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RESEARCH PAPER

RACK1 mediates multiple hormone responsiveness and developmental processes in *Arabidopsis*

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Abstract

The scaffold protein RACK1 (Receptor for Activated C Kinase 1) serves as an integrative point for diverse signal transduction pathways. The *Arabidopsis* genome contains three RACK1 orthologues, however, little is known about their functions. It is reported here that one member of this gene family, *RACK1A*, previously identified as the *Arabidopsis* homologue of the tobacco *arcA* gene, mediates hormone responses and plays a regulatory role in multiple developmental processes. *RACK1A* expresses ubiquitously in *Arabidopsis*. Loss-of-function mutations in *RACK1A* confer defects in multiple developmental processes including seed germination, leaf production, and flowering. *rack1a* mutants displayed reduced sensitivity to gibberellin and brassinosteroid in seed germination, hypersensitivity to abscisic acid in seed germination and early seedling development, and hyposensitivity to auxin in adventitious and lateral root formation. These results provide the first genetic evidence that RACK1A is involved in multiple signal transduction pathways.

Key words: Abscisic acid, auxin, brassinosteroid, flowering, gibberellin, RACK1, seed germination, signal transduction.

Introduction

Signal molecules, such as plant hormones, regulate diverse processes in plant growth and development. Classic genetic screens have yielded various components involved in signal transduction. Recently, microarray-based analyses have proven to be another productive approach for the discovery of genes in plant hormone signal integration. From these studies, it is now clear that numerous genes can be regulated by a single signal, such as auxin (Pufky *et al*., 2003). The reciprocal is true; a single gene can be regulated by many different biological or environmental stimuli. For example, some genes originally defined as auxin-inducible genes can also be induced by other plant hormones, such as brassinosteroids (Goda *et al*., 2004; Nemhauser *et al*., 2004). It has been recognized for many years that signals integrate and the term 'cross-talk' was coined to describe this complexity (see recent reviews by Gazzarrini and McCourt, 2003; Chinnusamy *et al*., 2004). However, it remains largely unclear how signalling components interact with each other, and how signals are integrated.

One mechanism by which eukaryotic cells are able to integrate multiple signals is through the organized proximity of signalling components using scaffold proteins. Scaffold proteins assemble active complexes with multiple

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signalling proteins/enzymes from the same or different pathways to facilitate the interaction and regulation of signal transduction networks. For a growing number of signalling pathways, scaffold proteins are shown to be central elements. For example, the yeast scaffold protein, Ste5p, simultaneously binds different members of the mitogen-activated protein kinase (MAPK) cascade to transduce signals controlling mating (Harris *et al*., 2001; Park *et al*., 2003). This same scaffold is used in the osmolarity response but concatenating a different set of MAPK kinases (Park *et al*., 2003).

The β propeller motif has emerged as a common platform for integrating various signals. Beta propeller proteins are diverse (van Nocker and Ludwig, 2003); one member of this large family of proteins is designated Receptor for Activated C Kinase 1 (RACK1). Mammalian RACK1 has a seven-bladed propeller structure with one Trp-Asp 40 (WD40) unit constituting each blade. The WD40 repeat is thought to be involved in protein–protein interactions, and the β propeller structural unit may enable the protein to form reversible complexes with its ligand proteins. Increasing evidence suggests that interaction domains, such as β propeller, have been evolutionarily selected for their flexibility, their ability to assemble multiprotein machines, and their potential to mediate sophisticated biological functions (Fulop and Jones, 1999; Pawson and Nash, 2003). Originally identified as an anchoring protein for protein kinase C (PKC) in mammals (Ron *et al*., 1994) which shuttles the active enzyme to different cellular sites, RACK1 is now viewed as a versatile scaffold protein, that can bind numerous signalling molecules from diverse signal transduction pathways in a regulated fashion (see review by McCahill *et al*., 2002). Some recent examples include cAMP signalling (Yarwood *et al*., 1999; Steele *et al*., 2001), cell cycle control (Mamidipudi *et al*., 2004), Ca^{2+} release (Patterson *et al*., 2004), ribosome assembly and mRNA translation regulation (Ceci *et al*., 2003; Nilsson *et al*., 2004; Sengupta *et al*., 2004), cytoskeleton remodelling (Osmanagic-Myers and Wiche, 2004), and proteasome degradation (Fomenkov *et al*., 2004).

RACK1 is a highly conserved protein found in both animals and plants. Positioning of RACK1 WD repeats is conserved in the alga *Chlamydomonas reinhardtii* which diverged from the forerunners of the plant kingdoms about 1 billion years ago, indicating that the biological function of RACK1 is ancient (Neer *et al*., 1994; McCahill *et al*., 2002). Although not recognized as such, the first RACK1 homologue in plants was identified in tobacco BY-2 suspension cells as an auxin-inducible gene, *arcA* (Ishida *et al*., 1993), and later in *Arabidopsis*, rice, rape, and alfalfa (Iwasaki *et al*., 1995; McKhann *et al*., 1997; Vahlkamp and Palme, 1997; Kwak *et al*., 1997). Indirect evidence suggested that plant RACK1 may be involved in hormone-mediated cell division, UV and salicylic acid responses (Ishida *et al*., 1993; McKhann *et al*., 1997; Perennes *et al*., 1999). However, direct genetic evidence has been lacking, thus no signal transduction pathway involving a RACK1 has been unequivocally identified in plants. The first genetic evidence that *Arabidopsis* RACK1 has a role in hormonal signalling pathways and may have a regulatory role in multiple developmental processes is provided here.

Materials and methods

Plant materials

Initially, deconvoluted pools of DNA from T-DNA transformed plants were used to screen for insertions in the *Arabidopsis RACK1A* gene (At1g18080). A total of 60 000 T-DNA insertion lines in the Columbia (Col-0) ecotype (Alonso *et al*., 2003) were screened by PCR using *RACK1A*- and T-DNA-specific primers. A putative insertion line was identified by using primers specific for the *RACK1A* 5′-UTR (5′-GGCATCTCCAGACACCGAAA-3′) or 3′-UTR (5′-GCAGAGAGCAACGACAGC-3′) together with a T-DNA left border-specific primer (5′-GGCAATCAGCTGTTGCCCGTCT-CACTGGTG-3′). A single insertion in the first exon of *RACK1A* was isolated, and the insertion was confirmed by sequencing. This *rack1a* mutant allele was designated *rack1a-1*. The second *rack1a* mutant allele (*rack1a-2*, SALK_073786) was obtained from the Salk Institute sequence-indexed insertion mutant collection (http://signal.salk.edu/cgi-bin/tdnaexpress). The T-DNA insertion site in this allele was at the first intron of *RACK1A*. Plants homozygous for *rack1a-2* were isolated, and the insertion was confirmed by sequencing. Loss of detectable *RACK1A* transcripts in *rack1a-1* and *rack1a-2* mutants was verified by RT-PCR. Total RNA was isolated from 10-d-old, light-grown seedlings by using the TRIzol reagent (Life Technologies). cDNA was synthesized using 1 μg of total RNA by Oligo$(dT)_{20}$-primed reverse transcription, using THERMOSCRIPT RT (Life Technologies). *RACK1A* primers described above that flank the insertion sites and *Arabidopsis ABP1* (At4g02980) primers (5′-TGATCGTACTTTCTGTTGGTTCC-3′ and 5′-CCAATAGTA-AGGGAACTTCAGCC-3′ were added together in each RT-PCR reaction. All mutant alleles are in Col-0 ecotype.

Molecular modelling

The sequence of RACK1A was submitted to 4 fold-recognition web servers to identify structural templates for homology modelling. The fold-recognition servers were bioinbgu (www.cs.bgu.ac.il/~bioinbgu/), 3D-PSSM (www.sbg.bio.ic.ac.uk/3dpssm/; Kelley *et al*., 2000), GenTHREADER (bioinf.cs.ucl.ac.uk/psipred/index.html; Jones, 1999), and FUGUE (www-cryst.bioc.cam.ac.uk/~fugue/prfsearch.html; Shi *et al*., 2001). The fold-recognition servers identified Gβ1 (PDB ID 1GOT) from the Protein Data Bank (Berman *et al*., 2000) as a template for modelling RACK1A. A model of RACK1A and human RACK1 was built using the modeller module of the InsightII molecular modelling system from Accelrys Inc. (www.accelrys.com). The homology model was evaluated for sequence-structure compatibility using the verify function of the Profiles-3D module from InsightII. The theoretical model for RACK1A was given a self-compatibility score of 150.2 by the Profiles-3D/Verify module of InsightII. The typical score expected for an experimentally-determined protein structure of 323 residues was 147.1, indicating the model is plausible. Similar results were obtained for the model of human RACK1 with a self-compatibility score of 135.8 and a typical score of 141.8 for a 311 residue globular protein. For comparison, the self-compatibility score for the 340 residue mammalian Gβ1 structural template was 165.9, with a typical

score of 154.4. Figures were created using PyMOL (http:// pymol.sourceforge.net/).

Germination assay

The procedure of germination assay was according to Chen *et al*. (2004). Briefly, sterilized wild-type and mutant seeds from matched lots were pretreated with 8 μM paclobutrazol (Chem Service, West Chester, PA) in the dark at 4 °C for 2 d, washed six times with deionized water, sown on MS/G plates consisting of ½ Murashige and Skoog (MS) basal medium with vitamins (plantmedia, Dublin, Ohio), 1% (w/v) sucrose, 0.5% (w/v) phytoagar (plantmedia, Dublin, Ohio), pH adjusted to 5.7 with 1 N KOH, and with different concentrations of gibberellic acid (GA_3) or brassinolide (BL). The plates were pretreated with 80 μmol m^{-2} s^{-1} light for 6 h. After 3 d in darkness at 23 °C, the percentage of germination was scored. Germination was defined as an obvious protrusion of the radicle through the seed coat. Each experiment was repeated three times. Minimally 50 seeds were scored for each treatment of each genotype.

For the ABA assay, sterilized wild-type and mutant seeds from matched lots were directly sown on MS/G plates with different concentrations of ABA, and cold-treated at 4 °C in the dark for 2 d. Two days after the plates were moved to 23 °C, with 14/10 h photoperiod at 120 μmol m^{-2} s^{-1}, the percentage of seed germination was scored. Another 3 d later, the percentage of green seedlings was measured. Each experiment was performed with three replicates.

Root assays

The assay for auxin-induced lateral root formation was according to the protocol of Ullah *et al*. (2003) with modifications. Seedlings were grown on MS/G plates with the addition of 5 μM naphthylphthalamic acid (NPA), an auxin polar transport inhibitor, for 9 d. The NPA-pretreated seedlings were then transferred to MS/G plates with or without 0.1 μM 1-naphthaleneacetic acid (NAA, plantmedia, Dublin, Ohio), and grown vertically under a 14/10 h photoperiod at 120 μmol m^{-2} s^{-1}. The numbers of lateral roots per length of primary root were scored 4 d later. The standard error of the mean was calculated based on at least 10 seedlings.

The assay for auxin-induced adventitious root formation was according to the protocol of Kubo and Kakimoto (2000) with modifications. Seedlings were grown for 6 d in dim light (5 μmol m^{-2} s^{-1}). Hypocotyls were excised aseptically and transferred to Petri dishes containing 1× MS basal medium with Gamborg's vitamins, 1% (w/v) sucrose, 0.3% (w/v) phytoagar, pH adjusted to 5.7 with 1 N KOH, with or without 0.5 μM of 1-NAA. Excised hypocotyls were grown for additional 14 d under a 14/10 h photoperiod at 80 μmol m^{-2} s^{-1}. The number of adventitious roots per length of hypocotyl was scored under a dissecting microscope. The standard error of the mean was based on at least 10 excised hypocotyls.

Yeast split-ubiquitin assay

Interaction between RACK1A and GPA1 was assessed by complementation of split ubiquitin domain fusion in yeast (Fetchko and Stagljar, 2004). The entire open-reading frame of RACK1A was cloned and fused in frame to the C-terminal domain of ubiquitin in pMet AtMlo1 Cub R-Ura3 bait vector (Kim *et al*., 2002), kindly provided by Dr Ralph Panstruga (Max-Planck-Institüt für Züchtungsforschung-Köln, Germany). The construction of *GPA1* prey vector, host strain used, yeast transformation and selection, and identification of interaction have been described previously (Chen *et al*., 2003). Interaction between RACK1A and GPA1 was analysed based on yeast growth in selective media containing 5-fluoro-orotic acid. The MLO1-calmodulin interaction (pAtMlo1 NuI-Hv CaM3) (Kim *et al*., 2002) was used as positive controls, and the point mutant MLO1 (pAtMlo1 NuG)-calmodulin interaction was used as a negative control (Kim *et al*., 2002).

RACK1 *promoter:β-glucuronidase (*GUS*) constructs*

Genomic DNA 2740 bp upstream of the *RACK1A* start codon, 2215 bp upstream of the *RACK1B* start codon, and 1091 bp upstream of the *RACK1*C start codon was amplified by PCR, and cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA) respectively, then moved into the Gateway plant transformation destination vector pBGWFS7 (Karimi *et al*., 2002) by LR recombination reactions. The binary vector was transformed into *Arabidopsis thaliana* (Col-0 ecotype) by *Agrobacterium*-mediated transformation (Bechtold and Pelletier, 1998). There are 4113 bp, 43 bp, and 371 bp separating *RACK1A*, *RACK1B*, and *RACK1C* genes from their nearest neighbour genes, respectively. The exact promoter regions for *RACK1A*, *RACK1B*, or *RACK1C* genes could not be determined. However, the regions chosen contain typical promoter-proximal elements such as CAAT box and GC box that are necessary for gene transcription, and probably contain most *cis*-acting regulatory elements that are important for the regulation of transcription.

Six-day-old whole seedlings and floral organs from transgenic *Arabidopsis* plants containing *RACK1A*, *RACK1B*, or *RACK1C* promoter:*GUS* fusion constructs were sampled for GUS histochemical staining. Briefly, seedlings or excised tissues were vacuum infiltrated for 1 min with freshly-prepared staining solution [100 mM Na_2HPO_4 and NaH_2PO_4, pH 7.0, 10 mM EDTA, 0.1% (v/v) Triton X-100, 20% (v/v) methanol, and 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc; Rose Scientific Ltd., Edmonton, Alberta, Canada)]. After incubation at 37 °C in the dark overnight, seedlings or tissues were cleared in 70% ethanol, examined with a dissecting or compound microscope, and photographed with a digital camera.

Results

Arabidopsis arcA *is not a heterotrimeric G-protein β subunit as previously proposed*

The first plant RACK1 orthologue was originally cloned as an auxin-responsive gene, *arcA*, in tobacco BY-2 suspension cells in a differential screen for genes involved in auxin-mediated cell division (Ishida *et al*., 1993). Vahlkamp and Palme (1997) further found such an *arcA* homologue in *Arabidopsis*. arcA was proposed to be a putative heterotrimeric G-protein β subunit (Gβ) (Ishida *et al*., 1993), a subgroup of the large family of WD40 repeat proteins (van Nocker and Ludwig, 2003). In order to be consistent with the nomenclatures in recent publications (Chang *et al*., 2005; Giavalisco *et al*., 2005), and for clarity hereafter, the *Arabidopsis arcA* gene (At1g18080), and *arcA* gene product will be referred to as *RACK1A* and RACK1A (described below), respectively. Compared with the genuine *Arabidopsis* Gβ, AGB1, RACK1A shares 26% identity and 43% similarity (Fig. 1A). Molecular modelling data revealed that both Gβ and RACK1A have a very similar circular seven-bladed propeller structure (Fig. 2). However, RACK1A lacks the N-terminal helix of Gβ which is critical for Gγ interaction (Fig. 2). Therefore, although RACK1A is similar to Gβ in structure, it is unlikely that it can function as a Gβ.

This was tested further by analysing the interaction of RACK1A and the sole *Arabidopsis* heterotrimeric G-protein α subunit, GPA1, in a yeast split-ubiquitin assay.

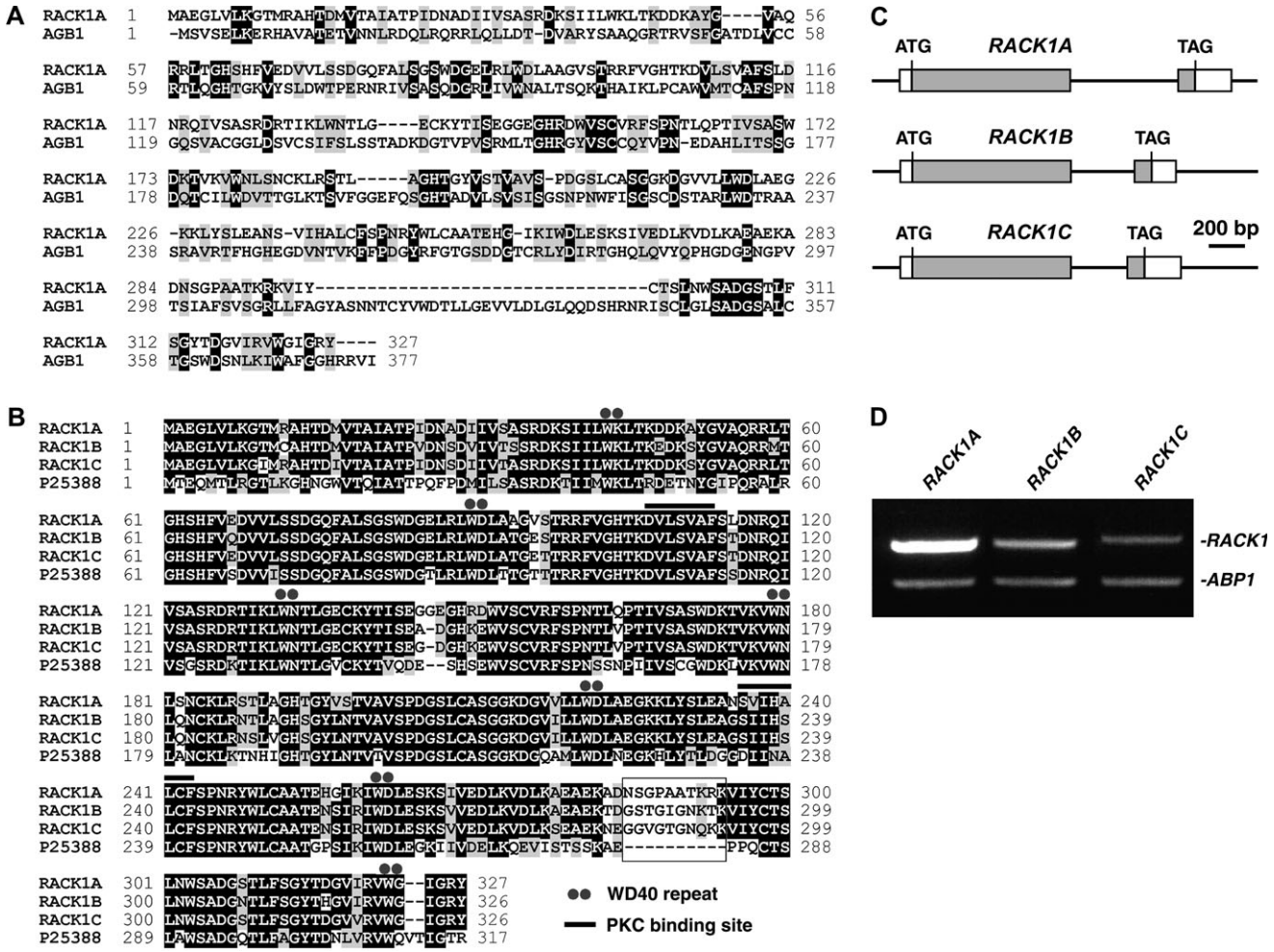

Fig. 1. RACK1 orthologues in *Arabidopsis*. (A) RACK1A (At1g18080, NP_173248) has similarity to *Arabidopsis* heterotromeric G-protein β subunit AGB1 (At4g34460, NP_195172). Amino acids that are identical or similar between RACK1A and AGB1 are shaded with black or grey, respectively. The sequence alignment was generated by the ClustalW multiple alignment of BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). (B) Protein sequence lineup between *Arabidopsis* RACK1 orthologues and mammalian RACK1. The *Arabidopsis* genome contains three RACK1 orthologues, At1g18080, At1g48630, and At3g18130. They are redesignated here as RACK1A, RACK1B, and RACK1C, respectively. The protein sequences of *Arabidopsis* RACK1 (NP_173248, NP_175296, and NP_188441) are compared with RACK1 (P25388) from human. The seven WD40 repeats are indicated by double dots, and the conserved PKC binding sites are delineated by a grey line above the sequence. The 10 aa insert near the C-terminus that is absent in the mammalian RACK1 is indicated with a box. The sequence alignment was generated by the ClustalW multiple alignment of BioEdit Sequence Alignment Editor. (C) Genes of *Arabidopsis RACK1*. *RACK1A*, *RACK1B*, and *RACK1C* have similar structures, each with two exons and one intron of similar size and position. (D) RT-PCR analysis of *Arabidopsis RACK1* transcripts. Total RNA was isolated from 10-d-old, light-grown seedlings. As a control, *Arabidopsis Auxin-binding protein 1* (*ABP1*) primers that amplify a 554 bp product were used in each PCR reaction with 28 cycles.

The assay that was established to test the interaction between GPA1 and AtRGS1, the sole Regulator of G-protein Signalling in *Arabidopsis* (Chen *et al*., 2003) was used. GPA1 and RACK1A were cloned into the N- and C-terminal domain of ubiquitin vector, respectively. As a control, the sole *Arabidopsis* heterotrimeric G-protein β subunit, AGB1, was used. The interaction was analysed based on yeast growth in selective media containing 5-fluoro-orotic acid. As shown in Fig. 3, AGB1 interacts with GPA1 whereas RACK1A does not interact in the yeast split-ubiquitin assay. Because RACK1A does not bind the sole *Arabidopsis* heterotrimeric G-protein α subunit in this configuration (Fig. 3), and because RACK1A lacks the critical N-terminal helix of Gβ required for binding to Gγ (Fig. 2), it was concluded that RACK1A is not a heterotrimeric G-protein β subunit.

RACK1 orthologues in Arabidopsis

The BLAST search of the completed *Arabidopsis* genome using the *Arabidopsis* arcA protein sequence (At1g18080, NP_173248) as a template revealed two homologues, At1g48630 (NP_175296) and At3g18130 (NP_188441). These three proteins are highly similar to each other (Fig. 1B). RACK1A shares approximately 87% identity and 94% similarity with encoded proteins at loci At1g48630 and At3g18130 and each shares 92% identity and 97%

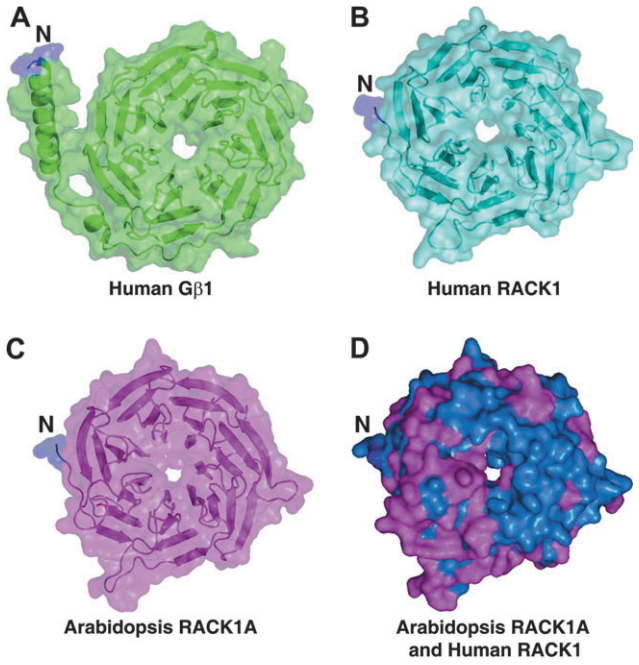

Fig. 2. Molecular models of RACK1A. RACK1A contains a seven-bladed propeller structure with one Trp-Asp 40 (WD40) unit constituting each blade, a conserved structure found in the heterotrimeric G-protein β subunit (Gβ), but RACK1A lacks the typical N-terminal helix that is present in Gβ. This N-terminus helix is required for interaction with Gγ helices. Homology models were built using the Insight II molecular modelling system. (A) Human Gβ1 (1GOT). Green with N-terminus (N) blue. (B) Human RACK1 (homology model). Cyan with N-terminus blue. (C) RACK1A (homologue model). Magenta with N-terminus blue. (D) RACK1A (cyan) overlayed with human RACK1, with identical residues coloured dark blue.

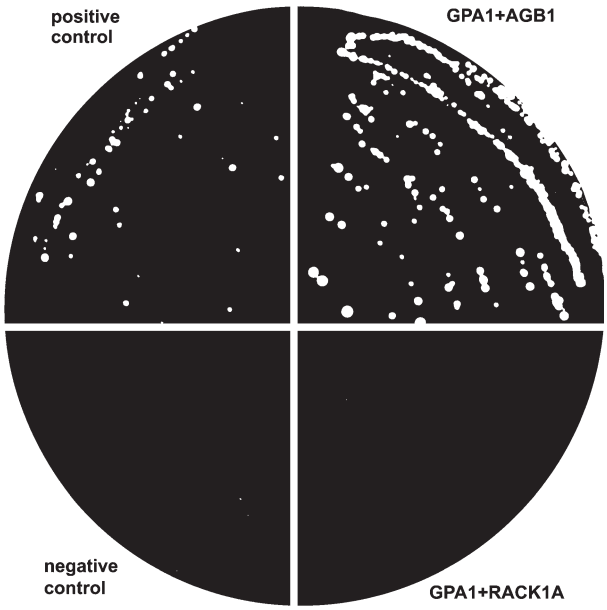

Fig. 3. RACK1A does not interact with the heterotrimeric G-protein α subunit GPA1 in a yeast split-ubiquitin, *in vivo* complementation assay. The interactions between RACK1A and GPA1 were analysed based on yeast growth in selective media. The MLO1-calmodulin interaction (pAtMlo1 NuI-Hv CaM3) was used as a positive control, and the point mutant MLO1 (pAtMlo1 NuG)-calmodulin interaction was used as a negative control (Kim *et al*., 2002). The interaction between AGB1 and GPA1 was also shown.

similarity to each other. These three *Arabidopsis* proteins are approximately 65% identical and 78% similar to mammalian RACK1. The number and position of WD40 repeats, and the PKC binding sites of mammalian RACK1 are also conserved in these *Arabidopsis* proteins (Fig. 1B). Therefore, hereafter, these three *Arabidopsis* proteins are designated RACK1A (At1g18080), RACK1B (At1g48630), and RACK1C (At3g18130), respectively. It should be noted that *RACK1A* was described as a single copy gene in *Arabidopsis* based on Southern blot analysis (Vahlkamp and Palme, 1997). These three genes also have very similar gene structure, consisting of two exons and one intron (Fig. 1C). Results of gel-based semi-quantitative RT-PCR indicated these three genes have different transcript levels in 10-d-old, light-grown seedlings, with *RACK1A* mRNA being the most predominant member (Fig. 1D).

RACK1 was originally identified in mammals as an anchoring protein for protein kinase C about a decade ago (Ron *et al*., 1994), but has now been recognized as a scaffold protein that is able to interact simultaneously with many signalling molecules to regulate diverse signal transduction pathways (see recent review by McCahill *et al*., 2002). RACK1 is highly conserved in a diverse range of species; besides mammals and plants, RACK1 orthologues were found in our analyses of fish, insects, and fungi EST databases and genomes (see supplementary Fig. 1 at JXB online). The phylogenetic analysis did not reveal a striking difference between *Arabidopsis* RACK1 and RACK1 proteins from other species (see supplementary Fig. 2 at JXB online). However, most plant RACK1 proteins are distinguishable from RACK1 proteins of non-plant species by an insert of 10-amino acid in the C-terminal portion of the protein (Fig. 1B; see supplementary Fig. 1 at JXB online). Moreover, *Arabidopsis* RACK1 proteins are also distinguishable from each other within this insert (Fig. 1B).

Within the current NCBI and Phytome databases, RACK1 homologues are present in other plant species such as rice, rape, soybean, tobacco, tomato, beech and alfalfa, as well as algae (see supplementary Fig. 3 at JXB online). Both tobacco and tomato have at least two RACK1 orthologues. Within plants, rape RACK1 (NCBI accession number Q39336) is mostly closed to RACK1A based on phylogenetic analysis (see supplementary Fig. 4 at JXB online).

RACK1 *expression*

To visualize tissue and organ distribution of *RACK1* expression, 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 each fused with a β-glucuronidase (*GUS*) reporter gene and transformed into *Arabidopsis* (Col-0)

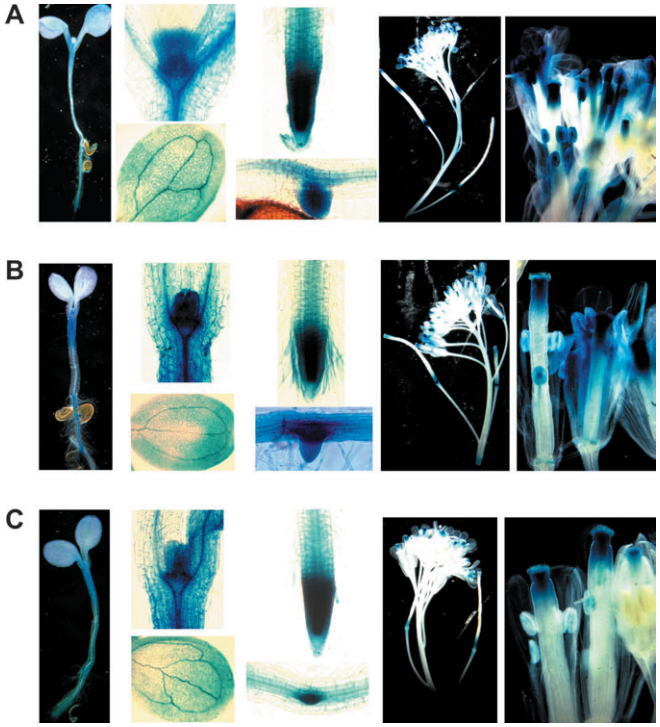

Fig. 4. Expression patterns of the *Arabidopsis RACK1* genes. Transgenic plants expressing *RACK1A:GUS* (A), *RACK1B:GUS* (B), and *RACK1C:GUS* (C) reporter gene were stained for GUS activity. Shown are GUS staining of 6-d-old, light-grown seedling with highlights in the shoot meristem, cotyledon, primary root meristem, and lateral root meristem, and of floral tissues.

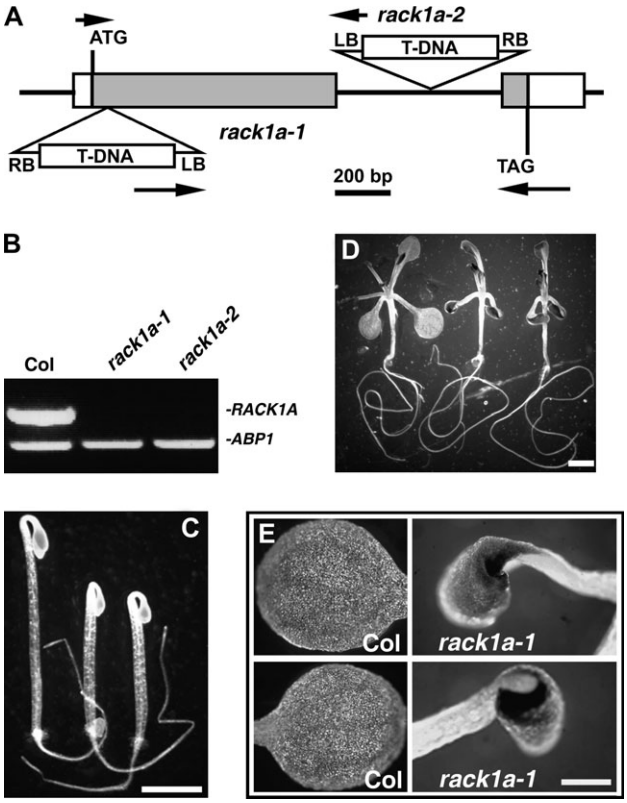

Fig. 5. T-DNA insertion mutant alleles of *RACK1A* in *Arabidopsis*. (A) T-DNA insertion sites in *RACK1A* gene. LB, T-DNA left border; RB, T-DNA right border. Grey boxes represent exons. The T-DNA insert is not drawn to scale. The arrows indicate the T-DNA left border primer, and the positions of *RACK1A* gene-specific primers used for mutant isolation. (B) RT-PCR analysis for *RACK1A* transcript. Total RNA was isolated from 10-d-old, light-grown seedlings. The *RACK1A* transcript was present in total RNA from wild-type *Arabidopsis* but absent in the *rack1a-1* and *rack1a-2* mutants. As a control, *Arabidopsis Auxin-binding protein 1 (ABP1)* primers that amplify a 554 bp product were added together with *RACK1A* primers in each PCR reaction with 28 cycles. (C) Two-day-old, dark-grown *rack1a* mutant seedlings. Left to right: wild type (Col-0), *rack1a-1, rack1a-2*. Scale bar, 2 mm. (D) Ten-day-old, light-grown *rack1a* mutant seedlings. Left to right: wild type (Col-0), *rack1a-1, rack1a-2*. Scale bar, 2 mm. (E) The shape of two cotyledons of 10-d-old, light-grown *rack1a* mutant seedlings. The cotyledons were taken from the Col-0 and *rack1a-1* seedlings in (D). Scale bar, 2 mm.

plants. *RACK1A:GUS*, *RACK1B:GUS*, and *RACK1C:GUS* displayed very similar expression patterns, and were expressed ubiquitously (Fig. 4A–C). The strongest GUS staining was detected in the meristems of the shoot, primary roots and lateral roots. In reproductive organs, GUS staining was detected in anthers, stigma, and the distal and proximal ends of siliques (Fig. 4A–C). The pattern of *RACK1A* expression in *Arabidopsis* based on the *RACK1A:GUS* transcriptional fusion reporter is largely consistent with that of *arcA* in tobacco plants analysed by northern analysis with one exception; *arcA* was detected at a relatively low level in the shoot tips of fully grown tobacco plants (Ishida *et al*., 1993).

The ubiquitous expression patterns of the *RACK1:GUS* transcriptional fusion reporter are also consistent with the microarray data from the Genevestigator *Arabidopsis thaliana* microarray database (http://www.genevestigator.ethz.ch/; Zimmermann *et al*., 2004). As shown in Fig. 4 (and in supplementary Fig. 5 at JXB online), *RACK1A*, *RACK1B*, and *RACK1C* transcripts showed very similar expression patterns in the various tissues and organs. However, based on the microarray data, the levels of the transcripts were distinguishable among *RACK1* genes, with *RACK1A* having highest transcript levels in this gene family (see supplementary Fig. 5 at JXB online). Such differences in transcript levels were also observed in 10-d-old, light-grown whole seedlings (Fig. 1D).

rack1a *mutants*

It has been shown by molecular modelling (Fig. 2) that the *Arabidopsis* arcA, RACK1A, is unlikely to function as a heterotrimeric G-protein β subunit and fails to interact with the Gα subunit in the yeast split-ubiquitin assay (Fig. 3), and the results from sequence and homologue research indicated that *Arabidopsis* arcA genes are mammalian RACK1 orthologues (Fig. 1). It is interesting to note that RACK1 is a single copy gene in other species whereas some plants have more than two RACK1 orthologues (see supplementary Figs 3 and 4 at JXB online). The focus here

was on the functional analysis of *RACK1A*, the most abundantly-expressed member of the *RACK1* gene family in *Arabidopsis*. T-DNA insertion-mutant alleles of *RACK1A* were isolated and characterized. *rack1a-1*, which harbours a T-DNA insertion in the first exon of the *RACK1A* gene, was obtained by screening deconvoluted pools of DNA from T-DNA transformed *Arabidopsis* (Col-0) plants as described in the Materials and methods. A second allele, *rack1a-2*, was obtained from the Salk Institute sequence-indexed insertion mutant collection (http://signal.salk.edu/cgi-bin/tdnaexpress), and the T-DNA insertion was confirmed to be in the first intron of the *RACK1A* gene (Fig. 5A). RT-PCR analysis using primers specific for *RACK1A* 5′- and 3′-UTR regions did not detect *RACK1* transcript in either allele (Fig. 5B), indicating that the T-DNA insertions probably result in loss-of-function alleles. This notion is supported by the facts that the T-DNA insertion in *rack1a-1* tagged a site 41bp downstream of the start codon, presumably from which no functional polypeptide or protein would be encoded, and that *rack1a-2* displayed identical phenotypes with *rack1a-1* mutant (Figs 5C, D, 6, 7).

rack1a mutants displayed pleiotropic phenotypes. In etiolated seedlings, the hypocotyls of *rack1a* mutants were shorter than those of the wild type (Fig. 5C). The short hypocotyl phenotype of *rack1a* mutants are similar to that of null mutants of *Arabidopsis* G-protein α subunit, *gpa1*, and β subunit, *agb1*, as reported previously (Ullah *et al*., 2001, 2003; Jones *et al*., 2003). However *rack1a* mutants had closed hooks whereas G-protein mutants had partially-opened hooks. In light-grown seedlings, the cotyledons of *rack1a* mutant seedlings were epinastic (Fig. 4D, E), resembling the phenotypes of some auxin mutants, such as *yucca* mutants (Zhao *et al*., 2001).

rack1a *mutants have reduced rate of rosette leaf production*

Mature *rack1a* mutant plants had a smaller rosette size than wild-type plants (Fig. 6C). Based on the number of days taken for bolting, *rack1a* mutants were late flowering, especially under short-day conditions (Fig. 6A, B). However, *rack1a* mutants are not classic flowering mutants, based on the well-established criteria for determining a flowering mutant (Weigel and Glazebrook, 2002). *rack1a* mutants are late flowering mutants in short-day conditions, because it took more than 120 d for *rack1a* mutants to bolt under short-day conditions, compared with approximately 80 d for wild-type plants. However, *rack1a* mutants produced about 27 rosette leaves in short-day conditions when bolting, compared with 41 rosette leaves for wild-type plants.

It was found further that *rack1a* mutants had defects in rosette leaf production. In the vegetative growth stage in prebolting plants, *rack1a* mutants produced approximately half the number of rosette leaves of wild-type plants (Fig. 6D), resulting in a decrease of the leaf production rate

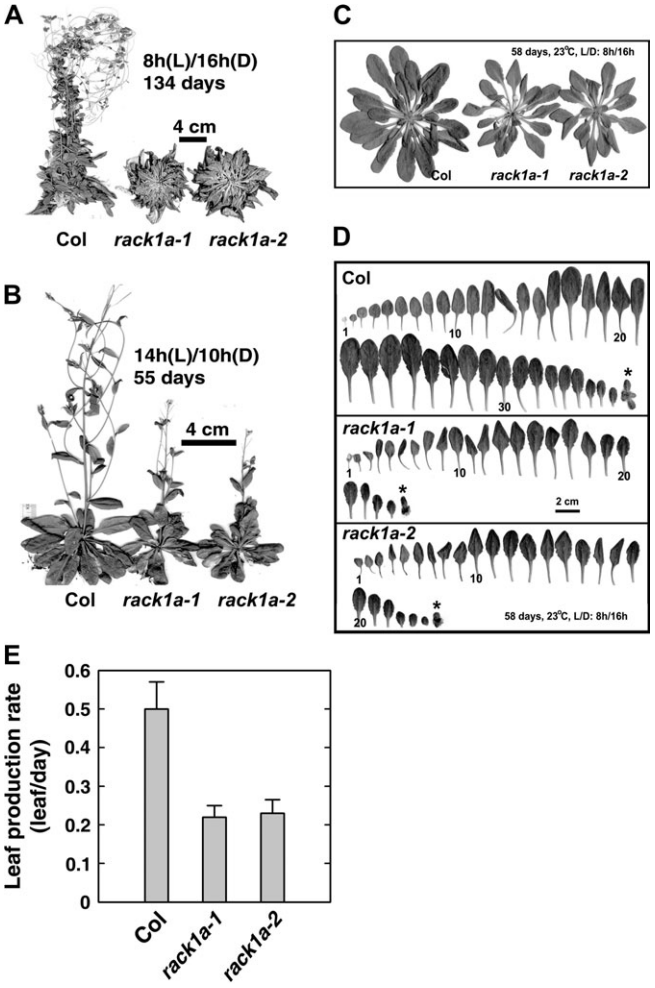

Fig. 6. *rack1a* mutants have reduced rate of rosette leaf production. (A) *rack1a* mutants grown in short-day conditions. Wild-type (Col-0) and *rack1a* mutants were photographed 134 d after being grown under 8/16 h photoperiod. (B) *rack1a* mutants grown in long-day conditions. Col-0 and *rack1a* mutants were photographed 55 d after being grown under 14/10 h photoperiod. (C) *rack1a* mutant phenotypes of preflowering plants. Col-0 and *rack1a* mutants were photographed 58 d after being grown under 8/16 h photoperiod. (D) Rosette leaves of *rack1a* mutant plants. The rosette leaves were taken from plants in (C). Numbers indicate the rosette leaf number starting from the one next to cotyledons. Asterisks indicate the leaf bud in shoot meristem. (E) Rate of rosette leaf production is reduced in *rack1a* mutants. The rates of rosette leaf production were calculated from plants grown under 8/16 h photoperiod.

by about 50% (Fig. 6E). These results indicate that leaf development in *rack1a* mutants is delayed. *rack1a* mutant leaves had near wild-type morphology although rosette leaves appeared to be slightly narrow and epinastic (Fig. 6D).

rack1a *mutants have altered sensitivities to hormones*

rack1a mutants displayed altered sensitivities to several plant hormones. The previously established germination assay (Ullah *et al*., 2002; Chen *et al*., 2004) was used to test the sensitivities of *rack1a* mutants to gibberellin (GA) and brassinosteroid. Wild-type and *rack1a* mutant seeds were pretreated with the GA biosynthesis inhibitor,

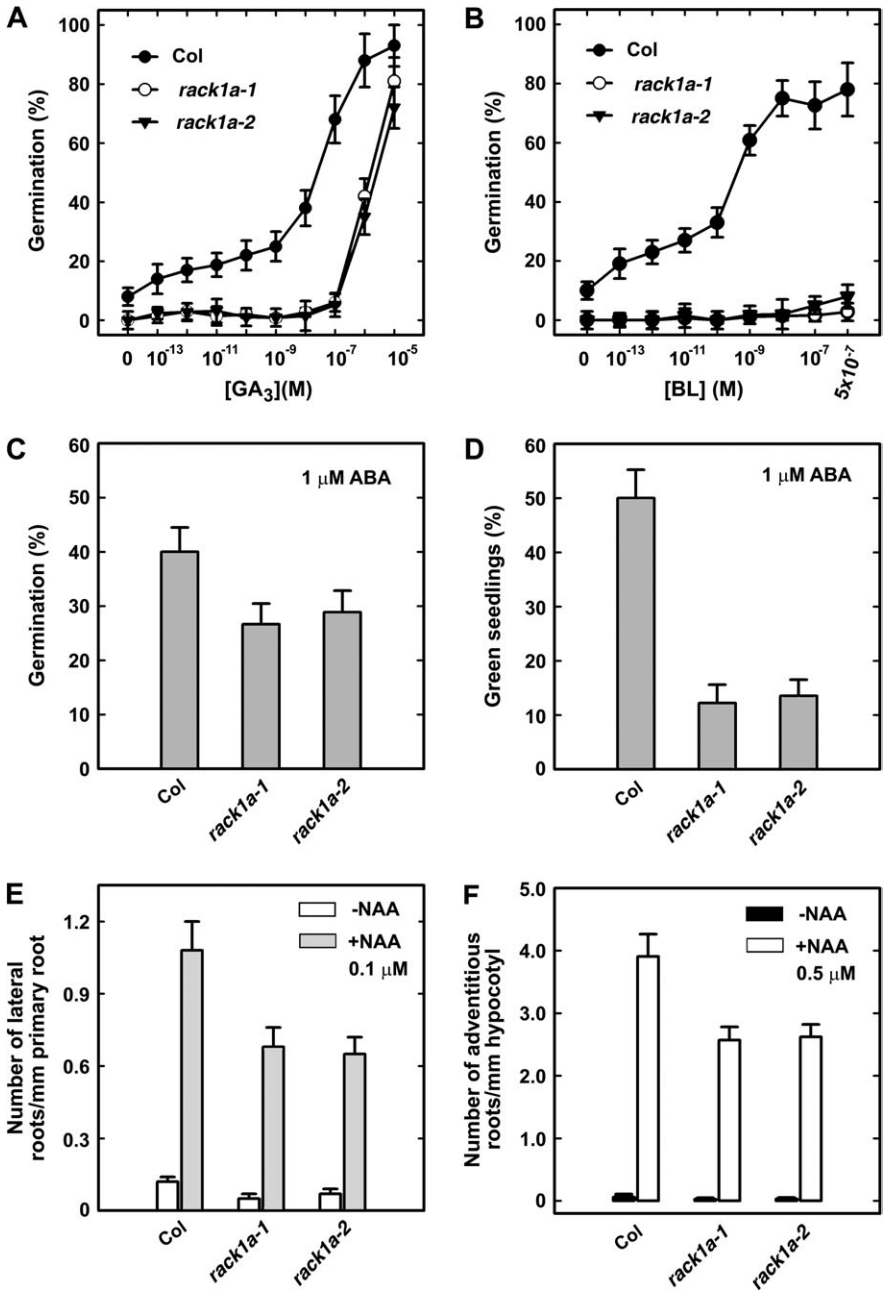

Fig. 7. *rack1a* mutants have altered responsiveness to hormones. (A) Responsiveness of *rack1a* mutant seeds to gibberellic acid (GA_3) in seed germination. (B) Responsiveness of *rack1a* mutant seeds to the brassinolide (BL) in seed germination. Sterilized wild-type (Col-0) and *rack1a* mutant seeds from matched seed lots in (A) and (B) were pretreated with 8 μM gibberellin (GA) biosynthesis inhibitor, paclobutrazol (PAC), to reduce endogenous GA. After 3 d at 23 °C in darkness, germination (protruded radicle) was scored and expressed as a percentage of total seeds. Shown are means of three replicates ±SE. (C) Responsiveness of *rack1a* mutants to abscisic acid (ABA) in seed germination. Col-0 and *rack1a* mutant seeds from matched seed lots were directly placed on MS medium with or without 1.0 μM ABA. After 2 d at 23 °C, the percentages of germination were scored. Shown are means of three replicates ±SE of at least 50 seeds each replicate of each genotype. (D) Responsiveness of *rack1a* mutants to abscisic acid (ABA) in early seedling development. Seeds in (C) were grown for an additional 3 d, and the percentages of green seedlings were scored. (E) Responsiveness of *rack1a* mutants to auxin-induced lateral root formation. Col-0 and *rack1a* mutant seedlings were grown on 5 μM naphthylphthalamic acid (NPA) for 9 d, transferred to MS medium with or without 0.1 μM 1-NAA. The numbers of lateral roots were scored 4 d later. Shown are the numbers of lateral roots per length of primary root. The standard error of the mean is based on at least 10 seedlings. (F) Responsiveness of *rack1a* mutants to auxin-induced adventitious root formation. Col-0 and *rack1a* mutant seedlings were grown for 6 d under dim light of 5 μmol m^{-2} s^{-1}. Hypocotyls were excised and transferred to MS medium with or without 0.5 μM of 1-NAA, and grown for an additional 14 d under 14/10 h photoperiod at 80 μmol m^{-2} s^{-1}. Shown are the numbers of adventitious roots per length of hypocotyls. The standard error of the mean is based on at least 10 excised hypocotyls.

paclobutrazol (PAC), to reduce endogenous GA, then sown on plates supplemented with defined concentrations of gibberellic acid (GA_3) or brassinolide (BL). As shown in Fig. 7, *rack1a* mutant seeds were less responsive to exogenous GA_3 and BL.

The assay of ABA-induced inhibition of seed germination and early seedling development was used to measure the responsiveness of *rack1a* mutants to ABA. It was found that the ABA responsiveness was enhanced in *rack1a* mutants in both seed germination and early seedling development (Fig. 7C, D). It was found that when exogenous ABA concentration was lower than 0.5 μM, both wild-type and *rack1a* mutant seeds could germinate and develop into seedlings, and no significant difference was observed. When ABA was applied at concentrations higher than 3.0 μM, germination and early seedling development was equally inhibited for both wild-type and *rack1a* mutants. Therefore, it appeared that such a difference in ABA responsiveness between wild-type and *rack1a* mutants occurred in a narrow range of ABA concentrations. It was also found that *rack1a* mutants were more sensitive to ABA in the arrest of early seedling development than in the inhibition of seed germination (Fig. 7C, D).

Because *arcA*, a tobacco RACK1 homologue, was originally identified as an auxin-induced gene, auxin sensitivities of *rack1a* mutants were examined further. Previously-established lateral and adventitious root formation assays (Ullah *et al*., 2003) were used to measure the responsiveness of *rack1a* mutants to auxin. Wild-type and *rack1a* mutant seedling were pretreated with the auxin transport inhibitor, naphthylphthalamic acid (NPA), then moved to medium containing 0.1 μM 1-NAA. As shown in Fig. 7E, *rack1a* mutants responded to exogenous 1-NAA, but produced fewer lateral roots compared with wild type. Excised hypocotyls were used for the adventitious root formation assay. The advantage of this assay is that there are no pre-existing root primordia in hypocotyls. It was found that, in response to exogenous 1-NAA, the excised hypocotyls of *rack1a* mutants produced about 40% fewer adventitious roots than the wild type (Fig. 7F). These results indicate that *rack1a* mutants are less sensitive to auxin.

In summary, *rack1a* mutants are less sensitive to GA and BR in seed germination, more responsive to ABA in seed germination and early seedling development, and less responsive to auxin in root formation. Based on the results of cytokinin-mediated root elongation inhibition, and ethylene-induced triple responses, *rack1a* mutants had near wild-type responsiveness to cytokinin and ethylene (data not shown).

Discussion

Mammalian RACK1 serves as a scaffold protein for a wide range of signalling molecules including kinases and membrane-bound receptors. RACK1 is a WD-repeat family protein and is predicted to have a β-propeller architecture with seven blades, similar to the heterotrimeric G-protein β subunit. RACK1 is evolutionally conserved in a wide range of species. In plants, the first RACK1 orthologue was cloned over a decade ago (Ishida *et al*., 1993). Recently, Chang *et al*. (2005) found that *Arabidopsis* orthologues of RACK1 associate with the 40S subunit of cytosolic ribosomes. In another independent study, Giavalisco *et al*. (2005) found that *Arabidopsis* RACK1 co-migrated with the 80S ribosomes suggesting a possible association with the 80S ribosomes. These studies providing the first biochemical evidence that the function of RACK1 is conserved in plants, because in yeasts and mammals, it has been shown that RACK1 associates with ribosomes (Link *et al*., 1999; Shor *et al*., 2003; Ceci *et al*., 2003; Nilsson *et al*., 2004; Sengupta *et al*., 2004). In the present report, the first direct genetic evidence that *Arabidopsis* RACK1 has a role in hormonal responses and affect multiple developmental processes is provided.

It was found that loss-of-function of one member of the *Arabidopsis RACK1* gene family, *RACK1A*, conferred developmental defects in both seedlings and mature plants, including hypocotyl and cotyledon growth, and rosette leaf production. *rack1a* mutants displayed reduced sensitivities to GA and BR in germination response, hypersensitivity to ABA in seed germination and early seedling development, and reduced sensitivity to auxin in adventitious and lateral root formation.

The involvement of RACK1A in multiple hormonal signalling is consistent with a scaffold function, and indicates that RACK1A may interact with a key signalling component in these signalling pathways. For example, *rack1a* mutants share several phenotypes with *Arabidopsis* heterotrimeric G-protein mutants, *gpa1* and *agb1*, such as reduced sensitivity to GA and BR in germination, increased sensitivity to ABA in germination, and reduced sensitivity to auxin in root formation (Ullah *et al*., 2002, 2003). It is possible that RACK1 and the heterotrimeric G-proteins may constitute a signalling complex to regulate these diverse responses. In mammals, RACK1 can physically interact with the Gβγ dimer (Dell *et al*., 2002; Chen S *et al*., 2004*a*, *b*), and regulate some specific processes mediated by the Gβγ dimer. Recently, it has been found that RACK1 protein levels are down-regulated in a heterotrimeric G-protein α subunit mutant in rice (Komatsu *et al*., 2005), implying that plant RACK1 may be indeed regulated by G-proteins, or vice versa. However, RACK1A may have roles in other developmental processes independent of the heterotrimeric G-protein complex. For example, *rack1a* mutants have reduced rosette leaf production whereas the *gpa1* and *agb1* mutants appeared to be wild type (data not shown). It is also known that mammalian RACK1 binds to the Gβγ dimer and has regulatory roles in some processes, such as the inhibition Gβγ-mediated activation of phospholipase C β2 and adenylyl cyclase II, but has

no effect on many other functions of Gβγ (Chen S *et al*., 2004*a*).

Alternatively, because *rack1a* mutants have a pleiotropic phenotype and RACK1 has been found to be associated with the 40S subunit of ribosomes in *Arabidopsis* (Chang *et al*., 2005), it is possible that RACK1A may have a general role in translational regulation.

A dual role of RACK1 in signalling and translational regulation has already been recognized in mammals and yeasts. RACK1 has been found to be a potential physical and functional linker between various signalling molecules (Nilsson *et al*., 2004). So far, RACK1 and its orthologues have been found to be associated with the 40S small ribosomal subunit in human (Link *et al*., 1999), yeasts (Inada *et al*., 2002; Shor *et al*., 2003), *Arabidopsis* (Chang *et al*., 2005), and algae (Manuell *et al*., 2005). A recent cryo-electron microscopy (Cryo-EM) study of the 80S ribosome located RACK1 on the head region of 40S subunit, in the immediate vicinity of the mRNA exit site of the ribosome (Sengupta *et al*., 2004). One of the signalling molecules that interact with RACK1 on the ribosome is the activated PKC (Ceci *et al*., 2003). Through its interaction with RACK1, activated PKC is able to phosphorylate a translation initiation factor eIF6 and dissociate the latter from free 60S ribosomal subunit. This release of eIF6 from the 60S ribosomal subunit allows the joining of 40S and 60S subunits to form the functional 80S ribosome and hence activate the translation. Another signalling protein Src kinase was also found to bind to ribosome-bound RACK1 (Sengupta *et al*., 2004). Furthermore, RACK1 was found to be present both in a ribosome-bound and ribosome-non-bound forms in humans (Ceci *et al*., 2003), and yeasts (Shor *et al*., 2003; Baum *et al*., 2004). It is not clear how many of the RACK1 interacting partners identified so far bind to ribosome-bound RACK1 and how many of them bind to non-ribosome-bound RACK1.

Despite three conserved RACK1 paralogues in *Arabidopsis*, disruption of a single gene, *RACK1A*, from this gene family is sufficient to cause defects in many developmental processes. This indicates that RACK1A may have a distinguishable function from the other two members, or that RACK1A is required for the function of the other two members. Because there are three RACK1 proteins in *Arabidopsis*, it is possible that these proteins can form heterodimers through the WD40 protein–protein interaction domains. Such interactions between WD40 repeat proteins have already been described. For example, bovine Gβγ binds at least three WD40 repeat proteins including RACK1 (Dell *et al*., 2002). Mammalian RACK1 binds to WD40 repeat protein FAN, a factor associated with neutral sphingomyelinase activation (Tcherkasowa *et al*., 2002). Two TATA-binding protein (TBP)-associated factors (TAF) TAF72 and TAF73, both of which are WD40 repeat proteins, co-immuoprecipitated in yeast (Mitsuzawa *et al*., 2001). Both RACK1A and RACK1B were found to be associated with the 40S subunit of cytosolic ribosomes in *Arabidopsis* (Chang *et al*., 2005). Such a heterodimerization among WD40 repeat proteins could have an important role for integrating signals from different cellular processes (Chen *et al*., 2004*b*), and could be required for the RACK1 function in *Arabidopsis*.

Mammalian RACK1 is known to interact with PKC. While no obvious PKC orthologue, the classic RACK1 interacting partner, is found in the sequenced plant genomes, it is possible that plant RACK1 interacts with a protein kinase that may share some property with PKC, such as regulation by lipids. For example, some members of the large plant AGC protein kinase family (S6 kinase, IRE, NDR) have a PKC-like kinase extension domain (Bogre *et al*., 2003).

In summary, these genetic data support that RACK1A mediates hormonal responses, and has a regulatory role in multiple developmental processes. RACK1A may either represent a critical integrative point for hormone signalling pathways, or is an essential component for translational regulation for genes that are involved in these hormone responses or developmental processes, or both. At this point, neither possibility could be ruled out. Future studies should focus on the identification of other RACK1 interacting partners and of RACK1 affected genes in plants.

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