Dynamic Aspects of Ion Accumulation by Vesicle Traffic Under Salt Stress in Arabidopsis

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The intracellular membrane dynamics of Arabidopsis cells under high salt treatment were investigated. When Arabidopsis was treated with high levels of NaCl in hydroponic culture, root tip cells showed rapid changes in the vacuolar volume, a decrease in the number of small acid compartments, active movement of vesicles and accumulation of Na\textsuperscript{+} both in the central vacuole and in the vesicles around the main vacuole observed with the Na\textsuperscript{+}-dependent fluorescence of Sodium Green. Detailed observation of Arabidopsis suspension-cultured cells under high salt treatment showed a similar pattern of response to that observed in root tip cells. Immunostaining of suspension-cultured cells with antibodies against AtNHX1 clearly showed the occurrence of dotted fluorescence in the cytoplasm only under salt treatment. We also confirmed the existence of AtNHX1 in the vacuolar membrane isolated from suspension-cultured cells with immunofluorescence. Knockout of the vacuolar Q\textsubscript{a}-SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein VAM3/SYP22 caused an increase in salt tolerance. In mutant plants, the distribution of Na\textsuperscript{+} between roots and shoots differed from that of wild-type plants, with Na\textsuperscript{+} accumulating more in roots and less in the shoots of the mutant plants. The role of vesicle traffic under salt stress is discussed.

Keywords: Arabidopsis suspension-cultured cells • Na\textsuperscript{+}/H\textsuperscript{+} antiporter • NHX • Salt tolerance • SNARE • Vacuole.

Abbreviations: GFP, green fluorescent protein; MS, Murashige and Skoog; PI, propidium iodide; PVC, pre-vacuolar compartment; ROS, reactive oxygen species; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor.

Introduction

Excessive soluble salts in soil inhibit growth of most higher plants. Salt stress usually causes dehydration, ion stress and production of reactive oxygen species (ROS). Under high salt conditions, plants display various salt tolerance mechanisms. To counter dehydration, cellular osmolality is adjusted by accumulating compatible solutes in the cytoplasm and salt in the vacuole. Ion extrusion from the cell or ion sequestration in the vacuole avoids ion stress in the cytoplasm, while ROS are dissipated by the enzymatic reactions (Niu et al. 1995, Hasegawa et al. 2000, Munns and Tester 2007).
It is well known that the ion accumulation in the vacuole is mediated by membrane transporters in the vacuolar membrane. Na$^+$ is actively transported into the vacuole by Na$^+$/H$^+$ antiporters (Blumwald and Poole 1988, Blumwald 2000), and Cl$^-$ is passively transported by Cl$^-$ channels (Nakamura et al. 2006). It has been shown that overexpression of the Na$^+$/H$^+$ antiporter increased the salt tolerance in Arabidopsis (Apse et al. 1999). Currently the Na$^+$/H$^+$ antiporter is one of the major targets for manipulating the salt tolerance of crop plants (Yamaguchi and Blumwald 2005).

In Arabidopsis, eight genes encoding Na$^+$/H$^+$ antiporters (AtNHX1–AtNHX8) have been found in the genome. AtNHX1–AtNHX4 are known to locate in the vacuolar membrane, and AtNHX5 localizes in the endosomal compartment (Pardo et al. 2006). The expression levels of NHX genes have been shown to increase under high salt treatment in various plants (Yokoi et al. 2002, Popova and Golldack 2007, Sottosanto et al. 2007). Sodium is not an essential nutrient for most terrestrial plants and the Na$^+$ content of plant tissues is also not high under normal growth conditions. The physiological role of NHX transporters in the vacuolar membrane under normal conditions (i.e. low salt) is not known. NHX proteins are phylogenetically similar to CHX proteins (cation/proton antiporter), which carry alkali cations (mainly K$^+$) other than Na$^+$ (Maser et al. 2001).

In Ipomea, control of the vacuolar pH is mediated by NHX during petal opening (Fukada-Tanaka et al. 2000), but no change in Na$^+$ level was detected; instead K$^+$ appeared to be transported (Yoshida et al. 2005). Functionally, NHX is likely to be involved in K$^+$ transport in plants.

Changes in cellular membrane components of Arabidopsis root cells

In the present study, we found that changes in vacuolar structure occur under salt stress in Arabidopsis cells, similar to those in mangrove or barley. In Arabidopsis, we could observe detailed changes in cellular structures using organelles markers. We also investigated how the dynamics of pre-vacuoles are involved in ion trafficking and accumulation in the vacuole using Qa-SNARE Vam3/SYP22 mutant plants. The relationship between vesicle dynamics in the cell and ion accumulation in the vacuole under salt stress is discussed.

In order to observe changes in the vacuolar structure in detail, we used Vam3 knockout Arabidopsis plants (atvam3 #3-1) transformed with proVAM3::GFP-VAM3. Vam3 protein is a Qa-SNARE protein from vacuolar and pre-vacuolar compartment (PVC) membranes (Sanderfoot et al. 1999, Uemura et al. 2004). Using this material, it is possible to observe more clearly than in the vacuolar membrane. Fig. 2 shows a fluorescence image of cortical cells at the root tip in which green fluorescent protein (GFP) fluorescence outlines the vacuolar structure in the cell. Salt treatment (200 mM NaCl, 24 h) increased the vacuolar volume as shown in Fig. 1. In Fig. 2, GFP fluorescence show a more complicated vacuolar system containing not only the central vacuole, but also the smaller vacuoles and partly the PVCs. Propidium iodide (PI), a non-penetrating red fluorescent dye, was used to show the outline of the cells. Under salt treatment, however, some cells showed strong red fluorescence throughout, suggesting that salt stress had destroyed the membrane integrity. High salt treatment causes not only ion stress, but also osmotic stress. In a previous report (Mimura et al. 2003), we confirmed that the change in the vacuolar volume was mainly caused by ion stress by using sorbitol treatment as the osmotic stress.
**Accumulation of Na⁺ in vacuoles of root cells**

To confirm a relationship between vacuolar structure and Na⁺ accumulation, root cells were stained with Sodium Green, which is a fluorescent indicator of Na⁺. Fig. 3 shows a fluorescence image of Arabidopsis root cells 12 h after 200 mM NaCl treatment. There were small structures with green fluorescence around the main vacuole, similar in appearance to the acidic particles observed under the light microscope. In control plants, no fluorescence was observed.
Figure 3

Fluorescence observation of cortical cells at the root tip stained with Sodium Green (5 µM) for 30 min. (A and B) Control. (C and D) Plants were treated with 200 mM NaCl for 12 h. (A and C) Differential interference contrast image of root cells. (B and D) Fluorescence of Sodium Green. Scale bar = 10 µm.

Changes in membrane structure and accumulation of Na\(^+\) in the vacuole in Arabidopsis suspension-cultured cells

The complex structure of a plant root makes it difficult to observe changes in cellular properties in detail. Thus, we also observed suspension-cultured cells of Arabidopsis (strain Deep). Figure 4 shows changes in the vacuolar structure of Deep cells transformed with p35S::GFP-Vam3 to enable the vacuolar membrane to be visualized. Before salt treatment, there were complex vacuolar systems in the cell. At 12 h after 75 mM salt treatment, an enlarged vacuole occupied almost all of the cell, and was surrounded by small membrane structures. As shown (Supplementary movies S3, S4), the vacuolar system under normal conditions moves slowly, but after high salt treatment small membrane structures actively move around the central vacuole.

The salt-treated suspension-cultured cells were also stained with Sodium Green. Figure 5 shows that Na\(^+\) was accumulated in the central vacuole, and there were smaller compartments showing green fluorescence around the vacuoles.

Immunofluorescent observation of NHX1 in suspension-cultured cells and in the vacuolar membrane

There have been several previous studies dealing with the subcellular distribution of members of the NHX family of proteins (Pardo et al. 2006). In Arabidopsis, NHX1 and NHX2 are the most abundant and they locate to the vacuolar membrane. In this study, to confirm the presence of NHX in vacuolar structures, cultured cells were immunostained with an antibody against NHX1 (Figure 6). After salt treatment, many dotted particles appeared in the cytoplasm, but we could not observe a clear membrane structure for the central vacuole. The existence of NHX1 and NHX2 in the vacuolar membrane has been confirmed using fluorescence protein fusion (Yokoi et al. 2002). Proteomic analysis of the vacuolar membrane found AtNHX4 proteins in the Arabidopsis vacuolar membrane (Jaquinod et al. 2007) and a homologous protein in the barley vacuolar membrane (Endler et al. 2006). Here we have confirmed the existence of NHX1 proteins on the vacuolar membrane.
Fig. 4 Fluorescence observation of vacuolar membrane transformed with 35S::GFP-VAM3 in suspension-cultured cells. (A and B) Control. (C and D) Cells were treated with 75 mM NaCl for 12 h. (A and C) Differential interference contrast images of suspension-cultured cells. (B and D) Fluorescence of GFP–VAM3 in the vacuole and PVC membrane. N, nucleus; V, vacuole. Scale bar = 10 µm.

by immunostaining of the vacuole isolated from suspension-cultured cells (Fig. 7A). The antibody recognizes the C-terminus of NHX1 on the cytoplasmic side and, when isolated vacuoles were treated with proteinase K, the immunofluorescence signal disappeared (Fig. 7C).

Involvement of membrane fusion in ion traffic
As shown above, under high salt treatment, many small membrane vesicles appear and actively move around the main vacuole in both root and cultured cells. Ultimately, they may fuse to the main vacuole, causing the vacuole to expand and occupy most of the cell. The fact that these structures can also be stained with Sodium Green seems to indicate a role in salt stress. In a previous article (Mimura et al. 2003), we stressed that the rapid changes in the vacuolar volume under salt stress might play an important role in mitigating the adverse effects of high salt.

SNARE proteins are known to be involved in vesicle fusion in the cell (Lipka et al. 2007). The role of these proteins in fusion of ion-accumulating vesicles with the central vacuole was investigated using VAM3/SYP22 (At5g46860) knockout plants. It is known that VAM3 operates as a Qa-SNARE between PVCs and vacuoles. When wild-type plants were treated with high salt, VAM3 protein increased slightly (data not shown). However, VAM3 knockout plants showed better tolerance against high salt treatment (Fig. 8). Interestingly, the Na\textsuperscript{+} content of shoots in knockout plants was lower than in wild-type plants, but the Na\textsuperscript{+} content of roots was higher than in wild-type plants. As a consequence, the shoots of knockout plants seemed more tolerant to salt stress.

Since the Na\textsuperscript{+} levels were clearly different between wild-type and mutant plants, the difference in AtNHX1 protein content was measured. Fig. 9 shows the Western blot data of AtNHX1 under salt treatment. In the shoot, irrespective of the presence of salt, there was more AtNHX1 in wild-type plants than in the mutant plants, but in the root there was more in the mutant plants.

No clear differences could be detected in vacuolar structure between VAM3 knockout plants and wild-type plants under 200 mM NaCl treatment (data not shown).
Rapid changes in the vacuolar volume in Arabidopsis cells

In mangrove suspension-cultured cells, we found rapid changes in vacuolar volume under salt stress and demonstrated that this was an active process correlated with salt adaptation. A similar process was also observed in root cells of barley, which is referred to as a semi-salt-tolerant plant (Mimura et al. 2003). In contrast, salt-sensitive higher plants, e.g. pea and tomato, did not show such large changes in the vacuolar volume under salt treatments. The increase in the vacuolar volume under salt stress is associated with a proportionate decrease in cytoplasmic volume. This would effectively increase the osmolality of the cytoplasm by concentrating non-toxic solutes such as K+ and compatible osmotic solutes, thereby osmotically balancing the increased Na+ in the expanding vacuole.

Arabidopsis is classified as a semi-salt-tolerant plant. In hydroponic culture, Arabidopsis continued to grow even in the presence of 100 mM NaCl. In the present study, we have confirmed that Arabidopsis has the ability to induce rapid changes in the vacuolar volume under salt stress, both in intact plants (Figs. 1, 2) and in suspension-cultured cells (Fig. 4). The changes in vacuolar volume caused by high salt could be clearly seen using GFP (Figs. 2, 4).

Evidence was also obtained for the involvement of small membrane structures in the salt tolerance mechanism. In root cells, small acidic compartments stained with neutral red only moved actively when the plant was treated with high salt. In suspension-cultured cells, these small vesicles tagged with VAM3–GFP also moved around the central vacuole. Although we were not able to observe any fusion of vesicles, it is highly likely that under high salt treatment, their fusion with the central vacuole is the cause of the increase in vacuolar volume.

Accumulation of Na+ in vesicles and vacuoles

Accumulation of ions in the vacuole is an important factor in countering salt stress. In Arabidopsis, it has been reported that among eight AtNHX proteins, AtNHX1–AtNHX4 localize mainly in the vacuolar membrane, and AtNHX5 and AtNHX6 localize in the endosomal membrane (Pardo et al. 2006). The vacuolar Na+/H+ antiporters function to accumulate alkali cations in the vacuole and also to control the vacuolar pH. Na+/H+ antiporters on the endosomal membranes control vesicle pH and vesicle trafficking, as has been shown in experiments with yeast. Na+/H+ antiporters in yeast localize to pre-vacuolar membranes, and mutation of Na+/H+ antiporters disrupts vesicle trafficking (Bowers et al. 2000).

Expression of AtNHX1 complements the lack of endogenous yeast NHX1 (ScNHX1) (Gaxiola et al. 1999, Hernandez et al. 2009).

The Sodium Green fluorescence observations showed that Na+ is accumulated not only in the main vacuole, but also in the small compartments around the vacuole. We have no direct evidence that these small acidic compartments around the vacuole, which actively move under salt stress, accumulate Na+, although the staining with Sodium Green makes this seem likely. It is also probable that these small vesicles accumulate Na+ and fuse to the main vacuole as observed in yeast cells (Nass and Rao 1998).
Fig. 6 Western blot of the vacuolar membrane with antibody against NHX1 (panel I). Immunofluorescence observation of AtNHX1 in suspension-cultured cells (panel II). (A and B) Control. (C and D) Cells were treated with 75 mM NaCl for 6 h. (A and C) Differential interference contrast images of suspension-cultured cells. (B and D) Fluorescence of antibodies against NHX1. Scale bar = 10 µm.

Fig. 7 Distribution of NHX1 on an isolated vacuole. The localization of NHX1 was observed by immunofluorescence staining with antibodies against NHX1. (A and B) A vacuole isolated from suspension-cultured cells. (C and D) Vacuoles were treated with proteinase K (20 µg ml−1) for 1 h at room temperature. (A and C) Confocal fluorescent images of the isolated vacuole. (B and D) Differential interference contrast images of the isolated vacuole. Scale bar = 5 µm.

Fig. 8 Growth of wild-type and mutants of vam3 Arabidopsis plants under 200 mM NaCl in hydroponic culture (A). Na⁺ levels of shoots and roots (B). Filled bar, Na⁺ levels of shoot; hatched bar, Na⁺ levels of root.
Immunostaining of NHX1 under salt stress showed an increase of small dots in the cytoplasm (Fig. 6). On the other hand, no immunostained structure could be found on the central vacuolar membrane under normal conditions, nor was NHX1 protein detected by proteomic analysis of the vacuolar membrane of Arabidopsis cells under low salt conditions (Carter et al. 2004, Shimaoka et al. 2004, Jaquinod et al. 2007). This appears to be the first report of localization of NHX1 on the vacuolar membrane by immunostaining (Fig. 7).

In root tip cells or suspension-cultured cells, the vacuole is immature under normal conditions, because the cells are actively dividing. When these cells are exposed to salt stress, many small vesicles appear and they accumulate ions. Subsequently, the vacuolar volume increases and cells become tolerant to salt stress. In our former observations (Mimura et al. 2003), salt-sensitive plants such as pea and tomato did not display rapid increases in vacuolar volume, which suggests that ion sequestration into the small vesicles in the initial phase of salt stress may play an important role in tolerance to salt stress.

Very recently, mutants of AtNHX1 were transformed into yeast cells and showed improved tolerance to salt stress. AtNHX1 in yeast cells localizes to the vesicle membrane where it must be involved in ion homeostasis (Hernandez et al. 2009). The present study suggests that a similar situation also occurs in higher plant cells.

Role of membrane fusion via the SNARE complex under salt stress

It is well established that the SNARE complex plays an important role in the membrane fusion process (Lipka et al. 2007). It has also been suggested that the SNARE proteins are involved in the control of ion transport (Leyman et al. 1999).

In the present study, in order to determine whether vesicle fusion to the vacuole is important under salt stress, we used knockout mutants of VAM3 (Qa-SNARE), a SNARE protein in the vacuolar and PVC membrane. Interestingly, these knockout mutants showed higher tolerance to salt stress compared with the wild type (Fig. 8a). This is similar to the increased salt tolerance as a result of suppression of Arabidopsis VAMP7C family proteins (R-SNAREs) reported by Leshem et al. (2006). These authors proposed that this mutation alters ROS in the cell, since fusion of H2O2-containing vesicles to the vacuolar membrane was inhibited. In the knockout mutants of VAM3, a similar physiological response may occur. However, in our analysis, the knockout of VAM3 also changed the distribution of Na+, so that the shoot Na+ level decreased and the root Na+ level increased in the mutant plants (Fig. 8b). It is likely that the protein levels of Na+/H+ antiporters increased in root tissue in mutants and more Na+ was therefore accumulated in the root cell (Fig. 9). More experiments are needed to clarify the role of SNARE complexes under salt stress.

In conclusion, we have found strong evidence that the accumulation of ions in vesicles and the fusion of these vesicles to the vacuole causing an increase in the vacuolar volume play a role in salt tolerance. Although the importance of ion segregation by membrane transport into the main vacuole under salt stress is a well-established phenomenon, the underlying mechanism of ion movement into the vacuole may be more complex than simple pumping across the vacuolar membrane.

Materials and Methods

Plant materials and culture

*Arabidopsis thaliana* plants of ecotype Col-0 and suspension-cultured cells [Deep: Arabidopsis Col-0 cell suspension supplied courtesy of Dr. Umeda (NAIST)] (Mathur et al. 1998) were used throughout the experiments. Plants (vam3-1) transformed with proVAM3::GFP-VAM3 were established by Dr. Uemura (Uemura et al. 2009). proVAM3::GFP-VAM3 was generated as follows. The cDNA encoding GFP was inserted in front of the start codon of VAM3 (3.2 kb of 5′- and 1.2 kb of 3′-flanking sequence) by the technique of fluorescent tagging of full-length proteins (Tian et al. 2004). The amplified chimera sequence was subcloned into the binary vector, which was used for transforming Arabidopsis. Transformation of Arabidopsis was performed by floral dipping using *Agrobacterium tumefaciens* (strain GV3101).

Seeds of knockout mutants atvam3-1 (SALK_060946, Ebine et al. 2008) and atvam3r3 (SALK_075924, Ueda et al. 2006) were donated by the Salk Institute Genome Analysis Laboratory. Plants were cultured in modified Murashige and
Skoog (MS) medium containing 1% sucrose and 0.2% gellan gum. They were first kept at 4°C for 3 d after sowing to break seed dormancy, then cultured in a growth chamber at 23.5°C in continuous light for around 10 d, then moved to hydroponic culture of MS medium without sucrose. One day later, the MS medium was supplemented with the appropriate NaCl concentration.

Suspension-cultured cells were cultured in MS medium supplemented with 4.5 µM 2,4-D and 3% sucrose at approximately 23°C in the dark with rotation at 125 r.p.m. (TAITEC, BioShaker BR-43FL, Nagoya, Japan). A transgenic line of Deep cells with 35S::GFP-VAM3 was established as described by Oda et al. (2005).

**Microscopic observation**

Images from a light microscope (BX51WI; Olympus, Tokyo, Japan) were captured with an imaging system (DP70; Olympus). The fluorescent images were obtained with a confocal laser microscope (FV-1000; Olympus) for GFP and PI fluorescence. For the detection of Na⁺ in the cell, Sodium Green™ tetraacetate cell permeant (S6901; Invitrogen, Carlsbad, CA, USA) was used. Cation-dependent Sodium Green fluorescence was emitted by 514 nm laser irradiation.

**Antibody and immunofluorescent observation**

Rabbit polyclonal antibodies against a peptide contained in Arabidopsis, an Na⁺/H⁺ antiporter (AtNHX1; AF106324) (Cys-FVPGSPTERNPPDLSKA), were prepared based on Yoshida et al. (2005) and purified using an antigenic peptide-conjugated column to remove non-specific antibodies.

For the immunofluorescence observation of AtNHX1 proteins, suspension-cultured cells were collected in a Buchner funnel, and were fixed with 1.5% paraformaldehyde in EMP buffer (10 mM EGTA, 5 mM MgSO₄, 20 mM PIPES). After washing in EMP buffer (three times for 5 min), cell walls were partially digested with a medium containing 0.1% cellulase Y-C (Kyowa Chemical Products Co., Ltd, Osaka, Japan) and 0.1% pectolyase Y-23 (Kyowa Chemical Products Co., Ltd.) for 30 min at 37°C. After washing with EMP buffer (three times for 5 min), the partially digested cells were treated with EMP buffer containing 0.1% NP-40 for 1 h. Specimens were treated with EMP buffer containing 1% bovine serum albumin (BSA) supplemented with the purified rabbit polyclonal antibody against AtNHX1 (1/500 dilution) for 1 h. After washing with EMP buffer (three times for 5 min), cells were incubated in EMP buffer supplemented with a goat Alexa488-conjugated anti-rabbit IgG (1/500 dilution; Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. After washing with EMP buffer (three times for 5 min), specimens were observed with a confocal laser scanning microscope (FV1000; Olympus).

**Isolation of intact vacuoles and fluorescent observation of vacuolar membrane proteins**

Vacuoles were isolated from suspension-cultured cells (7 d old) according to Shimaoka et al. (2004). Isolated vacuoles were stained with purified specific antibodies against NHX1. Fluorescently labeled F(ab)_2 fragment [Zenon Alexa Fluor 488 (or 594) Rabbit IgG Labeling Kit, Molecular Probes] was used as a secondary antibody. The purified antibody was incubated with the fluorescently labeled F(ab)_2 fragment for 10 min at room temperature. Isolated vacuoles were incubated with the labeled specific antibody for 30 min at 4°C. The stained isolated vacuoles were collected by Percoll density gradient centrifugation as described in Shimaoka et al. (2004).

**Measurement of ions**

For extraction of inorganic ions, Arabidopsis plants were separated into shoot and root after blotting on filter paper. Tissues were then frozen and ground with a grinding machine (SH-48, Kurabo, Osaka, Japan). Sample powder was dissolved in distilled water and centrifuged at about 5,000×g then the supernatant was diluted and boiled at 97°C for 7 min and then again centrifuged to remove debris. The supernatant was filtered through a 0.45 µm filter (EKICRODISC-AcroLC; Gelman Sciences Ltd, Tokyo, Japan).

Ion concentrations in samples were determined using an ion chromatograph (IC-7000S; Yokogawa, Tokyo, Japan) with a cation exchange column (IC-C6S; Hitachi Kasei, Tokyo, Japan) for Na⁺ determination. The ion concentrations in cells were calculated based on their fresh weight.

**Western blot analysis**

Arabidopsis plants were weighed and frozen in liquid nitrogen and then the frozen samples were ground with a grinding machine. Sample powder was dissolved in a buffer solution [50 mM Tris–HCl, 10 mM EDTA, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 105 µM leupeptin and 4 mM dithiothreitol (DTT) adjusted to pH 7.5 with KOH] and centrifuged at 10,000×g for 15 min at 4°C. For the collection of membrane proteins, the supernatant was again centrifuged at 40,000×g for 35 min at 4°C. The precipitate was dissolved in a membrane buffer solution (10 mM Tricine, 250 mM sucrose adjusted to pH 7.8 with KOH) and stored as the membrane fraction.

Proteins were subjected to SDS–PAGE and were transferred electrophoretically to a polyvinylidene difluoride membrane according to the manufacturer’s protocol (Biocraft, Tokyo, Japan). The membrane was incubated with the antibodies against AtNHX1. Alkaline phosphatase-conjugated rabbit antibodies was used as the secondary antibody (GE Healthcare Biosciences).
Supplementary data

Supplementary data are available at PCP online.

Funding

The Japanese Ministry of Education, Sports, Culture, Science, and Technology (Grant-in-Aid for Scientific Research of Priority Areas on ‘Organelle Differentiation as the Strategy for Environmental Adaptation in Plants’, No. 1685101); Japan Science and Technology Agency (Core Research for Evolutional Science and Technology).

Acknowledgments

We greatly appreciate Dr. Rob Reid (University of Adelaide, Adelaide, Australia) for his kind discussion and correction of this manuscript. We also thank Dr. Csaba Koncz (Max-Planck-Institut für Züchtungsforschung) and Dr. Masaaki Umeda (The University of Tokyo) for their kind supply of Arabidopsis suspension-cultured cells.

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(Received August 7, 2009; Accepted October 14, 2009)