

Dissection of the Relationship Between RACK1 and Heterotrimeric G-Proteins in Arabidopsis

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Mammalian receptor for activated C kinase 1 (RACK1) is a versatile scaffold protein, playing regulatory roles in multiple signal transduction pathways. Moreover, RACK1 interacts with the heterotrimeric G-proteins (G-proteins) and regulates some specific functions of G $\beta\gamma$. Although the protein sequences of both RACK1 and G-proteins are highly conserved in Arabidopsis, their relationship remains elusive. Here we provide genetic and biochemical evidence that Arabidopsis RACK1 and G-proteins may act through a mechanism that is distinct from their counterparts in mammals. Loss-of-function alleles of *RACK1A* (the most abundantly expressed RACK1 gene in Arabidopsis) do not appear to share morphological and developmental phenotypes with loss-of-function alleles of *GPA1* (encoding the sole G α in Arabidopsis) or *AGB1* (encoding the sole G β in Arabidopsis). The analysis of *gpa1 rack1a* and *agb1 rack1a* double mutants suggested that the effect of RACK1A on morphological and developmental traits may occur independently of the presence or absence of the G-protein subunits. Although both RACK1A and G-protein subunits are negative regulators of ABA responses in the ABA inhibition of early seedling development, an additive ABA hypersensitivity was observed in *gpa1 rack1a* and *agb1 rack1a* double mutants. Biochemical analysis suggested that unlike their counterparts in mammals, RACK1 may not physically interact with AGB1. Taken together, these findings revealed some fundamental differences in the relationship of RACK1 and G-proteins between Arabidopsis and mammals.

Keywords: ABA • AGB1 • Early seedling development • GPA1 • RACK1 • Seed germination.

Abbreviations: AGB1, Arabidopsis heterotrimeric G-protein β subunit; AGG1, Arabidopsis heterotrimeric G-protein γ subunit 1; AGG2, Arabidopsis heterotrimeric G-protein γ subunit 2; 3-AT, 3-amino-1,2,4-triazole; Co-IP, co-immunoprecipitation; FOA, fluoro-orotic acid; G α , G β and G γ , heterotrimeric G-protein α , β and γ subunit; GPA1, Arabidopsis heterotrimeric G-protein α subunit; G-protein, heterotrimeric GTP-binding protein; GUS, β -glucuronidase; HA, haemagglutinin; PVDF, polyvinylidene fluoride; RACK1, receptor for activated C kinase 1; RGS, regulator of G-protein signaling; RT-PCR, reverse transcription-PCR.

Introduction

Receptor for activated C kinase 1 (RACK1) was originally identified as a receptor for activated protein kinase C (PKC) in mammals (Mochly-Rosen et al. 1991, Ron et al. 1994). However, subsequent studies demonstrated that RACK1 can physically interact with many other proteins and facilitate their interactions (reviewed by McCahill et al. 2002, Sklan et al. 2006). Therefore, RACK1 is now regarded as a versatile scaffold protein that plays regulatory roles in multiple signal transduction pathways. RACK1 is structurally similar to the heterotrimeric GTP-binding protein (G-protein) β subunit (G β), both of which contain seven tryptophan-aspartic acid domain (WD40) repeats (Ullah et al. 2008). More importantly, RACK1 interacts with G β (Dell et al. 2002, Chen et al. 2004a, Chen et al. 2004b, Chen et al. 2005). The protein sequences of both RACK1 and G β are highly conserved in plants (Weiss et al. 1994, Chen et al. 2006b, Guo et al. 2007). However, little is known about the relationship between RACK1 and G-proteins in plants.

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The first plant *RACK1* gene was cloned from tobacco BY-2 cells as an auxin-induced gene, *arcA* (Ishida et al. 1993). Gene expression and induction studies implied that plant *RACK1* may be involved in hormone-mediated cell division (Ishida et al. 1993, McKhann et al. 1997), and ultraviolet and salicylic acid responses (Perennes et al. 1999). Rice *RACK1*, originally named *RWD* (Iwasaki et al. 1995), has recently been demonstrated to be a key regulator of innate immunity through interaction with multiple proteins in the Rac1 immune complex (Nakashima et al. 2008).

G-proteins consist of $G\alpha$, $G\beta$ and $G\gamma$ subunits. In Arabidopsis, the $G\alpha$ subunit is encoded by a single gene, *GPA1* (Ma et al. 1990); the $G\beta$ subunit is also encoded by a single gene, *AGB1* (Weiss et al. 1994); and the $G\gamma$ subunits are encoded by two genes, *AGG1* and *AGG2* (Mason and Botella 2000, Mason and Botella 2001). Substantial evidence indicated that G-proteins mediate multiple developmental processes and hormone signaling (reviewed by Fujisawa et al. 2001, Ma 2001, Assmann 2002, Jones 2002, Jones and Assmann 2004, Perfus-Barbeoch et al. 2004, Temple and Jones 2007, Chen 2008, Ding et al. 2008). *GPA1* and *AGB1* have also been shown to be negative regulators of ABA responses during seed germination and early seedling development (Ullah et al. 2002, Assmann, 2005, Pandey et al. 2006).

Three lines of evidence have prompted us to examine the relationship between *RACK1* and G-proteins in Arabidopsis. First, in mammalian cells, *RACK1* physically interacts with $G\beta$ (Dell et al. 2002, Chen et al. 2004a, Chen et al. 2004b, Chen et al. 2005). Secondly, in yeast, *RACK1/Asc1* functions as a $G\beta$ and interacts with one of the two $G\alpha$ subunits (Zeller et al. 2007). Thirdly, in rice, *RACK1* was one of the seven proteins whose expression was down-regulated in the *d1* mutant, a loss-of-function allele of the sole rice $G\alpha$ (Komatsu et al. 2005). We took several approaches to examine the relationship between *RACK1* and G-proteins in Arabidopsis. First, we took a genetic approach to generate and analyze double mutants between *rack1* and $G\alpha$ and $G\beta$ mutants. Secondly, we tested the physical interaction between *RACK1* and $G\beta$. Finally, we examined if the expression of *RACK1* is misregulated in G-protein subunit mutants. Our work revealed a different, G-protein-independent role for *RACK1* in Arabidopsis vs. mammals.

Results

Double mutants between *rack1a* and *gpa1* or *agb1*

There are three *RACK1* homologous genes in the Arabidopsis genome, designated as *RACK1A*, *RACK1B* and *RACK1C*, respectively (Chen et al. 2006b). Among these three *RACK1* genes, *RACK1A* is the most abundantly expressed member (Guo and Chen, 2008). Furthermore, loss-of-function mutations in *RACK1A*, but not in *RACK1B* or *RACK1C*, conferred defects in plant development (Guo and Chen 2008).

Therefore, we mainly focus on *RACK1A* in this study. There is a single $G\alpha$ (*GPA1*) and a single $G\beta$ (*AGB1*) in Arabidopsis (Ma et al. 1990, Weiss et al. 1994), and it has been demonstrated that *GPA1* interacts with *AGB1* physically, genetically and biochemically (Chen et al. 2004, Adjobo-Hermans et al. 2006, Chen et al. 2006a, Fan et al. 2008, Wang et al. 2008).

In order to gain an insight into the relationship between *RACK1* and the G-proteins, we first compared the phenotypes between loss-of-function alleles of *RACK1A*, *rack1a-1* (Chen et al. 2006b), and loss-of-function alleles of $G\alpha$ and $G\beta$ subunits, *gpa1-4* (Jones et al. 2003) and *agb1-2* (Ullah et al. 2003). All of these mutants are in the Columbia (Col) ecotype background. We reasoned that if *RACK1A* and G-proteins function genetically in the same pathway, we might observe shared phenotypic traits between these mutants. As has been reported previously (Ullah et al. 2001, Ullah et al. 2003, Chen et al. 2006a), both *gpa1* and *agb1* mutants had characteristic, round shaped rosette leaves, whereas the shape of rosette leaves of *rack1a* mutants was near that of the wild type (Fig. 1). On the other hand, *rack1a* mutants produced fewer rosette leaves due to a reduced rate of production (Chen et al. 2006b) whereas *gpa1* and *agb1* mutants have near wild-type traits of these phenotypes (Chen et al. 2004). Therefore, morphologically, *rack1a* mutants are different from *gpa1* and *agb1* mutants.

We then generated double mutants between *rack1a-1* and *gpa1-4* or *agb1-2* alleles. As shown in Fig. 1, *gpa1 rack1a* and *agb1 rack1a* double mutants showed the combined morphological traits of their parental single mutants. For example, the rosette leaves of *gpa1-4 rack1a-1* and *agb1-2 rack1a-1* double mutants were round shaped, similar to those of *gpa1-4* and *agb1-2* single mutants (Fig. 1A–C). On the other hand, compared with Col, or *gpa1-4* or *agb1-2* single mutants, the number of rosette leaves of *gpa1-4 rack1a-1* and *agb1-2 rack1a-1* double mutants was reduced, similar to the *rack1a-1* single mutant (Fig. 1A, D). We also noticed that although the overall shape of the rosette leaves of *gpa1-4 rack1a-1* and *agb1-2 rack1a-1* double mutants was round (evident by the increased leaf width/length ratio), these leaves appeared to be more wrinkled than those in *gpa1-4* and *agb1-2* single mutants (Fig. 1B). Taken together, these results suggested that the effects of *RACK1A* on these morphological and developmental traits probably occur independently of the presence or absence of the G-protein subunits.

An enhanced effect on ABA hypersensitivity was observed between *rack1a* and *gpa1* or *agb1* mutants in the ABA inhibition of cotyledon greening and root growth

Although the overall morphology of the *rack1a* and G-protein subunit mutants was different and independent effects on

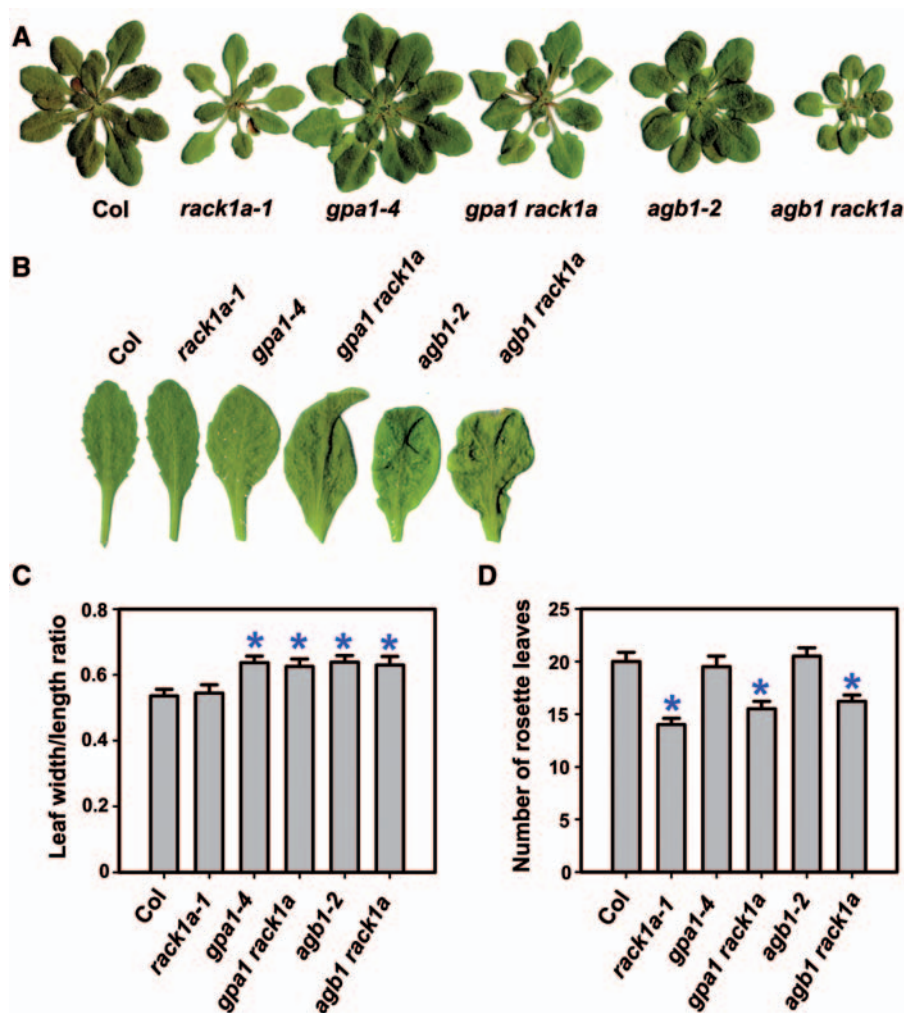


Fig. 1 *agb1 rack1a* and *gpa1 rack1a* double mutants. (A) Phenotypes of 43-day-old plants grown under a 8/16 h photoperiod. (B) Phenotypes of rosette leaves. Shown are the 10th rosette leaves taken from 58-day-old plants grown under a 8/16 h photoperiod. (C) The width/length ratio of fully expanded rosette leaves. Shown are the means \pm SE for 10 plants of each genotype. * $P < 0.05$, significantly different from Col. (D) Number of rosette leaves. The total number of rosette leaves of 43-day-old plants grown under a 8/16 h photoperiod was counted. Shown are means \pm SE for 10 plants of each genotype. * $P < 0.05$, significantly different from Col.

morphological and developmental traits were observed in the double mutants, we could not exclude the possibility that RACK1A and G-proteins may act together genetically in a specific process or under specific conditions. Therefore, we sought conditional phenotypes shared between *rack1a* and G-protein subunit mutants. Because one of the best known roles of G-proteins in Arabidopsis is that GPA1 and AGB1 function as negative regulators of ABA responses during seed germination and early seedling development (Ullah et al. 2002, Pandey et al. 2006) and we have previously shown that *rack1* mutants are hypersensitive to ABA (Chen et al. 2006b, Guo et al. 2009), we wanted to examine if RACK1A and GPA1 and AGB1 interact genetically in these ABA-mediated processes.

Although it has been shown that *gpa1*, *agb1* and *rack1a* mutants are hypersensitive to ABA in the ABA inhibition of seed germination and early seedling development (Ullah et al. 2002, Chen et al. 2006b, Pandey et al. 2006, Guo et al. 2009), a direct comparison of their ABA hypersensitivity had not been performed. Therefore, the seeds of *gpa1*, *agb1* and *rack1a* mutants were sown side by side in MS/G medium containing different concentrations of ABA. We used three different assays to examine and compare the ABA sensitivity of these mutants, and these results are presented in **Supplementary Fig. S1**. We found that compared with *gpa1* and *agb1* mutants, *rack1a* mutants displayed ABA hypersensitivity to a lesser extent (**Supplementary Fig. S1A, B**). For example, the hypersensitivity of *gpa1* and *agb1* mutants

could be observed at a concentration of ABA as low as 0.2 μM , whereas at this concentration, *rack1a* mutants had wild-type sensitivity to ABA (**Supplementary Fig. S1A**). At 1.0 μM ABA, a significant hypersensitivity was observed in *rack1a* mutants (**Supplementary Fig. S1A**). Further, the ABA hypersensitivity of *rack1a* mutants was more pronounced in the first 3 d after imbibed seeds had been transferred to germination conditions whereas *gpa1* and *agb1* mutants constantly displayed ABA hypersensitivity (**Supplementary Fig. S1B**).

Subsequently, we compared the ABA hypersensitivity of *rack1a* with *gpa1* and *agb1* mutants in the ABA inhibition of early seedling development. First, we scored the percentage of green seedlings in the presence of ABA, based on the obvious greening of cotyledons. We found that the ABA hypersensitivity of *rack1a* mutants was comparable with that of *gpa1* and *agb1* mutants. Consistent with the report by Pandey et al. (2006), *agb1* mutants displayed a stronger ABA hypersensitivity than *gpa1* mutants. Compared with G-protein subunit mutants, *rack1a* mutants appeared to be more hypersensitive to ABA than the *gpa1-4* mutant but less hypersensitive than the *agb1-2* mutant (**Supplementary Fig. S1C, D**). Secondly, we measured the length of the primary root with and without ABA application. Similar to the findings of the green seedling assay, the ABA hypersensitivity of *rack1a* mutants was comparable with that of *gpa1* and *agb1* mutants (**Supplementary Fig. S1E, F**). Because this ABA hypersensitivity (ABA inhibition of cotyledon greening and root growth) of the *rack1a* mutant is more comparable with the G-protein subunit mutants, in subsequent studies we used these assays to examine the ABA sensitivity of *agb1 rack1a* and *gpa1 rack1a* double mutants.

In the green seedling assay, without exogenously applied ABA, all single and double mutant seeds, like the wild type, could develop near 100% green seedlings (**Fig. 2A**). When exogenous ABA was applied, *gpa1 rack1a* double mutants were more hypersensitive to ABA than *gpa1-4* and *rack1a-1* single mutants (**Fig. 2B–D**). Similarly, *agb1 rack1a* double mutants were more hypersensitive to ABA than *agb1-2* and *rack1a-1* single mutants, and displayed the strongest ABA hypersensitivity among all genotypes tested (**Fig. 2B–D**).

In the root growth assay, without ABA application, the *rack1a* mutant had a slightly shorter primary root, *gpa1* mutants had near wild-type length of the primary root, whereas *agb1* mutants had a longer primary root (**Fig. 3A**). With ABA application, the length of the primary root was reduced in all genotypes (**Fig. 3B**). However, compared with their corresponding single mutants, *gpa1 rack1a* and *agb1 rack1a* double mutants were more hypersensitive to ABA (**Fig. 3B**). The strongest ABA hypersensitivity was observed in *agb1 rack1a* double mutants (**Fig. 3B–D**). Taken together, these results suggested that *rack1a* and G-protein subunit mutants may have an additive effect on ABA

hypersensitivity in the ABA inhibition of early seedling development.

RACK1 and AGB1 may not physically interact with each other

Genetic studies implied that RACK1A may or may not function in the same pathway with G-proteins to regulate ABA responses. Therefore, we sought additional evidence that may shed light on the relationship between RACK1 and G-proteins. Because RACK1 physically interacts with G-proteins in mammalian cells (Dell et al. 2002, Chen et al. 2004a, Chen et al. 2004b, Chen et al. 2005), it was necessary to examine if such an interaction is conserved in plants. Because in mammalian cells, the $G\beta$ of the heterotrimeric G-protein complex is responsible for binding RACK1 (Chen et al. 2004b), we used a conventional ProQuest yeast two-hybrid system (Invitrogen) to test the interaction between RACK1 and AGB1 directly. The known interactions between AGB1 and AGG1 or AGG2 (Mason and Botella 2000, Mason and Botella 2001) and the known interaction between TT8 and MYB75 (Zimmermann et al. 2004) were used as positive controls in our assays. As shown in **Fig. 4**, we detected no interaction between RACK1 (RACK1A, RACK1B or RACK1C) and AGB1.

Because in the conventional yeast two-hybrid system, the interactions inevitably take place in the yeast nucleus and it is known that the membrane localization is important for the proper function of G-proteins, it was necessary to test the interaction between RACK1 and AGB1 further using the split-ubiquitin yeast two-hybrid system in which the interactions take place at the plasma membrane (Johnsson and Varshavsky 1994, Stagljar et al. 1998). The known interaction between AGB1 and AGG1 (Mason and Botella 2000) was used as a positive control in our assays. Again, no interaction was detected between RACK1 (RACK1A, RACK1B or RACK1C) and AGB1 (**Fig. 4B**).

Because mammalian RACK1 prefers to bind to $G\beta\gamma$ dimer and $G\alpha\beta\gamma$ trimer (Dell et al. 2002, Chen et al. 2005) and AGB1 may not be able to use yeast $G\gamma$ to form a stable dimer to bind RACK1, we decided to test the interaction between RACK1A and G-proteins further using the yeast three-hybrid system (Clontech). Specifically, we wanted to test the interaction between RACK1A and AGB1–AGG1. In this assay, the expression of AGG1 was driven by a methionine-repressive promoter: in the absence of methionine, the expression of AGG1 was activated, whereas in the presence of methionine, the expression of AGG1 was repressed. As shown in **Fig. 5A**, we detected no interaction between RACK1A and AGB1–AGG1.

Because interactions detected or not detected in yeasts may not always reflect the true situation in living plant cells (Ehlert et al. 2006) and AGB1 may not be able to use yeast $G\gamma$ to form a stable dimer, we decided to test the interaction

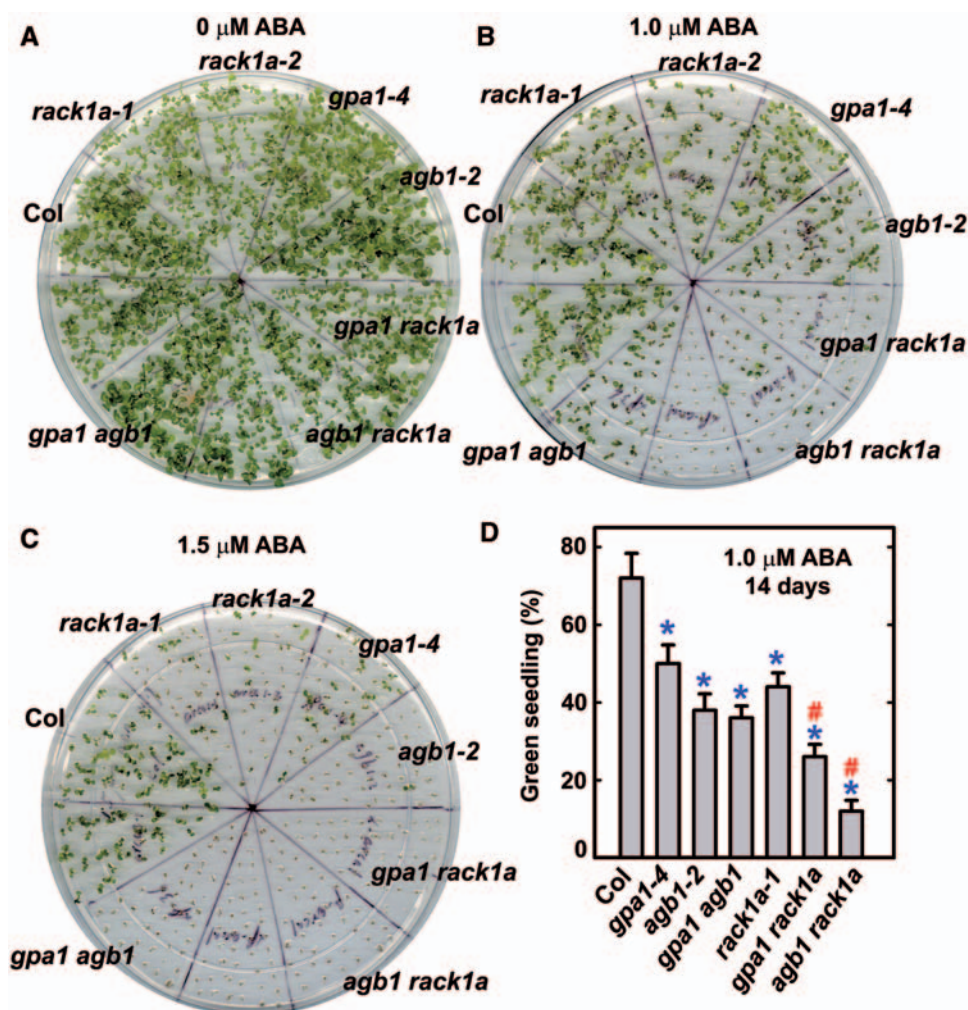


Fig. 2 ABA hypersensitivity of *agb1 rack1a* and *gpa1 rack1a* double mutants in the ABA inhibition of cotyledon greening assay. Sterilized wild-type (Col) and mutant seeds from matched seed lots were sown on MS/G medium containing 0 μM (A), 1.0 μM (B) or 1.5 μM (C) ABA, and cultured at 23°C, with a 14/10h photoperiod (120 $\mu\text{mol m}^{-2}\text{s}^{-1}$). After 14d, the percentage of green seedlings at 1.0 μM ABA was scored (D). Shown are mean values \pm SE of three replicates. * $P < 0.05$, significantly different from Col; # $P < 0.05$, significantly different from the corresponding single mutants.

between RACK1A and AGB1 in plant cells further using the Arabidopsis mesophyll protoplast transfection system. Our reverse transcription–PCR (RT–PCR) analysis indicated that endogenous *AGG1* and *AGG2* were present in Arabidopsis mesophyll protoplasts (Fig. 5B). In our assays, effector plasmids containing AGB1 fused in-frame with the Gal4 DNA-binding domain (GD) together with RACK1A fused in-frame with the VP16 transactivator were co-transfected with the Gal4::GUS (β -glucuronidase) reporter into Arabidopsis rosette leaf mesophyll protoplasts. We found that while *AGG1* or *AGG2* interact with AGB1 in these assays, RACK1A did not interact with AGB1 in the absence or the presence of exogenous ABA (Fig. 5B).

Finally, we used an *in vivo* co-immunoprecipitation (Co-IP) assay to test the interaction between RACK1A and AGB1 by transiently co-expressing Myc-RACK1A and AGB1-HA (hemagglutinin) tag fusion proteins in Arabidopsis mesophyll protoplasts. Although both tag-fused proteins were expressed well in Arabidopsis protoplasts, again we failed to detect an interaction between AGB1 and RACK1A (Fig. 5C).

The regulation of RACK1 expression by G-proteins

Because rice RACK1 protein was found to be one of the seven proteins whose expression was down-regulated in the *d1* mutant (Komatsu et al. 2005), a loss-of-function allele of

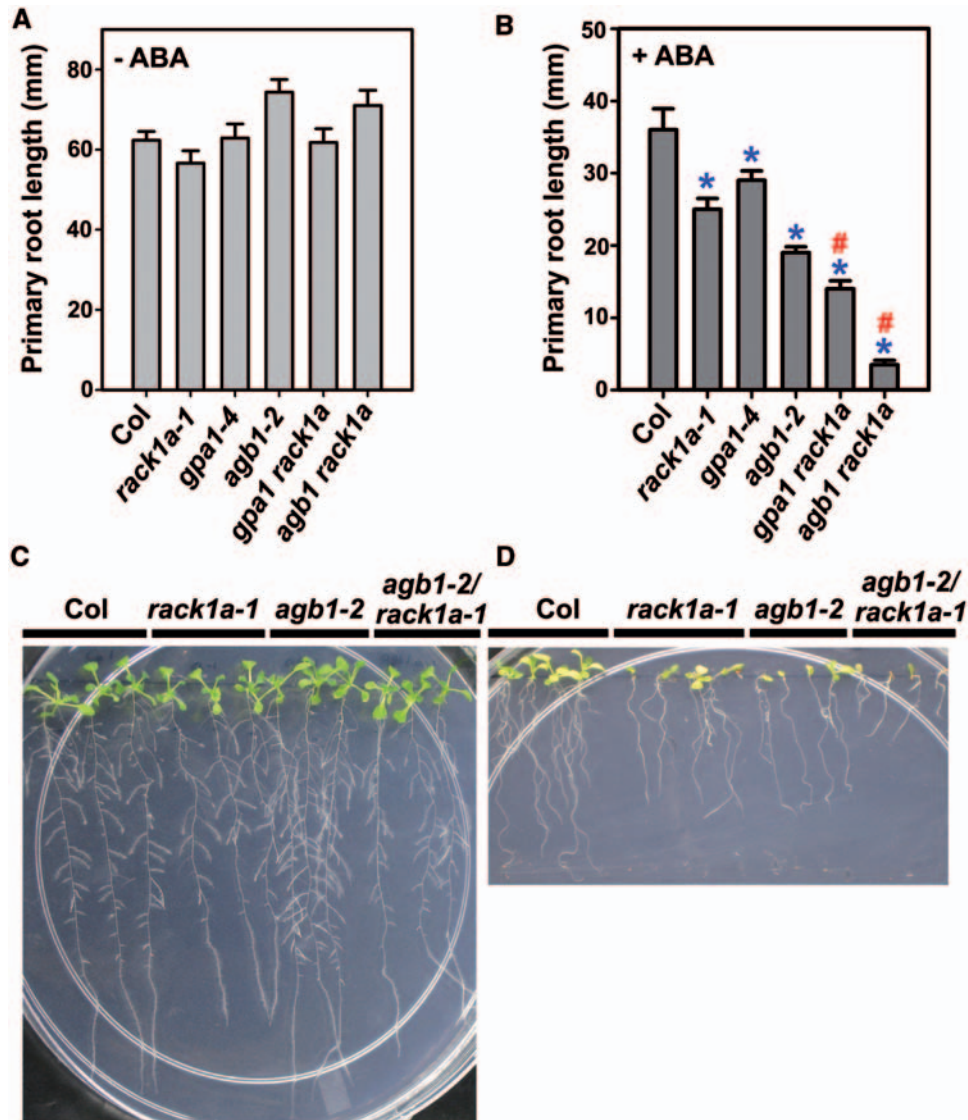


Fig. 3 ABA hypersensitivity of *agb1 rack1a* and *gpa1 rack1a* double mutants in the ABA inhibition of root growth assay. (A) The length of the primary root of seedlings grown on MS/G plates without ABA for 14 d. (B) The length of the primary root of seedlings grown on MS/G plates with 1.0 μ M ABA for 14 d. (C) Seedlings grown for 10 d on MS/G plates without ABA. (D) Seedlings grown for 14 d on MS/G plates with 1.0 μ M ABA. Shown in (A) and (B) are the means \pm SE from at least 10 seedlings. * P < 0.05, significantly different from Col; # P < 0.05, significantly different from the corresponding single mutants.

the sole $G\alpha$ subunit in rice (Fujisawa et al. 1999, Ueguchi-Tanaka et al. 2000), it was necessary to test if such regulation is conserved in Arabidopsis. First, we examined the transcript of *RACK1A* in the young seedlings of G-protein subunit mutants. As shown in **Fig. 6**, no apparent difference in the *RACK1A* transcript level was observed between the wild type and *gpa1-4* and *agb1-2* single and double mutants. Subsequently, we examined the *RACK1A* protein level in *gpa1* mutants. Again, no apparent difference in *RACK1A* protein level was detected between the wild type and two

independent loss-of-function alleles of *GPA1* (**Fig. 6B**). We extended our examination to other G-protein subunit and signaling mutants including the *agb1-2* single mutant, the *gpa1-4 agb1-2* double mutant and two independent loss-of-function alleles of *Regulator of G-protein Signaling* (*RGS*), *rgs1-1* and *rgs1-2* (Chen et al. 2003). Similarly, no apparent reduction of *RACK1A* protein was detected in any of these mutants (**Fig. 6C**). To test if *RACK1A* may regulate the transcription of *GPA1* and *AGB1*, we examined the transcripts of *GPA1* and *AGB1* in *rack1a* mutants by RT-PCR. We found

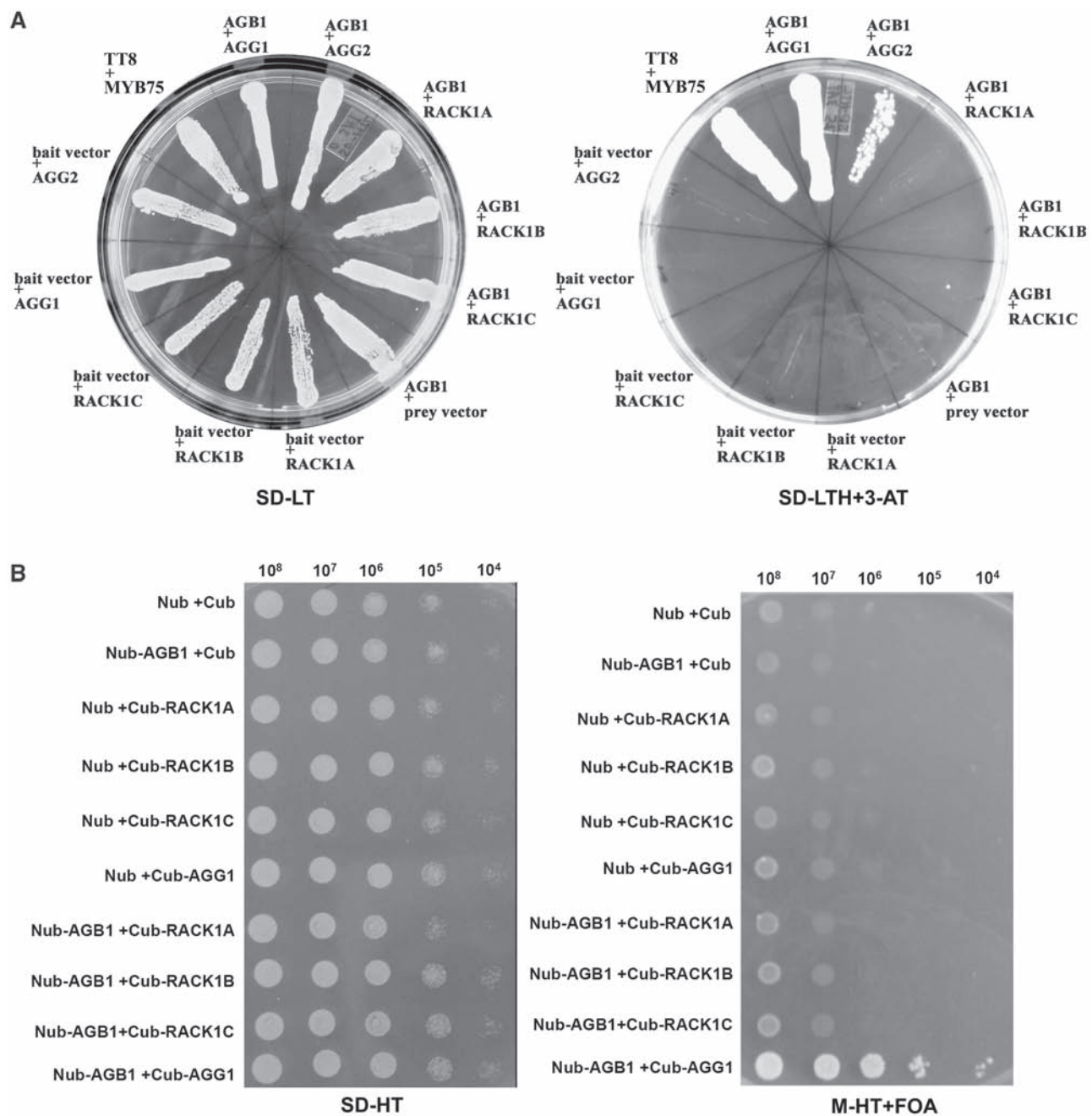


Fig. 4 Test of the physical interaction between RACK1 and AGB1 using the yeast two-hybrid assay. (A) Conventional yeast two-hybrid assay. AGB1 was cloned into the bait vector, and individual RACK1 genes (*RACK1A*, *RACK1B*, or *RACK1C*) were cloned into the prey vector. The interactions between AGB1 and AGG1 or AGG2 and the interaction between TT8 and MYB75 were used as positive controls. The interaction between AGB1 and empty prey vector, and the interaction between empty bait vector and RACK1 were used as negative controls. The ability of yeast cells to grow on double-selective plates (SD-LT, lacking leucine and tryptophan) is indicative of the presence of both prey and bait constructs. Positive interactions are indicated by the growth of yeast cells on the triple-selective plates (lacking leucine, tryptophan and histidine) supplemented with 25 mM 3-AT (SD-LTH+3-AT). (B) Split-ubiquitin yeast two-hybrid assay. AGB1 and RACK1 genes were fused with the N-half (Nub) and the C-half (Cub) of the *ubiquitin* gene, respectively. The known interaction between AGB1 and AGG1 was used as a positive control and the interaction between AGB1 and empty prey vector, and that between empty bait vector and RACK1 were used as negative controls. The ability of yeast cells to grow on double-selective plates (SD-HT, lacking histidine and tryptophan) is indicative of the presence of both prey and bait constructs. Positive interactions are indicated by the growth of yeast cells on the minimum yeast medium lacking histidine and tryptophan but supplemented with 0.1% (w/v) FOA (M-HT+FOA). The number on the top indicates the number of yeast cells used.

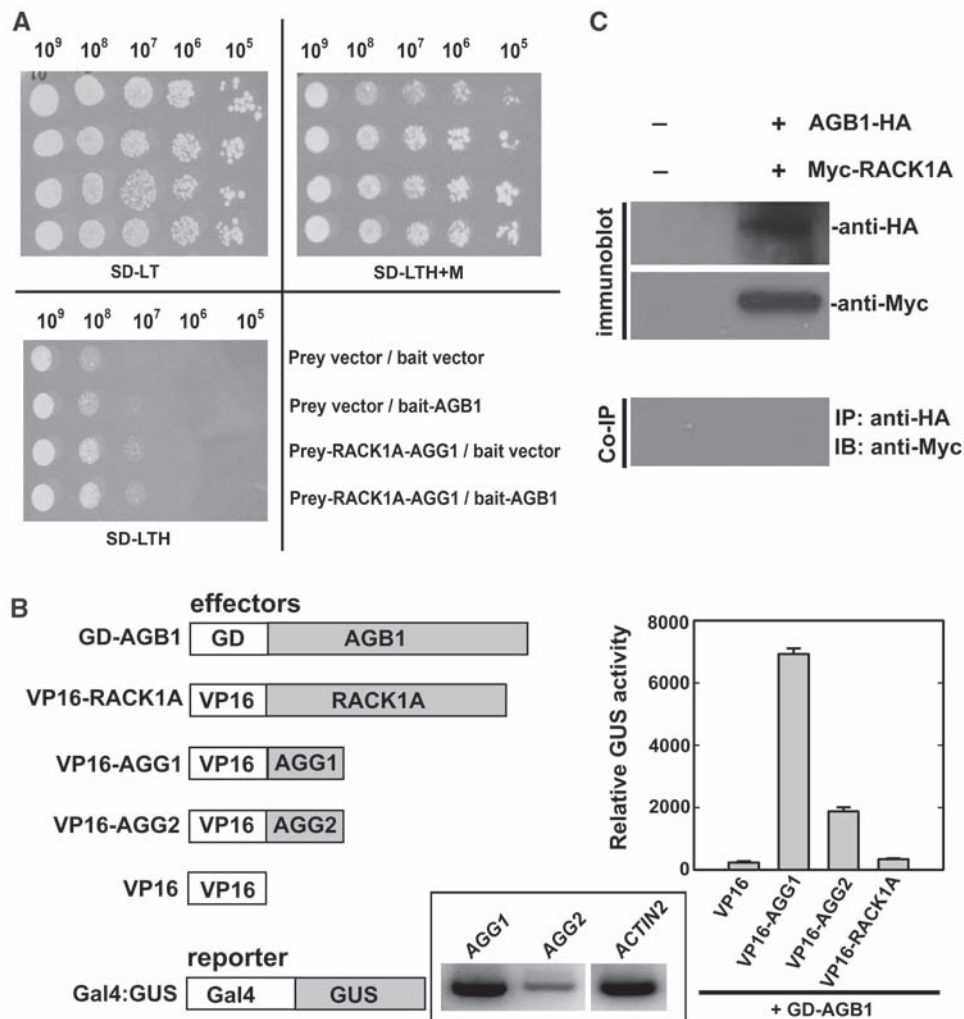


Fig. 5 Test of the interaction between RACK1A and AGB1 using the yeast three-hybrid assay, plant two-hybrid assay and the Co-IP assay. (A) Yeast three-hybrid assay. RACK1A and AGG1 were cloned into the pBridge vector in which RACK1A was placed in the N-terminus of the GAL4 BD domain and the expression of AGG1 was driven by a methionine-repressive promoter. AGB1 was cloned into the pACTGW-attR vector and fused with the AD domain. The ability of yeast cells to grow on double-selective plates (SD-LT, lacking leucine and tryptophan) is indicative of the presence of both prey and bait constructs. Positive interactions are indicated by the growth of yeast cells on the triple-selective yeast medium supplemented with methionine (SD-LTH+M, lacking leucine, tryptophan and histidine) or without methionine (SD-LTH). On SD-LTH+M plates, methionine was used at 1 mM to repress the expression of the AGG1 gene. On SD-LTH plates, the expression of the AGG1 gene was activated by methionine starvation. The number on the top indicates the number of yeast cells used. (B) Plant protein-protein two-hybrid assay. Shown on the left are effector and reporter constructs used in the transfection assays. Effector genes and reporter genes were co-transfected into protoplasts derived from Arabidopsis rosette leaves. Shown on the right are averages of the relative GUS activities of three replicates \pm SE in the absence of exogenous ABA. Similar results were obtained in the presence of 5 μ M exogenous ABA. Inset, RT-PCR analysis of AGG1 and AGG2 transcripts in Arabidopsis mesophyll protoplasts. The expression of ACTIN2 was used as a control. RT-PCR was performed for 35 cycles. (C) Co-IP assay. Constructs of 35S:AGB1-HA and 35S:Myc-RACK1A were co-transfected into Arabidopsis protoplasts. The expression of tag-fused proteins was examined by anti-HA polyclonal antibodies and anti-Myc monoclonal antibodies, respectively. In the Co-IP assay, anti-HA antibody was used for immunoprecipitation (IP), and anti-Myc antibody was used for immunoblotting (IB).

no apparent alteration in the level of *GPA1* and *AGB1* transcripts in two independent alleles of *rack1a* mutants, compared with that in the wild type (Fig. 6D).

Next, we wanted to examine specifically the transcript and protein levels of RACK1A in the mature seeds (e.g. with

mature embryos) of *gpa1* mutants because Komatsu et al. (2005) reported a significant reduction of the RACK1 protein level in rice embryos of the *G α* mutant. Only the transcripts of RACK1A and RACK1B, but not RACK1C, were detected in the mature seeds under our experimental

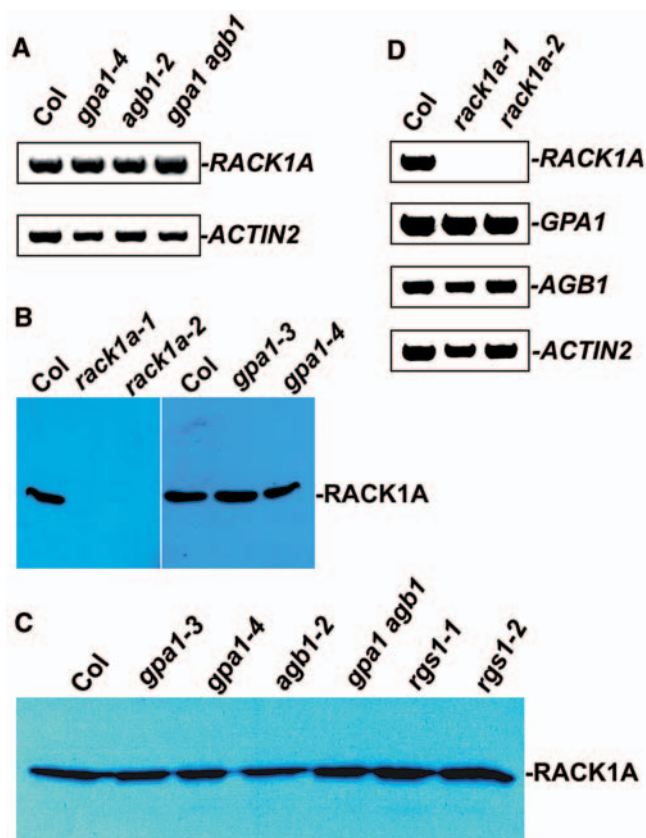


Fig. 6 Expression analysis of RACK1A in the young seedlings of G-protein mutants. (A) RT-PCR analysis of RACK1A transcript in *gpa1* and *agb1* mutants. (B) Western blot analysis of RACK1A protein in *gpa1* mutants. (C) Western blot analysis of RACK1A protein in *agb1* and *rgs1* mutants. (D) RT-PCR analysis of GPA1 and AGB1 transcripts in *rack1a* mutants. In (A) and (D), the expression of ACTIN2 was used as a control. RT-PCR was performed for 35 cycles.

conditions, and we found that the transcript levels of RACK1A and RACK1B were significantly reduced in *gpa1-4* mutants, compared with the wild type (Fig. 7A). While RACK1A protein could be readily detected in the wild-type seeds, we found that RACK1A protein was at an undetectable level in the seeds of *gpa1-4* mutants (Fig. 7B). Taken together, these results suggested that RACK1A expression is probably regulated by G-proteins in a tissue- or organ-specific manner and may be developmental stage dependent.

Discussion

Our genetic and biochemical analyses suggested that there may be some fundamental differences in the relationship of RACK1 and G-proteins between mammals and Arabidopsis. This view is directly or indirectly supported by the following four lines of evidence. (i) Loss-of-function alleles of RACK1A were morphologically different from those of *gpa1* and *agb1*

mutants (Fig. 1). (ii) Combined effects on morphological and developmental traits were observed in *gpa1 rack1a* and *agb1 rack1a* double mutants, compared with parental single mutants (Fig. 1). (iii) A direct physical interaction between RACK1 and AGB1 was not observed in yeast two-hybrid (conventional system or split-ubiquitin system) or yeast three-hybrid assays (between RACK1A and AGB1-AGG1) (Figs. 4, 5). (iv) A direct physical interaction between RACK1A and AGB1 was not observed in the plant two-hybrid assay or in vivo Co-IP assay (Fig. 5).

Although we have attempted several different assays to test the physical interaction between RACK1A and AGB1, we are aware that our investigation is not exhaustive. The lack of direct physical interaction between RACK1A and AGB1 in our assays does not exclude the possibility that these proteins may interact genetically or physiologically in a specific manner, such as under biotic or abiotic stress conditions. We observed an enhanced ABA hypersensitivity in *agb1 rack1a* and *gpa1 rack1a* double mutants, compared with single mutants (Figs. 2, 3). However, this genetic analysis does not allow us to draw a firm conclusion regarding the precise relationship between RACK1A and G-proteins in ABA signaling. In rice, RACK1A interacts with Rac1 (Nakashima et al. 2008) which acts downstream of $G\alpha$ in innate immunity (Suharsono et al. 2002). Therefore, in rice innate immunity, RACK1A may interact genetically with G-proteins via Rac1, although a direct, physical interaction between G-proteins and RACK1A or Rac1 has not been established. It remains unknown whether Arabidopsis RACK1 proteins have a role in innate immunity.

It is worth noting that the regulation of RACK1 expression by $G\alpha$ may occur in a tissue- or organ-specific manner or may be developmental stage dependent in Arabidopsis. Komatsu et al. (2005) found that the protein level of RACK1 was down-regulated in the rice embryos of the $G\alpha$ mutant. Initially, we examined the transcript and protein level of RACK1A in the young seedlings of Arabidopsis $G\alpha$ and other G-protein signaling mutants. However, we failed to detect any apparent differences (Fig. 6). Interestingly, when we specifically examined the transcript and protein levels of RACK1A in the mature seeds of $G\alpha$ mutants (*gpa1-4*), we found that the transcript and protein levels of RACK1A were dramatically reduced in *gpa1-4* (Fig. 7). Research on plant G-proteins has already revealed that G-proteins function in a cell type- or developmental stage-specific manner (reviewed by Perfus-Barbeoch et al. 2004, Ding et al. 2008). Here we showed that the regulation of RACK1 expression by G-proteins is also likely to be tissue or organ specific or developmental stage dependent.

In yeast, RACK1/Asc1 functions as a $G\beta$ and interacts with one of the two $G\alpha$ subunits, acting as a negative regulator of G-protein signaling in the glucose response (Zeller et al. 2007). However, Arabidopsis RACK1A did not physically

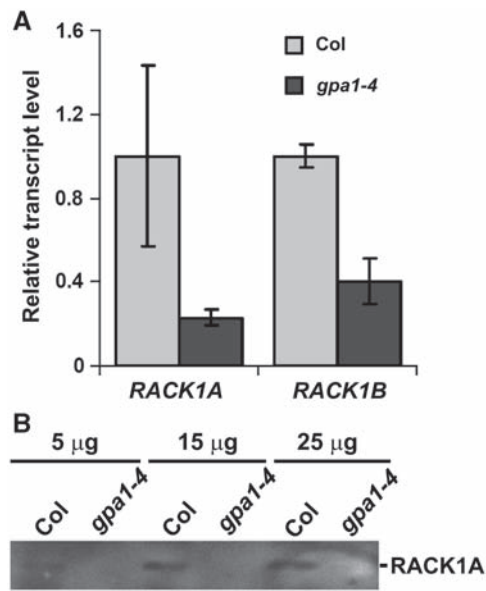


Fig. 7 Expression analysis of RACK1A in the mature seeds of *gpa1* mutants. (A) The transcript levels of RACK1A and RACK1B in the wild type (Col) and *gpa1-4* mutants analyzed by quantitative RT-PCR. The expression of *ACTIN2* was used as a control. All transcript levels are normalized against Col, with the value of the first biological replicate set as 1. Shown are the mean values of three biological replicates \pm SE. (B) The protein level of RACK1A in Col and *gpa1-4* mutants analyzed by Western blot. The experiment was repeated twice and similar results were obtained. Shown at the top is the amount of total proteins loaded in each well.

interact with the sole $G\alpha$, GPA1 (Chen et al. 2006). Here we provide genetic evidence that *rack1a* and *gpa1* mutants are morphologically different, and a combined effect on morphological and developmental traits was observed in *gpa1 rack1a* double mutants (Fig. 1). Further, *rack1a-1* and *gpa1-4* did not appear to be epistatic to each other in the ABA-mediated inhibition of early seedling development (Figs. 2, 3). Therefore, it appears that there may also be some fundamental differences in the relationship of RACK1 and G-proteins between Arabidopsis and yeast.

In summary, loss-of-function alleles of RACK1A and G-protein α and β subunits have distinct morphological and developmental traits, and a combined effect on these traits was observed in *gpa1 rack1a* and *agb1 rack1a* double mutants. Both RACK1A and G-proteins negatively regulate ABA responses in the ABA inhibition of early seedling development, but an additive effect on ABA hypersensitivity was observed in *gpa1 rack1a* and *agb1 rack1a* double mutants. Biochemical analysis indicated that RACK1 may not physically interact with G-proteins. Taken together, these findings revealed some fundamental differences in the relationship of RACK1 and G-proteins between Arabidopsis and mammals, and between Arabidopsis and yeast.

Materials and Methods

Plant materials and growth conditions

All mutants are in the Arabidopsis Col-0 ecotype background. The *rack1a-1* and *rack1a-2* mutants were reported by Chen et al. (2006a). The *gpa1-3* and *gpa1-4* mutants were reported by Jones et al. (2003). The *agb1-2* mutants were reported by Ullah et al. (2003). The *rgs1-1* and *rgs1-2* mutants were reported by Chen et al. (2003). The *gpa1-4 agb1-2* double mutants were reported by Chen et al. (2004).

Plants were grown in 5 \times 5 cm pots containing a moistened 1:3 mixture of Sunshine Mix #1 (Sun Gro Horticulture Canada Ltd., Seba Beach, Alberta, Canada) and Metro-Mix 220 (W.R. Grace & Co. of Canada, Ontario, Canada) with a 14/10 h or 8/16 h photoperiod at approximately 120 $\mu\text{mol m}^{-2}\text{s}^{-1}$ at 23°C.

Generation of *rack1a-1*, *gpa1-4* and *agb1-2* double mutants

Double mutants between *rack1a-1* and *gpa1-4* or *agb1-2* were generated by crossing *gpa1-4* or *agb1-2* into the *rack1a-1* single mutant, and isolated in the F_2 progeny by PCR genotyping. For simplicity, the *gpa1 rack1a*, *agb1 rack1a*, and *gpa1 agb1* double mutant nomenclatures in this report refer specifically to the *gpa1-4 rack1a-1*, *agb1-2 rack1a-1* and *gpa1-4 agb1-2* mutants, respectively.

ABA inhibition of seed germination and early seedling development assays

Seeds from matched lots were surface sterilized, sown on MS/G plates (Chen et al. 2006) that are supplemented with different concentrations of ABA, and stratified at 4°C for 2 d in the dark before they were transferred to germination conditions (23°C, with a 14/10 h photoperiod, 120 $\mu\text{mol m}^{-2}\text{s}^{-1}$). Germination was defined as an obvious protrusion of the radicle through the seed coat. The examination of the percentage of green seedlings was based on the presence of obvious green cotyledons.

For the root growth assay, seeds were germinated on MS/G plates covered with one layer of sterilized filter paper. After 24–60 h, the germinated seeds were transferred to MS/G plates supplemented with ABA, and the plates were placed vertically to monitor root growth. After 5–14 d, the length of the primary root was measured from each genotype.

RT-PCR and quantitative RT-PCR

Total RNA was isolated from the mature seeds, young seedlings or rosette leaf mesophyll protoplasts using the TRIzol reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada). cDNA was synthesized from 1 μg of total RNA by oligo(dT)₂₀-primed reverse transcription, using

THERMOSCRIPT RT (Invitrogen). *RACK1A*-specific primers (5'-GGCATCTCCAGACACCGAAA-3' and 5'-GCAGAGAGC AACGACAGC-3'), *GPA1*-specific primers (5'-ATGGGCTT ACTCTGCAGTA-3' and 5'-TCATAAAAGGCCAGCCTCCA GT-3') and *AGB1*-specific primers (5'-CTGCTGATGTACT AAGCGTCTCA-3' and 5'-CTGCATGTTCCATCGTCTGA-3') were used to amplify the transcripts of these genes. The expression of *ACTIN2* (amplified by primers 5'-CCAGAA GGATGCATATGTTGGTGA-3' and 5'-GAGGAGCCTCGGT AAGAAGA-3') was used as a control in PCRs. Quantitative RT-PCR analysis was performed as described by Guo and Chen (2008).

Western blot

The *RACK1A* protein level was examined by using immunoblot analysis. Briefly, 10-day-old, light-grown seedlings or mature seeds of the wild type (Col) and mutant were ground into powder with liquid nitrogen. Total protein was isolated by incubating the samples with 100 μ l of freshly made lysis buffer [50 mM Tris, 50 mM NaCl, 5 mM EGTA, 2 mM dithiothreitol (DTT), 1% Triton X-100 and 1 \times protease inhibitor cocktail (Sigma), pH 7.5] at 4°C for 30 min, followed by rocking at 4°C for another 30 min. Total proteins in the supernatant were collected by centrifuging at 14,000 r.p.m. for 15 min at 4°C. Protein samples were separated by SDS-PAGE, electroblotted onto a polyvinylidene fluoride (PVDF) membrane, and immunoblotted with 1:1,000 anti-*RACK1A* peptide antibodies (Chang et al. 2005). Goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Sigma) was used as secondary antibody. The blot was developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc., Rockford, IL, USA).

Yeast two-hybrid assay

The interaction between *AGB1* and *RACK1* was tested by using the ProQuest yeast two-hybrid system (Invitrogen). *AGB1* was cloned into the bait vector (pDEST32) and *RACK1* (*RACK1A*, *RACK1B*, or *RACK1C*) was cloned into the prey vector (pDEST22). The known interaction between *AGB1* and *AGG1* or *AGG2* (Mason and Botella 2000, Mason and Botella 2001) and the known interaction between *TT8* and *MYB75* (Zimmermann et al. 2004) were used as positive controls. The interaction between *AGB1* and the empty prey vector, and the interaction between the empty bait vector and *RACK1* were used as negative controls. The ability of yeast transformants to grow on minimal SD medium lacking both leucine and tryptophan (SD-LT) is indicative of the presence of both prey and bait constructs. Positive interactions are indicated by the growth of yeast cells on the triple-selective minimal SD medium lacking leucine, tryptophan and histidine but supplemented with 25 mM 3-AT (3-amino-1,2,4-triazole) (SD-LTH+3-AT).

In the second assay, the split-ubiquitin yeast two-hybrid system developed by Dr. Imre Somssich (Max Planck Institute for Plant Breeding Research, Cologne, Germany) was used. *AGB1* and *RACK1* genes were cloned into the Gateway entry vector using the pCR[®]8/GW/TOPO cloning kit (Invitrogen) and then subcloned into yeast expression vector Nul (with an N-terminal fusion of the N-half of the *ubiquitin* gene) and pMKZ (with an N-terminal fusion of the C-half of the *ubiquitin* gene), respectively. The known interaction between *AGB1* and *AGG1* was used as a positive control. The interaction between *AGB1* and the empty prey vector, and the interaction between the empty bait vector and *RACK1* were used as negative controls. The growth of yeast transformants on the plates lacking both histidine and tryptophan (SD-HT) indicates the presence of both bait vector and prey vector. Positive interactions are indicated by the growth of yeast cells on the minimum yeast medium lacking HT but supplemented with 0.1% (w/v) FOA (fluoro-otic acid) (M-HT+FOA).

Yeast three-hybrid assay

RACK1A and *AGG1* were cloned into the pBridge vector (Clontech, Mountain View, CA, USA) using restriction enzyme digestion and ligation methods. *RACK1A* was cloned to the N-terminus of the Gal4 BD domain and the expression of *AGG1* was driven by a methionine-repressive promoter, *P_{met25}*. *AGB1* was first cloned into the pCR[®]8/GW/TOPO cloning kit (Invitrogen) and then subcloned into the pACTGW-attR vector (Nakayama et al. 2002) fused with the AD domain. The growth of yeast transformants on double-selective medium SD-LT (lacking leucine and tryptophan) indicates the presence of both vectors. Positive interactions are indicated by the growth of yeast cells on the triple-selective yeast medium supplemented with methionine (SD-LTH+M, lacking leucine, tryptophan and histidine) or without methionine (SD-LTH). On SD-LTH+M medium, methionine was used at 1 mM to repress the expression of the *AGG1* gene. On SD-LTH medium, the expression of the *AGG1* gene is activated by methionine starvation. Vectors were co-transformed into the HF7c host strain (courtesy of Dr. Crisanto Gutierrez, Centro de Biología Molecular 'Severo Ochoa', Universidad Autónoma de Madrid, Madrid, Spain).

Plant two-hybrid protein-protein interaction assay

The test of interaction between *AGB1* and *RACK1A* in plant cells was conducted by using the two-hybrid protein-protein interaction assay (Ehlert et al. 2006). The procedures of *Arabidopsis* protoplast isolation, transfection and GUS activity assays were described previously (Wang et al. 2007). Plasmid DNAs for reporter and effector genes were isolated using Endofree Plasmid Maxi Kits (Qiagen, Mississauga, Ontario, Canada). A 10 μ g aliquot of each effector plasmid or reporter plasmid was used in co-transfection assays.

Arabidopsis mesophyll protoplasts were co-transfected with AGB1 fused with Gal4 DNA-binding domain (GD-AGB1) and RACK1A fused with VP16 transactivator (VP16-RACK1A), together with *Gal4:GUS* reporter in the absence or presence of 5 μ M exogenous ABA. As a positive control, GD-AGB1 was co-transformed with VP16-AGG1 or VP16-AGG2. Each transfection assay was performed in triplicate and each experiment was repeated at least twice. GUS activities were measured by using a Fluoroskan Finstruments Microplate Reader (MTX Lab Systems Inc., Vienna, VA, USA).

Co-immunoprecipitation (Co-IP)

The full-length open reading frames of RACK1A and AGB1 were cloned in-frame with an N-terminal Myc or C-terminal HA tag, respectively, into the pUC19 vector under the control of the double 35S enhancer promoter of cauliflower mosaic virus followed by the translational enhancer from the 5' leader of tobacco mosaic virus, and terminated by a 3'-untranslated region derived from the nopaline synthetase gene (Tiwari et al. 2003). Protoplasts were co-transfected with plasmid DNAs containing Myc-RACK1A and AGB1-HA. The expression of tag-fused proteins in transfected protoplasts was examined by immunoblotting using anti-Myc monoclonal antibody (Sigma) or anti-HA polyclonal antibodies (Abgent, San Diego, CA, USA). In the Co-IP assay, protein extracts prepared from the Myc-RACK1A and AGB1-HA co-transfected protoplasts were immunoprecipitated with anti-HA polyclonal antibodies, separated by SDS-PAGE, electroblotted onto a PVDF membrane and immunoblotted with anti-Myc monoclonal antibody.

Supplementary data

Supplementary data are available at PCP online.

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