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RESEARCH PAPER

RACK1 is a negative regulator of ABA responses in *Arabidopsis*

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Abstract

Receptor for Activated C Kinase 1 (RACK1) is viewed as a versatile scaffold protein in mammals. The protein sequence of RACK1 is highly conserved in eukaryotes. However, the function of RACK1 in plants remains poorly understood. Accumulating evidence suggested that RACK1 may be involved in hormone responses, but the precise role of RACK1 in any hormone signalling pathway remains elusive. Molecular and genetic evidence that *Arabidopsis* RACK1 is a negative regulator of ABA responses is provided here. It is shown that three *RACK1* genes act redundantly to regulate ABA responses in seed germination, cotyledon greening and root growth, because *rack1a* single and double mutants are hypersensitive to ABA in each of these processes. On the other hand, plants overexpressing *RACK1A* displayed ABA insensitivity. Consistent with their proposed roles in seed germination and early seedling development, all three *RACK1* genes were expressed in imbibed, germinating and germinated seeds. It was found that the ABA-responsive marker genes, *RD29B* and *RAB18*, were up-regulated in *rack1a* mutants. Furthermore, the expression of all three *RACK1* genes themselves was down-regulated by ABA. Consistent with the view that RACK1 negatively regulates ABA responses, *rack1a* mutants lose water significantly more slowly from the rosettes and are hypersensitive to high concentrations of NaCl during seed germination. In addition, the expression of some putative RACK1-interacting, ABA-, or abiotic stress-regulated genes was mis-regulated in *rack1a rack1b* double mutants in response to ABA. Taken together, these findings provide compelling evidence that RACK1 is a critical, negative regulator of ABA responses.

Key words: ABA, drought, early seedling development, RACK1, salt, seed germination.

Introduction

Receptor for Activated C Kinase 1 (RACK1) was originally identified as a receptor for activated protein kinase C (PKC) in mammalian cells (Mochly-Rosen *et al.*, 1991; Ron *et al.*, 1994), but now it is viewed as a multi-functional protein that plays regulatory roles in diverse signal transduction pathways (reviewed by McCahill *et al.*, 2002; Sklan *et al.*, 2006). The protein sequences and the structure of RACK1 are highly conserved in plants (Chen *et al.*, 2006; Guo *et al.*, 2007; Ullah *et al.*, 2008). However, the research on plant RACK1 lags behind its counterparts in mammals and yeasts, and the function of plant RACK1 remains poorly understood.

The first plant RACK1 was discovered as a G-protein β subunit-like protein in tobacco BY-2 cells (Ishida *et al.*, 1993). Subsequently, the *RACK1* gene was cloned from rice (Iwasaki *et al.*, 1995), alfalfa (McKhann *et al.*, 1997), rape (Kwak *et al.*, 1997), and *Arabidopsis* (Vahlkamp and Palme, 1997). The amino acid sequence homologues of RACK1 was found in all the plant species examined (Chen *et al.*, 2006; Guo *et al.*, 2007). In earlier studies, the characterization of plant RACK1 was mainly concentrated on gene expression and induction studies (Ishida *et al.*, 1993; McKhann *et al.*, 1997; Perennes *et al.*, 1999). Recent genetic studies using the model plant *Arabidopsis* revealed that

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RACK1 may have multiple functions in plants (Chen *et al.*, 2006; Guo and Chen, 2008). Like their counterparts in mammals and yeasts, plant RACK1 proteins are also associated with ribosomes (Chang *et al.*, 2005; Giavalisco *et al.*, 2005). Nakashima *et al.* (2008) demonstrated that RACK1 proteins are key regulators of innate immunity in rice. Furthermore, rice RACK1 physically interacts with multiple proteins in the Rac1 immune complex (Nakashima *et al.*, 2008), providing evidence that the scaffolding feature of RACK1 protein may be conserved in plants.

Accumulating evidence suggested that plant RACK1 may have a role in hormone responses. For example, the first plant *RACK1* gene was discovered as an auxin-induced gene in tobacco BY-2 cells (Ishida *et al.*, 1993). In these tobacco cells, salicylic acid (SA) can block the UV irradiation-induced *RACK1* expression (Perennes *et al.*, 1999). Interestingly, in alfalfa, *RACK1* was induced by cytokinin, but not by auxin (McKhann *et al.*, 1997). In rice, ABA can induce the protein expression of RACK1 in imbibed seeds (Komatsu *et al.*, 2005), whereas in rice cell cultures, the expression of *RACK1* can also be induced by methyl jasmonate, auxin, and ABA (Nakashima *et al.*, 2008). In *Arabidopsis*, loss-of-function mutations in *RACK1A* conferred altered sensitivities to auxin, ABA, gibberellin, and brassinolide (Chen *et al.*, 2006). Despite these findings, the exact role of RACK1 in any hormone response has not been well characterized. *Arabidopsis* RACK1 is defined here as a negative regulator of ABA responses.

Materials and methods

Plant materials and growth conditions

All mutants are in the Arabidopsis Columbia (Col-0) ecotype background. The *rack1a-1* and *rack1a-2* single mutants have been reported previously (Chen *et al.*, 2006). The *rack1b-1*, *rack1b-2*, *rack1c-1*, and *rack1c-2* single mutants, as well as *rack1a-1 rack1b-2*, *rack1a-1 rack1c-1*, and *rack1b-2 rackc-1* double mutants have been reported by Guo and Chen (2008).

For simplicity, the *rack1a rack1b, rack1a rack1c*, and *rack1b rack1c* double mutant nomenclatures in this report refer specifically to the *rack1a-1 rack1b-2*, *rack1a-1 rack1c-1* and *rack1b-2 rack1c-1* mutants, respectively. Unless specified elsewhere, wild-type and mutant plants were grown under identical conditions with 14/10 h photoperiod at approximately 120 μmol m^{-2} s^{-1} at 23 °C.

Generation of RACK1A *over-expression lines*

The whole open reading frame of *RACK1A* (At1g18080) was amplified by PCR from a cDNA library made from seedlings grown in light for 10 d and cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA) and then subcloned into Gateway plant transformation destination vectors pB2GW7 (Karimi *et al.*, 2002) by an LR recombination reaction. In this construct, the expression of *RACK1A* was driven by the 35S promoter of cauliflower mosaic virus. The *35S::RACK1A* binary vector was transformed into Col-0 by *Agrobacterium*-mediated transformation (Clough and Bent, 1998).

The RACK1A protein level in *35S::RACK1A* transgenic lines was examined by Western blot analysis using anti-RACK1A peptide antibodies (Chang *et al.*, 2005). Goat-anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) was used as a secondary antibody. The blot was developed using the SuperSignal West Pico Chemiluminescent Substrate (PIERCE Biotechnology Inc., Rockford, Illinois).

Generation of P_{RACK1}::GUS *reporter lines*

Previously, the genomic DNA 2740 bp upstream of the *RACK1A* start codon, 2215 bp upstream of the *RACK1B* start codon, and 1091 bp upstream of the *RACK1C* start codon were selected as putative promoter sequences for *RACK1A*, *RACK1B*, and *RACK1C*, respectively (Chen *et al.*, 2006). In this study, shorter genomic DNA sequences were selected, the regions between each *RACK1* gene and its nearest upstream gene. Specifically, the genomic DNA 1491 bp upstream of the *RACK1A* start codon, 682 bp upstream of the *RACK1B* start codon, and 371 bp upstream of the *RACK1C* start codon was amplified by PCR, respectively, and cloned into the PZP211 binary vector (Hajdukiewicz *et al.*, 1994) upstream of the GUS gene that was ligated into the vector earlier. The binary vectors containing P_{RACK1}::*GUS* constructs were transformed into Col-0 by *Agrobacterium*-mediated transformation (Clough and Bent, 1998). GUS staining revealed no significant difference in expression patterns between this new set of promoter::GUS reporter lines and the lines generated earlier. These short genomic DNA sequences were also used for generating the GFP/CFP/YFP reporter lines described below. Furthermore, expression of RACK1-GFP/CFP/YFP fusion proteins under the control of these short genomic DNA sequences complemented *rack1* mutants. Taken together, these results suggested that these short genomic DNA sequences probably contain most *cis*-acting regulatory elements that are required for the proper expression of *RACK1* genes.

Generation of P_{RACK1}::RACK1-GFP/CFP/YFP *reporter lines*

The genomic DNA starting from the beginning of the promoter region (the same regions as used for the P_{RACK1}::*GUS* constructs) prior to the stop codon of each *RACK1* gene was amplified by PCR and cloned into the Gateway entry vector using the pCR®8/GW/TOPO cloning kit (Invitrogen Inc.). The cloned fragments were then transferred into the Gateway compatible binary vectors pGWB4, pGWB43, and pGWB40 (Nakagawa *et al.*, 2007) by LR recombination reactions for constructing P_{RACK1A}::*RACK1A-GFP*, P_{RACK1B}::*RACK1B-CFP*, and P_{RACK1C}::*RACK1C-YFP*, respectively. These vectors allow the fusion

of the fluorescent proteins to the C-terminal of RACK1 proteins. The binary vectors containing P_{RACK1}::*RACK1-GFP/CFP/YFP* constructs were transformed into Col-0, *rack1a* single mutant, and *rack1a rack1b* or *rack1a rack1c* double mutants by *Agrobacterium*-mediated transformation (Clough and Bent, 1998).

ABA inhibition of seed germination and cotyledon greening assays

Wild-type and mutant seeds from matched lots were surface-sterilized and sown on MS/G plates consisting of half-strength Murashige and Skoog (MS) basal medium supplemented with vitamins (Plantmedia, Dublin, Ohio), 1% (w/v) sucrose, 0.6% (w/v) phytoagar (Plantmedia), pH adjusted to 5.7 with 1 N KOH, and supplemented with different concentrations of ABA. Imbibed seeds were cold-treated at 4 °C in dark for 2 d, and then moved to 23 °C, with 14/10 h photoperiod (120 μmol m^{-2} s^{-1}). Germination is defined as an obvious protrusion of the radicle through the seed coat. Green seedling is defined as the presence of two obviously green cotyledons.

ABA inhibition of root growth assay

For the root growth assay, sterilized seeds were sown on MS/G plates and cold-treated at 4 °C in the dark for 2 d. The plates were then moved to germination conditions (23 °C, 14/10 h photoperiod at 120 μmol m^{-2} s^{-1}) and placed vertically to allow the root to grow along the surface of the agar. Sixty hours later, the evenly-grown seedlings were transferred to MS/G plates supplemented with or without 5 μM ABA. The plates were placed under the same conditions with a vertical orientation for monitoring root growth. Seven days later, the length of the primary root was measured from each genotype.

Water loss assay

Water loss from the detached whole rosettes (with roots removed) of Col and *rack1a* single and double mutants was measured according to the method described by Tian *et al.* (2004) with minor modifications. Briefly, 20-d-old Col and *rack1a* mutants grown under short-day conditions (8/16 h photoperiod) were transferred from the growth chamber to the laboratory. The rosette from each plant was cut from its roots and weighed at different time points. The assay was performed at room temperature (~23 °C) under dim light conditions (6 μmol m^{-2} s^{-1}) with 35% relative humidity. Three plants of each genotype were used and the water loss was calculated as the percentage of initial fresh weight at each time point.

Salt stress germination assay

Sterilized wild-type and *rack1* mutant seeds were sown on MS/G plates supplemented with different concentrations of NaCl. Imbibed seeds were cold-treated at 4 °C in the dark for 2 d, and then moved to 23 °C, with a 14/10 h photoperiod (120 μmol m^{-2} s^{-1}). Germination is defined as an obvious protrusion of the radicle through the seed coat.

RT-PCR and quantitative real-time RT-PCR

For the analysis of transcripts of *RACK1* genes in imbibed, germinating, and germinated seeds, the procedure of sampling has been described by Gao *et al.* (2007). For extracting total RNA from imbibed, germinating, and germinated seeds, hot borate RNA extraction method (Wilkins and Smart, 1996) was used. cDNA was synthesized using 1 μg total RNA by Oligo(dT)-primed reverse transcription using OMNISCRIPT RT Kit (Qiagen, Mississauga, Ontario, Canada). Primers used for RT-PCR and quantitative RT-PCR are listed in Supplementary Table S1 at *JXB* online. The expression of *ACTIN2* was used as a control.

For the ABA induction assay, 4.5-d-old light-grown seedlings of the wild type, the *rack1a* single mutant, and the *rack1a rack1b* and *rack1a rack1c* double mutants were used. Seedlings grown vertically on MS/G plates were moved to MS/G liquid medium without phytoagar and grown for another 2 h prior to ABA induction in an orbital shaking incubator. ABA was added at 10 μM (for *RD29B* and *RAB18* induction) or 50 μM (for putative RACK1 interactors induction) for 2 h. Total RNA was isolated from ABA-treated and untreated whole seedlings using the RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized as described above. Gene-specific primers (see Supplementary Table S1 at *JXB* online) for the ABA-responsive marker genes, *RD29B* and *RAB18*, and putative RACK1 interacting, ABA- or abiotic stress-regulated genes, At1g54510 (*ATNEK1*), At2g35940 (*EDA29*), At3g24080, At4g23570 (*SGT1A*), At4g27560, At5g03730 (*CTR1*), At5g08415, and At5g08420 were used for quantitative RT-PCR analysis to compare the expression of these genes in the wild type and in *rack1a* mutants, with and without ABA treatment. The expression of *ACTIN2* was used to normalize the expression of each gene. The quantitative real-time PCR was performed using the MJ MiniOpticon real-time PCR system (Bio-Rad, http://www.biorad.com) and IQ SYBR Green Supermix (Bio-Rad).

Results

RACK1 *genes act redundantly to negatively regulate ABA responses during seed germination and early seedling development*

The *Arabidopsis* genome contains three *RACK1* genes, designated as *RACK1A*, *RACK1B*, and *RACK1C*, respectively, that encode three proteins with near 90% identity at the amino acid level (Chen *et al.*, 2006). Previously, it was shown that three Arabidopsis *RACK1* genes function in an unequally redundant manner to regulate rosette leaf production and root development (Guo and Chen, 2008). Because preliminary analysis using *rack1a* single mutants suggested that RACK1A also mediates the hormone

response (Chen *et al.*, 2006), it is likely that, similar to the situation in plant development, the three *Arabidopsis RACK1* genes may also function redundantly to regulate hormone responses, although this has not been tested experimentally. Therefore, in this study, the specific focus was on the characterization of the role of the *RACK1* genes in ABA responses.

Loss-of-function alleles of *RACK1A*, *rack1a-1*, and *rack1c-2*, are hypersensitive to ABA in seed germination and early seedling development (Chen *et al.*, 2006). Although loss-of-function alleles of *RACK1B* (*rack1b-1* and *rack1b-2*) and *RACK1C* (*rack1c-1* and *rack1c-2*) displayed wild-type morphology (Guo and Chen, 2008), it had remained unknown if *rack1b* and *rack1c* mutants have altered sensitivity to ABA. In order to address this question, seeds of two independent loss-of-function alleles of each *RACK1* gene were sown side-by-side on MS/G plates supplemented with different concentrations of ABA. Consistent with our previous findings (Chen *et al.*, 2006), *rack1a* mutants displayed ABA hypersensitivity in the seed germination assay (Fig. 1A, B). However, *rack1b* and *rack1c* mutants exhibited wild-type sensitivity to ABA (Fig. 1A, B). These results suggested that among the three *RACK1* genes, *RACK1A* is the prominent one that regulates the ABA response in seed germination.

Because it has previously been shown that loss-of-function mutations in *RACK1B* or *RACK1C* can enhance the developmental defects observed in *rack1a* mutants, it was necessary to examine if the ABA hypersensitivity of *rack1a* mutants in seed germination can also be enhanced by *rack1b* or *rack1c* mutations. Therefore, seeds of *rack1a-1 rack1b-2*, *rack1a-1 rack1c-1*, and *rack1b-2 rack1c-1* double mutants (Guo and Chen, 2008) were sown side-by-side with *rack1a* single mutant seeds on ABA plates. It was found that, similar to the scenario of developmental traits (Guo and Chen, 2008), *rack1a-1 rack1b-2* and *rack1a-1 rack1c-1* double mutants displayed much stronger ABA hypersensitivity than *rack1a-1* single mutants, whereas *rack1b-2 rack1c-1* double mutants exhibited wild-type sensitivity to ABA in the seed germination assay (Fig. 1C, D).

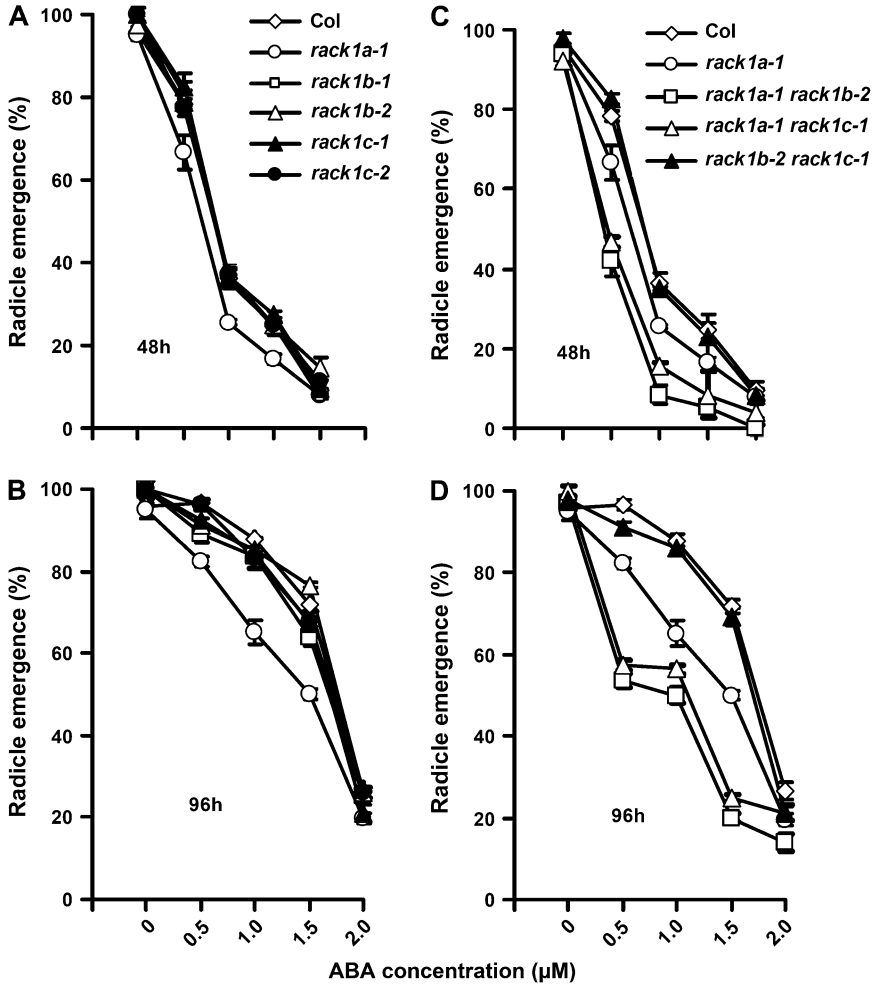

Fig. 1. ABA sensitivity of *rack1* single and double mutants in the seed germination assay. Sterilized wild-type (Col) and *rack1* single (A, B) and double mutant (C, D) seeds were sown on MS/G plates supplemented with different concentrations of ABA. The percentage of seeds with radicle emergence was scored 48 h (A, C) and 96 h (B, D) after the imbibed seeds were transferred from stratification conditions (4 °C, dark for 2 d) to germination conditions (23 °C, with 14/10 h photoperiod at 120 μmol m^{-2} s^{-1}). Shown are the averages of three replicates ±SE.

Next, the percentage of seedlings with green cotyledons in the presence of ABA was scored to measure and compare the impact of these mutations on the ABA responsiveness of seedlings during early development. Without ABA treatment, almost all seeds of *rack1* single and double mutants, similar to the wild type, could germinate and develop into green seedlings (Fig. 2). It was found that, similar to the situation of the seed germination assay, *rack1a-1 rack1b-2* and *rack1a-1 rack1c-1* double mutants displayed much stronger ABA hypersensitivity than *rack1a-1* single mutants whereas *rack1b-2 rack1c-1* double mutants exhibited wild-type sensitivity to ABA in the ABA inhibition of cotyledon greening assay (Fig. 2). These results suggested that *RACK1* genes function redundantly to regulate ABA responses in seed germination and early seedling development, and supported the view that the *RACK1A* gene is the prominent member of the *RACK1* gene family that regulates ABA responses in *Arabidopsis*.

The analysis of ABA sensitivity of *rack1* single and double mutants was extended to post-germination root growth. Wild-type and mutant seeds were imbibed on MS/G medium without ABA for 60 h under normal germination conditions (23 °C, 14/10 h photoperiod at 120 μmol m^{-2} s^{-1}). Germinated seeds with emerged radicles were then transferred to MS/G medium supplemented with ABA. This assay allows us specifically to examine the post-germination ABA sensitivity of these mutants. Because without ABA treatment, *rack1a* single mutants and *rack1a rack1b* and *rack1a rack1c* double mutants had shorter primary roots, compared with the wild-type (Guo and Chen, 2008), the percentage of root elongation inhibition (ABA treatment versus non-ABA treatment) was used to reflect the difference in ABA sensitivity in different genotypes more precisely. It was found that, as in the seed germination and cotyledon greening assays, *rack1a-1* single mutants were hypersensitive to ABA, *rack1a-1 rack1b-2* and *rack1a-1 rack1c-1* double mutants displayed enhanced ABA hypersensitivity than *rack1a* single mutants, and *rack1b-2 rack1c-1* double mutants exhibited wild-type sensitivity to ABA in this ABA inhibition of root growth assay (Fig. 3). Collectively, these results suggested that the three *RACK1* genes act redundantly to negatively regulate ABA responses in the ABA inhibition of seed germination, cotyledon greening, and root growth.

Over-expression of RACK1A *conferred ABA hyposensitivity*

To study the role of *RACK1* genes in ABA responses further, transgenic lines were generated over-expressing *RACK1A* (*35S::RACK1A*), the prominent member of the *RACK1* gene family. Western blot analysis indicated that the RACK1A protein level was elevated in three independent transgenic lines, designated as RACK1A Overexpressor

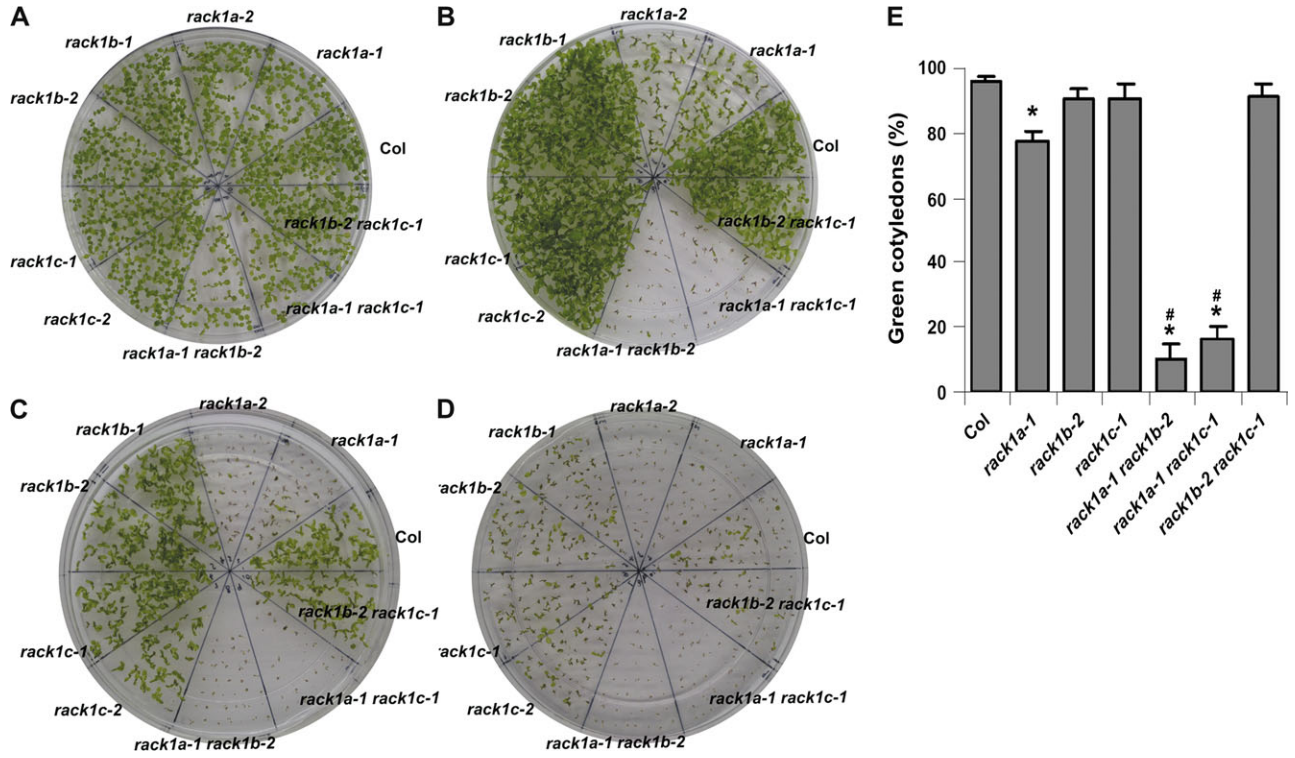

Fig. 2. ABA sensitivity of *rack1* double mutants in the cotyledon greening assay. Sterilized wild-type (Col) and mutant seeds from matched seed lots were sown on MS/G medium containing 0 μM (A), 1.0 μM (B), 1.5 μM (C), and 2.0 μM ABA (D), and cultured at 23 °C, with 14/10 h photoperiod (120 μmol m^{-2} s^{-1}). After 10 d, the percentage of seedlings with green cotyledons was scored. Shown in (E) are the mean values of the percentage of seedlings with green cotyledons ±SE. of three replicates at 1.0 μM ABA. * P <0.05, significantly different from Col; # P <0.05, significantly different from *rack1a-1* single mutant.

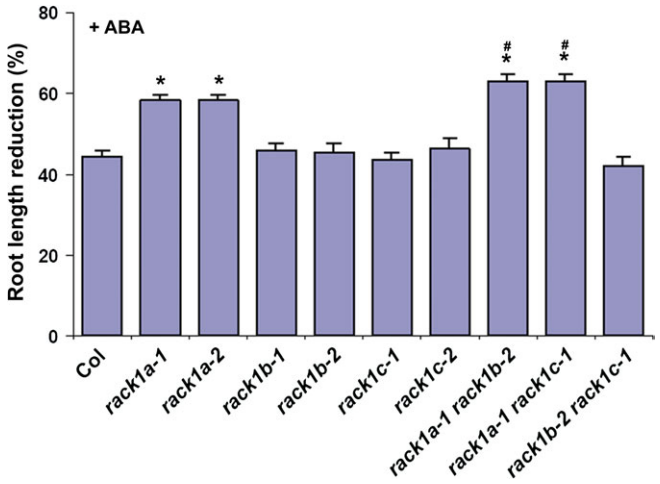

Fig. 3. ABA sensitivity of *rack1* double mutants in the root growth assay. Sterilized wild-type (Col) and mutant seeds from matched seed lots were sown and germinated on MS/G medium without ABA for 60 h. Then seedlings were transferred to MS/G plates with or without 5 μM ABA and grown for another 7 d before the root length was scored. Shown are mean values of the percentage of root length reduction ±SE (n=16). * P <0.05, significantly different from Col; # P <0.05, significantly different from *rack1a-1* single mutant.

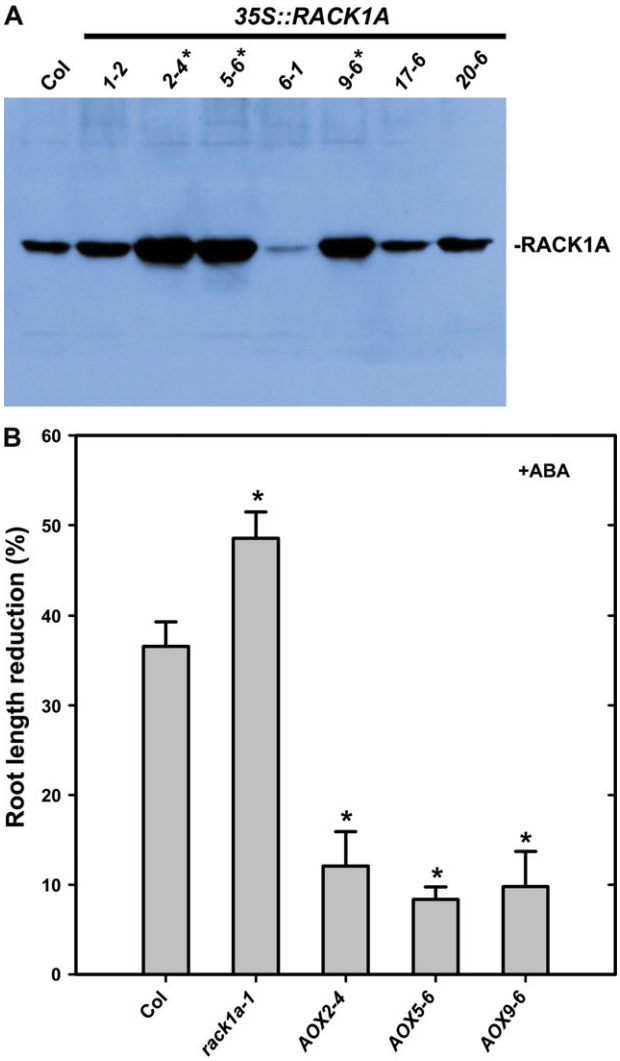

Fig. 4. Over-expression of *RACK1A* conferred ABA hyposensitivity. (A) Western blot analysis of RACK1A protein level in *35S::RACK1A* plants. Total proteins were isolated from 7-d-old, light-grown seedlings. Lines *1-2*, *2-4*, *5-6*, *6-1*, *9-6*, *17-6*, and *20-6* are independent *35S::RACK1A* transgenic lines. (asterisk) Lines in which RACK1A is over-expressed and are used in subsequent studies. These lines are designated as *AOX2-4*, *AOX5-6*, and *AOX9-6*. (B) ABA sensitivity of RACK1A over-expressors in the root growth assay. Sterilized wild type (Col), *rack1a-1* mutant and *35S::RACK1A* seeds from matched seed lots were sown and germinated on MS/G medium without ABA for 60 h. Then, seedlings were transferred onto MS/G plates with or without 5 μM ABA and grown for another 7 d before the root length was scored. Shown are means values of the percentage of root length reduction ±SE (n=16). * P <0.05, significantly different from Col.

lines 2-4, 5-6, and 9-6 (*AOX2-4*, *AOX5-6*, and *AOX9-6*), respectively (Fig. 4A). It was found that, consistent with the view that RACK1 functions as a negative regulator of the ABA response, these independent lines displayed a significantly reduced sensitivity to ABA in the root growth assay (Fig. 4B) and in the seed germination assay (see Supplementary Fig. S1 at *JXB* online).

Expression of RACK1 *in imbibed, germinating, and germinated seeds*

Because our genetic analyses demonstrated that *RACK1* genes regulate seed germination and early seedling development, it was necessary to examine whether the expression of *RACK1* genes is correlated with seed germination and early seedling development. Three different assays were used to examine the expression of the *RACK1* genes. First, RT-PCR was used to examine the presence of *RACK1* transcript in imbibed, germinating, and germinated seeds. It was found that the transcripts of the *RACK1A* gene could clearly be detected during the whole process of seed germination (Fig. 5A). The transcripts of *RACK1B* and *RACK1C* appeared to be only weakly expressed in imbibed seeds under stratification conditions, but were readily detectable in germinating and germinated seeds (Fig. 5A).

In the second assay, the promoter activity of each of the three *RACK1* genes was examined using the *RACK1* promoter::GUS (P_{RACK1}::*GUS*) reporter lines. Consistent with the RT-PCR results, the promoters of the *RACK1* genes were active in imbibed, germinating, and germinated seeds (Fig. 5B). The GUS staining was readily detected in the cotyledons of seeds 24 h after imbibition, and in the cotyledons and radicles of seeds 48 h after imbibition (under stratification conditions, 4 °C, dark). One day after the imbibed seeds had been transferred from stratification conditions to germination conditions (23 °C, 14/10 h photoperiod, 120 μmol m^{-2} s^{-1}), *RACK1* promoters were active in the protruding radicles of germinating seeds. Another day later, when radicle protrusion through seed

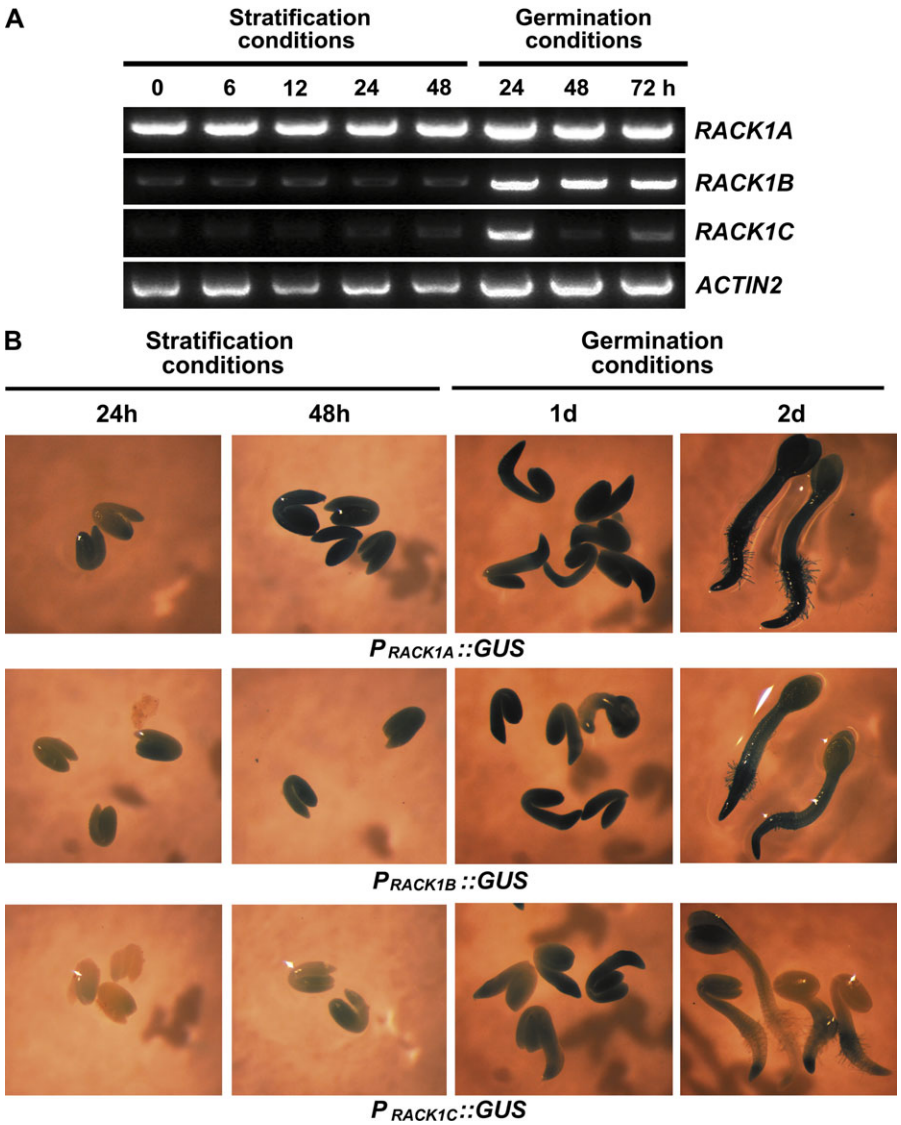

Fig. 5. Analysis of *RACK1* expression in imbibed, germinating, and germinated seeds by using RT-PCR and P_{RACK1}*::GUS* reporter lines. (A) RT-PCR analysis of the expression of *RACK1* genes. Sterilized seeds were placed under stratification conditions, and sampled 0, 6, 12, 24, or 48 h later. After being stratified for 48 h, seeds were then transferred to germination conditions (23 °C, 14/10 h photoperiod at 120 μmol m^{-2} s^{-1}) for 24, 48, or 72 h. The expression of *ACTIN2* was used as control. PCR was performed at 30 cycles. (B) Analysis of *RACK1* promoter activity. GUS staining was performed in seeds placed under stratification conditions for 24 h and 48 h, and in seeds that had been placed in stratification conditions for 48 h and subsequently transferred to germination conditions for 24 h and 48 h, respectively.

coats was apparent in most seeds, the GUS staining appeared to be stronger in roots (particularly, in the root apical meristem) than in shoots (Fig. 5B), consistent with a role of *RACK1* in root development (Guo and Chen, 2008).

In the third assay, RACK1 protein expression was examined during seed germination and early seedling development. Fusion proteins between RACK1 and green, cyan or yellow fluorescent protein (GFP, CFP or YFP) were generated. Specifically, *RACK1A promoter::RACK1A-GFP* (P_{RACK1A}*::RACK1A-GFP*), *RACK1B promoter::RACK1B-CFP* (P_{RACK1B}*::RACK1B-CFP*), and *RACK1C promoter::RACK1C-YFP* (P_{RACK1C}*::RACK1C-YFP*) lines were generated, in which the expression of the fusion proteins was driven by the native promoters of *RACK1A*, *RACK1B,* and *RACK1C*, respectively. Because among *rack1* mutants, only *rack1a* single mutants (but not *rack1b* or *rack1c* single mutants) and *rack1a rack1b* and *rack1a rack1c* double mutants displayed morphological and ABA phenotypes, the functionalities of these fusion proteins were tested by transforming the related constructs into *rack1a* single mutants (for P_{RACK1A}*::RACK1A-GFP*), *rack1a rack1b* (for P_{RACK1B}*::RACK1B-CFP*) or *rack1a rack1c* (for P_{RACK1C}*::RACK1C-YFP*) double mutants. In each case, it was found that the fusion proteins could function equivalently to the corresponding wild-type form of RACK1 protein (data not shown). By using these reporter lines, it was found that the GFP/CFP/YFP fluorescence could be

detected in the imbibed, germinating, and germinated seeds of P_{RACK1A}::*RACK1A-GFP*, P_{RACK1B}::*RACK1B-CFP*, and P_{RACK1C}::*RACK1C-YFP* lines (Fig. 6). Similar to the situation of P_{RACK1}::*GUS* reporter lines, RACK1-GFP/CFP/YFP proteins were expressed strongly in the protruding radicles of germinating seeds and the root apical meristem of germinated seeds. Taken together, the expression of *RACK1* genes, the activity of the *RACK1* promoter, and the expression of RACK1 proteins in imbibed, germinating, and germinated seeds are consistent with their proposed roles in seed germination and early seedling development.

ABA marker genes, RD29B *and* RAB18*, were up-regulated in* rack1a *mutants*

To get an insight into the role of *RACK1* in ABA responses, it was necessary to investigate if RACK1 is involved in the regulation of ABA-induced gene expression in young seedlings. The expression of two well-known ABA marker genes, *RESPONSIVE TO DESSICATION29B* (*RD29B*) and *RESPONSIVE TO ABA18* (*RAB18*) whose expressions are under direct regulation through ABA-responsive elements (ABRE) (Yamaguchi-Shinozaki and Shinozaki, 1994; Mantyla *et al.*, 1995; Uno *et al.*, 2000; Umezawa *et al.*, 2006) was chosen for examination. Quantitative RT-PCR analysis revealed that without ABA induction, the transcript levels of *RD29B* and *RAB18* were up-regulated 2–5-fold in the *rack1a* single mutant and *rack1a rack1b* and *rack1a rack1c* double mutants (Fig. 7). Upon ABA treatment, the transcripts of *RD29B* and *RAB18* were dramatically increased in both wild-type and in *rack1a* single and double mutants (Fig. 7). Although the differences in the transcript levels of *RD29B* and *RAB18* between wild-type and *rack1a* mutants with ABA treatment was not as large as those without ABA treatment, the *rack1a rack1b* and *rack1a rack1c* double mutants accumulated more *RD29B* and *RAB18* transcripts than the wild-type in response to ABA (approximately 50% increase) (Fig. 7). These results support the view that RACK1 negatively regulates ABA responses.

The transcription of three RACK1 *genes was down-regulated by ABA*

Our genetic and molecular analyses demonstrated that *RACK1* negatively regulates ABA responses in the ABA-mediated inhibition of seed germination, cotyledon greening and root growth, and ABA-induced gene expression. Because the expression of some negative regulators of ABA signalling, such as Rop 10 (Zheng *et al.*, 2002), is also negatively regulated by ABA, the possibility whether the

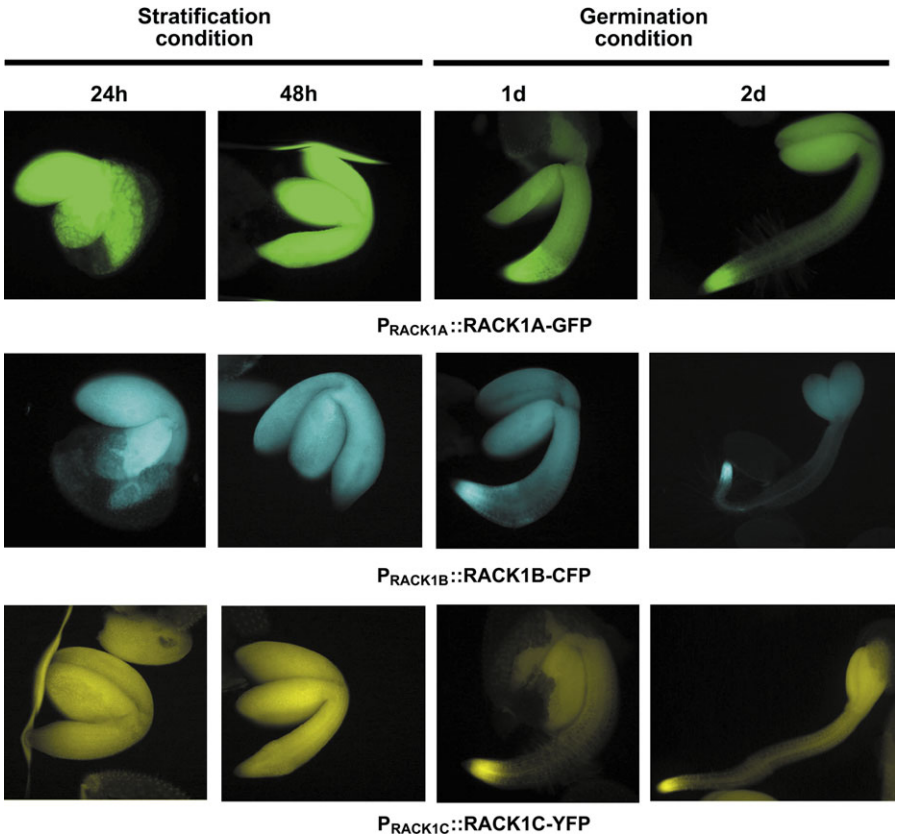

Fig. 6. Analysis of RACK1 protein expression in imbibed, germinating and germinated seeds using P_{RACK1}::*RACK1-GFP/CFP/YFP* reporter lines. GFP/CFP/YFP fluorescence was examined in seeds placed under stratification conditions for 24 h and 48 h, and in seeds that had been placed in stratification conditions for 48 h and subsequently transferred to germination conditions for 24 h and 48 h, respectively.

expression of *RACK1* genes themselves may be regulated by ABA was examined. Interestingly, it was found that the transcription of all three *RACK1* genes was significantly down-regulated by ABA treatment in young seedlings (Fig. 8), in contrast to that in rice cell cultures or in imbibed rice seeds where *RACK1A* transcripts or RACK1A protein were shown to be up-regulated by ABA (Komatsu *et al.*, 2005; Nakashima *et al.*, 2008).

rack1 *mutants display reduced water loss*

Our genetic and molecular analyses suggested that *RACK1* genes are negative regulators of ABA responses. Additional evidence was sought to support this conclusion. Because ABA is a critical regulator of stomatal movements (opening and closure), it was examined if *rack1* mutants may display alternations in water loss from rosettes. The water loss from the detached whole rosette (with root removed) of *rack1a* single mutant and *rack1a rack1b* and *rack1a rack1c* double mutants was measured and compared with the wild type. It was found that the *rack1a* single mutant and *rack1a rack1b* and *rack1a rack1c* double mutants lost water significantly slower than the wild type (Fig. 9), whereas the *RACK1A* over-expressors lost water significantly faster than the wild type (see Supplementary Fig. S2 at *JXB* online). These results implied that *rack1* mutants may have extensive stomatal closure, probably because of their hypersensitivity to ABA, although this has not been experimentally tested.

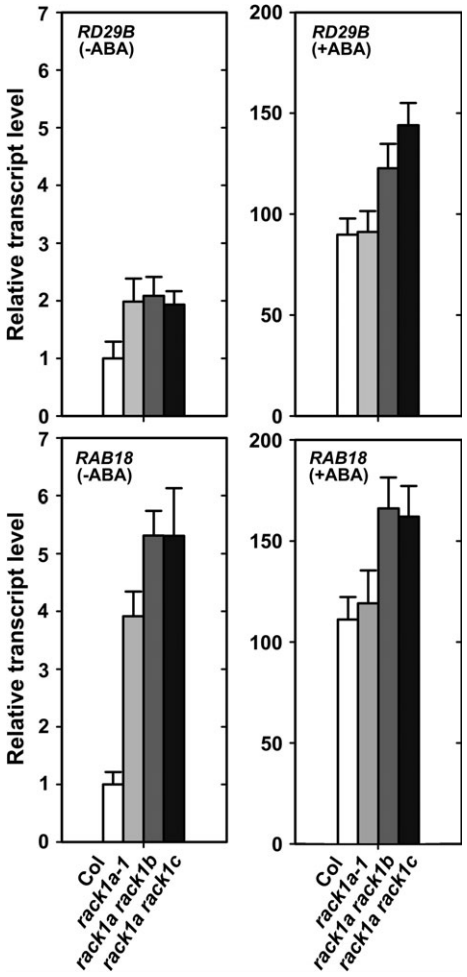

Fig. 7. Expression of ABA marker genes, *RD29B* and *RAB18*, in *rack1* mutants. The transcript levels of *RD29B* and *RAB18* in the wild-type and the *rack1a* mutant without or with ABA treatment (10 μM for 2 h) were analysed by quantitative RT-PCR. The expression of *ACTIN2* was used as a control. All transcript levels are normalized against Col without ABA treatment, with the value of the first biological replicate set as 1. Shown are the mean values of three biological replicates ±SE.

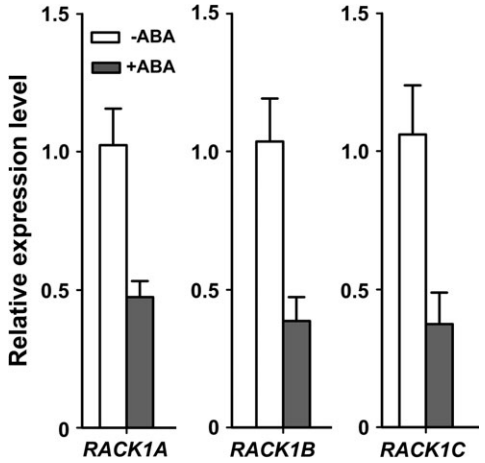

Fig. 8. Regulation of the transcription of *RACK1* by ABA. The transcript levels of *RACK1* genes in wild-type (Col) with ABA treatment (10 μM for 2 h), compared with no ABA treatment, were analysed by quantitative RT-PCR. The expression of *ACTIN2* was used as control. Each *RACK1* gene was normalized against Col without ABA treatment, with the value of the first biological replicate set as 1. Shown are the mean values of three biological replicates ±SE.

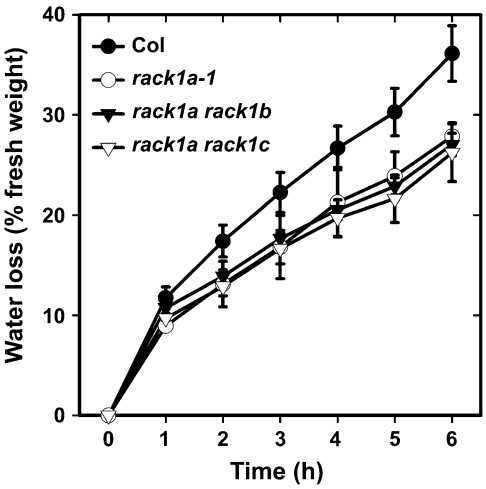

Fig. 9. Water loss assay of *rack1* mutants. Whole rosettes of 20-d-old plants grown under short day conditions (8/16 h photoperiod) were cut off from the base and used for water loss assay. Shown are the mean values of three replicates ±SE.

rack1 *mutants display hypersensitivity to salt during seed germination*

Both drought and salt stress signal transduction pathways involve osmotic homeostasis and ABA plays an important role in some of these processes (reviewed by Zhu, 2002; Xiong *et al.*, 2002). For example, many studies have observed that mutants with altered ABA sensitivity are affected in germination on salt-containing media. Therefore, it was necessary to extend our analysis of *rack1a* mutant to the salt stress response. The sensitivity of the *rack1a* single mutant and *rack1a rack1b* and *rack1a rack1c* double mutants to different concentrations of NaCl during seed germination was examined. It was found that the *rack1a* single mutant and the *rack1a rack1b* and *rack1a rack1c* double mutants displayed hypersensitivity to NaCl (Fig. 10), consistent with the view that these mutants are hypersensitive to ABA. As expected, *RACK1A* over-expressors displayed hyposensitivity to NaCl (see Supplementary Fig. S3 at *JXB* online).

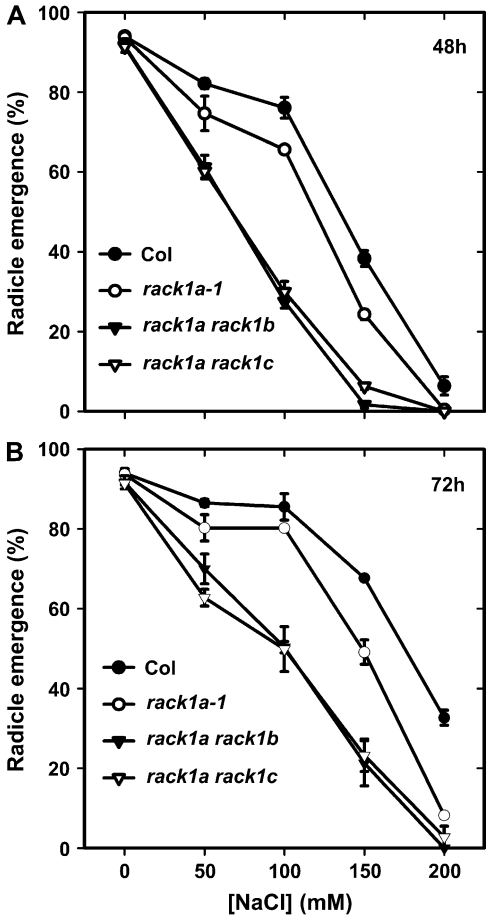

Fig. 10. Salt stress sensitivity of *rack1* mutants during seed germination. Sterilized wild-type (Col) and mutant seeds were sown on MS/G plates supplemented with different concentrations of NaCl. The percentage of seeds with radicle emergence was scored 48 h (A) and 72 h (B) after the imbibed seeds had been transferred to germination conditions (23 °C, with 14/10 h photoperiod at 120 μmol m^{-2} s^{-1}). Shown are the averages of three replicates ±SE.

RACK1 *interaction network*

Our genetic and molecular characterization provided strong evidence that RACK1 regulates ABA responses. In an attempt to understand the molecular mechanism by which RACK1 regulates ABA responses, proteins that may interact with RACK1 were sought. The RACK1 interaction network was generated using the BAR Arabidopsis Interactions Viewer (Geisler-Lee *et al.*, 2007). This tool predicts interactome of protein of interest in Arabidopsis. Only RACK1A and RACK1C are present in the BAR Arabidopsis Interactions Viewer database (http://bar.utoronto.ca/interactions/cgi-bin/arabidopsis_interactions_viewer.cgi). The database predicts 53 potential interactors for RACK1A (see Supplementary Table S2 at *JXB* online) and 68 potential interactors for RACK1C (see Supplementary Table S3 at *JXB* online). Among the 121 interactors indentified, 35 proteins interact with both RACK1A and RACK1C, whereas RACK1A has 18 unique interactors and RACK1C has 33 (Supplementary Table S2 and Supplementary Table S3 at *JXB* online).

To examine the possibility that RACK1 may work together with these potential RACK1 interactors regulating ABA responses, these 86 interactors identified through the BAR Arabidopsis Interactions Viewer were searched against the Genevestigator (Zimmermann *et al.*, 2004; https://www.genevestigator.com/gv/index.jsp) and the available literatures for their potential roles in ABA or abiotic stress responses. A total of eight genes was identified from these 86 candidates. These genes are briefly summarized here. At1g54510 (*ATNEK1*) encodes a member of the NIMA-related serine/threonine kinases that have been linked to cell-cycle regulation in fungi and mammals, and was shown to be up-regulated by ABA (Nishimura *et al.*, 2007). At2g35940 (*EDA29, embryo sac development arrest 29*) encodes a putative homeodomain transcription factor and was identified as an ABA-induced gene (Hoth *et al.*, 2002). At3g24080 was identified as a salt-induced gene in a differential subtraction screening (Gong *et al.*, 2001) and was shown to be up-regulated by ABA according to Genevestigator. At4g23570 (*SGT1A*, *suppressor of G2 allele of skp1*) is involved in plant disease resistance (Austin *et al.*, 2002; Nakashima *et al.*, 2008) and its rice orthologue physically interacts with RACK1 protein (Nakashima *et al.*, 2008). *SGT1* is slightly down-regulated by ABA according to Genevestigator. At4g27560 encodes a putative glycosyltransferase and was shown to be a salt-induced gene (Gong *et al.*, 2001). At5g03730 (*CTR1*, *CONSTITUTIVE TRIPLE RESPONSE 1*) encodes a serine/threonine protein kinase and is a negative regulator of ethylene signalling (Kieber *et al.*, 1993). At5g08415 belongs to the lipoic acid synthase family and is shown to be down-regulated by ABA according to Genevestigator. At5g08420 encodes an RNA-binding protein and is shown to be up-regulated by ABA according to Genevestigator.

Then, the ABA induction of these eight genes was examined in *rack1a rack1b* double mutant seedlings and compared with that in the wild type. Among these eight genes, the transcript levels of two genes, At1g54510 (*ATNEK1*) and At3g24080, were reduced in response to ABA in the *rack1a rack1b* mutant background, compared with the wild type (Fig. 11). The transcript levels of five genes, At2g35940 (*EDA29*), At4g23570 (*SGT1A*), At5g03730 (*CTR1*), At5g08415, and At5g08420 were increased in response to ABA in the *rack1 rack1b* mutant background (Fig. 11). One gene, At4g27560, responded to ABA similarly in the wild type and in *rack1a-1 rack1b-2* mutant background (Fig. 11). The alternation of ABA responses of these genes in the *rack1a rack1b* mutant background implied that RACK1 may be involved in the ABA signalling route towards induction of these genes.

Discussion

Accumulating evidence suggested that RACK1 regulates plant development and that RACK1 may be involved in hormonal responses in plants. However, the role of RACK1 in any hormone signalling pathways has not been defined

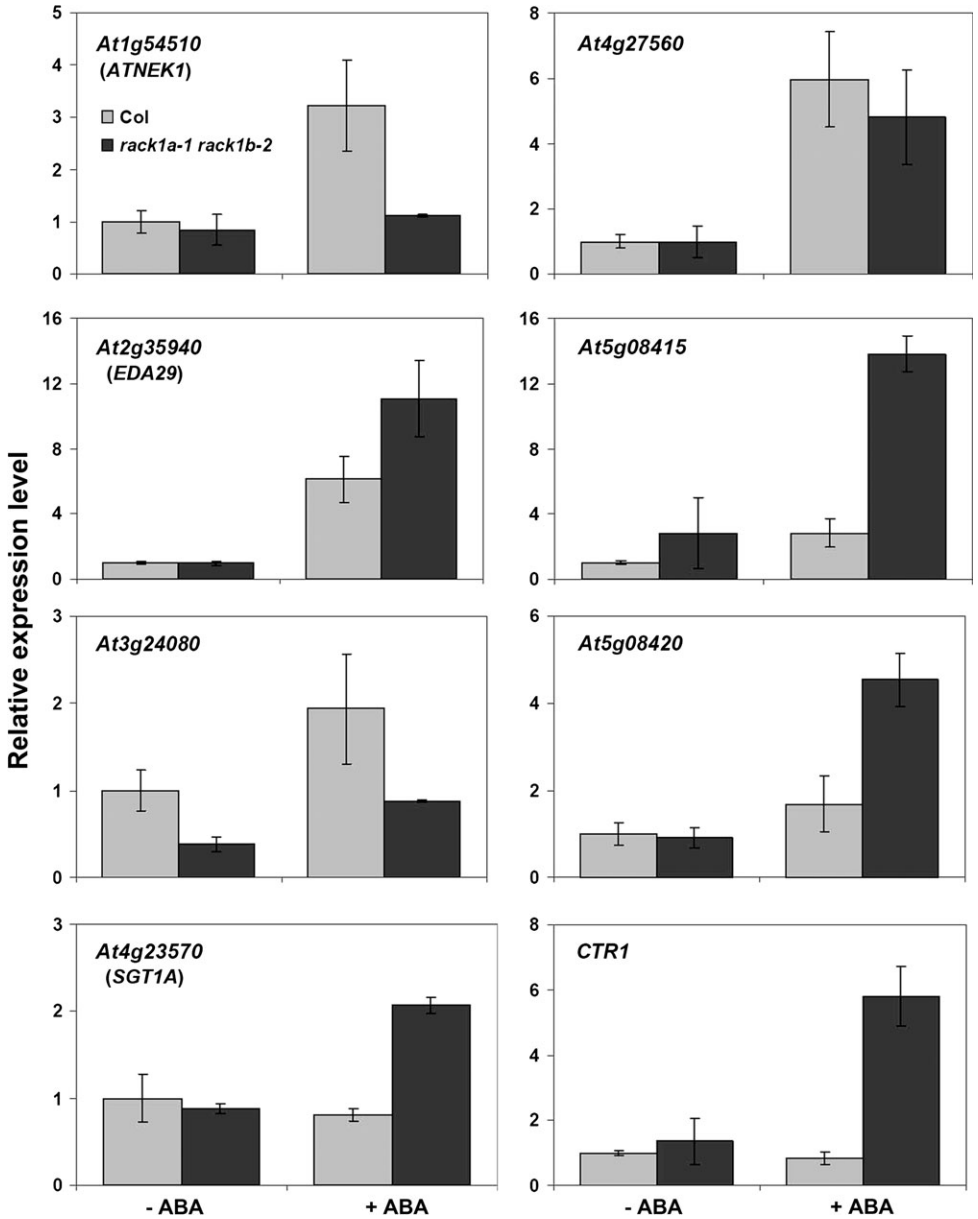

Fig. 11. Quantitative RT-PCR analysis of the expression of selected putative RACK1 interactors in response to ABA. The transcript levels of At1g54510 (*ATNEK1*), At2g35940 (*EDA29*), At3g24080, At4g23570 (*SGT1A*), At4g27560, At5g03730 (*CTR1*), At5g08415, and At5g08420 in wild-type (Col) and *rack1a rack1b* double mutants with ABA treatment (50 μM for 2 h), compared with no ABA treatment, were analysed by quantitative RT-PCR. The expression of *ACTIN2* was used as control. The transcript level of each gene was normalized against that in Col without ABA treatment, with the value of the first biological replicate set as 1. Shown are the mean values of three biological replicates ±SE.

prior to this study. Six lines of evidence directly or indirectly support the conclusion that Arabidopsis RACK1s are negative regulators of ABA responses. (i) *rack1a* single mutants and *rack1a rack1b* and *rack1a rack1c* double mutants were hypersensitive to ABA in the ABA inhibition of seed germination, cotyledon greening, and root growth. (ii) Over-expression of *RACK1A* conferred ABA hyposensitivity. (iii) The expression of ABA marker genes, *RD29B* and *RAB18*, was up-regulated in the young seedlings of *rack1a* single mutants and *rack1a rack1b* and *rack1a rack1c* double mutants. (iv) The expression of three *RACK1* genes was down-regulated by ABA. (v) *rack1a* single mutants and *rack1a rack1b* and *rack1a rack1c* double mutants lose water from detached rosettes significantly slower than wild-type plants. (vi) *rack1a* single mutants and *rack1a rack1b* and *rack1a rack1c* double mutants were hypersensitive to NaCl during seed germination.

Our discovery of RACK1 as a negative regulator of ABA signalling in seed germination and early seedling development expanded the long list of negative regulators of ABA signalling. ABA INSENSITIVE 1 (ABI1) and ABI2, both protein phosphatase 2Cs (PP2Cs), are among the first negative regulators of ABA signalling identified through genetic screens (reviewed by Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003; Hirayama and Shinozaki, 2007; McCourt and Creelman, 2008; Wasilewska *et al.*, 2008). Subsequently, other PP2Cs, including ABA HYPERSENSITIVE GERMINATION1 (AHG1) (Nishimura *et al.*, 2004, 2007) and AHG3/AtPP2CA (Nishimura *et al.*, 2004; Yoshida *et al.*, 2006), HYPERSENSITIVE TO ABA 1 (HAB1) (Saez *et al.*, 2004, 2006) and HAB2 (Saez *et al.*, 2004; Yoshida *et al.*, 2006) have also been demonstrated as negative regulators of ABA signalling in seed germination and early seedling development (reviewed by Hirayama and Shinozaki, 2007). Many other negative regulators of ABA signalling in seed germination and early seedling development have been discovered through reverse genetics or gene expression studies (Finkelstein *et al.*, 2002). For example, through reverse genetics, the heterotrimeric G-proteins are proposed to be negative regulators of ABA signalling in seed germination and early seedling development (reviewed by Perfus-Barbeoch *et al.*, 2004; Assmann, 2005; Chen, 2008), because the loss-of-function alleles of *Arabidopsis* heterotrimeric G-protein α (Gα) and β (Gβ) subunits are hypersensitive to ABA in these processes (Ullah *et al.*, 2002; Pandey *et al.*, 2006). Similarly, a putative G-protein-coupled receptor (GPCR) in *Arabidopsis*, *GCR1*, is a negative regulator of ABA signalling in seed germination and early seedling development (Pandey and Assmann, 2004; Pandey *et al.*, 2006). A small GTPase, Rop10, was also characterized as a negative regulator of ABA signalling in *Arabidopsis* (Zheng *et al.*, 2002).

Genetic screens have also yielded many other critical components of ABA signalling, including ABI3, ABI4, and ABI5 (reviewed by Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003; McCourt and Creelman, 2008). ABI3 is a B3 domain transcription factor. ABI4 is an APETALA2 domain transcription factor. ABI5 is a basic leucine zipper transcription factor. It is believed that these transcription factors mediate ABA responses by controlling ABA-induced gene expression. Although at least three different types of proteins have been proposed as ABA receptors in the last three years, including FLOWERING TIME CONTROL PROTEIN A (FCA) (Razem *et al.*, 2006), a nuclear RNA-binding protein, the H subunit of Mg-chelatase (CHLH) (Shen *et al.*, 2006), a chloroplast protein, and G-Protein-Coupled Receptor 2 (GCR2), a proposed seven-transmembrane GPCR (Liu *et al.*, 2007), as reviewed by McCourt and Creelman (2008), none of these proposed ABA receptors appeared to function as the major receptor mediating ABA signalling in seed germination and early seedling development. Furthermore, the structure and functionality of GCR2 have been challenged by other studies (Gao *et al.*, 2007; Johnston *et al.*, 2007; Guo *et al.*, 2008; Illingworth *et al.*, 2008; Risk *et al.*, 2009). Subsequent studies also do not support the claim that FCA is an ABA receptor (Jang *et al.*, 2008; Razem *et al.*, 2008; Risk *et al.*, 2008). At the cell surface, there are a few candidate ABA receptors perceiving the ABA signal. For example, a leucine-rich repeat (LRR) receptor-like kinase 1, RPK1, has been shown to function as a positive regulator of ABA signalling in seed germination, early seedling growth, stomatal closure, and ABA-induced gene expression in *Arabidopsis* (Osakabe *et al.*, 2005). Recently, the *Arabidopsis* A4 subfamily of lectin receptor kinases, LecRKs, has been shown to function as negative regulators of the ABA response in seed germination (Xin *et al.*, 2008). However, the ability of these candidate receptors to bind ABA has not been established. In 2009, two new types of ABA receptors have been proposed, including two novel GPCR-type G-proteins, GTG1 and GTG2 (Pandey *et al.*, 2009), and the PYR/PYL (RCAR) family of START proteins (Ma *et al.*, 2009; Park *et al.*, 2009), which has led to new discussions on ABA receptors (Pennisi, 2009).

As discussed above, molecular and genetic studies have already identified a rich collection of signalling components, positive or negative regulators, involved or required in ABA signalling (reviewed by Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003; Hirayama and Shinozaki, 2007; McCourt and Creelman, 2008; Wasilewska *et al.*, 2008). However, it remains elusive how these signalling components are co-ordinated to regulate ABA responses. The identification of RACK1 proteins, whose mammalian counterparts function as multi-functional scaffold proteins, as redundant, negative regulators of ABA responses may help to provide new insights into the complex ABA signalling networks. In a preliminary analysis, a RACK1 interaction network was generated through a bioinformatics approach (see Supplementary Table S2 and Supplementary S3 at *JXB* online). The transcription of a total of eight genes among those 86 candidate interactors has been shown to be regulated by ABA or abiotic stresses. It was found that ABA induction of seven of these eight ABA-regulated genes was altered in the *rack1a rack1b* double mutant background, implying that RACK1 may be involved in the ABA signalling route towards the induction of these genes. One of the well

characterized proteins in our list is CTR1 which has been shown to be a negative regulator of ethylene signalling (Kieber *et al.*, 1993). CTR1 was down-regulated by ABA in the wild type whereas it was up-regulated in the *rack1a rack1b* double mutant background (Fig. 11). Because ethylene is considered to be a negative regulator of ABA signalling in seed germination and a positive regulator of ABA signalling in root growth (Gazzarrini and McCourt, 2001; Wang *et al.*, 2007), it raises the possibility that RACK1 may serve as a nexus for these two hormone signalling pathways. This will be investigated further in future studies. On the other hand, the relationship between RACK1 and other known components in the ABA signalling pathway is unknown. The next major challenge is to position RACK1 precisely in the intricate ABA signal transduction network.

In summary, it is demonstrated that *rack1a* single and double mutants are hypersensitive to ABA in the ABA inhibition of seed germination, cotyledon greening, and root growth whereas over-expression of *RACK1A* confers ABA hyposensitivity. It was shown that the expression of ABA-responsive marker genes, *RD29B* and *RAB18*, are up-regulated in *rack1a* mutants and the *RACK1* genes are down-regulated by ABA. Consistent with ABA hypersensitivity, *rack1a* mutants lose water significantly more slowly from the rosettes and are hypersensitive to high concentrations of NaCl during seed germination. Furthermore, the expression of some known ABA- or abiotic stress-regulated genes which encode putative RACK1 interactors was altered in the *rack1a rack1b* mutant background, in response to ABA. Collectively, these results have defined RACK1s as critical regulators of ABA signalling. Because RACK1 functions as a scaffold protein in mammalian cells, our work may help provide new insights into the complex ABA signalling network.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. ABA sensitivity of *RACK1A* over-expression lines in the seed germination assay.

Supplementary Fig. S2. Water loss assay of *RACK1A* over-expression lines.

Supplementary Fig. S3. Salt stress sensitivity of *RACK1A* over-expression lines during seed germination.

Supplementry Table S1. Primers used in this study.

Supplementry Table S2. RACK1A-interacting proteins identified by using the BAR *Arabidopsis* Interactions Viewer.

Supplementary Table S3. RACK1C-interacting proteins identified by using the BAR *Arabidopsis* Interactions Viewer.

Acknowledgements

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