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Plasma Membrane–Associated ROP10 Small GTPase Is a Specific Negative Regulator of Abscisic Acid Responses in Arabidopsis

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Abscisic acid (ABA) is an important plant hormone that modulates seed germination and plant growth and stress responses, but its signaling remains poorly understood. We investigated the role of ROP10, a member of the Arabidopsis Rop subfamily of Rho GTPases, in ABA signaling. A null *rop10* mutant exhibits enhanced responses to ABA in seed germination, root elongation, and stomatal closure assays and in the induction of expression of the transcription factor MYB2, but it shows wild-type levels of ABA and normal responses to other hormones. Consistently, transgenic expression of a constitutively active form of *ROP10* reduces ABA inhibition of seed germination, whereas dominant-negative mutants of ROP10 enhance ABA response and partially suppress *abi2*. Furthermore, ABA specifically downregulates *ROP10* transcription in root tips. ROP10 is localized to the plasma membrane (PM), and PM localization is crucial for its function. These results suggest that ROP10 is a PM-localized signaling molecule that is involved specifically in the negative regulation of ABA signaling.

INTRODUCTION

Abscisic acid (ABA) is a phytohormone that modulates a variety of growth and developmental processes and responses to the environment, ranging from the establishment of seed dormancy and stomatal movement to drought, salt, and cold stress responses (for a recent comprehensive review, see Finkelstein et al., 2002). ABA signaling pathways remain poorly defined, although several candidate genes involved in ABA signaling have been identified through either forward or reverse genetic approaches. These include genes that encode intracellular signaling proteins such as heterotrimeric G-proteins, protein kinases and protein phosphatases, and transcription factors, and proteins involved in post-translational protein modification, RNA processing, and inositol trisphosphate metabolism (Finkelstein and Lynch, 2000; Li et al., 2000; Hugouvieux et al., 2001; Merlot et al., 2001; Sanchez and Chua, 2001; Wang et al., 2001; Xiong et al., 2001a, 2001b; reviewed by Finkelstein et al., 2002). However, pleiotropic effects of their mutant alleles and cross-talk between ABA and other pathways most likely account for the difficulty of elucidating ABA signal transduction pathway(s).

Among the pleiotropic genes, the *ERA1* gene that encodes the β-subunit of protein farnesyltransferase (FTase) is particularly interesting (Cutler et al., 1996; Pei et al., 1998). FTase attaches a farnesyl lipid to the Cys (C) residue of a CaaX motif (where a indicates usually aliphatic amino acids and X indicates any amino acid except Leu [L]) located at the C terminus of target proteins. Farnesylation and other lipid modifications, such as geranylgeranylation, myristoylation, and palmitoylation, allow proteins to be targeted to membranes; thus, it is a prerequisite for the function of many signaling proteins, including the small GTPase superfamily (Resh, 1999; Yang, 2002). Ras GTPases are the best-studied FTase targets in mammals, but they are absent in plants. However, among 11 members of the Rop subfamily of Rho GTPases in Arabidopsis, ROP9 and ROP10 contain a putative farnesylation motif, whereas others contain either the CaaL motif for geranylgeranylation (ROP1 to ROP8) or no prenylation motif (ROP11) (Zheng and Yang, 2000; Yang, 2002).

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The function of ROPs has been studied using transgenic expression of constitutively active (CA) and dominant-negative (DN) mutants of ROPs (Yang, 2002). This approach has implicated two putative geranylgeranylation targets (ROP2 and ROP6/AtRac1) in the negative regulation of ABA responses (Li et al., 2001; Lemichez et al., 2001), but dominant mutants of ROP2 and ROP6 also caused many other phenotypes (Li et al., 2001; Molendijk et al., 2001; Fu et al., 2002; Jones et al., 2002). Recent studies using loss-of-function mutants of Rac in *Drosophila* demonstrate that dominant mutants caused certain phenotypes unrelated to Rac signaling (Hakeda-Suzuki et al., 2002). Therefore, it is not clear whether the observed changes in ABA responses induced by the dominant mutants of ROP2 and ROP6 reflect their function in ABA signaling.

Here, we report the functional study of ROPs using a loss-of-function mutant. We show that a null mutation in *ROP10* specifically enhances various ABA responses and gene expression and that the localization of ROP10 to the plasma membrane (PM) is critical for its function. Together with dominant mutants of *ROP10*, our results provide genetic evidence that ROP10 acts as a negative regulator of a PM-associated ABA signaling pathway.

RESULTS

The *rop10-1* Mutation Specifically Enhances ABA Sensitivity

To test the possibility that ROP10 is an ERA1 target, we isolated a T-DNA insertional mutant of *ROP10*, designated *rop10-1*. The *rop10-1* mutant contains a T-DNA insertion at the 5′ end of the second exon that encodes the 36th amino acid, Asp; thus, the mutation would likely truncate the conserved effector domain, three GTP/GDP binding or GTP hydrolysis domains, and the C-terminal membrane localization motifs (Zheng and Yang, 2000). Indeed, reverse transcriptase–mediated (RT) PCR analysis failed to detect any full-length *ROP10* transcript in the mutant (Figure 1A), suggesting that no intact protein is produced. Given the importance of the major domains, particularly the C terminus, of ROP10 in GTPase localization and function, the abnormal transcript that resulted from the T-DNA insertion, if expressed stably, is unlikely to be functional. Therefore, the *rop10-1* mutant is likely null.

Because *rop10-1* does not have any visible whole plant phenotype, we investigated various ABA responses. We first characterized the root elongation response. As shown in Figure 1B, *rop10-1* dramatically enhanced the ABA inhibition of root elongation. Application of 1 μM ABA did not inhibit root elongation in the wild-type ecotype Wassilewskija (Ws) but caused ∼45% reduction in root elongation in *rop10-1*. Higher levels of ABA (10 and 50 μM) inhibited the elongation of Ws roots, but the inhibition was much stronger in *rop10-1*. To confirm that the enhanced ABA response in *rop10-1* is attributable to the loss of *ROP10* function, *rop10-1* plants were transformed with a *35S*:*ROP10* cDNA construct. Seedlings from three independent transgenic lines expressing the *ROP10* transgene (Figure 1A) showed similar ABA inhibition of root elongation as Ws (Figure 1C), demonstrating that ROP10 is involved in the regulation of ABA responses. The ABA content in *rop10-1* was similar to that in Ws (seeds, 127.2 ± 12.2 and 135.0 ± 9.1 ng/g fresh weight; seedlings, 1.9 ± 0.2 and 1.7 ± 0.1 ng/g fresh weight, for the wild type and *rop10-1*, respectively [$n = 3$]), showing that ROP10 affects ABA sensitivity rather than accumulation.

Because several hormones are known to affect root elongation and because of cross-talk between ABA and other hormones (reviewed by Gazzarrini and McCourt, 2001; Finkelstein et al., 2002), we tested whether *rop10-1* also was altered in response to other hormones. Interestingly, *rop10-1* and Ws responded similarly to four hormones—auxin, cytokinin, ethylene, and brassinolide—that are known to inhibit root elongation (Figures 1D to 1G). Given the interaction of ABA with ethylene and brassinolide signaling in root elongation responses (Ephritikhine et al., 1999; Beaudoin et al., 2000; Ghassemian et al., 2000), these results suggest that ROP10 is a specific negative regulator of ABA signaling.

ROP10 Regulates Various Aspects of ABA Responses

The hormonal specificity of ROP10 function indicates that ROP10 might act early in ABA signaling. If this is the case, *rop10-1* would be expected to be altered in other ABA responses. We characterized the ABA response in guard cell movement. RT-PCR analysis (Figure 2A) and transgenic expression of a *ROP10* promoter:GUS fusion protein (see below) confirmed *ROP10* expression in guard cells. The *rop10-1* mutant exhibited hypersensitivity to ABA stimulation of stomatal closure (Figure 2B). Interestingly, the dose–response curve of *rop10-1* to ABA was very similar to that for the *abh1* mutant (Hugouvieux et al., 2001). Because ABA-stimulated stomatal closure in guard cells is responsible for reducing transpiration under water-deficit stress, we measured water loss in detached rosette leaves. As expected, *rop10-1* exhibited reduced water loss compared with the wild type, indicating a possible increase in sensitivity to endogenous ABA (Figure 2C).

ABA also modulates seed dormancy and germination. As shown in Figure 2D, freshly harvested *rop10-1* seeds germinated at significantly lower rates than Ws seeds but showed an identical germination rate after 4-day cold treatments, suggesting that dormancy is enhanced in *rop10-1* seeds. In the presence of 0.6 to 1.2 μM ABA, the germination rate of cold-treated *rop10-1* seeds was ∼50% that of Ws seeds (Figure 2E), demonstrating that the mutation also enhances ABA inhibition of seed germination.

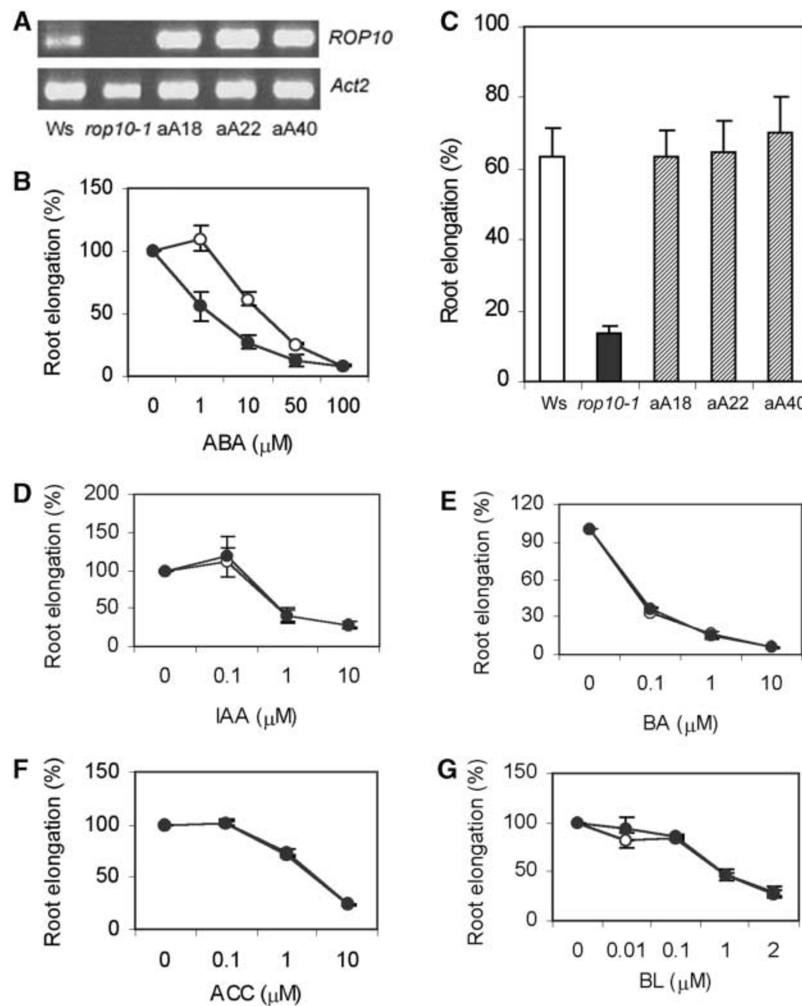

Figure 1. The *rop10-1* Mutant Specifically Enhances the ABA Sensitivity of Root Elongation Inhibition.

(A) RT-PCR analysis of *ROP10* mRNA expression in seedlings of the wild type (Ws), the *rop10-1* mutant, and three transgenic lines (aA18, aA22, and aA40) expressing the *35S:ROP10* transgene in *rop10-1*. *Actin2* (*Act2*) was used as a reference.

(B) The *rop10-1* mutant exhibits hypersensitivity of root growth inhibition to ABA. Root elongation was calculated as the percentage of root length increase compared with roots grown on ABA-free medium, which was designated 100%. Data represent means ± SD of three replicates, each with 10 seedlings. Open circles, Ws; closed circles, *rop10-1*.

(C) Complementation of *rop10-1* by *35S:ROP10*. The three transgenic lines (aA18, aA22, and aA40) showed similar root elongation inhibition in the presence of 8 μM ABA compared with Ws. Inhibition of root elongation was expressed as the percentage of root length increase compared with roots grown on ABA-free medium.

(D) to **(G)** *rop10-1* does not affect root elongation inhibition responses to the hormones indoleacetic acid (IAA; an auxin) **(D)**, benzyladenine (BA; a cytokinin) **(E)**, 1-aminocyclopropane-1-carboxylic acid (ACC; an ethylene precursor) **(F)**, and brassinolide (BL) **(G)**. Bars show the SD from three replicates, each with 10 to 16 seedlings. Open circles, Ws; closed circles, *rop10-1*.

Arabidopsis MYB2, which is induced by ABA and is a member of the large family of Myb transcription factors, has been shown to activate drought- and ABA-induced *RD22* gene expression (Urao et al., 1993; Abe et al., 1997). We investigated whether *MYB2* expression was altered in *rop10-1* using real-time PCR to quantify the relative levels of *MYB2* mRNA. Compared with Ws, *rop10-1* contained higher levels of *MYB2* mRNA both with and without ABA treatments, and *MYB2* expression in *rop10-1* at 1 μM was similar to that in Ws at 50 μM (Figure 2F). This result suggests that ROP10 suppresses ABA-induced *MYB2* expression. Therefore, it is likely that MYB2 acts as a transcriptional effector of ROP10 in ABA signaling.

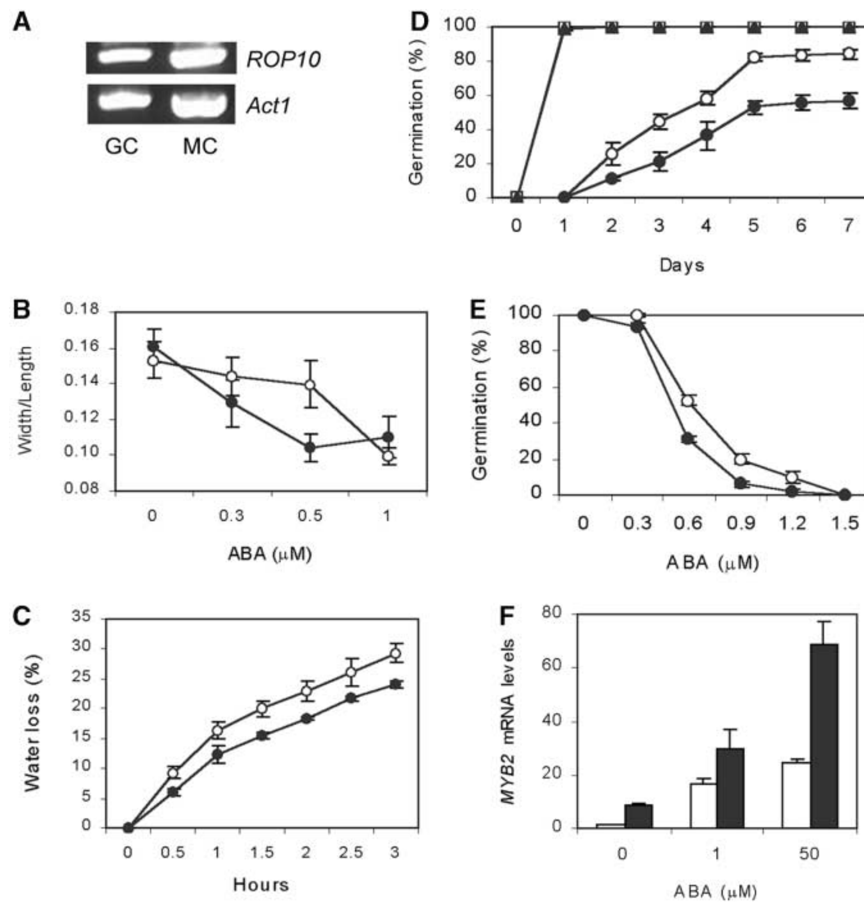

Figure 2. ROP10 Regulates Various Aspects of ABA Responses.

(A) to **(C)** The *rop10-1* mutant exhibits hypersensitivity to the ABA stimulation of stomatal closure.

(A) RT-PCR analysis of *ROP10* expression in guard cells (GC) and mesophyll cells (MC). *Act1* was used as a reference.

(B) Hypersensitivity of *rop10-1* in guard cell response to ABA. The width and length of each stomate were measured, and the ratio of width to length was determined. Bars represent the ±SD of at least three replicates, each with 20 stomata. Open circles, Ws; closed circles, *rop10-1*.

(C) *rop10-1* reduces water transpiration on detached leaves. Bars represent the ±SD of three plants, each with four leaves. Open circles, Ws; closed circles, *rop10-1*.

(D) and **(E)** Enhanced seed dormancy and germination inhibition by ABA in *rop10-1*.

(D) Seed dormancy is enhanced in *rop10-1*. Without cold treatment, *rop10-1* seeds (closed circles) germinated more slowly and less completely than Ws seeds (open circles). Cold treatments at 4°C for 4 days eliminated the germination differences (open squares, Ws; closed triangles, *rop10-1*; the two symbols overlap in the curves). Data represent means ± SD values of three replicates, each with 70 to 80 seeds.

(E) Enhanced responses of *rop10-1* to ABA inhibition of seed germination. Values represent means ± SD of seed germination percentage from three replicates (each with ~60 seeds) measured 3 days after cold treatment. Open circles, Ws; closed circles, *rop10-1*.

(F) Enhanced expression of *MYB2* mRNA in *rop10-1*. Seven-day-old liquid-cultured seedlings were treated with ABA for 4 h, and RNA was subjected to real-time RT-PCR analysis. *MYB2* mRNA levels were normalized with those of the reference (*Act2*), and the relative *MYB2* level in non-treated Ws was set at 1 ($n = 3$). Open columns, Ws; closed columns, *rop10-1*.

Constitutively Active and Dominant-Negative rop10 Mutations Reduced and Enhanced ABA Response, Respectively

Because dominant mutants of *ROP2* and *ROP6* are pleiotropic (Lemichez et al., 2001; Li et al., 2001; Molendijk et al., 2001; Fu et al., 2002; Jones et al., 2002), we were interested in whether similar mutants of *ROP10* would possess functional specificity. Consistent with the role of ROP10 as a negative regulator, the ectopically expressed constitutively active mutant (Q66L) of ROP10, designated *CA-rop10*, reduced ABA sensitivity. In all three independent lines (in the Ws background) that express high levels of *CA-rop10* mRNA (Figure 3A), inhibition of seed germination by ABA

was reduced (Figure 3B), whereas overexpression of wild-type *ROP10* did not alter ABA responses (data not shown). These results suggest that downregulation of ROP10 activity is critical for ABA signaling, whereas *ROP10* gene expression is not rate limiting in its negative regulation of ABA responses. Conversely, ABA sensitivity was enhanced in the *DN-rop10* (D123A) mutant that locks GTPase in the GDP-bound form and thus blocks the GTPase from signaling by sequestering the GTPase activators. Three independent homozygous lines of *DN-rop10* expressing high levels of *DN-rop10* mRNA (Figure 3C) all displayed a strong response to the ABA inhibition of seed germination (Figure 3D). Two lines (DN7 and DN31) with higher expression levels showed slightly stronger inhibition responses than *rop10-1*, indicating that one or more other ROPs might be involved in the negative regulation of ABA responses. Importantly, unlike dominant mutants of *ROP2* and *ROP6* (Lemichez et al., 2001; Li et al., 2001; Molendijk et al., 2001; Fu et al., 2002; Jones et al., 2002), both DN- and CA-rop10 showed no other developmental phenotypes (data not shown), further confirming the functional specificity for ROP10 in ABA signaling.

We next determined whether *ROP10* interacts genetically with the well-characterized ABA signaling gene *ABI2*, which encodes a protein phosphatase type 2C, another negative regulator (Leung et al., 1997; Merlot et al., 2001). *DN-rop10* was introduced by transformation into *abi2-1*, which is insensitive to germination inhibition by high concentrations of ABA (Koornneef et al., 1984). Expression of *DN-rop10* (Figure 3E) partially suppressed the ABA insensitivity of *abi2-1* to the inhibition of both seed germination (Figure 3F) and root elongation (Figure 3G). The partial suppression of *abi2-1* by *DN-rop10* is similar to that of the *ear1 abi1*, *era1 abi2*, and *abh1 abi1* double mutants (Cutler et al., 1996; Pei et al., 1998; Hugouvieux et al., 2001), indicating that ROP10 might function in parallel with or downstream of ABI2.

Transcription of *ROP10* in Root Tips Was Downregulated Specifically by ABA

Results from both loss-of-function and dominant mutants of *ROP10* suggest that the downregulation of ROP10 activity is critical for ABA responses. To determine whether ABA also regulates *ROP10* gene expression, we first analyzed *ROP10* transcript levels by RT-PCR analysis. We found that treatments with various concentrations of ABA did not induce obvious changes in *ROP10* mRNA in whole seedlings (data not shown). We next determined whether ABA affects the tissue-specific expression of *ROP10* using transgenic expression of the *ROP10* promoter fused to the β-glucuronidase (GUS) reporter gene. GUS activity was detected in most tissue types of untreated plants, including root tips, vascular tissues, cotyledons, leaves, gynoecium, and germinating seeds; however, very low levels were detected in male reproductive organs and petals (Figures 4A to 4D). GUS activity also was present in both guard cells and mesophyll cells (Figure 4E). Very strong staining was observed in the radicles of germinating seeds (Figures 4C and 4D) and root tips (Figures 4F and 4H), which appear to correspond to root apical meristematic regions. Importantly, treatment with 10 μM ABA for 24 h dramatically decreased GUS activity in the root tip (Figure 4I), and with 100 μM ABA, *ROP10* expression was suppressed completely (Figure 4J). However, these treatments did not affect *ROP10* expression in other parts of the seedlings (Figures 4F and 4G). The ABA concentrations that effectively suppressed *ROP10* expression were similar to those that inhibited root elongation in wild-type seedlings, suggesting that ABA suppression of *ROP10* expression is important for ABA responses in roots. Interestingly, treatment with other hormones, including auxin, cytokinin, ethylene, brassinolide, and gibberellins, did not affect GUS staining patterns in root tips (Figures 4K to 4O) or elsewhere in the seedlings (data not shown). The ABA-specific downregulation of *ROP10* transcription further confirms the specific function of ROP10 in the regulation of ABA responses.

Localization of ROP10 to the PM Is Required for ROP10 Function

Because both *rop10-1* and *era1* mutants are ABA hypersensitive, we addressed the possibility that ROP10 is an ERA1 target involved specifically in ABA responses. We first mutated the C residue to Ser (S) in the CGKN motif (C205S-ROP10) and determined the localization of the mutant, because the C residue usually is involved in prenylation. Green fluorescent protein (GFP) alone was localized to both the cytosol and the nucleus (Figure 5D). Like maize ROP6 and ROP7 (Ivanchenko et al., 2000), GFP-ROP10 was localized to the cell periphery and absent from the nucleus (Figure 5A), suggesting that ROP10 is localized to the PM. We found that GFP-C205S-ROP10 was localized uniformly to the nucleus and the cytoplasm (Figure 5C), similar to GFP alone (Figure 5D). To assess whether the PM targeting is critical for the function of ROP10, we tested the complementation of the *rop10-1* phenotype by *35S:C205S-ROP10*. Transgenic lines expressing high levels of *C205S-ROP10* mRNA (Figure 5F) did not complement the root elongation inhibition response to ABA (Figure 5G). This result provides evidence that the PM localization of ROP10 is critical for its function.

We next tested whether the PM localization of GFP-ROP10 is dependent on ERA1, the only β-subunit of FTase in Arabidopsis, as would be expected if ROP10 were an ERA1 target. As shown in Figures 5B and 5E, PM localization was affected weakly in *era1-2*, a genomic deletion mutant (Cutler et al., 1996). Quantitative analysis showed that the effect of the *era1-2* mutation on the PM localization of ROP10 was statistically significant (Figure 5E). This finding

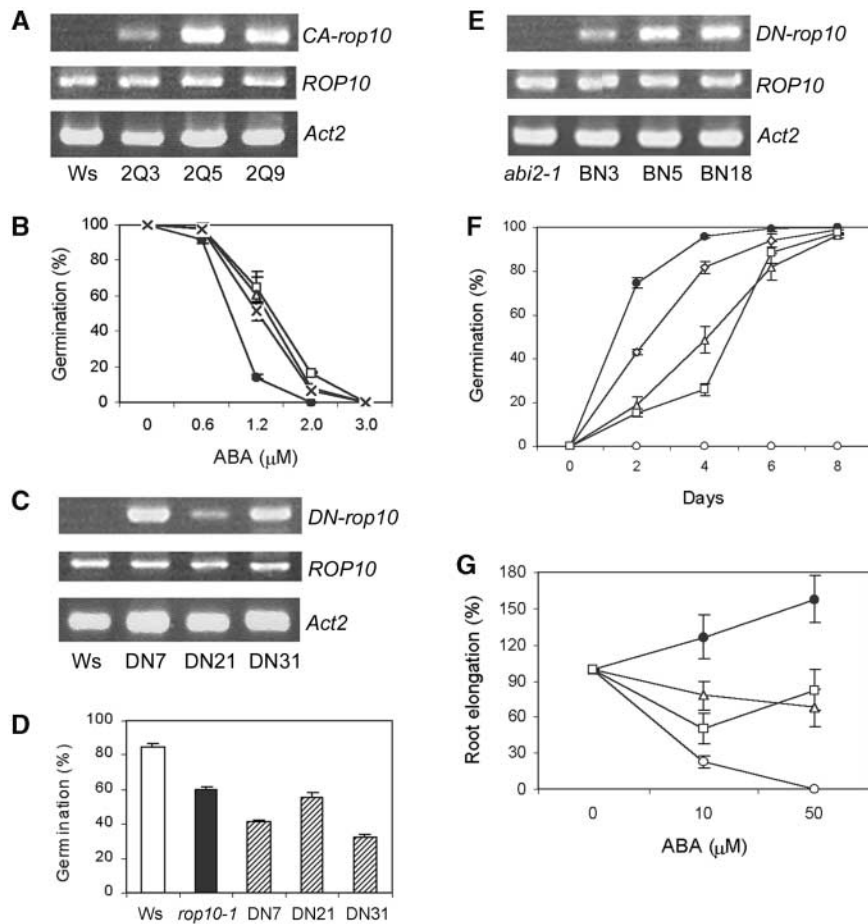

Figure 3. *CA-rop10* and *DN-rop10* Mutants Exhibit Relative Insensitive and Hypersensitive ABA Responses, Respectively.

(A) and **(B)** *CA-rop10* mutants reduce the ABA sensitivity of seed germination.

(A) RT-PCR analysis of *CA-rop10* and wild-type *ROP10* mRNA in Ws and three *CA-rop10* lines (2Q3, 2Q5, and 2Q9), using *Act2* as a reference.

(B) The three lines showed reduced sensitivity to seed germination inhibition by ABA. Bars represent SD values of three replicates ($n \approx 60$ seeds). Closed circles, Ws; open squares, 2Q3; open triangles, 2Q5; crosses, 2Q9.

(C) and **(D)** *DN-rop10* mutants enhance ABA responses.

(C) RT-PCR analysis of *DN-rop10* and wild-type *ROP10* mRNA in three *DN-rop10* lines (DN7, DN21, and DN31), using *Act2* as a reference.

(D) The three lines showed enhanced sensitivity to seed germination inhibition by 0.6 μM ABA, similar to or stronger than *rop10-1*. Bars represent SD values of three replicates ($n \approx 70$ seeds).

(E) to **(G)** *DN-rop10* mutants partially suppress the *abi2-1* mutant phenotypes.

(E) RT-PCR analysis of *DN-rop10* mRNA in transgenic *abi2-1*/*DN-rop10* lines (BN3, BN5, and BN18).

(F) Seed germination percentages in the presence of 5 μM ABA were reduced in *abi2-1*/*DN-rop10* seeds. Bars represent SD values of three replicates ($n \approx 80$ seeds).

(G) Root elongation inhibition is enhanced in BN3 and BN18 lines compared with *abi2-1*. Bars represent SEM values of 10 seedlings.

Symbols in **(F)** and **(G)** are as follows: open circles, Landsberg *erecta*; closed circles, *abi2-1*; open triangles, BN3; open diamonds, BN5; open squares, BN18.

is consistent with in vitro prenylation results showing that ROP10 was weakly farnesylated (data not shown). These results suggest that the efficient PM localization of ROP10 likely requires an additional modification of the C residue with the CGKN motif by a different type of lipid.

DISCUSSION

The observations described here convincingly show that ROP10 acts specifically as a negative regulator of ABA re-

sponses in Arabidopsis. Several sets of genetic and molecular data demonstrate an important role for ROP10 in the negative regulation of ABA signaling. First, a loss-of-function mutation in *ROP10* enhanced various ABA responses, including increased seed dormancy, inhibition of seed germination and root elongation, and stimulation of stomatal closure, but it did not affect ABA levels. Second, this mutation affected the ABA-mediated expression of the *MYB2* gene. Third, expression of *CA-rop10* and *DN-rop10* dominant mutants reduced and increased, respectively, the ABA responses in Arabidopsis. Finally, *DN-rop10* expression partially suppressed *abi2-1* phenotypes.

Several lines of evidence indicate that ROP10 functions specifically in ABA signaling. First, unlike CA or DN mutants of *ROP2* and *ROP6*, which, in addition to affecting ABA responses exhibit pleiotropic phenotypes (Lemichez et al., 2001; Li et al., 2001; Molendijk et al., 2001; Fu et al., 2002; Jones et al., 2002), similar mutants of *ROP10* do not show any abnormal development. Second, the *rop10-1* knockout mutant displays hypersensitivity to ABA but not to other hormones,

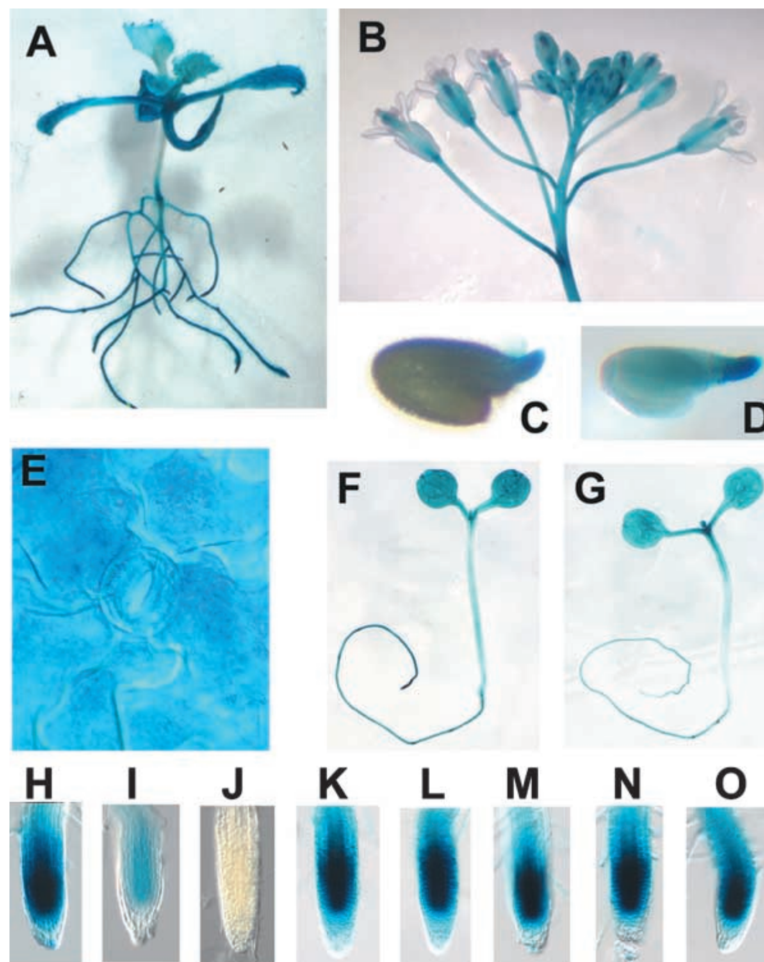

Figure 4. ABA-Specific Downregulation of *ROP10* Transcription in Root Tips.

(A) to **(E)** Expression patterns of *ROP10* revealed by histochemical examination of *ROP10* promoter activity using the *GUS* reporter. Shown are a 3-week-old plant **(A)**, an inflorescence **(B)**, germinating seeds with **(C)** and without **(D)** seed coats, and a leaf in which a guard cell also is stained **(E)**.

(F) to **(O)** ABA-specific downregulation of *ROP10* promoter activity. Four-day-old seedlings grown in liquid Murashige and Skoog (1962) medium plus 1% Suc were treated with water **(F)** or 100 μM ABA **(G)**. Close-ups are shown of root tips treated for 24 h with water **(H)**, 10 μM ABA **(I)**, 100 μM ABA **(J)**, 1 μM indoleacetic acid **(K)**, 1 μM benzyladenine **(L)**, 1 μM brassinolide **(M)**, 100 μM gibberellic acid **(N)**, and 10 μM 1-aminocyclopropane-1-carboxylic acid **(O)**. Note that 0.1 μM indoleacetic acid, benzyladenine, and brassinolide, 10 μM gibberellic acid, and 1 μM 1-aminocyclopropane-1-carboxylic acid resulted in similar staining patterns (data not shown).

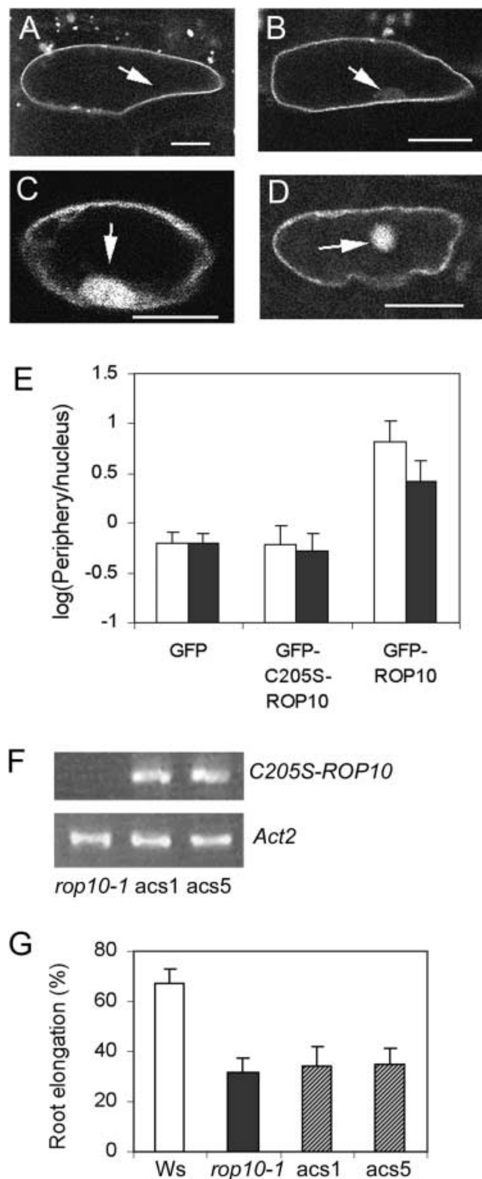

Figure 5. Functional Requirement for the PM Localization of ROP10.

(A) to **(D)** Transient expression of GFP-ROP10 and GFP-C205S-ROP10 in Arabidopsis leaf epidermal cells. Shown are single optical sections of GFP-ROP10 **(A)**, GFP-C205S-ROP10 **(C)**, and GFP alone **(D)** in the wild type (Columbia ecotype) and GFP-ROP10 in *era1-2* **(B)**. Arrows indicate the nucleus. Bars = 20 μm.

(E) Quantitative analysis of subcellular localization for various GFP constructs. The periphery/nucleus ratios were log transformed; 0 represents equal intensity, and >0 or <0 indicates more peripheral or more nuclear fluorescence distribution, respectively. Data are means ± SD of eight transformed cells. Open columns, wild type (Columbia ecotype); closed columns, *era1-2*. Statistical analysis showed that GFP and GFP-C205S-ROP10 were not different, but they were significantly different from GFP-ROP10. Also, there was a significant difference for GFP-ROP10 in the wild type and *era1-2*.

(F) and **(G)** PM localization is required for ROP10 function.

(F) RT-PCR analysis of *C205S-ROP10* mRNA in transgenic lines (acs1 and acs5) transformed with *35S*:*C205S-ROP10* in the *rop10-1* background.

(G) In the presence of 8 μM ABA, the acs1 and acs5 lines displayed root elongation inhibition responses similar to that of *rop10-1*. Bars show SD values of three experiments.

including auxin, cytokinin, ethylene, and brassinolide, even though the latter two hormones have been shown to interact with ABA (Ephritikhine et al., 1999; Beaudoin et al., 2000; Ghassemian et al., 2000). Third, the downregulation of *ROP10* transcription in the root tips is specific to ABA but not to other hormones.

The specific effect of ROP10 on various ABA responses indicates that ROP10 may act in an early step of ABA signaling or in a common pathway of the ABA signaling network. Interestingly, the hormonal specificity resembles that of both *ABH1* and *SAD1*, two other genes thought to be involved specifically in the negative regulation of ABA signaling and shown to encode proteins that potentially participate in RNA processing (Hugouvieux et al., 2001; Xiong et al., 2001a). ROP-related Cdc42 GTPases in mammals regulate RNA processing by stimulating the phosphorylation of the RNA cap binding protein (Erickson and Cerione, 2001). It will be interesting to determine whether or not ROP10 and ABH1/SAD act in the same pathway in ABA signaling or in the post-transcriptional regulation of an ABA signaling component.

A potential role for ROP10 at an early step of ABA signal transduction is supported by the PM localization of ROP10. The fact that PM localization is required for ROP10 function is significant, given that none of the ABA signaling proteins has been shown convincingly to localize specifically to the PM. Several biochemical studies suggest the presence of unidentified ABA receptors on both the PM and the cytoplasm (reviewed by Finkelstein et al., 2002). Based on the functional requirement for the PM localization of ROP10 and the observation that a ROP-like protein is associated with the active complex of the CLV1 receptor Ser/Thr kinase (Trotochaud et al., 1999), ROP10 could be situated in close proximity to and inactivated by a PM-localized ABA receptor in response to ABA. The suppression of *ROP10* expression also is likely to be important in sustained ABA action. The inactivation and/or suppression of ROP10 release the inhibition of *MYB2* expression, leading to the induction of target genes such as *RD22*.

The mechanism for targeting ROP10 to the PM needs to be investigated further. ROP10 contains a putative CaaX motif for farnesylation, but the *era1* loss-of-function mutation affects its PM localization only weakly. This result does not formally exclude the possibility that ROP10 is a key ERA1 target involved in the negative regulation of ABA responses. However, it raises the possibility that another type

of lipid modification may play an important role in targeting ROP10 to the PM. During the course of our experiments, we became aware that palmitoylation may be important for ROP10 localization to the PM (S. Yalovsky, personal communication). The importance of palmitoylation for localization also has been speculated in maize ROP6 and ROP7, which have C-terminal sequences similar to that of Arabidopsis ROP10 (Ivanchenko et al., 2000). We have observed that the palmitoylation inhibitor (2-bromopalmitate) caused a localization shift similar to that seen in GFP-C205S-ROP10 (data not shown). Palmitoylation may offer plant cells dynamic control of ABA signaling as a result of the reversible nature of this modification (Resh, 1999). Palmitoylation alone or together with farnesylation may target ROP10 to the PM. In mammals, palmitoylation is thought to stabilize the membrane localization of prenylated proteins. Further work is necessary to address the relative contribution of each of these modifications in ROP10 PM localization.

Our results provide genetic evidence that changes in ROP10 activity are involved in the negative regulation of ABA responses in various tissues. We also show that *ROP10* transcription is downregulated specifically by ABA in root tips. The regulation of ROP10 activity appears to be an early ABA signaling event, because it is involved in all ABA responses, whereas the ABA suppression of *ROP10* transcription may be crucial for sustained ABA action, specifically in its inhibition of root elongation. This hypothesis is supported by the much more dramatic enhancement of ABA inhibition of root elongation (Figure 1B) in *rop10-1* compared with its weaker effect on other ABA responses (Figures 2B to 2E). Indeed, high concentrations (10 to 100 μM) of ABA are required for the inhibition of root elongation and the suppression of *ROP10* expression, in contrast to other more sensitive ABA responses (Figures 2B to 2E).

Together, our results provide strong evidence that ROP10 GTPase is an important PM-associated negative regulator that participates in an early event of ABA signal transduction or a common pathway of the ABA signaling network. Future work should further reveal the mode of ROP10 regulation and action in ABA signaling and how it interacts with other known components of ABA signaling.

METHODS

ROP10 Mutant Isolation

The *rop10-1* T-DNA knockout mutant of *Arabidopsis thaliana* was isolated by screening the ABRC collection of 12,000 T-DNA insertional mutant lines (generated by T. Jack and K. Feldman) maintained at Ohio State University (Columbus). Two degenerate *ROP* primers were used: ConS2, 5′-GTCGG(A/C)GATGGTGC(A/T/C)GTAGGA-AAAACTTG-3′, and ConA2, 5′-CCC(G/A/T)GCAGTATCCCACAAT-CC(A/C)AG(A/G)TT(G/C)A-3′. Sequencing of the T-DNA–*ROP10* junction showed that the T-DNA was present at the 5′ end of the second exon.

Construction of *35S:ROP10* and *35S:C205S-ROP10*

The *ROP10* cDNA was amplified using primers A8NCOIS (5′-GCA-TTGGCCATGGCTTCGAGTGCTTCAA-3′) and A8ECO72A (5′-GAT-TGGCACGTGTCAATTCTTCCCACACAGA-3′). The *C205S-ROP10* cDNA fragment was amplified using primers A8NCOIS and A8SECO72A (5′-GATTGGCACGTGTCAATTCTTCCCAGACAGAAT-3′, incorporating the C205S mutation). Both PCR fragments were cloned into the pCAMBIA3301 vector, which contains a BASTA resistance selection marker and a 35S promoter of *Cauliflower mosaic virus*.

Abscisic Acid Response Assays

Seed germination was performed on basal medium (half-strength Murashige and Skoog [1962] salts, without Suc) as described previously (Li et al., 2001). For assays of root elongation, 4-day-old seedlings grown vertically in basal medium were transferred to the abscisic acid (ABA)–supplemented medium, or seeds were germinated and grown vertically in basal medium supplemented with other hormones. The increase in primary root length was measured after 7 days. Stomatal closure assay was performed as described (Pei et al., 1998). For water loss assay, young leaves from each plant at the same stage were detached and fresh weights were measured. The water loss was expressed as the percentage of water lost over the initial fresh weight.

Measurement of ABA Content

Radioimmunoassay was performed to determine the ABA content of 3-week-old seeds and 6-day-old seedlings of both wild-type Wassilewskija and *rop10-1*, as described previously (Bray and Beach, 1985).

Construction of the *CA-rop10* and *DN-rop10* Mutations

The *CA-rop10* mutation was constructed using two sets of primers to amplify two cDNA fragments: A8NCOIS (described above) and A8CAA (5′-ATAGTCCTCGAGCCCGGCAGTGTCCCA-3′, containing the Q66L mutation); and A8CAS (5′-CGGGCTCGAGGACTATAACAG-ACTAAG-3′) and A8ECO72A (described above). The *DN-rop10* mutation was constructed using two sets of primers to amplify two cDNA fragments: A8NCOIS (described above) and A8DNA (5′-CAT-TTTGGTACCAACAAGCAC-3′); and A8DNS (5′-GTTGGTACCAAA-ATGGCTCTTCGTGAAGAT-3′, containing the D123A mutation) and A8ECO72A. The *CA-rop10* and *DN-rop10* fragments were joined by cloning into pUC19 vector and then subcloned into the modified pCAMBIA3301 vector in which the 35S promoter was replaced with a dual enhancer–35S promoter.

Reverse Transcriptase–Mediated PCR

Reverse transcriptase–mediated PCR analysis was performed using total RNA from young seedlings as described previously (Li et al., 2001). For endogenous *ROP10*, two primers, A8NCOIS and A8UTR3′ (5′-GGGTCAAACCAAATCGAAACA-3′) were used. For transgenes, A8NCOIS and CAM (5′-TGGTCACCAATTCACACGTG-3′, specific to the 3′ untranslated region in the pCAMBIA3301 vector) were used. The internal control, *Act2*, has been described (Li et al., 2001).

For reverse transcriptase–mediated PCR analysis in guard cells,

RNA was extracted from guard cell and mesophyll cell protoplasts prepared from ecotype Columbia leaves. *ROP10* cDNA was amplified with two primers (sense, 5′-CATCACTGTGAACTTAGGCCTTTG-3′; antisense, 5′-TTACGAGAGACATCCGCTGCGAGG-3′), and *Actin1* was amplified using two primers (sense, 5′-GGCCGATGGTGAGGATATTCAGCCACTTG-3′; antisense, 5′-TCGATGGACCTGACTCATCGTACTCACTC-3′).

Real-Time PCR

Real-time PCR for quantification of *MYB2* mRNA expression was performed according to the instructions provided for the ABI7700 Sequence Detector and with TaqMan Universal PCR Master Mix (Perkin-Elmer Applied Biosystems). Primers for *MYB2* were MYB2SDS (5′-TGCCGAGATTAGTGGAACGAA-3′) and MYB2SDA (5′-CGGTGATCATTGACTCCACTTGT-3′), and the TaqMan probe for *MYB2* was 6FAM 5′-AACGCCCAATCATTACCCACCACGT-3′ TAMRA. The reference *Act2* primers were ActSDS (5′-GCACCAAGCAGCATGAAGATT-3′) and ActSDA (5′-GGAACCACCGATCCAGACACT-3′), and the *Act2* probe was VIC 5′-ACTTCCTTTCAGGTGGTGCAACGACCT-3′ TAMRA.

ROP10 Promoter:β-Glucuronidase Construction and β-Glucuronidase Assay

A *ROP10* genomic DNA fragment containing a 2.2-kb region upstream of the ATG codon and 300 bp of coding DNA from ATG to the beginning of the second exon was amplified by primers A8HINDIIIS (5′-TCGAAGCTTGATGGATCATTTGCCTTGTC-3′) and A8BAMHIA (5′-TGCGGATCCTATGTAGTCCTGATACAATCCA-3′) and then cloned into the pBI101.1 vector. Homozygous transgenic plants were used for β-glucuronidase assays as described by Li et al. (2001).

Green Fluorescent Protein–ROP10 Construction and Transient Expression

For green fluorescent protein (GFP)–C205S-ROP10, the *ROP10* cDNA was fused to mGFP (Li et al., 1999), and then the 3′ region of cDNA was replaced by a 285-bp fragment cut from the *C205S-ROP10* cDNA as described above. The resulting GFP-C205S-ROP10 was cloned into pBI221 with a 35S promoter of *Cauliflower mosaic virus*. For the GFP-ROP10 construct, *ROP10* cDNA was cloned into the GFP vector yy217 (Yamamoto et al., 2001). Transient expression in leaves was determined as described previously (Fu et al., 2002). The fluorescence intensity was quantified using a series of 10 to 15 sections covering the nucleus, each 1 μm thick, as described by Ivanchenko et al. (2000).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for noncommercial research purposes.

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