Inactivation of AtRac1 by abscisic acid is essential for stomatal closure

Emmanuel Lemichez,[1] Yan Wu,[1] Juan-Pablo Sanchez,[1] Amel Mettouchi,[2] Jaideep Mathur,[3] and Nam-Hai Chua[1,4]

[1]Laboratory of Plant Molecular Biology, Rockefeller University, New York, New York 10021-6399, USA; [2]Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, USA; [3]Laboratory of Plant Cell Biology, Institute of Molecular Agrobiology, National University of Singapore, Kent Ridge 117604, Singapore

Plant water homeostasis is maintained by the phytohormone abscisic acid (ABA), which triggers stomatal pore closure in response to drought stress. We identified the *Arabidopsis* small guanosine triphosphatase (GTPase) protein AtRac1 as a central component in the ABA-mediated stomatal closure process. ABA treatment induced inactivation of AtRac GTPases and disruption of the guard cell actin cytoskeleton. In contrast, in the ABA-insensitive mutant *abi1-1*, which is impaired in stomatal closure, neither AtRac inactivation nor actin cytoskeleton disruption was observed on ABA treatment. These observations indicate that AtRac1 inactivation is a limiting step in the ABA-signaling cascade leading to stomatal closure. Consistent with these findings, expression of a dominant-positive mutant of AtRac1 blocked the ABA-mediated effects on actin cytoskeleton and stomatal closure in wild-type plants, whereas expression of a dominant-negative AtRac1 mutant recapitulated the ABA effects in the absence of the hormone. Moreover, the dominant-negative form of AtRac1 could also restore stomatal closure in *abi1-1*. These results define AtRac1 as a central element for plant adaptation to drought.

The closure of stomatal pores in aerial tissues is an important mechanism by which higher plants respond rapidly to water loss. This essential homeostatic response is regulated by the stress phytohormone ABA (Van Overbeek et al. 1967). ABA-induced stomatal closure requires different signaling components (Leung and Giraudat 1998; Leckie et al. 1998; Pei et al. 1998; Leyman et al. 1999; Li et al. 2000), one of which is ABI1, a member of the PP2C serine/threonine phosphatase family, which acts as an upstream regulator of the ABA pathway (Leung et al. 1994; Meyer et al. 1994; Gosti et al. 1999). The *Arabidopsis abi1-1* mutant displays a wilty phenotype caused by impaired stomatal closure. Stomatal pores are bordered by a pair of guard cells. Under conditions of adequate water status, when the guard cells are fully turgid, constraints on their inner walls maintain the pore open. Closure of stomata has been ascribed to a release of the guard cell turgor pressure primarily caused by Cl⁻ and K⁺ efflux (Blatt 2000). Additionally, a reorganization of the actin cytoskeleton of guard cells has been observed after ABA treatment (Eun and Lee 1997). Despite this observation, the precise role of the actin cytoskeleton in regulating stomatal relaxation has remained elusive because of the lack of characterized regulators of the plant actin cytoskeleton (Blatt 2000).

The family of Rho GTPases has emerged as a key regulator of the actin cytoskeleton in yeast and animal cells (Hall 1998). Through their interaction with multiple regulators and effectors, Rho GTPases in these organisms transduce signals from cell surface receptors to reorganize actin architecture in the cytoplasm. Small GTPases exist either in an active form bound to GTP or in an inactive form bound to GDP. The transition between the two forms is regulated by either GTPase-activating proteins (GAP) or GTP/GDP exchange factors (GEF), which inactivate or activate the GTPases, respectively (Bishop and Hall 2000). The association of GTP-bound Rho GTPases with plasma membranes is stabilized by a carboxy-terminal prenyl extension. In contrast, soluble Rho GDP is maintained in the cytoplasm through its interaction with a GDP dissociation inhibitor (GDI).

The *Arabidopsis* database contains at least 13 distinct putative genes coding for Rho-related GTPases, some of which have been cloned (Xia et al. 1996; Winge et al. 1997; Li et al. 1998). Rho-related plant proteins have been described to regulate a complex array of biological processes, such as the regulation of pollen tube growth and plant cell death (Lin and Yang 1997; Kost et al.

1999a,b; Kawasaki et al. 1999). Our study here defines a new involvement of Rho-related plant proteins in the control of stomatal closure and therefore plant water homeostasis.

Results

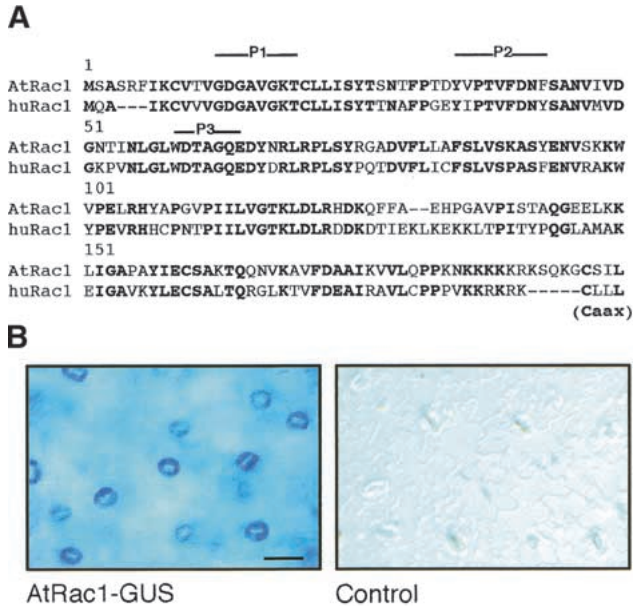### Arabidopsis *AtRac1* is a plant Rho GTPase homolog

We have cloned a Rho-related *Arabidopsis* GTPase by screening for induction of abnormal cell shape after over-expression of *Arabidopsis* cDNAs in *Schizosaccharomyces pombe* (Xia et al. 1996). AtRac1 is identical to ARAC3 (Winge et al. 1997) and Rop6At (Li et al. 1998). AtRac1 shares 60.9%, 47.7%, and 51.85 homology with human Rac1, RhoA, and Cdc42, respectively, and is thus more related to mammalian Rac proteins than to RhoA and Cdc42. Rho-related GTPases bind to and hydrolyze GTP. The key amino acids involved in GTP binding and hydrolysis in mammalian small GTPases are conserved in AtRac1 (Fig. 1A). In particular, the three major residues that coordinate magnesium in the GTP bound form of Ras (T17, T35, and D57), as well as those involved in the binding of phosphate important for the intrinsic GTPase activity (G12, A59, and Q61), are strictly conserved (Pai et al. 1989). Based on the conservation of key

Figure 1. Amino acid sequence of AtRac1 and *AtRac1* expression in guard cells. (*A*) AtRac1 amino acid sequence alignment with its close homolog human Rac1 Rho GTPase (huRac1). Conserved amino acids are shown in bold. The guanine nucleotide-binding domains are indicated by P1 to P3. The conserved prenylation motif, Caax, is noted. GenBank accession nos.: At-Rac1 (U62746) and huRac1 (M29870). (*B*) GUS expression pattern in leaves of *Arabidopsis* WT (*right*) and *AtRAC1* promoter–GUS (*left*) transgenic seedlings. Three independent transgenic lines were analyzed, and all showed elevated GUS staining in guard cells over the epithelial background. Bar, 50 µm.

amino acids, we predict that AtRac1 should have similar biochemical characteristics to its mammalian Rho homologs. AtRac1 is ubiquitously expressed, having a stronger expression in vascular tissues (Kost et al. 1999a). Using *AtRAC1* promoter β-glucuronidase (GUS) transgenic *Arabidopsis* plants (Kost et al. 1999a), we found that AtRac1 is expressed at a much higher level in guard cells than in the surrounding epithelial cells (Fig. 1B). This observation and the known role of mammalian Rho GTPases in regulating the actin cytoskeleton led us to investigate whether the *Arabidopsis* small GTPase AtRac1 is involved in the regulation of the actin cytoskeleton in guard cells.
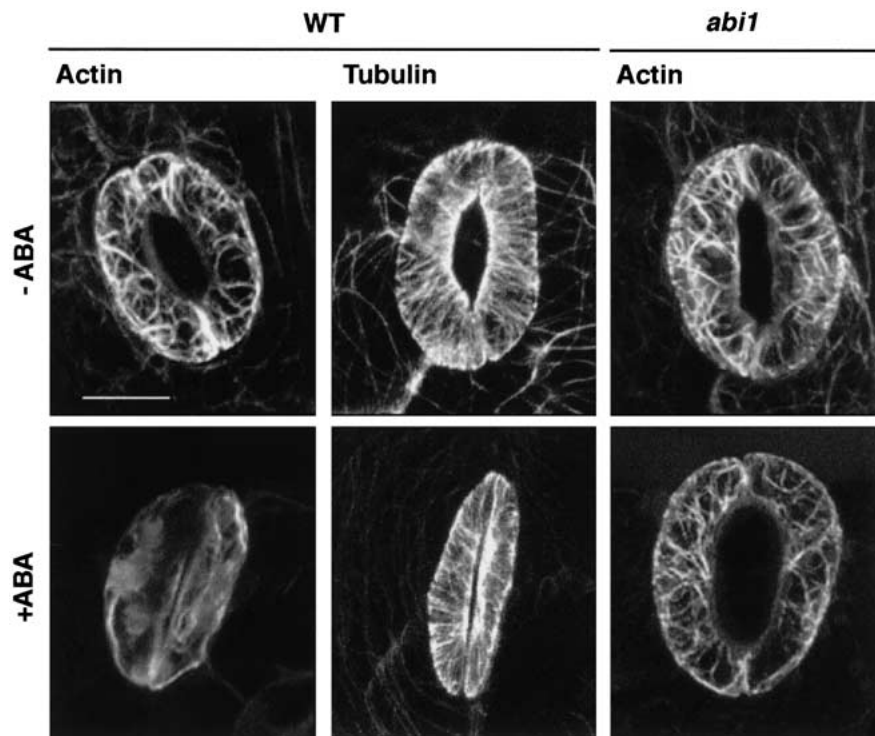
ABA-induced actin cytoskeleton disruption is impaired in *abi1-1* plants

Until recently, the extreme sensitivity of guard cells to biochemical treatments has seriously hampered studies of the actin cytoskeleton. Fixation techniques artificially release the guard cell turgor pressure and thus cause stomatal closure. The diffuse arrangement of actin that results has permitted only a correlation of actin reorganization with ABA treatment but not with stomatal closure per se (Eun and Lee 1997). To circumvent this technical difficulty and to simultaneously visualize both the state of the stomatal aperture and the organization of the actin cytoskeleton, we made use of transgenic *Arabidopsis* lines expressing a green fluorescent fusion protein (GFP-mTn) targeted to the actin cytoskeleton (Kost et al. 1998). We have previously generated two transgenic plant lines, WT/*GFP-mTn* and WT/*MAP4-GFP*, expressing GFP fusion proteins targeted to the actin (Kost et al. 1998) and tubulin (Mathur and Chua 2000) cytoskeletons, respectively. In WT/*GFP-mTn* guard cells of open stomata, we observed thick radial actin cables bridging the cell wall aperture to the dorsal side of the guard cell and a few tangential cables (Fig. 2). The microtubule cables of WT/*MAP4-GFP* guard cells were organized in a more regular fan-shaped pattern compared with that of actin (Fig. 2). On ABA addition, unlike microtubule cables, actin cables became rapidly disorganized and shortened. After 15 min of ABA treatment, 88% of the guard cells were closed and contained substantially fewer actin cables (Fig. 2). The guard cell actin cytoskeleton is thus specifically disorganized on ABA treatment.

To further study the link between actin reorganization and ABA-induced stomatal closure, we analyzed the response of the actin cytoskeleton in the mutant *abi1-1*, which is impaired in ABA-induced stomatal closure. We generated *abi1-1*/*GFP-mTn* transgenic plants by crossing WT/*GFP-mTn* with the *abi1-1* mutant. T3 *abi1-1*/*GFPmTn* homozygous plants were analyzed for the effects of ABA on the actin cytoskeleton. We observed that during ABA treatment, both stomatal aperture and the actin cytoskeleton remained unaffected (Fig. 2). Together, our observations indicate that ABA induced a disorganization of actin cables in guard cells during stoma-

Figure 2. Actin and tubulin cytoskeleton reorganization in *Arabidopsis* guard cells on abscisic acid (ABA)-induced stomatal closure. Transgenic *Arabidopsis* WT and *abi1-1* plants expressing GFP-mTn (Kost et al. 1998) or MAP4-GFP (Mathur and Chua 2000) were analyzed for cytoskeleton reorganization before (–ABA) and after (+ABA) 15 min of treatment with 50 μM ABA. Using confocal microscopy, actin was visualized with GFP-mTn, whereas tubulin was visualized with MAP4-GFP. Pictures represent the projection of serial confocal optical sections and are representative of at least 100 guard cells analyzed for each condition. Bar, 10 μm.

tal closure, a process impaired in the ABA-signaling mutant *abi1-1*. These results confirm and extend previous reports (Eun and Lee 1997; Eun et al. 2001).

Conditional expression of AtRac1 mutants in transgenic Arabidopsis plants

To further investigate the role of AtRac1 in regulating the guard cell actin cytoskeleton, we generated dominant-negative (AtRac1-T20N) and dominant-positive (AtRac1-G15V) mutants, corresponding to the mammalian Rac-T17N (Ridley et al. 1992) and Rac-G12V (Diekmann et al. 1991), respectively. These types of mutants have been successfully used to uncover their specific effects on actin regulation in other systems (Hall 1998). WT and mutant AtRac1s were purified as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* (Self and Hall 1995a) and released from GST (Fig. 3A). WT AtRac1 and mutant proteins were further characterized biochemically. AtRac1 has a low intrinsic GTPase activity characteristic of small GTPases (Self and Hall 1995a). The half time of GTP hydrolysis was 17 min, giving a rate constant (k) of 0.04 min^{-1}, which is more similar to that of RhoA (k = 0.039 min^{-1}) than to that of Rac1 (k = 0.069 min^{-1}; Self and Hall 1995b). As expected, AtRac1-G15V displayed a 10-fold reduction in its intrinsic GTPase activity (Fig. 3B), whereas AtRac1-T20N showed a dramatic loss of its affinity for guanine nucleotides (Fig. 3C). Both mutants were tagged with the myc-epitope so they could be differentiated from the endogenous AtRac on immunoblots. Transgenic *Arabidopsis* plants harboring the myc-tagged AtRac1 mutants, under

the control of a dexamethasone (dex)-inducible promoter (Aoyama and Chua 1997), were generated. Expression of both AtRac1 mutant proteins was followed by immunoblotting. Figure 3D shows the typical sustained expression of AtRac1 mutants during the first 48 h of dex treatment for two independent lines.

Effect of AtRac1 mutants on guard cell actin cytoskeleton and stomatal closure

Given their sequence homology with mammalian Rho family GTPases, it seems feasible that *Arabidopsis* Rho homologs might regulate the plant cell actin cytoskeleton. To address this question, we analyzed the effect of AtRac1 mutant expression on the guard cell actin cytoskeleton. WT/*AtRac1* mutant transgenic lines were crossed with WT/*GFP-mTn* transgenic lines to generate *AtRac1-T20N/GFP-mTn* and *AtRac1-G15V/GFP-mTn* transgenic lines. T2 transgenic plants were verified for mutant AtRac1 induction and the GFP-mTn expression level (data not shown). On 48 h of dex-induction in continuous white light (WL) without ABA treatment, we observed a breakdown of actin cables in the majority of guard cells of *AtRac1-T20N/GFP-mTn* transgenic plants compared with those of the control. In contrast, we could not detect any significant changes in the guard cell actin cytoskeleton of *AtRac1-G15V/GFP-mTn* transgenic lines induced with dex (Fig. 4A). AtRac1-T20N expression can thus induce a breakdown of actin cables in guard cells, in a manner resembling the effects of ABA on the actin cables of WT guard cells.

We also found that expression of AtRac1-T20N could
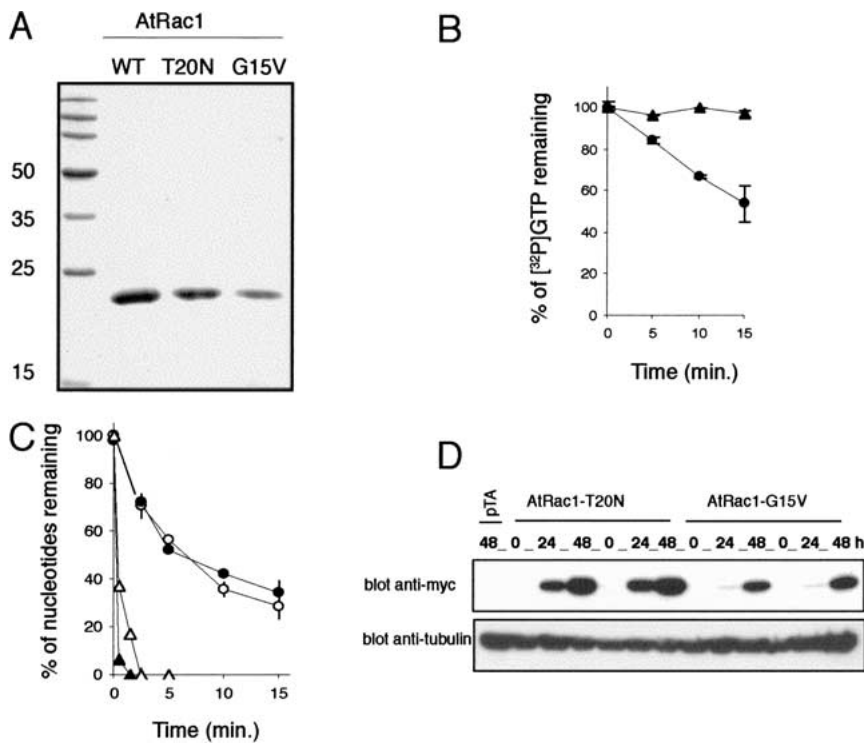
Figure 3. Biochemical characterization of AtRac1, and mutants and transgenic *Arabidopsis* plants showing inducible expression of AtRac1-mutants. (*A*) Coomassie blue staining of purified WT AtRac1 (lane *1*), AtRac1-T20N (lane *2*), and AtRac1-G15V (lane *3*). Molecular mass markers are indicated on the left (kD). (*B*) Kinetics of [γ-^{32}P]GTP hydrolysis by WT AtRac1 (solid circles) and AtRac1-G15V (solid triangles). (*C*) Measurements of the [^{3}H]GTP released by WT AtRac1 (solid circles) and AtRac1-T20N (solid triangles) and [^{3}H]GDP released by WT AtRac1 (hollow circles) and AtRac1-T20N (hollow triangles). (*D*) Anti-myc immunoblot showing the kinetics of dexamethasone-induced expression of AtRac1-mutants in two independent *Arabidopsis* transgenic lines for each mutant. pTA corresponds to transgenic plants carrying the empty vector. The amount of tubulin in the sample, as visualized by anti-tubulin antibodies, was used as a loading control.

induce stomatal closure, as observed on ABA-treatment of WT plants (Fig. 4A). To further analyze this observation, we measured the effect of *AtRac1* mutant expression on stomatal aperture (Fig. 4B). WT/*AtRac1-T20N* plants were induced with dex for 48 h under WL. One leaf of each seedling was used to measure stomatal apertures, whereas the remainder of the seedling was processed for quantitation of AtRac1 mutant expression levels by Western blotting. We first verified that *Arabidopsis* transgenic lines containing the empty vector (pTA7002) remained sensitive to ABA, even after dex induction. Using WT/*AtRac1-T20N* transgenic plants, we observed that expression of the AtRac1 dominant-negative mutant induced a closure of stomata, which is correlated with the expression level of *AtRac1-T20N* (Fig. 4B). In contrast, no stomatal closure was seen in either vector control or *AtRac1-G15V* plants after dex treatment (data not shown).

We next examined whether the constitutively active mutant of AtRac1 would display opposite effects on stomatal apertures. To this end, we compared the efficiency of ABA in inducing stomatal closure in dex-induced or noninduced WT/*AtRac1-G15V* transgenic plants under WL conditions. We observed that noninduced WT/*AtRac1-G15V* plants were as sensitive as vector control transgenic plants to ABA with respect to stomatal closure. In contrast, expression of *AtRac1-G15V* interfered with the normal ABA-induced stomatal closure. Furthermore, this blocking effect by AtRac1-G15V was stronger in transgenic plants with a higher expression level of the transgene (Fig. 4B). In conclusion, we found that AtRac1 mutants have opposing effects on stomatal dynamics. Expression of a dominant-negative AtRac1 mutant mim-

icked the ABA-induced actin disruption and stomatal closure, whereas a constitutively active AtRac1 mutant blocked the ABA-induced stomatal closure. In both cases, the physiological effect was related to the expression level of the AtRac1 mutant.

ABA induces AtRac inactivation in WT but not in abi1-1 cells

Because AtRac1-T20N recapitulates the ABA effects on actin cytoskeleton and stomatal closure, we investigated whether ABA acts by inactivating endogenous AtRac. We took advantage of the finding that only activated Rac can bind to the p-21 activated kinase (PAK). This feature was used to pull down active Rac using GST fused to PAK (residues 70 to 106; Manser et al. 1998). Using transgenic plants expressing AtRac1 mutants, we determined that AtRac1-G15V bound 62.8-fold more to GST-PAK$^{70–106}$ than AtRac1-T20N, showing the specificity of this assay in plants (Fig. 5A). Because the binding of GTP-loaded AtRac1 to GST-PAK$^{70–106}$ was 100-fold lower than that of mammalian Rac1, this biochemical assay required the use of higher protein amounts (Fig. 5B). To overcome this technical difficulty, we generated cell cultures from *Arabidopsis* WT and *abi1-1* mutant. ABA sensitivity of the cell lines was verified by following the induction of the early ABA responsive gene *KIN2* (Kurkela and Borg-Franck 1992). *KIN2* transcript was detected in WT cells 30 min after ABA addition, whereas no *KIN2* transcript was detected in similarly treated *abi1-1* cells (Fig. 5C). Having established the specificity of AtRac1 binding to GST-PAK$^{70–106}$ and the sensitivity
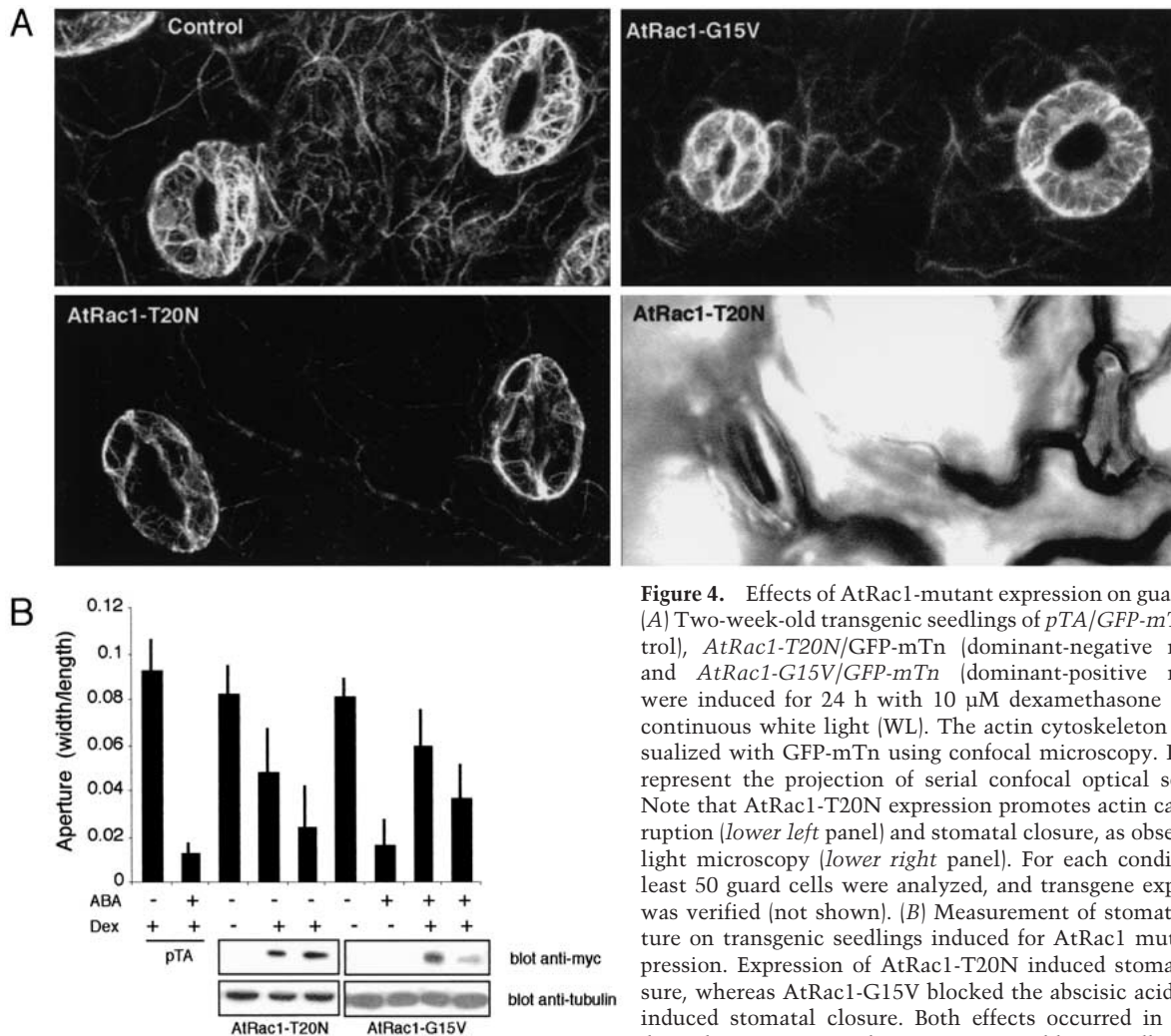
Figure 4. Effects of AtRac1-mutant expression on guard cells. (*A*) Two-week-old transgenic seedlings of *pTA/GFP-mTn* (control), *AtRac1-T20N*/GFP-mTn (dominant-negative mutant) and *AtRac1-G15V*/GFP-mTn (dominant-positive mutant) were induced for 24 h with 10 µM dexamethasone (dex) in continuous white light (WL). The actin cytoskeleton was visualized with GFP-mTn using confocal microscopy. Pictures represent the projection of serial confocal optical sections. Note that AtRac1-T20N expression promotes actin cable disruption (*lower left* panel) and stomatal closure, as observed by light microscopy (*lower right* panel). For each condition, at least 50 guard cells were analyzed, and transgene expression was verified (not shown). (*B*) Measurement of stomatal aperture on transgenic seedlings induced for AtRac1 mutant expression. Expression of AtRac1-T20N induced stomatal closure, whereas AtRac1-G15V blocked the abscisic acid (ABA)-induced stomatal closure. Both effects occurred in a dose-dependent manner, as shown on immunoblots. Seedlings were incubated with 10 µM dex for 48 h in WL conditions, followed by a 1-h ABA(10 µM)-treatment when indicated. One leaf of each seedling was used to measure the stomatal aperture. The graph represents the mean width of 60 stomata. The effects plotted in the graph were verified in three independent experiments. Error bars show the standard deviations. The remaining tissues were used to quantify transgene expression using Western blotting (*lower* panels). The anti-myc immunoblot shows the level of dex-induced expression of AtRac1 mutants, whereas the anti-tubulin immunoblot was used as a loading control. Measurements were performed on different trangenic lines and plotted on the graph to show that the effects on stomatal aperture are related to the expression levels of AtRac1 mutants.

of *Arabidopsis* cells to ABA, we tested the effects of ABA on AtRac biochemical activity. We observed that addition of ABA to WT *Arabidopsis* cells inactivated AtRac in a time-dependent manner (Fig. 5D), with a half time of 15 min (Fig. 5F). This ABA effect on AtRac inactivation was dose dependent (Fig. 5E). In contrast to WT cells, we never observed AtRac inactivation in *abi1-1* cells, which are insensitive to ABA (Fig. 5F,G). These results established that AtRac is specifically inactivated in *Arabidopsis* WT cell lines on ABA-treatment and indicate that AtRac1 is likely inactivated on ABA-treatment of plants. Together with the observation that AtRac1-dominant mutants interfere with the stomatal closure (Fig. 4B), our results indicate that AtRac1 inactivation could represent a limiting step in ABA-induced stomatal closure.

Expression of AtRac1 dominant-negative mutant can rescue stomatal closure in abi1-1

To investigate whether AtRac1 inactivation was required to allow the ABA-induced stomatal closure, we made use of our previous observations that ABA induces neither actin cable disruption nor AtRac inactivation in the *abi1-1* mutant. To test whether AtRac1 inactivation limits ABA-induced stomatal closure in *abi1-1* we crossed *abi1-1* plants with inducible *AtRac1* mutant transgenic plants. T2 lines were screened for *abi1-1* homozygosity using PCR amplification (Fig. 6A). T3 *abi1-1/AtRac1* mutant transgenic lines were tested for AtRac1 effects on stomatal aperture (Fig. 6B). We first verified that in the absence of the inducer *abi1-1/At-*
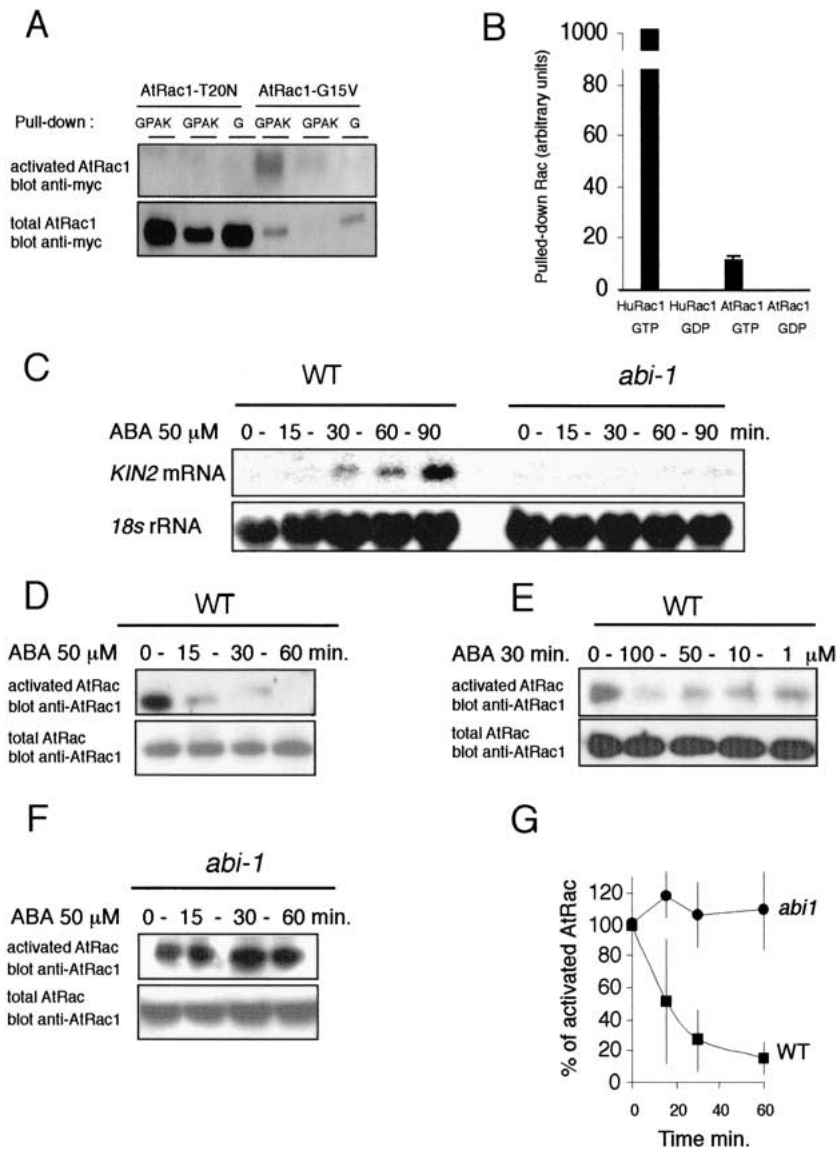
Figure 5. Abscisic acid (ABA) induces inactivation of AtRac in WT but not in *abi1-1* cells. (*A*) Activated AtRac1 (AtRac1-G15V) binds specifically GST-PAK$^{70-106}$. WT/AtRac1-mutants transgenic plants were dexamethasone-induced for 48 h and processed for the GST-PAK$^{70-106}$ pull-down assay. (*Upper* panel) Anti-myc immunoblots showed that AtRac1-G15V (active) but not AtRac1-T20N (inactive) bound to GST-PAK$^{70-106}$ (GPAK) and not to GST (G). (*Lower* panel) 10 µL of each lysate was used to compare the amount of AtRac1-mutant engaged for each pull down. (*B*) In vitro affinity comparison of HuRac1 and AtRac1 binding to GST PAK$^{70-106}$. Recombinant activated AtRac1 (GTP-loaded) binds specifically to GST-PAK$^{70-106}$, with a 100-fold lower affinity than HuRac1. (*C*) ABA-induced *KIN2* transcript levels in Arabidopsis WT and *abi1-1* suspension cells. (*Lower* panel) 18S rRNA loading control. (*D–G*) Effects of ABA on AtRac biochemical activity, using ABA-sensitive WT and ABA-insensitive *abi1-1* cells. (*D*) Kinetics of ABA-mediated inactivation of AtRac1. Cells were treated with 50 µM ABA and active AtRac1 determined by pull-down experiments. (*E*) Dose response of the ABA-induced AtRac inactivation after 30 min of ABA-treatment. (*F*) AtRac1 is not inactivated by ABA in *abi1-1* cells. (*G*) Quantitation of the ABA-induced inactivation of AtRac1 in WT and *abi1-1* suspension cells measured by GST-PAK$^{70-106}$ pull down. Autoradiographs were quantified using N.I.H. Image 1.6 (average of four independent experiments with standard deviations).

Rac1-T20N plants remained insensitive to ABA. Using these plants, we observed that AtRac1-T20N expression induced stomatal closure, the extent of which was dependent on the expression level of the AtRac1 dominant negative mutant.

Discussion

ABA-induced actin cable breakdown in guard cells is impaired in abi1-1

Stomatal closure caused by guard cell relaxation is a dynamic process, allowing a plant to adapt rapidly to changes in environmental conditions. Recent studies have pointed to the importance of guard cell membrane dynamics during ABA-induced stomatal closure (Homann and Thiel 1999; Blatt 2000). Another related regulatory process of guard cell dynamics has emerged with the observation that the actin cytoskeleton might be disor-

ganized during ABA-induced stomatal closure (Eun and Lee 1997). Notwithstanding this initial observation, the role of the actin cytoskeleton has remained elusive because of technical difficulties in imaging cytoskeletons in living guard cells. We have obviated this problem by using transgenic lines of *Arabidopsis* expressing either GFP-mTn (Kost et al. 1998) or MAP4-GFP (Mathur and Chua 2000), which allow noninvasive imaging of actin or tubulin cytoskeleton dynamics. We found that actin cables in guard cells were disrupted on ABA treatment. This breakdown was specific to the actin cytoskeleton because the microtubule cytoskeleton remained unchanged. Moreover, the actin cytoskeleton was unaffected by ABA treatment in the ABA-insensitive mutant *abi1-1*, which is impaired in stomatal closure. These observations, which confirm and extend earlier work (Eun and Lee 1997; Eun et al. 2001), reinforce the hypothesis that the actin cytoskeleton disruption is linked to stomatal closure.
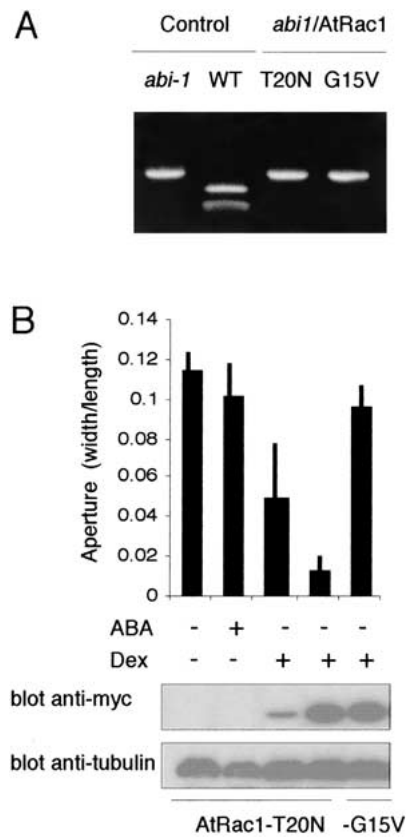
Figure 6. AtRac1-T20N expression can rescue stomatal closures in *abi1-1*. (*A*) Inducible AtRac1-mutant constructs were introduced into *abi1-1* plants by crossing. The ethidium bromide DNA gel electrophoresis shows the endonuclease *Nco*I polymorphism on *abi1-1* PCR products obtained from genomic DNAs of WT *Arabidopsis* ecotype Columbia, *abi1-1* and the generated F_2 plants *abi1-1*/AtRac1-mutants. (*B*) Dexamethasone (dex)-induction of AtRac1-T20N but not AtRac1-G15V can rescue stomatal closure in *abi1-1*. Seedlings were incubated with 10 µM dex for 48 h in continuous white light. One leaf of each seedling was used to measure the stomatal aperture. The graph represents the mean width value of 60 stomata with standard deviations. The effects plotted in the bar charts were verified in three independent experiments. The remaining tissues were used to quantify transgene expression using Western blotting (*lower* panels). Immunoblot anti-tubulin was used as a loading control. Note that AtRac1-T20N restores stomatal closure in *abi1-1* in a dose-dependent manner.

AtRac1 mutants interfere with ABA-induced actin cable disorganization and stomatal closure

The finding that ABA treatment leads to actin cable disorganization raises the question of how the ABA signal is transduced and the identity of the components of the pathway for this process. In yeast and animal cells, Rho proteins are known to be upstream regulators of actin networks (Hall 1998). This finding, along with our observation that AtRac1 is expressed in *Arabidopsis* guard cells, led us to investigate the possible roles of AtRac1 in regulating both guard cell actin cytoskeleton

dynamics and stomatal closure. One important feature of RhoGTPases is that dominant-positive and dominant-negative mutants can be generated to decipher their signaling roles (Bishop and Hall 2000). Dominant-negative mutants are thought to poison their exchange factors, thereby blocking activation of downstream effectors, whereas dominant-positive mutants remain insensitive to GTPase activating proteins, resulting in constitutive activation of their effectors.

Several lines of evidence presented in this paper support the notion that AtRac1 is a key player in ABA-triggered guard cell actin disorganization and stomatal closure. First, expression of the dominant-negative mutant AtRac1-T20N can recapitulate the effects of ABA (Fig. 4). Second, In WT plants, the effects of ABA can be blocked by the dominant-positive mutant AtRac1-G15V (Fig. 4). Third, in the ABA-insensitive mutant *abi1-1*, AtRac1-T20N, but not AtRac1-G15V, can induce stomatal closure (Fig. 6). In all three cases, the effect was correlated with the expression level of the appropriate AtRac1 mutant indicating that this small GTPase regulates a rate-limiting step.

ABA treatment inactivates AtRac1

Because AtRac1-T20N can recapitulate the effects of ABA on actin cable disorganization and stomatal closure, we hypothesize that one consequence of ABA signaling in guard cells is to inactivate AtRac1. Rho GTPases in other systems have been shown to be activated by many different growth factors, but almost nothing is known concerning external factors that may be responsible for their down-regulation. As it is technically difficult to obtain sufficient quantities of guard cells for biochemical analyses of AtRac1, we resorted to the use of *Arabidopsis* tissue culture cells. We first monitored expression of the ABA-responsive gene *KIN2* in our WT and *abi1-1* cell cultures to ensure that these cultures retain their appropriate ABA responses (Fig 5C). In addition, we modified a biochemical assay to measure the level of activated AtRac in *Arabidopsis*. In vitro, 1% of AtRac1 GTP-loaded molecules bind to GST-PAK[70–106]. This low affinity binding probably reflects the two substitutions V/I33 and F/Y40 in the effector domain of AtRac1 compared with that of the human Rac. This low interaction affinity can be overcome by using high protein concentrations of *Arabidopsis* cell lysates. Using this biochemical assay, we showed that indeed AtRac is inactivated on ABA treatment. The AtRac inactivation was dependent on the ABA concentration used and occurred with a half time of 15 min. The time course of AtRac inactivation strongly correlated with the kinetics of stomatal closure induced by ABA. Moreover, ABA did not inactivate AtRac in *abi1-1*, which is insensitive to ABA (Fig. 5). Taken together, these results support the hypothesis that ABA induces stomatal closure through inactivation of AtRac1 and that a block of AtRac1 inactivation by ABA may be responsible for the impairment of stomatal closure in the *abi1-1* mutant. Our results reveal a new pathway for the down-regulation of small

Rho GTPases by ABA, which is dependent on the protein phosphatase IIC, ABI1. Given the emergence of guard cells as a popular system with which to dissect complex signaling mechanisms within a single cell, it will be of considerable interest to identify additional molecular components that govern stomatal opening and thus plant water status. Changes in guard cell volume require large adjustments in the surface area of the guard cell plasma membrane. Recent evidence indicates that extensive and rapid exocytic and endocytic events can account for the osmotically driven changes in guard cell surface area, which regulates stomatal aperture (Homann and Thiel 1999). Because the vesicles that add or remove membrane material must traffic along the actin cytoskeletal network, the possible involvement of ABA-mediated inactivation of AtRac1 in preventing the efficient shuttling between cytoplasmic vesicles and the plasma membrane requires further investigation.

Materials and methods

Arabidopsis transgenic plants and suspension cell culture

The *AtRac1-GUS* construct was previously described (Kost et al. 1999a). *AtRac1-G15V* and *AtRac1-T20N* mutants were generated by targeted mutagenesis on pAt043 encoding AtRac1 (Xia et al. 1996) and were tagged with sequences encoding myc by PCR amplification. cDNAs were subcloned into the *Xho*I–*Spe*I sites in the dex-inducible vector pTA7002 derived from pBI101/121 (Clontech) and into the *Eco*RI site of pGEX-2T (Pharmacia). WT *Arabidopsis thaliana* ecotype Columbia was transformed with pTA7002-*AtRac1* mutants or the empty vector pTA7002 (pTA) by vacuum-infiltration (Bechtold et al. 1993). Transformants were selected on 100 μg/mL hygromycin containing MS medium supplemented with 3% sucrose and 0.8% agar. Plants were transferred onto MS medium containing 10 μM dex (D-4902, Sigma) to induce expression of AtRac1 mutants. Transgenic plants carrying *AtRac1* mutant and pTA vector control (Fig. 2C) were crossed with plants carrying the *GFP-mTn* construct (Kost et al. 1998) to generate *GFP-mTn*/AtRac1 mutant or *GFP-mTn*/pTA transgenic plants. Transgenic *abi1*/*AtRac1* mutant plants were obtained by crossing WT/*AtRac1* mutants with *abi1-1*. Transgenic *abi1*/*GFP-mTn* plants were obtained by crossing WT/*GFP-mTn* with *abi1-1*. All transgenic *abi1-1* homozygous plant lines were screened by PCR amplification using DNA from T2 and T3 lines as described previously (Pei et al. 1998). Cell suspension cultures from WT and *abi1-1 A. thaliana* were generated as previously described (Mathur and Koncz 1998). Cells were propagated at 22°C in darkness in MS medium supplemented with twofold B_5-vitamins, 3% sucrose, 1 mg/L 2,4-dichlorophenoxyacetic acid, and 0.5 mg/L 6-(γ,γ-dimethylallylamino)-purine riboside.

Histochemical and fluorescent microscopy analyses

AtRac1-GUS transgenic seedlings were incubated overnight at 37°C with gentle rolling in 50mM Tris-HCl (pH 7.0) containing 0.2% X-Gluc (Jersey Lab and Gloves Supply), 1% Triton X-100, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide. Seedlings were treated 48 h with 70% ethanol and mounted in 50% glycerol to prevent tissue dehydration. GUS staining was analyzed by bright-field transmitted light microscopy using an Axioscope (Carl Zeiss Inc.) microscope. Images were taken by 35-mm photography (63t film; Eastman Kodak). Confocal analysis of GFP expression was performed using an LSM410 inverted microscope (Carl Zeiss) as previously described (Kost et al. 1998). Transgenic *GFP-mTn* and *GFP-MAP4* seedlings were submerged intact in 10 mM MES-Tris (pH 6.1), 10 mM KCl supplemented with 50μM ABA whenever indicated and observed.

GTPase activity and nucleotide exchange assays

Recombinant AtRac1 WT and mutants were affinity-purified from *E. coli* lysates as GST fusion proteins and released from GST using thrombin as previously described (Self and Hall 1995a). The intrinsic AtRac1 and AtRac1-G15V GTPase activity was measured using the nitrocellulose filter-binding method (Self and Hall 1995b). The intrinsic nucleotide exchange activity of AtRac1 and AtRac1-T20N was measured at high magnesium concentrations using the nitrocellulose filter-binding method (Self and Hall 1995b).

Pull-down methods

AtRac inactivation was assessed using the GST-PAK[70–106] pull-down method (Manser et al. 1998). For the in vitro affinity comparison, AtRac1 and huRac1 proteins were loaded with GTP or GDP (Self and Hall 1995b) and assessed for GST-PAK[70–106] binding as previously described (Benard et al. 1999). To measure the in vivo AtRac biochemical activities, 1 mL of pelleted suspension cells or ten 2-week-old seedlings were ground in 1.5 mL of the IP buffer (40 mM HEPES at pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 4% glycerol, 0.5% Triton X-100, 10 mM NaF, 20 mM β-glycerophosphate supplemented freshly with 2 mM Na-Vanadate, 5 mM DTT, 1 mM PMSF, and protease inhibitors complete EDTA-free; Roche); 2.5 mg of the clarified lysates was used in each pull-down assay. In parallel, 25 μg of each lysate was used to determine the amount of total-AtRac by Western blotting. Western blots were performed after SDS-PAGE fractionation and transfer to immobilon-P membranes (Millipore) at a constant voltage 35 V, using carbonate buffer. Anti-Myc monoclonal 9E10, anti-β-tubulin (Amersham Life Science), and immunopurified AtRac1 polyclonal antibodies were used at 1 μg/mL, followed by anti-mouse or anti-rabbit horseradish peroxidase incubation (1:3000, Amersham Life Science). Immunoreactive bands were visualized using ECL-plus (Amersham Life Science). Polyclonal antiserum against GST-AtRac1 was immunopurified on recombinant AtRac1. Immunopurified anti-AtRac1 used at 1 μg/mL enables detection of 5 fmoles of purified AtRac1. Anti-AtRac1 could detect other AtRac members, referred to as AtRac when Western blots were performed on total cell extracts.

Stomatal aperture measurements

Measurements were performed on 2-week-old seedlings induced for 48 h with 10 μM dex in WL conditions. Seedlings were incubated for 30 min in 10 mM MES-Tris (pH 6.1), 10 mM KCl and supplemented with 10 to 50 μM ABA ([±]-*cis*,*trans*-ABA; Sigma) when indicated. Widths and lengths of stomatal pores were measured using a LSM410 inverted confocal microscope (Carl Zeiss). Each value corresponds to the average of 60 stomata measured on one leaf, along with the corresponding standard deviation. The remainder of each seedling was processed for Western blot analysis of AtRac1 mutants to correlate the expression level with the effects on stomatal aperture. One representative experiment out of three was plotted for each graph.

Northern blotting

Total RNA was isolated from 1 mL of cell pellet using the QIAGEN purification kit. Each lane contained 10 μg total RNA. Northern blot hybridization was performed as described previously (Ausubel et al. 1994).

Acknowledgments

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