerized (Fig. 3B) and cortical microtubules whose array was restored (Fig. 3B). According to our observations presented above, the treatment of oryzalin has a promoting effect on the tolerance of *Arabidopsis* under salt stress; the seedlings with disrupted cortical microtubules or with normal 'restored' cortical microtubules were then selected from those cultured in the absence and presence of 0.1 μM oryzalin under salt stress, and cultured separately in the same conditions as before the selection. The surviving *Arabidopsis* seedlings for each treatment were counted at 56 h after the culture (Table 3). All the salt-stressed seedlings in which the cortical microtubules were initially disrupted and then recovered survived. In contrast, most of the salt-stressed seedlings in which the cortical microtubules remained disrupted died (Table 3). Therefore, the recovery of cortical microtubules after salt stress-induced depolymerization is an indispensable step for *Arabidopsis* survival and tolerance to salt stress.

Our data demonstrate that reorganization of the cortical microtubules made *Arabidopsis* seedlings more tolerant to salt stress. The addition of microtubule-disrupting drugs increased the number of seedlings in which recovery of the salt stress-induced cortical microtubule occurred and enhanced seedling tolerance to salt stress. In contrast, the addition of microtubule-stabilizing drugs hindered salt stress-induced depolymerization of the cortical microtubules and reduced seedling tolerance to salt stress. Therefore, both depolymerization and reorganization of the cortical microtubules are important processes involved in a plant's responses to salt stress. Interfering with either of these processes affects the tolerance of *Arabidopsis* to salt stress.

Depolymerization of cortical microtubules elevates cytoplasmic calcium concentration in response to salt stress

Calcium is thought to be involved in the plant's responses to salt stress. Furthermore, depolymerization of the cortical microtubules may up-regulate the activity of calcium channels in plant cells (Thion et al. 1996, Thion et al. 1998). To investigate the interaction of calcium and depolymerization of the cortical microtubules in salt stress, we counted the surviving salt-stressed *Arabidopsis* seedlings that were treated with oryzalin after calcium was removed from the medium using the calcium chelator, EGTA, or after calcium channels were blocked by lanthanum chloride ($LaCl_3$).

Removal of $CaCl_2$ from the MS medium by the addition of 1 mM EGTA had a serious effect on the survival of salt-stressed *Arabidopsis* seedlings. The percentage of surviving seedlings dropped from 100% when grown in normal MS medium to $13 \pm 3.0\%$ when grown in medium that contained 150 mM NaCl. No seedlings survived when the NaCl concentration in the medium was 200 mM NaCl (Fig. 4A). Unlike the results obtained from previous experiments, the addition of 0.1 μM oryzalin had no rescuing effect on the rate of survival of salt-stressed seedlings when calcium was removed from the medium (Fig. 4A). We also assessed the effects of treatment with $LaCl_3$ in salt-stressed *Arabidopsis* seedlings (Fig. 4B). All seedlings survived when grown in normal MS medium that did not contain NaCl after the addition of either 100 or 200 μM $LaCl_3$, and/or 0.1 μM oryzalin (Fig. 4B). Increasing concentrations of $LaCl_3$ worsened the rate of survival in a concentration-dependent manner (Fig. 4B). No obvious rescue effect of oryzalin on the survival of seedlings was observed in the 200 mM NaCl media containing 100 or 200 μM $LaCl_3$ (Fig. 4B). Therefore, the removal of extracellular calcium or blocking the entry of calcium into cells severely reduced the survival of *Arabidopsis* seedlings and eliminated the rescue effect associated with depolymerization of the cortical microtubules. This suggests that the oryzalin-mediated rescue and depolymerization of the cortical microtubules involves calcium influx.

We further measured the $[Ca^{2+}]_{cyt}$ in cotyledon pavement cells before and after the depolymerization of the cortical microtubules induced by salt stress (Fig. 4C). Because our observations indicated that depolymerization of the cortical microtubule does not occur at 12 hpt but occurs between 16 and 18 hpt, $[Ca^{2+}]_{cyt}$ was measured at 12 and 18 hpt, using *Arabidopsis* seedlings that expressed the calcium indicator, yellow cameleon 3.6 (YC3.6). The $[Ca^{2+}]_{cyt}$ of cotyledon pavement cells in seedlings grown on normal MS medium is approximately 1.24×10^{-7} M at 12 hpt and 1.46×10^{-7} M at 18 hpt (Fig. 4C and Table 4). When the seedlings were treated with 200 mM NaCl, $[Ca^{2+}]_{cyt}$ decreased to approximately 0.75×10^{-7} M at

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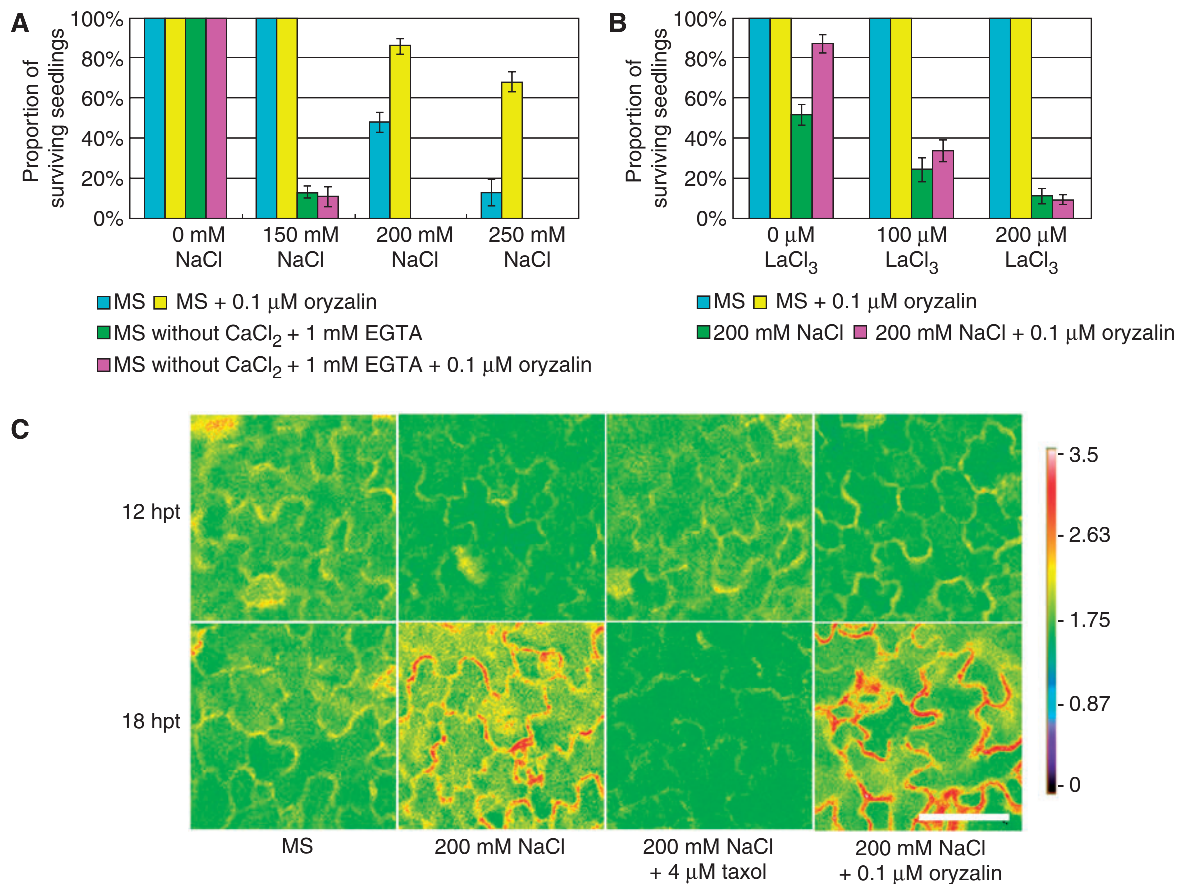

Fig. 4 Depolymerization of the cortical microtubule raises $[Ca^{2+}]_{cyt}$. (A) The survival of salt-stressed *Arabidopsis* seedlings that were grown for 4 d in MS medium that either contained or did not contain calcium and/or treated with oryzalin. Data presented are mean ± SE. Each experimental group involved at least 100 seedlings and each experiment was repeated three times. (B) The survival of salt-stressed *Arabidopsis* seedlings that were grown for 4 d in MS medium, containing $LaCl_3$ and oryzalin. Data presented are means ± SE. Each experimental group involved at least 100 seedlings and each experiment was repeated three times. (C) Ratio images made at 12 or 18 h post-transfer of *Arabidopsis* seedlings that expressed YC 3.6 that were grown on normal MS medium or MS medium containing 200 mM NaCl and/or microtubule-targeting drugs. Each experimental group involved at least 20 seedlings and each experiment was repeated three times. Bar = 30 μm.

12 hpt (Fig. 4C and Table 4), but increased to about 2.56×10^{-7} M at 18 hpt (Fig. 4C and Table 4). The addition of 0.1 μM oryzalin caused a further increase in $[Ca^{2+}]_{cyt}$ in the salt stress-induced cells at 18 hpt. In fact, the $[Ca^{2+}]_{cyt}$ was six times higher than the concentration measured in cells at 12 hpt ($\sim 4.21 \times 10^{-7}$ M vs. $\sim 0.69 \times 10^{-7}$ M). This difference is statistically significant when the data were analyzed using a paired Student's *t*-test (Table 4). In contrast, the addition of 4 μM paclitaxel had a remarkable effect in that the drug completely inhibited the increases in $[Ca^{2+}]_{cyt}$ (Fig. 4C and Table 4).

The number of seedlings that displayed depolymerization of the cortical microtubule at 18 hpt after salt treatment with addition of an extra 3 mM $CaCl_2$ to or depleting calcium from the medium was also measured. Addition or depletion of calcium had no obvious effect on salt stress-induced depolymerization of the cortical microtubules (Table 5). This result suggests that the increase of $[Ca^{2+}]_{cyt}$ occurred after depolymerization of the cortical microtubules and that the increase probably does not play a role in microtubule depolymerization. However, our data suggest that $[Ca^{2+}]_{cyt}$ plays a role in seedling survival. Thus $[Ca^{2+}]_{cyt}$ is likely to be involved in the recovery of the cortical microtubules and seedling survival.

To investigate whether the increase in $[Ca^{2+}]_{cyt}$ mediates the recovery of the cortical microtubules, we observed the cortical microtubules at 40 hpt after the salt treatments with addition of an extra 3 mM $CaCl_2$ to or depleting calcium from the medium (Table 5). In MS medium that contained 200 and 250 mM NaCl, 40 ± 5.0 and 17 ± 3.8% of seedlings observed, respectively, exhibited recovery of the cortical microtubules. The addition of

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Table 4 Calcium concentrations in root cells after treatments

NaCl concentration in MS medium		In the absence of microtubule drugs	In the presence of microtubule drugs	
			4 μM paclitaxel	0.1 μM oryzalin
0 mM	YFP/CFP ratio	1.81 ± 0.04 (12 h); 1.85 ± 0.04 (18 h)	–	—
	$[Ca^{2+}]_{cyt}$	1.24×10^{-7} M (12 h); 1.46×10^{-7} M (18 h)	–	–
200 mM	YFP/CFP ratio	1.72 ± 0.08 (12 h); 2.02 ± 0.06 (18 h)*	1.78 ± 0.06 (12 h); 1.70 ± 0.12 (18 h)	1.71 ± 0.10 (12 h); 2.17 ± 0.07 (18 h)*
	$[Ca^{2+}]_{cyt}$	0.75×10^{-7} M (12 h); 2.56×10^{-7} M (18 h)	1.08×10^{-7} M (12 h); 0.63×10^{-7} M (18 h)	0.69×10^{-7} M (12 h); 4.21×10^{-7} M (18 h)

$[Ca^{2+}]_{cyt}$ was measured by the emission ratio of YFP to CFP, and converted into $[Ca^{2+}]_{cyt}$. Data presented are means ± SE. Each experimental group involved at least 20 seedlings and each experiment was repeated three times. A paired Student's *t*-test was performed to compare the values at 12 and 18 h. $^{*}P = 0.05$.

Table 5 The depolymerization of cortical microtubules, but not the recovery of cortical microtubules, occurs independently of changes in cytoplasmic calcium concentrations under salt stress

NaCl concentrations in MS medium	MS medium	MS medium without $CaCl_2$ + 1 mM EGTA	MS medium supplemented with 3 mM $CaCl_2$
0 mM	100% (16 h); 100% (40 h)	$95 \pm 5.0\%$ (16 h); $87 \pm 7.3\%$ (40 h)	100% (16 h); 100% (40 h)
200 mM	$16 \pm 3.8\%$ (16 h); $40 \pm 5.0\%$ (40 h)	$10 \pm 2.5\%$ (16 h); 0% (40 h)	$23 \pm 3.8\%$ (16 h); $67 \pm 6.0\%$ (40 h)
250 mM	$12 \pm 2.5\%$ (16 h); $17 \pm 3.8\%$ (40 h)	$7 \pm 3.8\%$ (16 h); 0% (40 h)	$20 \pm 5.0\%$ (16 h); $45 \pm 6.6\%$ (40 h)

Data presented are the percentage of *Arabidopsis* seedlings with intact cortical microtubules ± SE (hours post-treatment). Each experimental group involved at least 20 seedlings and each experiment was repeated three times.

3 mM $CaCl_2$ to the two salt-loaded media increased the number of seedlings with recovered cortical microtubules to 67 ± 6.0 and $45 \pm 6.6\%$. In contrast, when calcium was removed by the calcium chelator from the two salt-loaded media, the cortical microtubules did not recover (Table 5). These observations indicate that the addition of calcium increases the number of salt-stressed seedlings in which recovery of the cortical microtubules occurs, whereas the removal of calcium did not have an effect on the recovery of the cortical microtubules. These results indicate that calcium is required for the process of recovery of the cortical microtubules under salt stress.

Discussion

In the present study, we tested the hypothesis that cortical microtubules are involved in plant tolerance under salt stress. Our observations demonstrated that cortical microtubules disassemble and reorganize in response to salt stress in *Arabidopsis*. Suppression of microtubule disassembly impairs the salt tolerance, while disassembly of microtubules promotes it. The disassembly and reassembly of cortical microtubules are related to calcium influx. Our study provides evidence to show that cortical microtubules play an active role in plant tolerance under salt stress, and suggests that microtubules might have a sensory role during the response to salt stress.

Cortical microtubules play a role in plant tolerance to salt stress

Multiple processes and cellular components are involved in the responses of plants to abiotic stresses (Zhu 2002). Several studies have indicated that the cortical microtubules play a role in the adaptation of plants to environmental stresses. Depolymerization of the cortical microtubules has been described during cold acclimation

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in wheat. In cold-tolerant wheat cultivars, the cortical microtubules partially disassemble during cold acclimation to form cold-stable microtubules, which are absent in cold-sensitive cultivars. Transient disassembly of the cortical microtubules by pronamide can also induce the cultivars can survive in freezing cold temperatures (Abdrakhamanova et al. 2003). In addition, the functional activity of the cortical microtubules may be involved in the response to cold acclimation because cortical microtubules in cold-resistant winter wheat cultivars are more sensitive to the microtubule-disrupting drug, oryzalin than those in cold-sensitive cultivars (Olinevich et al. 2002). Depolymerization and reorganization of the cortical microtubules are involved in the conversion of existing arrays into new arrays (Smith and Oppenheimer 2005). Osmotic stress also causes reorganization of the cortical microtubules in maize root cells, although their reorientation is a consequence of growth inhibition (Balancaflor and Hasenstein 1995). Although it has been suggested that cortical microtubules may be involved in the plant's response to salt stress (Chinnusamy and Zhu 2003, Shoji et al. 2006), it remains an open question whether they play a role in the plant's ability to withstand salt stress. In the present study, we provide the first evidence that reorganization of the cortical microtubules is an important process in the plant's ability to withstand salt stress. In salt stress, the plant responds to the stress by re-polymerizing the cortical microtubules and thereby facilitates the survival of the salt-stressed plant.

The underlying function of the dynamic changes of the cortical microtubules remains to be elucidated. Calcium is an important second messenger and is involved in triggering the plant's responses to stress (Xiong et al. 2002). In the study reported herein, we showed that the depolymerization of cortical microtubules usually occurs before the elevation of $[Ca^{2+}]_{cyt}$, and the removal of calcium from the growth medium had no obvious effect on salt stress-induced depolymerization of the cortical microtubules. Therefore, the depolymerization of cortical microtubules is an upstream event that occurs before the amount of $[Ca^{2+}]_{cyt}$ increases. Depolymerization of the microtubules has been reported to be involved in the regulation of calcium channels in plant cells and causes the channels to open (Thion et al. 1996, Thion et al. 1998). Therefore, salt stress-induced depolymerization of the cortical microtubules may affect calcium channel activity and thus lead to an increase in $[Ca^{2+}]_{cyt}$. However, the experiments carried out by Thion et al. (1996, 1998) used only protoplasts of *Arabidopsis* and carrot; therefore, the question of whether the properties of plasma membrane are affected by interaction with the cell wall should be addressed in future studies.

Suppression of cell growth may facilitate plant survival in salt stress. For example, the growth-repressing DELLA proteins of *Arabidopsis*, which restrain cell proliferation and expansion, have been shown to be beneficial and promote the survival of salt-stressed plants (Achard et al. 2006). That cortical microtubules are involved in the determination of plant cell growth is well established. Different arrays of cortical microtubules are usually present in the various phases of plant cell growth. Reorganization of the arrays of cortical microtubule occurs as the cell moves through its growth phases (Dixit and Cyr 2003, Hashimoto and Kato 2006). Thus, depolymerization and re-polymerization of the cortical microtubules might be involved in the regulation of plant cell growth in salt stress. It is possible that salt stress-induced reorganization of cortical microtubules may affect cell growth directly or by regulating the activity of calcium channels. Our data show that salt stress-induced depolymerization of the cortical microtubules still occurs, while the increase of plant survival that is made possible by microtubule-targeting drugs was eliminated when calcium was removed from the growth medium or calcium channels were blocked. This result suggests that calcium is a crucial factor for the survival of salt-stressed plants.

Nevertheless, there must be signals that are triggered or activated in salt stress to cause depolymerization of the cortical microtubules. Several signaling molecules have been shown to play roles in the responses of plants to salt stress, such as ABA, phosphatidic acid and ROS (Zhu 2002, Chinnusamy et al. 2005). Most of these molecules are also associated with the organization of the cortical microtubules. For instance, ABA, which is involved in plant responses to most environment stresses, affects the organization of cortical microtubules, such as the orientation of the cortical microtubules (Sakiyama and Shibaoka 1990, Shibaoka 1994, Jiang et al. 1996). PLD, whose expression is increased when plants respond to salt stress, is a microtubule-associated protein that is involved in the rearrangement of cortical microtubules (Katagiri et al. 2001, Dhonukshe et al. 2003). ROS are usually produced in salt-stressed plants (Chinnusamy et al. 2005) and can cause the fragmentation of microtubules in vitro (Xu et al. 2006) and mitotic arrest in plant cells (Dixit and Cyr 2003). It is possible that ROS production induced by salt stress may also damage cortical microtubules in cells. Thus, there is ample evidence that salt stress alters the functional activity of the cortical microtubule. It will be of considerable interest to conduct further investigations on how these molecules interact with the cortical microtubules in the response to salt stress.

Cortical microtubules may be involved in sodium homeostasis under salt stress

Salt stress has multiple effects on cells. However, little is known about the initial cue or signal that activates the responses of plants to salt stress. Although osmotic stress

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plays a role in sensing salt stress, a specific sensor for sodium may exist in plant cells (Zhu 2003).

In the present study, we demonstrated that the depolymerization of cortical microtubules is a response to salt stress, but not to osmotic stress caused by mannitol. In the presence of relatively low concentrations of NaCl, the cells of the *sos1* mutant displayed a greater extent of depolymerization of cortical microtubule than that observed in wild-type cells. Because the intracellular sodium concentration in the *sos1* mutant is higher than that in the cells of the wild-type *Arabidopsis* in the presence of low concentrations of NaCl (Shi et al. 2002), depolymerization of the cortical microtubules in the *sos1* mutant may be due to an increase in the intracellular sodium concentration. The results of a recent study on *sos1* and *spiral1* mutants show that sodium imbalance is related to the organization of cortical microtubules in *Arabidopsis* cells (Shoji et al. 2006). Therefore, the depolymerization of cortical microtubules could be triggered by a sodium-specific sensor.

How cells sense sodium imbalance and reorganize the cortical microtubules is still an open question. The evidence collected from in vitro studies indicates that sodium has no direct effect on depolymerization of the microtubules. Tubulin polymerizes into microtubules in 200 mM NaCl, and the addition of NaCl does not stimulate microtubule depolymerization (Li et al. 2007). Therefore, it is most likely that sodium indirectly induces depolymerization of the cortical microtubules in salt stress.

Zhu (2003) proposed that the cytoplasmic C-terminal end of SOS1 might contain a sensor domain for sodium. It is also possible that the cortical microtubules bind to this region to regulate the activity of SOS1. Other investigators have reported that the C-terminal end of NHE1 (a homolog of SOS1 in fibroblasts) interacts with the actin cytoskeleton, which suggests that the ion exchanger has an independent structural function (Denker et al. 2000, Shoji et al. 2006). However, further studies are needed to clarify whether the microtubular cytoskeleton regulates sodium channels in plants directly.

Calcium is important for cortical microtubule reorganization in response to salt stress

It is known that calcium is involved in the plant's ability to withstand salt stress (Sanders et al. 2002, White and Broadley 2003, Lecourieux et al. 2006). The results from the present study demonstrate that the removal of calcium from the growth medium reduces the survival of salt-stressed seedlings, thereby confirming the involvement of calcium. However, most previous observations indicated that various abiotic stresses stimulate transient increases in $[Ca^{2+}]_{cyt}$, which itself can trigger other cell responses to stress (Rudd and Franklin-Tong 2001, Sanders et al. 2002).

By using yellow cameleon (YC.3.6) as a calcium reporter, we showed that $[Ca^{2+}]_{cyt}$ was markedly increased after depolymerization of the cortical microtubules that occurred in response to salt treatment. These increases in $[Ca^{2+}]_{cyt}$ were inhibited when paclitaxel was added. However, the increased level of $[Ca^{2+}]_{cyt}$ that was induced by salt stress occurred at some time point after the salt treatments and persisted for a relatively long period. Relatively high concentrations of calcium may be required for the plants to adapt to salt stress. For instance, the SOS2–SOS3 complex may need relatively high levels of calcium to maintain its activity under salt stress, and for the subsequent regulation of the activity of SOS1 to cause sodium efflux. In addition, other calcium-binding proteins, such as Ca^{2+}-dependent protein kinases, calcineurin B-like proteins and calmodulin, have been identified as being involved in the plant's response to salt stress (White and Broadley 2003).

Although our observations indicate that depolymerization of the cortical microtubules may function in the regulation of calcium channel activity at an early stage in salt stress, the increase in levels of $[Ca^{2+}]_{cyt}$ is important for the reorganization of cortical microtubules after their salt stress-induced depolymerization. The organization of the cortical microtubules was not restored following the removal of calcium from the growth medium, whereas the addition of calcium to the medium increased the recovery of cortical microtubules under salt stress. However, we know little about how calcium is involved in microtubule reassembly. Some possibilities may be considered. First, calcium triggers defense responses so that cells recover from the impact of salt stress and microtubules are reassembled. In this case, calcium has no direct involvement in microtubule reassembly. Secondly, calcium may have some regulatory effect on microtubule reassembly, such as involvement in the regulation of the activity of microtubule-associated proteins, which further may affect the stability of cortical microtubules. Nevertheless, the mechanism of action of calcium in the reorganization of cortical microtubules under salt stress is a worthy topic for future study.

Moreover, addition of oryzalin has a promoting effect on microtubule reassembly. This effect may result from the increase in the level of $[Ca^{2+}]_{cyt}$ due to the depolymerization of cortical microtubules. It also suggests that cortical microtubules need to reassemble from existing arrays into new arrays to withstand salt stress. As we discussed above, depolymerization and reorganization of the cortical microtubules are involved in both cold and osmotic stress, suggesting a common mechanism involved in plant tolerance in stress conditions. We hypothesize that the reassembled cortical microtubules are more stabilized to withstand stress conditions, although future study is needed to obtain evidence for this.

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In conclusion, the results of the present study provide the first evidence that cortical microtubules are involved in the plant's ability to withstand salt stress. The depolymerization of cortical microtubules is not just a passive consequence of salt stress, but plays an active role in regulating cytoplasmic calcium concentrations and sensing sodium in salt stress. Our findings also provide novel data relating to the adaptation of plants to salt stress and may allow us to gain further insights into how plants respond to environmental cues.

Materials and Methods

Plant material

Seeds of wild-type *A. thaliana* (Columbia), its *sos1-1* mutant (Columbia) (Shi et al. 2000) and *Arabidopsis* seedlings expressing the fluorescent calcium indicator YC3.6 (Columbia) were sown in Petri dishes that contained Murashige and Skoog basal medium (MS), 1% (w/v) agar and 3% (w/v) sucrose, pH 6.0, and kept at 4°C in the dark for 3 d. The plates were then moved to a growth chamber at 22°C with 16 h light/8 h dark cycles. The 3-day-old seedlings were then transferred to other plates for treatments, according to the requirements of the specific experiments. Seedlings were photographed using a digital camera (Cyber-shot DSC-S85, Sony, Japan).

Measurement of skewed root growth and survival of Arabidopsis seedlings

The skewed angle of roots was measured with an LSM 5 Image Browser (Zeiss, Germany).

For the purpose of measuring the skewed angle of wild-type *Arabidopsis* seedling root growth under salt stress, the 3-day-old wild-type seedlings grown in MS medium were transferred to 0, 25, 50, 75, 100 or 125 mM NaCl on 1.5% (w/v) agar plates on a 45° incline, and treated for 4 d before observation. To investigate whether the right-handed pattern of root growth is regulated by the organization/dynamics of cortical microtubules, the 3-day-old seedlings grown in MS medium were transferred to 0, 50 or 100 mM NaCl, containing various concentrations of paclitaxel (Taxol®, Sigma, USA) or oryzalin (3,5-dinitro-N^4, N^4-dipropylsulfanilamide, Ps-410, Sigma, USA), on 1.5% (w/v) agar plates on a 45° incline for 4 d. At least 20 seedlings in each experiment were observed, and each experiment was repeated three times.

To determine which factor(s) might play a role in the depolymerization of cortical microtubules in their response to salt stress, the 3-day-old wild-type seedlings grown in MS medium were transferred to 100 or 200 mM mannitol, on 1.5% (w/v) agar plates on a 45° incline, and treated for 4 d before observation. The 3-day-old seedlings of the *sos1* mutant grown in MS medium were transferred to 50 mM NaCl on 1.5% (w/v) agar plates on a 45° incline, and treated for 4 d before observation. At least 20 seedlings in each experiment were observed, and each experiment was repeated three times.

In order to investigate the effect of microtubule-targeting drugs on *Arabidopsis* seedling survival in salt stress, the 3-day-old wild-type seedlings grown in MS medium were transferred to the MS media containing 0, 150, 200 or 250 mM NaCl with or without the addition of 4 μM paclitaxel, 0.1 μM oryzalin or 0.5 μM PPM [Pestanal, 3,5-dichloro-*N*- (1,1-dimethyl-2-propynyl) benzamide, Sigma, USA], then cultured for 4 d before observation. For the experiments using *sos1* mutant seedlings, the 3-day-old seedlings of the *sos1* mutant grown in MS medium were transferred to MS medium containing 100 mM NaCl with or without the addition of 4 μM paclitaxel or 0.1 μM oryzalin, then cultured for 4 d before observation. At least 100 seedlings in each experiment were observed, and each experiment was repeated three times. The bleaching of leaves was used as the indicator of seedling death.

Cortical microtubule observation

The cortical microtubules in root cells were observed by immunofluorescence microscopy, according to the protocol described by Sugimoto et al. (2000).

To confirm that skewed root growth under conditions of salt stress is caused by depolymerization of the cortical microtubules, seedling roots were fixed with 4% (v/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde. The primary antibody was a mouse monoclonal antibody against β-tubulin (Sigma, USA) at 1 : 800 dilution and the secondary antibody was Alexa Fluor®488-conjugated donkey antibody against rabbit IgG (Molecular Probes, Eugene, OR, USA) at 1 : 600 dilution. Immunofluorescence images were collected with a Zeiss LSM 510 META confocal microscope (Jena, Germany) using the following objective lenses: 100 × (Plan-APOCHROMAT, NA 1.4), 63 × (Plan-APOCHROMAT, NA 1.4) and 40 × oil (Plan-APOCHROMAT, NA 1.3). The samples were excited at 488 nm with a krypton–argon laser, and the emission from the Alexa 488® fluorochrome was detected using a 505–530 nm bandpass filter.

To investigate salt stress-induced dynamics and the organization of the cortical microtubules, the cortical microtubules in cotyledon pavement cells were observed in *Arabidopsis* seedings expressing GFP–tubulin using confocal microscopy as described above.

Calcium measurements

To investigate the interaction of calcium and depolymerization of the cortical microtubules under salt stress, *Arabidopsis* expressing the fluorescent calcium indicator YC3.6 were used in the experiments for measuring cytoplasmic free calcium concentrations $[Ca^{2+}]_{cyt}$. Yellow cameleons 3.6 (YC3.6) have cyan and yellow fluorescent proteins (CFP and YFP) as the FRET donor and acceptor, respectively. Images were acquired with a 10 × objective (Olympus Plan, NA 0.25) on an Olympus BX51 microscope equipped with a color CCD camera (Olympus Cool SNAP HQ, Japan). The measurements were made in cotyledon pavement cells with a MetaFour CCD (Molecular Devices, Sunnyvale, CA, USA) according to the method described by Yu and Hinkle (2000). Briefly, The cells that expressed YC3.6 were excited at a wavelength of 436 ± 5 nm and the emitted fluorescence was collected alternately at 465 ± 15 nm (CFP) and 530 ± 20 nm (YFP). The $[Ca^{2+}]_{cyt}$ was calculated from the emission ratio of YFP : CFP, which was collected from 60 cells from three seedlings, using the equation: $[Ca^{2+}]_{cyt} = K'_d \times [(R - (R_{min} + 14/100 \times (R_{max} - R_{min}))/(R_{max} - R)]^{(1/n)}$ (Foyouzi-Youssefi et al. 2000). The R_{max} value of 2.39 was obtained by measuring the emission ratio of YFP : CFP of cotyledon pavement cells of 3-day-old seedlings grown for 20 h on MS medium that contained 20 mM $CaCl_2$, while the R_{min} value of 1.48 was obtained by measuring the emission ratio of YFP : CFP of cotyledon pavement cells of 3-day-old seedlings grown for 20 h on MS medium that contained 2 mM EGTA. We used the values of 2.5×10^{-7} M for the apparent dissociation constant, K'_d, of YC3.6, and 1.7 for the Hill coefficient of the fitted Ca^{2+} calibration curve (n) according to Nagai et al. (2004).

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To investigate the interaction of calcium and depolymerization of the cortical microtubules under salt stress, the 3-day-old wild-type seedlings grown in MS medium were transferred to MS medium without $CaCl_2$, containing 0, 150, 200 or 250 mM NaCl with or without the addition of 1 mM EGTA, respectively, and treated for 4 d before observation. In addition, the 3-day-old wild-type seedlings grown in MS medium were transferred to MS medium containing 200 mM NaCl, or plus 100 or 200 μM $LaCl_3$, and treated for 4 d before observation. At least 100 seedlings in each experiment were observed, and each experiment was repeated three times. The bleaching of leaves was used as the indicator of seedling death.

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