Induction of Salt and Osmotic Stress Tolerance by Overexpression of an Intracellular Vesicle Trafficking Protein AtRab7 (AtRabG3e)

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Adaptation to stress requires removal of existing molecules from various cellular compartments and replacing them with new ones. The transport of materials to and from the specific compartments involved in the recycling and deposition of macromolecules is carried out by an intracellular vesicle trafficking system. Here, we report the isolation of a vesicle trafficking-regulating gene, *AtRabG3e* (formerly *AtRab7*), from Arabidopsis. The gene was induced during programmed cell death after treatment of intact leaves with superoxide and salicylic acid or infection with necrogenic pathogens. Transgenic plants that expressed the *AtRabG3e* gene under the constitutive 35S promoter from cauliflower mosaic virus exhibited accelerated endocytosis in roots, leaves, and protoplasts. The transgenic plants accumulated sodium in the vacuoles and had higher amounts of sodium in the shoots. The transgenic plants also showed increased tolerance to salt and osmotic stresses and reduced accumulation of reactive oxygen species during salt stress. These results imply that vesicle trafficking plays an important role in plant adaptation to stress, beyond the housekeeping function in intracellular vesicle trafficking.

Plants are constantly exposed to changes in the environment that results in development of stress, compelling them to adjust to the new conditions. The perturbations in the surrounding environmental conditions often cause an oxidative stress. A mild oxidative stress usually induces antioxidant defenses, whereas a severe stress causes rapid necrosis. Intermediate levels of reactive oxygen species (ROS) often trigger a programmed cell death (PCD) cascade, which eliminates the compromised cells (Datt et al., 2003). The induction and execution of PCD are tightly controlled processes and can be modulated by signaling molecules, such as jasmonic acid, salicylic acid (SA), and ethylene (Lam et al., 1999).

Little information exists on the role of intracellular vesicle trafficking in resistance to environmental stresses. Endocytosis has been viewed traditionally as a constitutive housekeeping function in both animal and plant cells. Recently, however, a syntaxin-related protein, NtSyr1, which is one of the central components of the vesicle trafficking machinery in eukaryotes, was implicated in abscisic acid-mediated responses in tobacco (*Nicotiana tabacum*; Leyman et al., 1999). In yeast (*Saccharomyces cerevisiae*), we found that vesicle trafficking between cytosol and the plasma membrane was inhibited by oxidative stress. Overexpression of an Arabidopsis synaptobrevin homolog partially restored the traffic and provided tolerance to lethal concentrations of hydrogen peroxide (H_2O_2; Levine et al., 2001). A link between regulation of endocytosis by GDI:Rab5 complex and the p38-dependent stress response was described recently in animal cells (Cavalli et al., 2001).

We have shown previously that a low concentration of superoxide in the presence of nontoxic concentration of SA-induced PCD in intact Arabidopsis leaves (Mazel and Levine, 2001). The latter treatment has been used to isolate genes induced during PCD (Mazel and Levine, 2002). Here, we report the isolation of a small Rab GTPase that was induced during this PCD response and describe the effects of its ectopic expression in plants.

The Rab family of monomeric GTPases is conserved from yeast to animals and has been implicated in intracellular vesicle trafficking and in the organization of membranes (Zerial and McBride, 2001). Rab proteins continuously cycle between the GTP- and GDP-bound states and between cytosol and membrane compartments. The Rab proteins contain isoprenylation and GTP-binding sites, which mediate Rab activity and localization to the cytoplasmic side of the membrane, respectively (Randall and Crowell, 1999). Analysis of Rab sequences in animals and yeast have identified regions that couple the cycle of GTP binding and GTP hydrolysis to vesicle formation, targeting, and docking. These processes are regulated through interaction with a range of effector molecules that are recruited to the Rab proteins through the so-called effector regions that are specific for the individual Rabs (Rutherford and Moore, 2002).

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The Rab protein family of Arabidopsis consists of more than 57 members and constitutes one of the largest Rab families among fully sequenced organisms (Pereira-Liel and Seabra, 2001). The Rab GTPases are considerably more diverse in plants and mammals than in yeast (Rutherford and Moore, 2002). Until recently, Rabs were thought to primarily assist the SNAREs in vesicle docking and fusion, but lately, Rab proteins have emerged as key regulators of the vesicle targeting and specificity (Zerial and McBride, 2001). In fission yeast (*Schizosaccharomyces pombe*), mutation in any of the seven members of the *Rab* genes produces a distinct phenotype, indicating lack of redundancy and existence of a specific function for each gene (Pereira-Liel and Seabra, 2001). Among the different members of the Rab protein family, the Rab7 controls delivery of the internalized material into degradative compartments and the acquisition of lysosomal hydrolases (Bruckert et al., 2000).

In this paper, we analyze the role of Arabidopsis *Rab7* gene (*AtRabG3e*, At1g49300, GenBank accession no. AC007504) in salt and osmotic stress tolerance. About 30% of agricultural lands are affected by high salinity. High concentrations of NaCl arrest plant development and lead to plant cell death by disrupting ion and water homeostasis, inhibition of metabolism, and damage to membranes (Huh et al., 2002). One of the protection mechanisms against salt stress is transport of ions to vacuoles. Overexpression of a vacuolar Na^+-H^+ antiporter in Arabidopsis plants enabled the transgenic plants to grow in 200 mM NaCl (Zhang and Blumwald, 2001). Another major strategy for increased salt tolerance is accumulation of osmolytes (Bartels and Nelson, 1994; Hasegawa et al., 2000). Synthesis of metabolically compatible molecules that balance the osmotic stress imposed by salinity enables plants to reestablish the water and osmotic homeostasis (Zhu, 2001b).

In addition, resistance to many abiotic stresses, including salt, is improved by protection against oxidative stress (Zhu, 2001a). Arabidopsis mutants that were able to grow in high salt showed superior antioxidant potential (Tsugane et al., 1999). Likewise, transgenic plants that have been engineered to express antioxidant genes are more tolerant to high salinity (Van Camp et al., 1996).

To study the effects of *AtRabG3e* on plant stress resistance we prepared transgenic plants that overexpress this gene and tested their tolerance to several abiotic stresses associated with PCD. The transgenic plants exhibited accelerated endocytosis and showed increased tolerance to ionic (salt) or osmotic (sorbitol) stresses but not to oxidative stress. We present evidence on sequestration of sodium in the vacuole and reduced production of ROS in these plants. Our results suggest involvement of endocytosis in regulation of stress responses.

RESULTS

Induction of AtRab7 (AtRabG3e) Gene during Oxidative Stress

We have shown previously that superoxide in the presence of a moderate concentration of SA triggered PCD in Arabidopsis leaves (Mazel and Levine, 2001). A differential display method was used to isolate genes that were expressed during such oxidative stress-induced PCD. Arabidopsis (Wassilewskija ecotype) leaves were infiltrated with an $O_2^{\cdot -}$-producing mixture of xanthine (X) plus xanthine oxidase (XO) and SA. Total RNA was isolated 3 h after treatment, a time point that precedes the beginning of ion leakage or visual cells death symptoms by at least 2 h (Mazel and Levine, 2001). Several bands that showed increased expression after the combined treatment (X/XO + SA) were isolated and used to verify the expression of the corresponding genes by northern blotting. The latter analysis confirmed strong induction of one of the isolated fragments by the PCD-inducing treatment of $O_2^{\cdot -}$ + SA (Fig. 1). Considerably weaker induction was seen after treatment with either $O_2^{\cdot -}$ or SA.

The induced fragment was sequenced and analyzed by the BLAST search against the Arabidopsis genome database (Altschul et al., 1997). A 100% identity was found with the C-terminal region of an Arabidopsis *Rab7* gene (At1g49300, *AtRabG3e*). The *Rab* genes constitute a family of small GTPases involved in membrane trafficking. The *Rab7* gene was shown to participate in the late steps of endocytosis in eukaryotic cells (Feng et al., 1995; Vitelli et al., 1997; Bruckert et al., 2000; Bucci et al., 2000).

The full-length sequence of the *AtRabG3e* gene was isolated from cDNA of leaves treated with $O_2^{\cdot -}$ + SA by RT-PCR. Primers corresponding to the putative 5′ and 3′ ends of the gene were selected from the Arabidopsis genome database and were used to amplify an approximately 600-bp fragment. The isolated gene differed from the Columbia-0 ecotype in the Arabidopsis database by only a single-nucleotide mis-

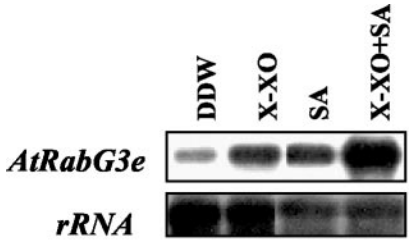

Figure 1. Expression of *AtRab7* (*AtRabG3e*) gene during stress. Leaf RNA was isolated 5 h after treatment with a superoxide generating mixture of 1 mM X and 0.75 units mL^{-1} XO with or without 0.75 mM SA. The substances were infiltrated into interstitial space of intact Arabidopsis leaves. RNA was prepared and used as template for radioactive reverse transcription (RT)-PCR as described in "Materials and Methods." Randomly labeled 18S rRNA from pea (*Pisum sativum*) was used for RNA loading control.

match (Fig. 2). BLAST analysis of the full-length gene against GenBank sequences revealed very high (>90%) homology to another Arabidopsis gene, *AtRabG3f* (At3g18829, formerly *AtRab71*, GenBank accession no. BAB68371). Among plants, the highest homology was to the *LjRab7C* gene from *L. japonicus* and a slightly lower homology to putative *Rab7* genes from other plant species, such as tobacco and pea (data not shown). High homology exists even with mammals, including humans (GenBank accession no. AFO050175). Surprisingly, a relatively low degree of homology (approximately 70%) exists between two *Rab7* genes from *L. japonicus*: the *LjRab7C* (>90% similar to *AtRabG3e*), which is a nodule-specific gene, and the *LjRab7A* gene (GenBank accession no. CAA98168), which is expressed in leaves. The *AtRabG3e* gene sequence contains the specific domains for GTP binding and hydrolysis that is conserved in all Rab proteins. The predicted protein sequence also has a specific effector domain, characteristic of the Rab7 subfamily (Fig. 2).

Analysis of the *AtRabG3e* gene expression in Arabidopsis cell culture by northern blotting showed that the *AtRabG3e* gene was induced by a high but not low concentration of H_2O_2 (Fig. 3A). To mimic continuous production of H_2O_2 as occurring in plants, we used a mixture of Glc and Glc oxidase. We have shown previously that the dose of 10 units mL^{-1} of enzyme produces about 400 μM H_2O_2 in Arabidopsis culture and induced PCD (Tiwari et al., 2002). *AtRabG3e* was also induced in plants infected with avirulent *P. syringae* bacteria or with a *B. cinerea* mold, both of which induce hypersensitive reaction (Fig. 3B; Govrin and Levine, 2000). No induction was seen after treatment with methyl jasmonate, cold, or wounding, and only minor induction was seen after salt stress (data not shown). Analysis of *AtRabG3e* gene in different Arabidopsis organs showed a basal level of expression, although higher expression was detected in older roots (Fig. 3C), in line with the extensive developmental PCD in this tissue (Yang et al., 1999).

Production of Plants Overexpressing AtRab7 (AtRabG3e)

To study the function of the *AtRabG3e* gene in plants, the gene was cloned behind a constitutive 35S cauliflower mosaic virus promoter and introduced into Arabidopsis plants via *Agrobacterium tumefaciens*-mediated transformation. Sixteen independent T_1 lines were obtained, which showed different level of overexpression of the *AtRabG3e* gene. Most transgenic lines had significantly greener leaves and a higher amount of chlorophyll (data not shown) when observed 7 d after germination, but the difference decreased later. The transgenic plants also had somewhat longer petioles. In soil, the transformed plants grew slightly faster than wild type and looked normal throughout development. The flowering time of

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                                          **************
AtRabG3e 1 MPSRRRTLLKVIILGDSGVGKTSLMNQYVNKKFSNQYKATIGADFLTKEVQFEDRLFTLQIWDTAGQERFQ
AtRabG3f 1  ...........................................................................
Lj-Rab7C 1 ..............................R..........................................
Lj-Rab7A 1 .SL.........V................H....Q..........V...L.ID...V..............
Hs-Rab7  1 .T..KKV.....................................I...MVD...V.M.............
Hs-Rab9  1 -M.GKSL.....L...G....S....R..TN..DS.AFH...VE..NRDLEVDG.FV.............K

                                                (N)
AtRabG3e 72 SLGVAFYRGADCCVLVYDVNSAKSFEDLSNWREEFLIQASPSDPENFPFVVIGNKIDVDGGSSRVVSEKKA
AtRabG3f 72 ......................M....N.N.....................L....V...D.N.........
Lj-Rab7C 72 ......................M...DN.N.............................I...N.........
Lj-Rab7A 72 ......................VM...DT.D..H....K..N....RT...ILL.....I...N.........
Hs-Rab7  72 ................F..TAPNT.KT.DS..D........R.........L.....LE---N.Q.AT.R.
Hs-Rab9  71 ..RT P........L.TFS.DDRQ...NNG..QK..IYY.DVK...H.....L...V.KE---D.Q.TTEE.

AtRabG3e 143 RAWCASKGNIPYYETSAKVGTNVEDAFLCITTNAMKSGEEEEMYLPDTIDVGTSNPQRS----TGCEC
AtRabG3f 143 K...........F...........E..Q..AKD.L.......L............Q.----------.
Lj-Rab7C 143 ............F.....G.I...E..Q..AK..L.......L.........N.SQ..A----S....
Lj-Rab7A 143 KD..........F.....EDY..DA.....AKT.LANEHDQDI.FQAIPEAAPE.E.K-----G..Q.
Hs-Rab7  143 Q...Y..N....F.....EAI...Q..QT.AR..L.QET...L.NEFPEPIKLDKND.AKASAES.S.
Hs-Rab9  142 QT..MEN.DY..L.....DD...TV..EEAVRQVLAVE.QL.HCMLGHTIDLN.GSKAG----SS.C-
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Figure 2. *AtRabG3e* gene analysis. The *AtRabG3e* gene was amplified by RT-PCR from RNA prepared from X/XO + SA-treated leaves using the primers from both ends of the gene. The gene was fully sequenced and subjected BLAST analysis against the GenBank database (Altschul et al., 1997). Alignment was done with ClustalW and Boxshadow programs. At, Arabidopsis; Lj, *Lotus japonicus*; Hs, human (*Homo sapiens*). Numbers indicate position of amino acids from the N terminus. The effector region of the Rab7 subfamily is indicated by asterisks. The mismatch between the isolated gene sequence and the GenBank record is at position 100 (resulting in exchange of N to S). The regions involved in GTP binding and hydrolysis are lined on top. The *AtRabG3e* fragment isolated by differential display corresponds to the last 234 bp.

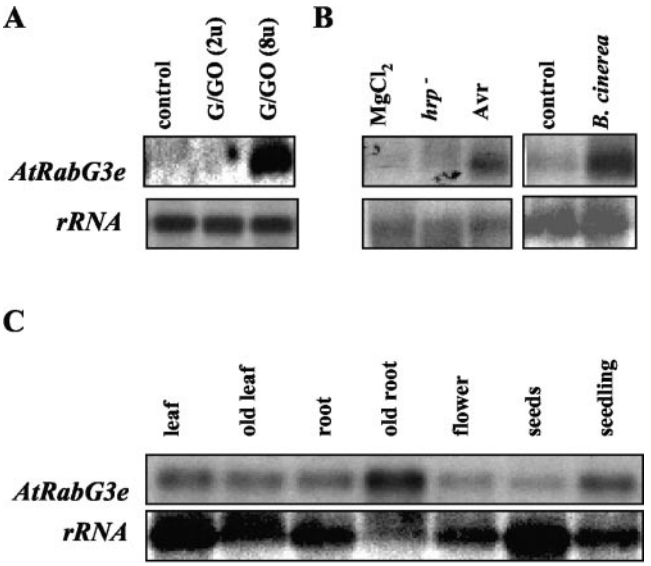

Figure 3. Northern analysis of *AtRabG3e* gene expression. A, *AtRabG3e* gene expression in Arabidopsis cell cultures was tested 2 d after cell transfer. Cultures were treated with 5 mM Glc (G) and either a low (2 units mL^{-1}) or high (8 units mL^{-1}) dose of Glc oxidase (GO) for 3 h. B, *AtRabG3e* expression in Arabidopsis leaves that were inoculated for 3 h with 10^8 colony forming units of avirulent (hypersensitive reaction-inducing) strain of *Pseudomonas syringae* carrying the *avrRpm1* avirulence gene (Avr), nonvirulent *hrp*$^-$ mutant strain, or buffer control (10 mM $MgCl_2$). *Botrytis cinerea* treatment was done by inoculation of leaves with 5 μL of 10^5 mL^{-1} pregerminated spores for 24 h. Mock treatment with inoculation buffer served as control. C, Tissue-specific expression of *AtRabG3e*. Total RNA was prepared from 1-week-old seedlings or from 6-week-old roots (old root), or leaves (old leaf), flowers, or seeds. RNA from other tissues (roots and leaves) was from 4-week-old plants.

the transgenics was accelerated by 3 to 6 d. Two homozygous lines (designated *AtRab7-7* and *AtRab7-9*) that exhibited an average level of transgene overexpression were chosen from T_3 progenitors for more detailed analysis (Fig. 4). Additional lines were included for some experiments; 11 transgenic lines from 13 tested showed similar results with respect to stress tolerance (see below). The opposite approach of suppressing the *AtRabG3e* expression by antisense constructs was not successful, probably due to the large size of the *Rab* gene family in Arabidopsis. Also, no T-DNA insertion mutants were found in the Arabidopsis stock centers.

Accelerated Endocytosis in Transgenic Plants

Given the involvement of the Rab7 in endocytosis in several systems (Marcote et al., 2000), we tested the rate of internalization of a lipophilic membrane probe FM1-43 into the cytosol (Smith and Betz, 1996). This dye is virtually nonfluorescent in aqueous medium but becomes fluorescent after coating the plasma membrane and internalization by the nonspecific general endocytotic pathway. FM1-43 was shown to be taken up by plant cells from the plasma membrane via the nonspecific endocytotic pathway in a time-dependent manner and finally accumulated within the vacuoles (Emans et al., 2002). The rate of the probe uptake was examined in leaf sections and in roots of wild-type and transgenic plants. Seedlings were placed on a microscope slide in a solution of FM1-43 and analyzed over a period of 20 min under a fluorescent microscope. Rapid dye uptake into roots of the transgenic plants was observed (Fig. 5, A–F). In addition, FM1-43 internalization was analyzed in cross sections of Arabidopsis leaves (Fig. 5, H–M). The dye accumulated much faster inside the cells of transgenic plants in both root and leaf tissues (Fig. 5). The accelerated uptake of the dye in the transgenic plants was also retained in protoplasts prepared from mature leaves (Fig. 5N).

The Effect of AtRab7 (AtRabG3e) Overexpression on Salt Stress Tolerance

To analyze the impact of constitutive high AtRab7 expression on stress tolerance, the wild-type and transgenic plants were subjected to oxidative stress-triggered PCD by infiltration of a superoxide-producing mixture together with SA (Mazel and Levine, 2001). The treatment resulted in necrotic lesions, but no significant differences were observed between the wild-type and the transgenic plants (data not shown).

To test resistance to other, less toxic stresses, such as salt, 2-week-old plants were irrigated with 200 mM NaCl. The treatment inhibited growth in both wild-type and transgenic plants, but the transgenic plants were much less sensitive (Fig. 6A) and achieved higher fresh weight (Fig. 6B). Significant salt tolerance was also observed in mature 25-d-old transformants (Fig. 6C). Virtually all of the wild-type plants died 15 d after beginning of treatment with 200 mM NaCl, whereas many of the transformed plants still had green leaves (data not shown). Constant irrigation with 100 mM NaCl had a smaller effect, and some of the transgenic plants even began flowering (data not shown). The effect of the transgene was even more pronounced when plants were grown in agar plates (Fig. 6D). About 20% of the transformed

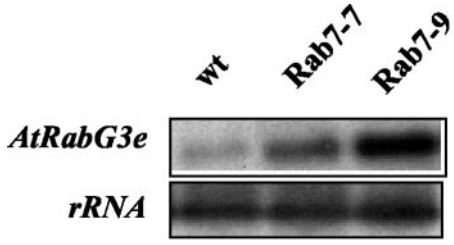

Figure 4. *AtRab7* expression in wild-type and transgenic Arabidopsis. Northern-blot analysis of *AtRab7* gene expression in wild-type (wt) and *AtRab7* transformed Arabidopsis plants (lines *AtRab7-7* and *AtRab7-9*). Total RNA was extracted, and 5 μg was used to probe with *AtRab7* or with 18S rRNA (as loading control).

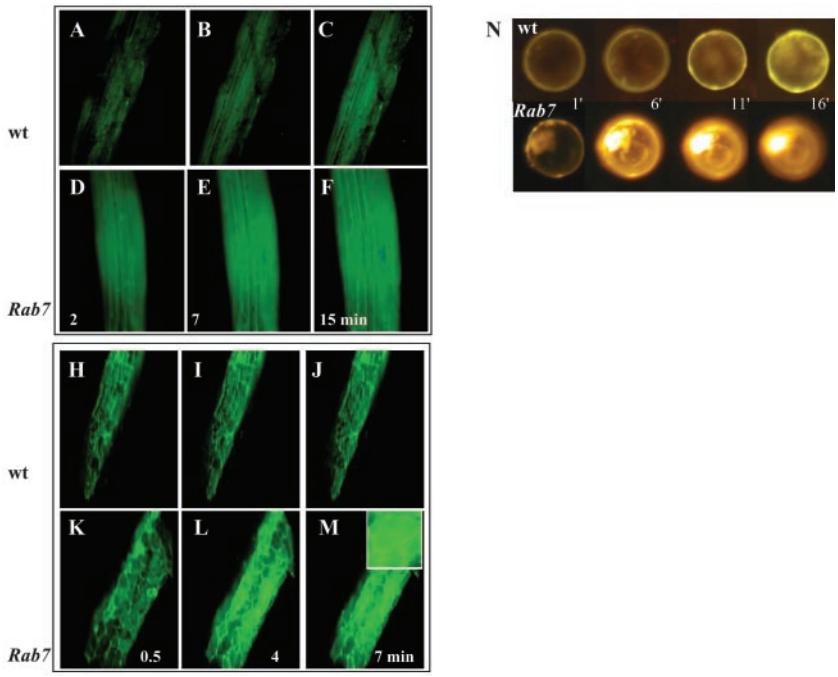

Figure 5. Uptake of the membrane probe FM1-43 in transgenic Arabidopsis. A to F, Wild-type (A–C) and *AtRab7-7* transgenic (D–F) seedlings were grown in Gelrite for 12 d. Plants were placed on microscope slides without cover glass and overlaid with solution of FM1-43. Pictures were taken in an epi-fluorescent microscope at the indicated times. H to M, Leaves of 24-d-old wild-type (H–J) and transgenic plants (K–M) growing in soil were cut through a potato tuber to obtain thin cross sections that were incubated in FM1-43 and photographed at the indicated times. Inset, Magnified view that shows the spreading of the dye within cells. N, Protoplasts were prepared from leaves of 5-week-old wild-type (wt) and *AtRab7-7* transgenic plants. Protoplasts were placed in an etched microscope slide, and the FM1-43 dye was added. Numbers indicate time after dye addition in minutes.

plants remained viable on 150 mM NaCl for more than 2 months.

Enhanced Accumulation of Sodium in Transgenic Plants

High salt concentration imposes both a hyperosmotic and a hyperionic stress. To resist the salt stress, plants use ions for osmotic adjustment. Ion accumulation in the vacuole keeps sodium away from the cytosol, while at the same time facilitating water uptake (Hasegawa et al., 2000). Intracellular compartmentation of Na^+ within vacuoles was found to have a decisive effect on plant salt tolerance (Apse et al., 1999; Xiong et al., 2002).

Sodium distribution in the plants was probed by using a cell-permeable sodium indicator, SodiumGreen diacetate, which is not fluorescent in the apoplast but becomes fluorescent after entering the cell and binding the sodium ions inside the cell (Szmacinski and Lakowicz, 1997). Diffuse fluorescence was detected in the wild-type plants treated with sodium, but in the transgenic plants, strong signal was detected in the vacuoles, suggesting accumulation of sodium in these organelles (Fig. 7, A and B).

To test directly whether the vacuolar compartmentalization of sodium in the transgenic plants resulted in a higher sodium uptake, we measured accumulation of sodium using a radioactive sodium tracer. Plants were germinated on agar plates and transferred after 7 d to plates containing 150 mM NaCl. Sodium uptake was assayed by addition of 5 μCi ^{22}NaCl. Three days later, the aerial parts of plants were removed, and the amount of the radioactive sodium was measured in a gamma counter. Significantly higher sodium concentration was detected in the transgenic plants (Fig. 7C).

Production of ROS during Salt Stress

One of the major causes of almost any stress-dependent damage is the development of an associated secondary oxidative stress. Increased antioxidant potential has been shown to improve tolerance to many abiotic stresses, including salt (Van Camp et al., 1996; Borsani et al., 2001). We tested ROS production in wild-type and transgenic plants during salt stress. High amounts of ROS were observed in wild-type but not in the transgenic plants after overnight exposure to 200 mM NaCl (Fig. 8, A and B). The NaCl-induced ROS production was inhibited by DPI, implying activation of the NADPH oxidase by salt (Fig. 8C).

Increased Tolerance of Transgenic Plants to High Concentration of Sorbitol

To test whether the transgenic plants were resistant to the ionic or the osmotic components of the NaCl stress, the transgenic plants were subjected to an osmotic stress produced by a nonionic osmolyte sorbitol. Four days after germination, plants were transferred to plates containing 500 mM sorbitol. The treatment caused severe growth retardation in both the wild-type and the transgenic plants, but the effect was more pronounced in the wild-type plants (Fig. 9). Some wild-type plants became bleached and died. The recovery from osmotic stress was assayed by transferring the plants from sorbitol-containing plates after 1 week into plates without sorbitol. The

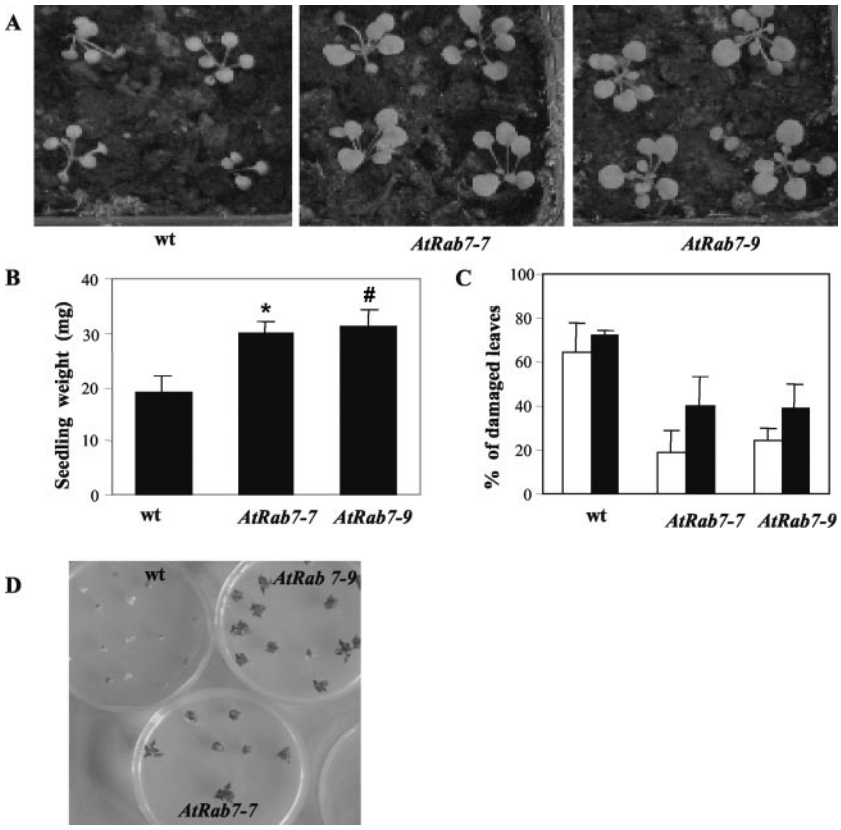

Figure 6. Salt stress tolerance in wild-type and *AtRabG3e* transgenic Arabidopsis. A, Salt tolerance in young plants. Both wild-type and transgenic plants were germinated on agar plates and transferred after 7 d to soil. After another 7 d, plants were irrigated every 3 d with 200 mM NaCl from below (by placing the pots into salt solution) and from above (by adding 20 mL of solution per pot). The photograph was taken 1 week after beginning of treatment. The representative experiment from two performed is shown ($n = 8$). B, Fresh weight of the plants shown in A. The values are means ± SE of the representative experiment, $n = 8$. *, $P = 0.013$; #, $P = 0.009$ compared with wild type. Two experiments were performed with similar results. C, Leaf damage during salt stress in plants grown in soil for 25 d and then treated with salt as described in A. The percentage of damaged leaves was scored on d 5 (white bars) and 7 (black bars) after the treatment. Leaves were classified as damaged if more than 50% of a leaf's surface was bleached, or if they appeared severely wilted. Shown is one of three experiments with similar results. No damage was seen in plants irrigated with water. D, Salt tolerance in plants grown on agar. Seedlings were germinated on agar plates containing one-half-strength Murashige and Skoog salts and transferred after 7 d to plates supplemented with 150 mM NaCl. The photograph was taken 4 weeks after transfer to high salt.

majority of wild-type plants failed to resume growth and died, whereas 100% of the transgenic plants fully recovered (Fig. 9C). The effect of the *AtRabG3e* transgene was also clearly seen in the weight attained after 7 d in sorbitol (Fig. 9A).

DISCUSSION

Isolation of the AtRab7 (AtRabG3e) Gene and Characterization of Its Expression

Here, we report isolation of *AtRabG3e* gene that was induced during PCD, triggered by superoxide and SA (Fig. 1). The Rab7 proteins are important component of the vesicle trafficking system in all eukaryotes (Zerial and McBride, 2001). Based on animal and yeast studies, the role of AtRab7 protein in eukaryotes seems to be associated with the late endocytosis, where it functions in the fusion of late endosomes to lysosomes or vacuoles.

Little is known about the molecular function of the individual Rab proteins in plants. Rab5 protein, which is one of the more studied members, was shown to participate in the early endocytic pathway in legumes, and elevated expression of the *Rab5* and *Rab7* genes was found in developing root nodules (Marcote et al., 2000). Our analysis of the Arabidopsis *AtRabG3e* (*Rab7*) gene expression shows that this gene was induced during severe biotic or abiotic stresses. It was induced by combined treatment with superoxide and SA (Mazel and Levine, 2001; Fig. 1) and by pathogens, such as avirulent *P. syringae* bacteria or *B. cinerea* fungus (Fig. 3), i.e. treatments that induced hypersensitive cell death (Govrin and Levine, 2000). However, because the expression of this gene was not analyzed in situ, it is not possible to conclude whether it is induced in the damaged tissue or in the surrounding cells proximal to the necrotic areas (Levine et al., 1994). Hence, it may be involved either in the induction or execution of cell death or in cell-protective responses.

The lack of *AtRabG3e* gene induction by cold, wounding, or methyl jasmonate treatments suggests that *AtRabG3e* is not a general stress-regulated gene. The *AtRabG3e* gene also was not strongly induced during salt treatment. Interestingly, induction of a related *Rab5b* gene was detected in *Mesembryanthemum crystallinum* plants treated with salt (Bolte et al., 2000).

Accelerated Membrane Endocytosis in AtRab7 (AtRabG3e) Transgenic Plants

Analyses of general membrane endocytosis in transgenic plants revealed accelerated uptake of the membrane dye FM1-43 in root and leaf cells (Fig. 5). The increased endocytosis in plants that overexpress the *AtRabG3e* gene may stem from faster vesicle trafficking at the end point, i.e. endosome-vacuole fu-

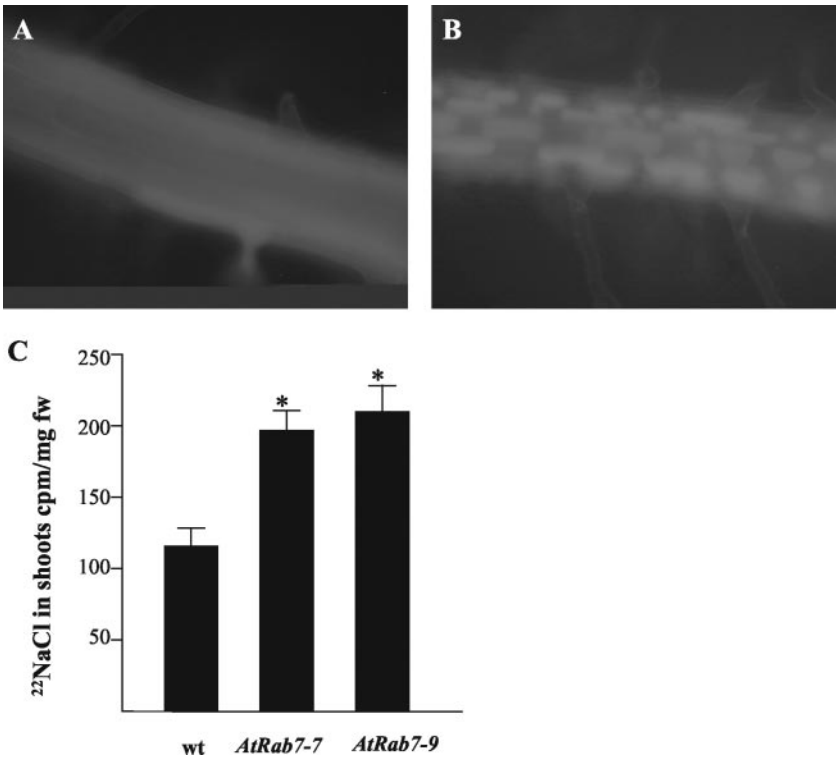

Figure 7. Sodium localization in roots and in shoots. A and B, Wild-type (A) and transgenic (B) plants were germinated on agar plates. After 8 d, both plants were transferred to Whatman 3M paper soaked with 100 mM NaCl. SodiumGreen dye was added to the medium 2 d later, and plants were examined under fluorescent microscope after 36 h. C, Wild-type and transgenic plants were germinated on agar plates in one-half-strength Murashige and Skoog medium. After 7 d, the plants were transferred to plates containing 150 mM NaCl. One day later the plates were air dried (by opening the cover of the petri dish for 6 h in a laminar flow hood) and filled with 8 mL of water containing 0.81 μCi ^{22}NaCl (Amersham, Buckinghamshire, UK). The aerial parts of the seedlings were harvested after 3 d and measured in a γ-counter (5 min sample^{-1}). Samples ($n = 5$) were analyzed according to unpaired Student's t test. *, P value $<$ 0.03 (compared with wild type). Representative experiment from three similar tests is shown.

sion. An increase in Rab7-dependent vacuolation by merging of late endosomes has been described in *Dictyostelium discoideum* and in HeLa cells (Papini et al., 1997; Bruckert et al., 2000). Vacuoles play a critical role in salt tolerance by accumulating sodium, which is removed from the cytosol, where it is toxic (Apse et al., 1999; Xiong et al., 2002). Interestingly, overexpression of the constitutively active form of Rab5, which functions in the early steps of endocytosis in HeLa cells, led to enhanced early endosome fusion that resulted in the enlargement of early endosomes (Ceresa et al., 2001). Further research, however, is needed to determine the rate-limiting step of the inwards vesicular traffic.

The Effects of AtRab7 (AtRabG3e) on Salt Localization

The nature of the damage inflicted by high salt and the molecular mechanism that mediates salt tolerance are not entirely clear (Hasegawa et al., 2000; Zhu, 2001b). Arabidopsis is a glycophytic plant that is sensitive to moderate levels of NaCl. Several mechanisms that confer salt tolerance in plants have been identified: (a) extrusion of Na^+ from the cytosol, (b) accumulation of osmolytes, (c) repair of salt-induced membrane damage, and (d) induction of antioxidant defenses (Zhu, 2001a). Moreover, each mechanism can be accomplished in several different ways. For example, extrusion of Na^+ from the cytosol can be attained by sodium transport into vacuole (Apse et al., 1999) or by preventing its entry into plant roots (Rus et al., 2001).

When exposed to salinity, Arabidopsis plants accumulate Na^+ in shoots. Plants that are mutated in the *sas1* gene, which causes overaccumulation of sodium in the shoots, exhibit a severely repressed growth phenotype (Nublat et al., 2001). However, although the *AtRabG3e* transgenic plants showed increased sodium content in the shoots (Fig. 7C), they grew better than wild-type plants in saline conditions (Fig. 6). The positive effect of the transgene is probably due to the preferential accumulation of sodium in vacuoles, as observed in the transgenic plant roots (Fig. 7B). These results are in agreement with work in fission yeast that showed involvement of the yeast homolog of Rab7 in vacuolar fusion in cellular responses to osmotic stress (Bone et al., 1998).

The Effects of AtRab7 (AtRabG3e) on Osmolyte and ROS Production

Reduction in damage to cellular components can also be achieved by several mechanisms: production of different/additional osmolytes or by antioxidant mechanisms. We measured Pro concentration in wild-type and transgenic plants but did not find significant difference (data not shown). This result is consistent with the predicted function of Rab7 in vacuolar fusion, whereas the preferential localization of Pro is in the cytosol (Leigh et al., 1981; Pahlich et al., 1983).

One of the factors affecting plant stress resistance in general and salt stress, specifically, is development of a secondary oxidative stress (Fig. 8; Zhu, 2001a).

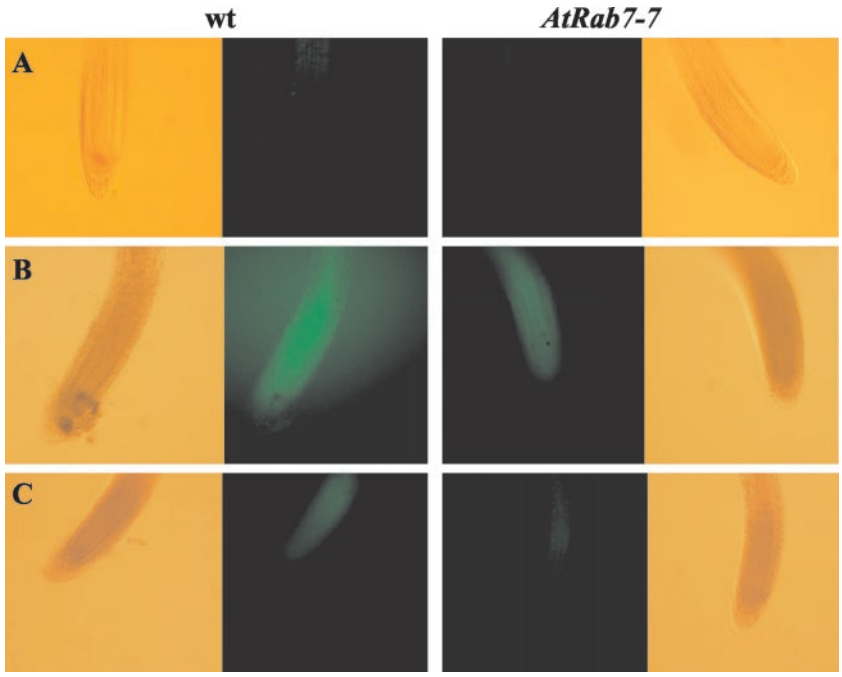

Figure 8. ROS production in salt-stressed Arabidopsis seedlings. A and B, Wild-type and transgenic plants were germinated on agar plates containing one-half-strength Murashige and Skoog medium. After 7 d, seedlings were transferred to one-half-strength Murashige and Skoog liquid medium with (B) or without (A) 200 mM NaCl for 16 h. ROS production was detected in an epi-fluorescent microscope (IX70, Olympus, Tokyo) by addition of 10 μM 2′,7′-dichlorofluorescin. Pictures were taken 5 min after addition of the dye. The corresponding bright-field photographs are at the sides. C, Involvement of NADPH oxidase in ROS production of salt-treated seedlings was tested by addition of an NADPH oxidase inhibitor, diphenylene iodonium (DPI), in two steps: 20 μM at the onset of the salt treatment and 30 min before the photography. A representative experiment from three with similar results is shown. Similar results were also obtained with *AtRab7-9* transgenic plants (data not shown).

ROS can directly damage cellular components, such as membrane lipids. Lipid peroxidation causes malfunctioning of the membrane, resulting in increased permeability to ions. ROS generation during salt stress can result from electron leakage toward oxygen, caused by defected electron flow due to altered membrane ionic interactions (Koyro, 1997; Borsani et al., 2001; Slesak et al., 2002). Alternatively, ROS production can result from induction of an NADPH oxidase activity (Kawano et al., 2001). Inhibition of NaCl-triggered ROS production in plants by DPI (Fig. 9C) supports the latter mechanism. Further work is needed to elucidate the mechanism that reduced the NADPH oxidase activity in transgenic plants. GTPases from the Rho family that is related to Rabs have been shown to regulate many cellular

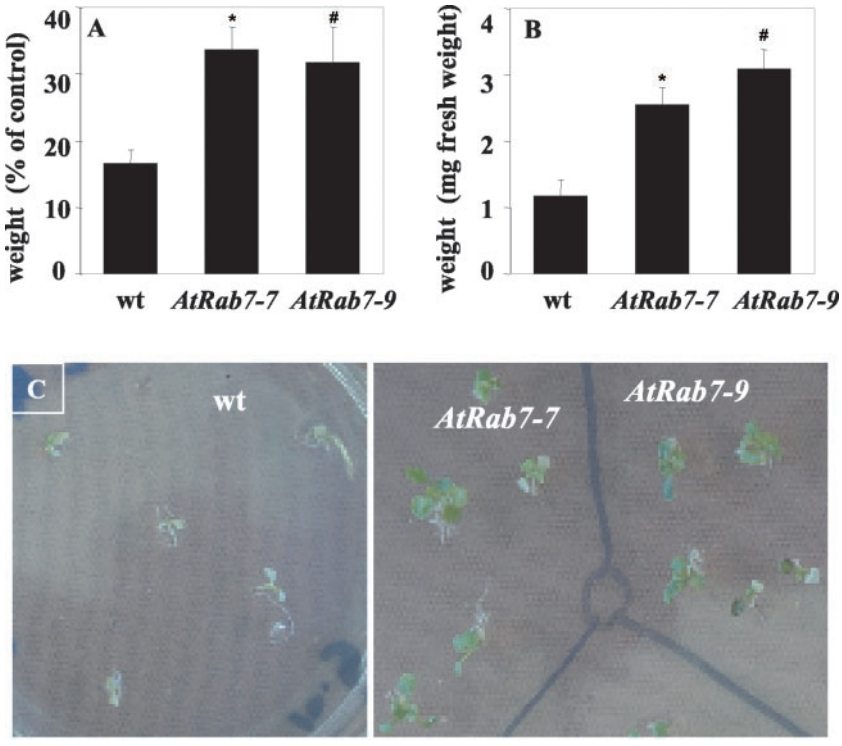

Figure 9. Tolerance of wild-type and *AtRabG3e* transgenic plants to osmotic stress. A, Wild type (wt) and transgenic plants were germinated on agar plates, and after 4 d, the seedlings were transferred to plates containing 500 mM sorbitol. After 7 d of growth in sorbitol, the aerial parts of the plants were cut off and their fresh weight measured. Because the transgenic plants develop slightly slower, the data are presented as a percentage of shoot weight of untreated plants (in each line separately). The values are means ± SE ($n = 5$) of a representative experiment from three performed. *, $P = 0.003$; #, $P = 0.03$ compared with wild type. B, Recovery of wild-type and transgenic plants after sorbitol stress. After growth of plants for 1 week on the sorbitol-containing agar, plants were transferred back to one-half-strength Murashige and Skoog salts and grown for an additional 7 d. The values are mean fresh weight of shoots ± SE of two experiments ($n = 5$). *, $P = 0.0009$; #, $P < 0.0001$ compared with wild type. C, Photograph of plants from the representative experiment described in B, 7 d after transfer of plants back to one-half-strength Murashige and Skoog medium.

processes, including cytoskeletal organization, pathogenesis signal transduction, and activation of the NADPH oxidase (Tolias et al., 1998). Studies in mammalian systems show that superoxide is released from stimulated neutrophils through exocytosis (Sengelov et al., 1992; Kobayashi et al., 1998). Interestingly, neutrophils NADPH oxidase activity is blocked by endogenous adenosine, which increased endocytosis in these cells (Swain et al., 2003).

The Involvement of Intracellular Vesicle Trafficking in Salt and Osmotic Stress Signaling

Increase in the vacuolar volume during salt stress can also serve as salt tolerance mechanisms in plant cells (Mimura et al., 2003). The latter process requires delivery of membrane material to the vacuole by an intracellular vesicle trafficking system, which is in line with the predicted function of *AtRabG3e*.

A link between endocytosis and salt tolerance was suggested by screening of yeast mutants of endocytosis for susceptibility to salt stress (Whitacre et al., 2001). In that study, a high correlation was found between increased susceptibility to salt and several additive defects in endocytosis. Reduced endocytosis was also implicated in excess susceptibility to salt stress of yeast that was defective in vacuolar H^+-ATPase (Munn and Riezman, 1994). Taken together, the emerging function of the Rab7 proteins in the vacuolar biogenesis of eukaryotic cells (Bruckert et al., 2000; Bucci et al., 2000) with the role of vacuoles in salt tolerance (Blumwald, 2000), we suggest that vesicle trafficking can act as a salt tolerance determinant.

The tolerance of the *AtRabG3e* transgenic plants to high salinity and to high sorbitol concentration indicate that they can tolerate both hyperosmotic and hyperionic stresses. The osmotic stress signal transduction can also be affected by intracellular vesicle trafficking, as inferred from the established role of phosphatidylinositol signaling in the vesicle trafficking and resistance to abiotic stresses (Levine, 2002; Zhu, 2002). Rapid increase in synthesis and accumulation of phosphatidylinositol(4,5)P_2 and (1,4,5)P_3 is a common early molecular response to both hyperosmotic and hyperionic stresses (Pical et al., 1999; DeWald et al., 2001; Meijer et al., 2001). Also, the levels of inositol triphosphate (IP3) in whole plants increase significantly within minutes of treatment with salt or with sorbitol. In addition to direct signaling activities, IP3 and other phosphoinositols influence the release of calcium, which mediates the osmotic and/or ionic stress responses (Knight et al., 1997; Zhu, 2002).

Salt stress induced the NtSyr1 protein at the plasma membrane of tobacco, suggesting its involvement in salt responses (Leyman et al., 1999). Interestingly, unlike other syntaxin proteins, NtSyr1 harbors a putative EF hand Ca^{2+}-binding sequence that may function in transduction of Ca^{2+} signals (Geelen et al., 2002). A similar EF hand-type calcium-binding domain also exists in the SOS3 protein, which functions in the signal transduction of salt tolerance (Zhu, 2002). Moreover, rapid endocytosis of the FM1-43 membrane probe was stimulated by calcium in the plant pollen tube apex cells (Camacho and Malho, 2003).

Vesicle trafficking and especially endocytosis were usually considered as housekeeping activities. However, it was shown recently that NtSyr1 is involved in abscisic acid-mediated responses (Geelen et al., 2002). Moreover, Rab5 was shown to regulate endocytosis in HeLa cells by a stress-responsive p38 MAP kinase, providing a molecular mechanism linking endocytosis with stress signal transduction (Cavalli et al., 2001).

In summary, we show profound effects on membrane endocytosis and stress tolerance in plants overexpressing the Arabidopsis *Rab7* gene. Overexpression of *AtRabG3e* in plants resulted in several protective mechanisms against ionic and/or osmotic stresses, such as increased sodium accumulation in the vacuole and reduced generation of ROS. Crossing the *AtRabG3e* transformants with plants engineered for increased tolerance by other mechanisms may further improve plant salt tolerance by combining several independent mechanisms (Hasegawa et al., 2000; Zhu, 2001b).

MATERIALS AND METHODS

Biological Material and Plant Treatment

Arabidopsis seeds (ecotype Wassilewskija) and *Pseudomonas syringae* pv *tomato* bacteria were from Jeffrey Dangl (University of North Carolina, Chapel Hill). Plants were grown as described by Mazel and Levine (2001). The Arabidopsis cell culture was maintained as described by Tiwari et al. (2002). *Botrytis cinerea* (strain INRA) was from the Alfred Mayer collection (Hebrew University, Israel). The fungal and bacterial infections and the substance infiltrations into leaves was done with syringe as described by Govrin and Levine (2002).

Differential Display and Cloning

Total RNA was isolated with the RNAeasy kit (Qiagen, Hilden, Germany) and stored with RNAase inhibitor (5Prime → 3Prime Inc., Boulder, CO). RNA was treated with 0.05 units μL^{-1} DNAase (Promega, Madison, WI) for 1 h at 37°C. RT was done with at 37°C for 1 h using 0.1 μg of total RNA, 0.1 μM T11-C (GenHunter, Nashville, TN) and 200 units of Moloney murine leukemia virus-RT (Gibco BRL, Grand Island, NY). Radioactive RT-PCR was performed with the RNAimage kit (Genhunter Corp., Brookline, MA) using [^{35}S]dCTP, Supernova *Taq* polymerase (JMR Holdings, Kent, UK), and primers T_{11}-G and AP-5. The full-length *AtRabG3e* cDNA was isolated by RT-PCR from plants treated with X/XO + SA by using the following primers: forward, ATGCCTTCTCGTAGAAGAACTCTCCTC; and reverse, TCAGCATTCACATCCTGTTGACCT. The PCR cycle was: 94°C, 1 min (94°C, 30 s; 70°C, 1 min (−1°C per cycle); and 72°C, 2 min) × 14; and (94°C, 30 s; 56°C, 1 min; and 72°C, 2 min) × 30.

Northern Blotting

Five micrograms of total RNA were separated on 1.2% (w/v) agarose gel and transferred to nylon Hybond N^+ membrane (Amersham). Blots were

probed with the *AtRabG3e* fragment obtained from differential display. The gene-specific probes were prepared by unidirectional PCR (10 ng of DNA; 2 μM reverse primers; 0.25 units of Supernova *Taq* polymerase; 15 μM each of dATP, dGTP, and dTTP; and 5 μL of 110 TBq $mmol^{-1}$ [^{32}P]dCTP). The PCR protocol was: 94°C, 1 min (94°C, 45 s; 54°C, 2 min; 72°C, 2.5 min) × 20; and 72°C, 7 min.

Construction of Transgenic Plants

The *AtRabG3e* gene was cloned into *Sal*I and *Sac*I sites of the PMD1 binary vector (Chris Lamb, Salk Institute, La Jolla, CA). The gene was introduced into Arabidopsis plants by a floral dip method via *Agroacterium tumefaciens* (Clough and Bent, 1998).

Analysis of Endocytosis

Whole plants or leaf cross-sections were incubated with the lipophilic membrane probe FM1-43 (Molecular Probes, Eugene, OR) directly under the epi-fluorescent microscope (Olympus IX70) equipped with a narrow band filter cube (excitation/emission 485DF22/535DF35) from Omega Optical, Inc. (Brattleboro, VT). Pictures were taken with a Coolpix 950 camera (Nikon Corporation, Tokyo) using identical exposure settings, as previously described (Govrin and Levine, 2000). Protoplasts were prepared according to Hawes and Satiat-Jeunemaitre (2001).

NaCl Localization in Plants

Eight-day-old plants were transferred to Whatman 3M paper (Whatman, Clifton, NJ) soaked with water or with 100 mM NaCl. After 2 d of growth in low-light conditions, SodiumGreen dye (Molecular Probes) was added to the petri dishes containing the Whatman 3M paper. The Sodium Green-dependent fluorescence was observed in the fluorescent microscope using narrow-band excitation (485DF22) and emission (535DF35) filters as described by Szmacinski and Lakowicz (1997). No autofluorescence was detected in control plants.

ROS Detection in Plants

Seedlings were taken out of agar after 7 d, washed, and transferred to one-half-strength Murashige and Skoog medium with or without 200 mM NaCl. ROS were detected with a fluorescent microscope 16 h later by addition of 10 μM 2′,7′-dichlorofluorescin (similar results were obtained also after 42 h). DPI (20 μM) was added at the onset of salt treatment and again 30 min before observations.

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