Running Title: RACK1 and protein translation

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**Research Category:** Signal Transduction and Hormone Action
Involvement of Arabidopsis RACK1 in Protein Translation and Its Regulation by Abscisic Acid 1[w]

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ABSTRACT

Earlier studies have shown that RACK1 functions as a negative regulator of ABA responses in Arabidopsis, but the molecular mechanism of the action of RACK1 in these processes remains elusive. Global gene expression profiling revealed that approximately 40% of the genes affected by ABA treatment were affected in a similar manner by the rack1 mutation, supporting the view that RACK1 is an important regulator of ABA responses. On the other hand, co-expression analysis revealed that >80% of the genes co-expressed with RACK1 encode ribosome proteins, implying a close relationship between RACK1’s function and the ribosome complex. These results implied that the regulatory role for RACK1 in ABA responses may be partially due to its putative function in protein translation, which is one of the major cellular processes that mammalian and yeast RACK1 is involved in. Consistently, all three Arabidopsis RACK1 homologous genes, namely RACK1A, RACK1B and RACK1C, complemented the growth defects of the S. cerevisiae cpc2/rack1 mutant. In addition, RACK1 physically interacts with Arabidopsis Eukaryotic Initiation Factor 6 (eIF6), whose mammalian homologue is a key regulator of 80S ribosome assembly. Moreover, rack1 mutants displayed hypersensitivity to anisomycin, an inhibitor of protein translation, and displayed characteristics of impaired 80S functional ribosome assembly and 60S ribosomal subunit biogenesis in a ribosome profiling assay. Gene expression analysis revealed that ABA inhibits the expression of both RACK1 and eIF6. Taken together, these results suggest that RACK1 may be required for normal production of 60S and 80S ribosomes and that its action in these processes may be regulated by ABA.
INTRODUCTION

Living organisms need to maintain their cellular homeostasis while dealing with various environmental stresses. This process involves multiple regulatory mechanisms including the regulation of protein translation. Protein translation is regulated at three steps: initiation, elongation and termination (Scheper et al., 2007). Most signaling events regulate translation at the initiation stage (Sonenberg and Hinnebusch, 2009). Translation initiation is a complex multi-reaction process. Briefly, in mammalian cells, a pre-initiation complex (containing the 40S ribosome subunit) first binds to the 5’-cap of target mRNA and scans for the AUG start condon. Subsequently, the 60S subunit joins to assemble a functional 80S ribosome complex, which is ready to accept the appropriate aminoacyl-tRNA and form the first peptidyl bond and thereby initiate translation elongation (Sonenberg and Hinnebusch, 2009).

Early studies in plants identified a variety of abiotic stresses, including drought, cold and salt stresses, that could lead to inhibition of global protein translation (Ben-Zioni et al., 1967; Aspinall, 1986; Kawaguchi and Bailey-Serres, 2002; Kawaguchi et al., 2003). Although the regulation of gene expression at the translation initiation stage plays an important role in adaptation of organisms to various environmental stresses (Brostrom and Brostrom, 1998; Yamasaki and Anderson, 2008), there has also been one report on the effect of stress conditions on regulating protein translation at the elongation and termination stages (Shenton et al., 2006). In addition, ribosome biogenesis, one of the major energy consuming cellular processes, is also under tight regulation in response to environmental signals (Martin et al., 2004). Despite the widely observed direct regulation of environmental stress on protein translation in plants, the identity of the specific molecular players that link stress responses, the stress-signaling hormone abscisic acid (ABA), and the regulation of global translation has remained elusive.

Mammalian RACK1 was initially identified as a Receptor for Activated C Protein Kinase 1 (Ron et al., 1994) and later found to interact with numerous proteins involved in various signal transduction pathways (reviewed by McCahill et al., 2002; Sklan et al., 2006; Guo et al., 2007). In plants, RACK1 homologues appear to play multiple roles. The first RACK1 homologue was initially identified as an auxin-responsive gene in tobacco BY-2 cells (Ishida et al., 1993) and a related gene was subsequently isolated from alfalfa (McKhani et al., 1997). The tobacco RACK1 homologue was found to mediate cell cycle arrest triggered by salicylic acid and UV...
irradiation (Perennes et al., 1999). More recently, RACK1 was identified as a component of the plant 40S ribosome subunit (Chang et al., 2005; Giavalisco et al., 2005), and as an interacting partner within a rice Rac1 immune complex that mediates the innate immune response (Nakashima et al., 2008). The crystal structure of the Arabidopsis RACK1A protein was also recently resolved (Ullah et al., 2008).

In earlier studies, we found that a loss-of-function mutation in one of the three RACK1 genes in Arabidopsis, RACK1A, conferred altered responses to multiple plant hormones (Chen et al., 2006). Later, we provided evidence to support the view that the three RACK1 genes regulate plant development in a manner of unequal genetic redundancy (Guo and Chen, 2008). More recently, we found that RACK1 genes work redundantly as negative regulators of ABA responses and mediate stress responses (Guo et al., 2009a). Interestingly, although Arabidopsis possesses homologues of both mammalian RACK1 and heterotrimeric G-proteins, the plant homologues appear to act through a mechanism that is distinct from their counterparts in mammals (Guo et al., 2009b).

One of the best characterized roles for RACK1 in Arabidopsis is acting as a regulator of ABA and abiotic stress responses (Guo et al., 2009a), and in this study, we investigate its molecular mechanism of action. Through a combination of molecular, genetic, biochemical and pharmacological approaches, we show that RACK1 is involved in protein translation and 60S ribosome biogenesis, and that its action in these processes may be regulated by ABA. These findings provide new insights into the molecular mechanism of action of RACK1 in modulating ABA responses, and into the regulation of protein translation, a fundamental cellular process in plants.

RESULTS

Many genes are co-regulated by ABA and rack1 mutation

To characterize the role of RACK1 in ABA responses in more detail, a global gene expression profiling assay was conducted using rack1a rack1b double mutants. We specifically looked for genes that are up- or down-regulated ≥2.0 fold in the rack1a rack1b mutant background, and
compared these responses with the list of genes that are up- or down-regulated by ABA treatment in wild-type (Col-0) background. Three biological replicates were used for each sample. This analysis identified a total of 1,254 genes that were up-regulated ≥2.0-fold in the rack1a rack1b mutant plants and a total of 1,312 genes that were down-regulated (Figure 1).

Under our experimental conditions, a total of 968 genes were up-regulated, and 1,253 genes were down-regulated, by ABA treatment in the wild-type plants (Figure 1). Functional categorization of the genes that were differentially expressed in rack1a rack1b mutant background revealed a relatively high percentage of genes whose predicted biological function is involved in stress responses (4.7% of up-regulated genes and 4.6% of down-regulated genes), in response to abiotic and biotic stimulus (4.1% of up-regulated genes and 4.8% down-regulated genes), in protein metabolism (6.7% of up-regulated genes and 5.8% of down-regulated genes), and in developmental processes (4.3% of up-regulated genes and 4.9% down-regulated genes) (Figure S1), suggesting an important role for RACK1 genes in mediating these biological processes.

Furthermore, when the gene profile between rack1a rack1b and Col after ABA treatment was compared, we found that the expression of many genes that are known to respond to stress (6.952%), abiotic or biotic stress stimulus (6.245%) were further up-regulated in rack1a rack1b mutant (Figure 2; Figure S2; Table S1). This coincides with the earlier observation that rack1a rack1b mutants displayed enhanced physiological response to ABA (Chen et al., 2006; Guo et al., 2009a).

Interestingly, we found that approximately 41% (400 out of 968) of the ABA-up-regulated genes in wild-type plants were also up-regulated in the rack1a rack1b double mutant background even in the absence of ABA treatment (Figure 1A, Table S2). Similarly, we found that approximately 41% (519 out of 1253) of the ABA-down-regulated genes in wild-type plants were also down-regulated in the rack1a rack1b mutant plants without ABA treatment (Figure 1A, Table S2). In contrast, only seven ABA-down-regulated genes were up-regulated in rack1a rack1b mutant and 26 ABA-up-regulated genes were down-regulated in the rack1a rack1b background.

Consistently, when analyzing all the significantly up- and down-regulated genes (genes whose expression level was significantly changed at 95% confidence interval with no regard to fold-change), the changes in gene expression resulting from the rack1a rack1b mutation and
from ABA treatment also showed considerable similarity (Figure 1B). Quantification of this similarity using the Pearson correlation coefficient showed moderate correlation (p=0.494) between the effect of the *rack1a rack1b* mutation and ABA treatment. These results indicate an important role for RACK1 in mediating ABA-regulated transcriptional responses.

**Co-expression analysis of RACK1 genes**

To gain further insights into the biochemical/molecular function of RACK1 in Arabidopsis, we performed global co-expression data analysis (PRIME, [http://prime.psc.riken.jp/](http://prime.psc.riken.jp/)) to identify genes that are co-expressed with all three *RACK1* genes. Surprisingly, we found that more than 80% (128 out of 154) of the genes that are co-expressed with *RACK1* encode ribosomal proteins (Figure S3 and Table S3), implying a potential relationship between *RACK1* function and the ribosome complex. RACK1 proteins were previously reported to be physically associated with ribosomes in Arabidopsis (Chang et al., 2005; Giavalisco et al., 2005) and one of the major functions of RACK1 in mammalian cells and yeast is to regulate translation initiation at the stage of ribosome assembly (Ceci et al., 2003; Shor et al., 2003). These findings prompted us to examine the function of Arabidopsis *RACK1* in ribosome assembly and translation initiation, and the relationship, if any, between such a role and cellular responses to ABA.

**Arabidopsis RACK1 complements the S. cerevisiae cpc2/rack1 mutant**

Because a large amount of information has been accumulated about the molecular function of RACK1 in mammals and yeast, and many of the signaling pathways and cellular processes that RACK1 is involved in appear to be conserved across eukaryotic kingdoms (McCahill et al., 2002; Sklan et al., 2006; Guo et al., 2007), we asked whether the Arabidopsis *RACK1* genes can rescue the yeast *cpc2/rack1* mutant phenotypes.

Diploid *S. cerevisiae* strains of the genetic Σ1278b background are dimorph and develop from single spherical yeast cells to filament-like pseudohyphal cells under nitrogen starvation.
conditions (Gimeno et al., 1992). A homozygous deletion of CPC2 results in the loss of pseudohyphae development under nitrogen starvation condition and formation of a smooth-border round colony (Figure 3A, B, Valerius et al., 2007). We first expressed the full length of yeast CPC2 gene in the cpc2 mutant using the yeast expression vector p424MET25 (Mumberg et al., 1994) and observed the restoration of pseudohyphae growth (Figure 3C). With this validated system, we found that when any of the three Arabidopsis RACK1 genes was expressed in the yeast cpc2 diploid mutant background, the transformant regained the ability to produce the filament-like structures (pseudohyphae) (Figure 3D-F). These results demonstrated that the Arabidopsis RACK1 genes are functional equivalent to the yeast CPC2/RACK1. In an earlier study, Gerbasi et al. (2004) demonstrated that the mammalian RACK1 is also a functional orthologue of the yeast CPC2 gene. In agreement with this genetic data, both the amino acid sequences (Chen et al., 2006) and crystal structure (Ullah et al., 2008) of RACK1 are also highly conserved in different eukaryotic organisms. Taken together, these results supported the view that some functions of RACK1 gene are likely to be conserved in mammals, yeast and Arabidopsis. In this study, we focused on the possible role of Arabidopsis RACK1 in ribosome assembly and protein translation.

RACK1 physically interacts with Eukaryotic Initiation Factor 6 (eIF6)

In mammalian ribosomes, it has been proposed that RACK1 acts as a scaffold protein to bring together activated protein kinase C (PKC) and Eukaryotic Initiation Factor 6 (eIF6). eIF6 is then phosphorylated by PKC and subsequently dissociates from the 60S ribosome subunit, which allows the 40S and 60S ribosome subunits to form the functional 80S ribosome (Ceci et al., 2003). Despite the lack of obvious PKC homologues in the Arabidopsis genome, two homologues of eIF6, encoded by loci At3g55620 (hereafter named as eIF6A) and At2g39820 (hereafter named as eIF6B), are present, which led us to test whether physical interaction can be detected between the Arabidopsis RACK1 and eIF6 proteins.

When these interactions were tested in a yeast two-hybrid system, each of the three RACK1 proteins was found to physically interact with each of the two eIF6 proteins (Figure 4A). To establish whether the physical interaction also occurs in plant cells, a bimolecular
fluorescence complementation system (BiFC) (Citovsky et al., 2006) was used in combination with an Arabidopsis leaf mesophyll protoplast transient expression assay (Yoo et al., 2007). Again, positive interactions were detected for each pair of RACK1 and eIF6 proteins (Figure 4B). The interaction was primarily detected in the cytoplasm and nucleus, which is consistent with the respective subcellular localization of each protein (Figure S4) and resembles the subcellular localization patterns of their mammalian counterparts (Ceci et al., 2003). To determine whether ABA could influence the interaction between RACK1 and eIF6, the BiFC experiment was also conducted in the presence of 50 μM ABA. No obvious difference was observed for the YFP signal (Figure S5), implying that the interaction between RACK1 and eIF6 is not ABA dependent.

**eIF6 homologues in Arabidopsis**

The proteins predicted to be encoded by the two Arabidopsis eIF6 genes share 86% sequence similarity at the amino acid level and are 72% identical (Figure S6A, B). The protein sequence of eIF6A also appears to be highly conserved within the plant kingdom. Moreover, Arabidopsis eIF6A shares about 73% identity and 85% similarity with its homologues in humans (*Homo sapiens*) and yeast (*Saccharomyces cerevisiae*) (Figure S6A, B) whereas eIF6B is somewhat more divergent and shares about 60% identity and 78% similarity to its homologues in human and yeast. RT-PCR analysis revealed that the expression of eIF6A is ubiquitous across various tissues and organs in Arabidopsis whereas eIF6B is only expressed in flower buds (Figure 5A). These results are largely consistent with the in silico data from the Genevestigator *Arabidopsis thaliana* microarray database (Zimmermann et al., 2004) (Figure 5B). The higher amino acid sequence homology of eIF6A to its counterparts in other organisms, as well as its ubiquitous expression pattern, implies that eIF6A may be the predominant functional copy of the two eIF6 genes.

To further study the function of eIF6 genes in Arabidopsis, we obtained two independent T-DNA insertional alleles for each eIF6 gene, all in the Columbia (Col-0) ecotype background. The two mutant alleles of eIF6A were designated as *eif6a-1* (GABI_817H01) and *eif6a-2* (*emb* 1624, Syngenta) and the two mutant alleles of eIF6B as *eif6b-1* (SALK_017008) and *eif6b-2*
RT-PCR analysis indicated that \textit{eif6b-1} allele is a full-transcript null allele whereas \textit{eif6b-2} is a knock-down allele (Figure 6B). All insertion positions were validated by DNA sequencing.

When we examined the phenotypes of these mutant alleles, we were unable to recover plants homozygous for either the \textit{eif6a-1} or \textit{eif6a-2} alleles. We found that within the siliques of the \textit{eif6a\textsuperscript{+/-}} parent plants, the ratio of white seeds (containing developmentally-halted embryos) to green seeds (containing normally developing embryos) was approximately 1:3 (n=500), indicative of a homozygous embryo-lethal outcome (Figure 6C). By examining the white seeds microscopically, we found that the development of the embryo was arrested at the globular stage (Figure 6C). These results are consistent with the fact that \textit{eif6a-2/emb1624} allele was originally identified in a collection of mutants defective in embryo development (Tzafrir et al., 2004). We have observed such defects in both T-DNA insertional alleles of \textit{eIF6A} gene. The \textit{eif6b-1} and \textit{eif6-2} alleles, on the other hand, did not display any apparent developmental defects (Figure 6D), which supports the view that \textit{eIF6A}, but not \textit{eIF6B}, may be the predominant member of the small \textit{eIF6} gene family in Arabidopsis.

\textbf{rack1 mutants are hypersensitive to anisomycin, an inhibitor of protein translation}

Our co-expression analysis indicated that the majority of genes co-expressed with RACK1 encode ribosomal proteins (Figure S3 and Table S3), and we have shown that RACK1 physically interacts with eIF6 (Figure 4), a key protein regulating functional 80S ribosome assembly in mammals. We therefore sought additional evidence that might support a role for RACK1 in protein translation. Anisomycin is a drug that inhibits peptide bond formation, presumably by competing with amino acids for access to the peptidyltransferase centre (A-site, the entry point of amino acid-charged tRNA) (Meskauskas et al., 2005). This drug has been used in other eukaryotic cells to functionally implicate specific proteins in the translation process (Nelson et al., 1992; Spence et al., 2000; Regmi et al., 2008), although no study of the effect of anisomycin in plants has been reported. When we used Arabidopsis primary root elongation as the metric to assay the effect of anisomycin on plant growth, we established that the half-maximal inhibitory concentration (IC\textsubscript{50}) of anisomycin for root elongation is \(\sim 5 \mu\text{M}\) (Figure 7A), and that the growth of the primary root was completely halted by 15 \(\mu\text{M}\) anisomycin (Figure 7A). We then compared the sensitivity of wild-type and \textit{rack1} mutant plants to anisomycin. Because \textit{rack1a}
single mutants and \(\textit{rack1a rack1b}\) and \(\textit{rack1a rack1c}\) double mutants already displayed shorter primary roots without any treatment (Figure 7C; Guo and Chen, 2008), we used the percentage of root growth reduction to compare the relative sensitivity of each genotype to anisomycin. We found that the \(\textit{rack1a}\) single and \(\textit{rack1a rack1b}\) and \(\textit{rack1a rack1c}\) double mutants all displayed hypersensitivity to anisomycin (Figure 7A, B). The \(\textit{rack1b rack1c}\) double mutants also displayed hypersensitivity to anisomycin, but to a lesser extent (Figure 7B). Among all the genotypes examined, the \(\textit{rack1a rack1b}\) double mutants displayed the greatest hypersensitivity to anisomycin (Figure 7A, B). These results are consistent with a role for RACK1 in protein translation in Arabidopsis.

**RACK1 might be involved in functional 80S ribosomal subunit assembly and 60S ribosome biogenesis**

To assess the role of RACK1 in protein translation \textit{in vivo}, we compared the polyribosome profile of extracts prepared from wild type (Col-0) and \(\textit{rack1a rack1b}\) double mutant plants. This assay provides a relative measurement of efficiency in mRNA translation, as controlled by ribosome biogenesis and assembly (Lorsch, 2007). The profiling assay revealed a decrease in the abundance of both 60S ribosomal subunits and 80S monosomes (Figure 8A) in the \(\textit{rack1a rakc1b}\) double mutant plants, compared with Col, but no significant difference was observed at the level of polysomes, indicative of an important role for \textit{RACK1} in maintaining the normal 60S ribosome biogenesis and 80S monosome assembly.

**ABA inhibits global protein translation**

In view of the fact that \textit{RACK1} genes are negative regulators of ABA responses (Guo et al., 2009a), that our global gene expression profiling had revealed a convergent group of genes co-regulated by both ABA and \textit{rack1} mutation (Figure 1), and that RACK1 appeared to be involved in ribosomal subunit assembly and 60S ribosome biogenesis (Figure 8A), we next asked whether ABA might also affect translation initiation in Arabidopsis. By using the ribosome profiling assay, we found that 50 \(\mu\text{M}\) ABA caused a dramatic reduction in the relative abundance of
polysomes (Figure 8B). An increase in 80S monosome abundance was also observed, probably as a consequence of reduced progression into the elongation step (Naranda et al., 1997). These data agree with what was reported much earlier in soybean hypocotyls (Bensen et al., 1988), and support a model in which ABA plays a direct role in regulating protein translation.

To further understand the role of RACK1 in ABA-regulated protein translation, we compared the ribosome profiling between WT and rack1a rack1b mutant after ABA treatment. As can be seen from Figure 8C, the accumulation of both 60S ribosome subunit and the 80S monosome was reduced in the rack1a rack1b plants treated with ABA when compared to ABA-treated Col. The observed further reduction of ribosome/monosome peaks might be due to the inhibitory effect of ABA on the expression of RACK1C in rack1a rack1b mutant, which will be further examined below.

**ABA regulates the expression of both RACK1 and eIF6 genes**

Since both ABA and RACK1 appear to be involved in the regulation of protein translation (Figure 8A, B), we further investigated the functional relationship between ABA and RACK1 in these processes. A preliminary experiment had shown that ABA negatively regulates the expression of RACK1 genes (Guo et al., 2009a), leading us to hypothesize that ABA might regulate ribosome assembly and translation initiation through down-regulation of RACK1 genes. Using quantitative RT-PCR, we conducted a detailed analysis of the expression of RACK1 gene family members in response to ABA treatment. The level of transcripts for all three RACK1 genes was down-regulated as early as 1 h after ABA treatment, and remained suppressed thereafter (Figure 9A). Consistent with these direct measurements of expression, the promoter activities of all three RACK1 genes in the root tip were inhibited by ABA treatment (Figure 9B). We then extended our analysis to examine the possible regulation of eIF6 expression by ABA. We found that a reduction of eIF6A expression could be detected as early as 15 min after ABA treatment, and expression of eIF6A continued to decline for up to six hours (Figure 9A). The expression of eIF6B gene was too low to be detected in seedlings used for qRT-PCR. These results suggested that ABA might regulate translation initiation at least in part through the regulation of expression of RACK1 and eIF6.
DISCUSSION

To answer the question of how \textit{RACK1} gene products are involved in ABA responses in plants, we employed a combination of experimental approaches. First, by using global gene expression profiling, we detected a strong correlation between gene expression patterns invoked by ABA treatment and those associated with loss of function at the \textit{RACK1} loci. Second, yeast genetic complementation assays demonstrated that the function of \textit{RACK1} genes can be conserved across different kingdoms. Third, gene co-expression analysis provided evidence that \textit{RACK1}’s function might be associated with the ribosome complex. Therefore, we specifically focused on investigation of the role of \textit{RACK1} in protein translation as a candidate mechanism through which \textit{RACK1} negatively regulates ABA responses.

Five lines of evidence directly or indirectly support the idea that \textit{RACK1} regulates protein translation, and that these regulatory processes involve ABA. Firstly, \textit{RACK1} physically interacts with eIF6 (Figure 4), a homologue of a key regulator of the ribosome assembly reaction of translation initiation in mammals. Secondly, \textit{rack1} mutants are hypersensitive to anisomycin (Figure 7), a known protein translation inhibitor. Thirdly, a decrease in the relative abundance of 60S ribosome subunits and 80S ribosome was observed in \textit{rack1a rakclb} plants (Figure 8A). Fourthly, ABA itself inhibits protein translation at the initiation stage (Figure 8B), and finally, ABA inhibits the expression of both \textit{RACK1} and \textit{eIF6} (Figure 9).

\textit{Arabidopsis RACK1 genes are functionally equivalent to S. cerevisiae CPC2}

\textit{RACK1} is a versatile scaffold protein that is involved in numerous signaling pathways and cellular processes in mammals and yeast (McCahill et al., 2002; Sklan et al., 2006; Guo et al., 2007). A few earlier studies have implied that Arabidopsis \textit{RACK1} could be a functional orthologue of mammalian \textit{RACK1} and the yeast \textit{CPC2}/\textit{RACK1} gene. For example, the amino acid sequence of \textit{RACK1} is highly conserved between Arabidopsis and other taxa (Chen et al., 2006; Figure S7), as is the protein structure (Ullah et al., 2008). That proposed close relationship
has been confirmed in the present study where Arabidopsis RACK1 was found to complement a genetic lesion at the yeast CPC2 locus (Figure 3). These results provide a rationale for utilizing the vast information available in the mammalian and yeast systems to probe the function of RACK1 in Arabidopsis. However, we are cautious that our findings do not exclude the possibility that some aspects of RACK1’s function are not conserved across different kingdoms. Indeed, the majority of the identified RACK1 interacting partners in mammals and yeast do not have obvious homologues in Arabidopsis (Guo et al., 2007). Even for those with obvious plant homologues, there is evidence that their interaction with RACK1 is not necessarily conserved in Arabidopsis. For example, in mammals, RACK1 interacts with the β subunit of the heterotrimeric G-proteins and mediates a subset of the downstream signaling events in mammals (Dell et al., 2002; Chen et al., 2004b; Chen et al., 2004a; Chen et al., 2005). However, genetic and biochemical analyses indicate that RACK1 probably does not directly interact with G proteins in Arabidopsis (Guo et al., 2009b).

**RACK1 may be required for the normal production of 60 ribosome subunits and 80S monosomes in Arabidopsis**

RACK1’s multifaceted molecular function is mainly manifested via its physical interaction with many different signaling molecules in eukaryotes (Guo et al., 2007). Significantly, RACK1 was repeatedly identified as being associated with the ribosome in different species, using different approaches (Ceci et al., 2003; Shor et al., 2003; Gerbasi et al., 2004; Nilsson et al., 2004; Sengupta et al., 2004; Chang et al., 2005; Giavalisco et al., 2005; Manuell et al., 2005; Yu et al., 2005; Regmi et al., 2008; Coyle et al., 2009). Our co-expression data also indicated that RACK1 genes are co-ordinately regulated with many ribosome proteins encoding genes (Figure S3 and Table S3). These observations point to a phylogenetically conserved function of the RACK1 protein in its association with the ribosome complex. It has been proposed in other taxa that the function of RACK1 mostly directly related to its association with ribosomes is its regulatory effect on translation initiation at the functional 80S ribosome assembly reaction (Ceci et al., 2003). In mammalian cells, this regulatory role involves RACK1’s interaction with activated PKC and eIF6 (Ceci et al., 2003). By using yeast two-hybrid assays and the BiFC assay, we
showed that Arabidopsis RACK1 physically interacts with eIF6 (Figure 4). This conserved interaction between RACK1 and eIF6 likely mediates ribosome assembly, as is seen with their counterparts in mammalian cells. In addition, we found that a translation inhibitor, anisomycin, displayed a synergistic effect with the rack1 mutation in inhibiting root elongation (Figure 7). A significant role for RACK1 in protein translation regulation is also supported by the polysome profiling data, where the rack1 mutation led to reduced levels of 60S ribosome subunits and 80S monosomes (Figure 8A). Interestingly, the RACK1 homologue in S. cerevisiae is also known to play a role in ribosome biogenesis (Shor et al., 2003). Consistent with such an essential contribution of the RACK1 genes to the translation process, and the potentially same essential contribution of the eIF6 genes, the rack1 triple mutant is seedling lethal (Guo and Chen, 2008) whereas the knock-out mutant of eIF6A is embryo-lethal (Figure 6C). Intriguingly, it has been demonstrated that the eIF6 gene is also involved in 60S ribosome biogenesis in yeast (Basu, 2001). It would be interesting to know whether such impaired 80S ribosome assembly and reduced ribosome subunits biogenesis can also be observed in eif6a knock-down mutants generated using RNAi techniques. In mammals, PKC plays an important role within the PKC-RACK1-eIF6 complex in regulating ribosome assembly. Although no apparent PKC orthologue has been found in plants, searching for other plant protein kinases (e.g. those possessing a C2 domain) that can phosphorylate eIF6 and interact with RACK1 might help identify a functionally equivalent protein complex that regulates the same essential process in Arabidopsis.

ABA might inhibit ribosome biogenesis and monosome assembly by inhibiting RACK1 expression

Plants are sessile and subject to constant biotic and abiotic stresses from the environment. ABA is one of the major phytohormones that regulate plant abiotic stress responses and also plays a role in plant growth (Zhu, 2002). Global inhibition of protein translation in plants under stress conditions has been recognized for some time (Kawaguchi et al., 2004). However, little is known about the signaling mechanism responsible for linking abiotic stress signaling, ABA signaling and the inhibition of protein translation machinery. In the present study, we found that RACK1 genes, earlier identified as negative regulators of ABA responses (Guo et al., 2009a), may also
be required for normal production of 60S ribosome subunits and 80S monosomes (Figure 8). In addition, ABA exerts a constant, inhibitory effect on RACK1 gene expression (Figure 9) although it had no effect on the interaction between RACK1 and eIF6 (Figure S5). These data point to a scenario in which ABA might inhibit 60S ribosome subunit biogenesis and 80S monosome assembly via its inhibitory effect on the expression of RACK1 genes. However, the ribosome profile of ABA treatment displayed reduced polysome levels and concomitant accumulation of 80S ribosomes (Figure 8B), whereas the profile of rack1a rack1b mutant plants displayed wild-type polysome levels and reduced 60S ribosome subunit and 80S monosome accumulation (Figure 8A). Our interpretation is that ABA likely inhibits protein translation at multiple points (as summarized in Figure S8). On one hand, ABA inhibits protein translation at the 60S ribosome biogenesis and 80S ribosome assembly steps, which may be mediated by RACK1 (and potentially also by eIF6); on the other hand, ABA inhibits the entry point of the translation elongation stage. The latter effect may not be mediated by RACK1 based on the ribosome profiling results. This model is supported by the finding that ABA inhibits the expression of RACK1 and eIF6 over an extended period (Figure 9), that the “knockdown” mutant (rack1a rack1b) of RACK1 genes displayed characteristics of impaired 60S ribosome subunit biogenesis and 80S ribosome assembly (Figure 8A), and by reports of similar functions for RACK1 and eIF6 homologues in these two processes in other organisms (Basu et al., 2001; Ceci et al., 2003; Shor et al., 2003). These data together support a model in which RACK1 serves as a molecular link between ABA signaling and its effect on 60S ribosome subunit biogenesis and 80S monosome assembly. The inability to discern an obvious inhibitory effect of ABA on the accumulation of 60S subunits and 80S monosomes in the ribosome profiling assays (Figure 8B) probably reflects masking of a relative mild ABA-induced reduction in 60S and 80S ribosome accumulation by the vast accumulation of 80S ribosomes and ribosome subunits resulting from ABA blocking ribosome entry into the translation elongation phase. Consistent with our findings, an evolutionarily-conserved protein kinase TOR, which is known to regulate ribosome biogenesis in mammalian cells, is reported to be responsive to abiotic stress (Martin et al., 2004; Deprost et al., 2007). In addition, protein translation initiation efficiency was found to be reduced in tobacco leaves subjected to drought stress (Kawaguchi et al., 2003), while in soybean, ABA treatment increased the level of polysomes in hypocotyl tissue (Bensen et al., 1988). Nevertheless, in light of its multifaceted roles in mammal and yeast biology, we cannot rule out
the possibility that RACK1 may mediate ABA responses indirectly through its involvement in other signaling pathways and cellular processes.

Since one of the best characterized physiological targets of ABA is the control of stomata aperture, we also measured the response of the guard cells to ABA in Col and the 
\textit{rack1a rack1b} mutant. Although the stomata aperture was wider in 
\textit{rack1a rack1b} plants than in Col plants in the absence of ABA treatment, addition of 50 \textmu M ABA led to the closure of stomata to a similar aperture width in both Col and the 
\textit{rack1a rack1b} mutant (Figure S9). In addition, because we only tested the ABA hypersensitivity using 
\textit{rack1a rack1b} double mutants (weak 
\textit{rack1} mutant), it is likely that we may observe stronger ABA hypersensitivity in 
\textit{rack1a rack1b rack1c} triple mutant (\textit{rack1} knockout mutant). However, 
\textit{rack1} triple mutant is seedling lethal (Guo and Chen, 2008), making it difficult to assess its ABA hypersensitivity.

It should be noted that we used \textit{RACK1A, RACK1B} and \textit{RACK1C} nomenclatures to describe the three \textit{RACK1} homologous genes in Arabidopsis because their gene products are highly similar to mammalian RACK1 (encoded by a single gene) at the amino acid level and so are the protein structure, although the exact biological/biochemical function of Arabidopsis RACK1 has not yet been established.

Taken together, our study supports the view that RACK1 is required for normal production of 60S ribosome subunits and 80S monosome and protein translation in Arabidopsis. We further propose that the negative influence of RACK1 on plant response to ABA may result, in part, from its molecular function in ribosome biogenesis and protein translation. RACK1 may therefore represent a novel molecular link between ABA signaling and regulation of protein translation initiation.

\textbf{Experimental procedures}

\textit{Plant materials and growth conditions}
All mutants are in the Arabidopsis Columbia (Col-0) ecotype background. Plants were grown in 5 x 5 cm pots containing Sunshine Mix #4 (Sun Gro Horticulture Canada Ltd., http://www.sungro.com) with 14/10 hr photoperiod at approximately 120 μmol m⁻²s⁻¹ at 23°C.

**DNA microarray assay**

Seeds of Columbia (Col-0) and rack1a rack1b mutant were germinated on ½ Murashige & Skoog (MS) basal medium with vitamins (Plantmedia, Dublin, Ohio, http://www.plantmedia.com), 1 % (w/v) sucrose, 0.6 % (w/v) phytoagar (Plantmedia), pH adjusted to 5.7 with 1N KOH. The plates were vertically placed to allow root growth along the surface of the agar. Four and half day-old seedlings were harvested and then incubated in either liquid ½ MS medium containing 50 μM ABA or solvent only for 4 h before they were snap-frozen in liquid nitrogen. Microarray analysis was performed using custom-made full-genome (30K) Arabidopsis 70-mer oligo arrays (Douglas and Ehlting, 2005; Ehlting et al., 2005). A detailed description of DNA microarray experiment design, procedure and data analysis was provided in the supplementary material (Protocol S1).

**S. cerevisiae strains and plasmids used in yeast complementation experiment**

The *S. cerevisiae* strains of Σ1278b background used were RH2656 (wild type diploid, MAT a/α ura3-52/ura3-5 trp1::hisG/TRP1, Braus et al., 2003) and RH3264 (homozygous diploid cpc2/rack1 mutant, MATa/α GCRE6-lacZ::URA3/ura3-52 trp1::hisG/ trp1::hisG leu2::hisG/leu2::hisG cpc2Δ::LEU2/cpc2Δ::LEU, Valerius et al., 2007). The plasmids used were p424MET25, a TRP1-marked centromere vector (Mumberg et al., 1994). The protein coding sequences of CPC2, RACK1A, RACK1B and RACK1C were cloned into p424MET25 using restriction enzyme digestion and ligation method. A lithium acetate mediated transformation method was used to transfer the plasmid into host yeast strain and the successful transformants were selected on appropriate nutrient-selective media. For the pseudohyphal growth assay, the transformed yeast strains were grown on nitrogen starvation plates (0.15% (w/v) yeast nitrogen base (w/o amino acids and ammonium sulfate, BD Difco. http://www.bd.com/ds/), 50 μM ammonium sulfate, 2% (w/v) glucose, 2.5% (w/v) agar (Sigma. http://www.sigmaaldrich.com)
and (350 mg/L) Uracil for 5 days at 30°C before the morphology of individual yeast colonies was examined and photographed under compound light microscope.

**Isolation of eif6a and eif6b T-DNA insertional mutants**

All the T-DNA insertional mutants of *RACK1* genes have been described previously (Chen et al., 2006; Guo and Chen, 2008). The T-DNA insertional mutant of *eIF6A* (At3g55620), *eif6a-1* (GABI_817H01), and the T-DNA insertional mutants of *eIF6B* (At2g39820), *eif6b-1* (SALK_017008), *eif6b-2* (SALK_057424), were identified from the SALK T-DNA Express database (http://signal.salk.edu/cgi-bin/tdnaexpresses). The second mutant allele of *eIF6A*, *emb1624* (Tzafrir et al., 2004) was originally identified within a collection of mutants defective in embryo development, and was here renamed *eif6a-2*. For each SALK T-DNA insertional mutant (Alonso et al., 2003), the insertion locus was confirmed by PCR and sequencing using *eIF6B*-specific primers (5’-ATGGCGACTCGTCTTCTCAGTTTGTGAACAAAC-3’ and 5’-TATCGATCGAAGACTTCTCATCATTCCACTAC-3’) and a T-DNA left border-specific primer JMLB1 (5’-GGCAATCAGCTGTGCCCCTTCATGCGTG-3’). For the GABI-Kat T-DNA insertional mutant *eif6a-1* (Rosso et al., 2003), the *eIF6A*-specific primers (5’-AGGCTAACGTACACCTGCGTAG-3’) and another T-DNA left border-specific primer Gabi-LB-o2588 (5’-CGCCAGGGTTTTCCAGTCACGACG-3’) were used to confirm the insertion position by PCR and sequencing. For *eif6a-2* (*emb 1624*), the *eIF6A*-specific primers (5’-CTCTACAATACCTCATTTTACATGCTCC-3’ and 5’-AGGCTAACGTACACCTGCGTAG-3’) and T-DNA left border-specific primer LB3 (5’-TAGCATCTGATTACATCACATACTCAG-3’) (McElver et al., 2001) were used to confirm the insertion position by PCR and sequencing.

**Yeast two-hybrid assay**

The interactions between eIF6s and RACK1s were tested by using the ProQuest yeast two-hybrid system (Invitrogen Canada Inc. http://www.invitrogen.com). *eIF6* genes were cloned into bait vector pDEST32 and *RACK1* genes were cloned into prey vector pDEST22. The yeast
transformants that contain both prey and bait were able to grow on minimum SD (Synthetic Dextrose) drop-out medium lacking both leucine and tryptophan (SD-LT). A positive interaction between two proteins is indicated by the growth of yeast colony on the minimum SD medium lacking leucine, tryptophan and histidine and containing 10 mM 3-amino-1,2,4-triazolium.

**Bi-molecular Fluorescence Complementation (BiFC) assay in Arabidopsis mesophyll protoplasts**

The coding sequences of *RACK1* genes were cloned into *pSAT1A-nEYFP-N1* and fused to the N-terminal half of the YFP (yellow fluorescent protein) molecule. *eIF6* genes were cloned into *pSAT4A-cEYFP-N1* and fused to the C-terminal half of the YFP molecule. The coding sequences of *RACK1* genes and *eIF6* genes were also cloned into the *pSAT6-EYFP-N1* vector in which the full-length YFP is fused to the C-terminus of the proteins for studying subcellular localization of each protein (Citovsky et al., 2006).

The isolation and transfection of Arabidopsis leaf mesophyll protoplasts were conducted the same way as previously described (Wang et al., 2005; Yoo et al., 2007). Briefly, protoplasts were isolated from rosette leaves of 3-week-old plants. Constructs prepared as described above were transfected (for subcellular localization) or co-transfected (for BiFC) into protoplasts and incubated in the dark for 6 h to allow expression of the introduced genes. The double 35S:HY5 (LONG HYPOCOTYL 5)-mCherry was used as a control for nuclear localization. For testing the effect of ABA on the interaction between *RACK1* and *eIF6*, co-transfected protoplasts were incubated with or without 50 μM ABA for 6h before observed under microscope. The YFP fluorescence was examined and photographed using a Leica DM-6000B upright fluorescent microscope with phase and differential interference contrast (DIC) equipped with a Leica FW4000 digital image acquisition and processing system (Leica Microsystems, www.leica-microsystems.com).

**Root growth assay with anisomycin**

Seeds of Col and *rack1* mutants were germinated on ½ MS media plates for 60h at 14/10h photoperiod. The seedlings were then transferred to ½ MS media containing various
concentrations of anisomycin and grown vertically for another five days before data were collected. The Image J software was used to measure the primary root length from pictures of each plate.

**Analysis of embryo development**

Siliques at different developmental stages from heterozygous *eif6a-1* and *eif6a-2* mutants were opened under a dissecting microscope with a fine-tip pin. Since all the seeds from the same silique are at the same developmental stage, the numbers of white seeds and green seeds in each silique were scored and the seeds were then individually immersed in fixation/clearing solution (chloral hydrate:H2O:glycerol=8:2:1). The cleared green seeds were then examined under a compound microscope to assess their developmental stage. For each representative developmental stage of the green seeds, the white seeds from the same silique were observed microscopically and photographed.

**Ribosome profiling assay**

The procedure used for the ribosome profiling assay was essentially the same as previously described (Kawaguchi et al., 2003). In summary, a 2 g sample of 4 1/2-day-old seedlings were ground to fine powders under liquid nitrogen. For each sample, 750 µl frozen ground tissue was quickly homogenized in 750 µl ribosome extraction buffer (Kawaguchi et al., 2003) and incubated on ice for 10 min. The supernatant (500 µl) was layered on top of a 5 ml (20%-60%) sucrose gradient (Fennoy and Bailey-Serres, 1995) and centrifuged for 90 min at 45,000 rpm at 4 °C. Gradient fractions (200 µl) were collected manually, starting from top of the gradient and the OD260 for each fraction was measured using a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., http://www.biotek.com). The baseline absorbance of a gradient loaded only with extraction buffer was subtracted and the profiles were normalized to equal total OD absorption units to allow for comparison between samples.
For ABA treatment, 4 ½-day-old Col seedlings were incubated in ½ MS liquid medium containing 50 µM ABA for 4 h or 8 h before they were snap-frozen in liquid nitrogen and assayed later.

**Gene expression analysis**

For the quantitative RT-PCR assay, Col seeds were germinated on ½ MS medium and plates were placed vertically to allow the roots grow along the surface of the agar. Col seedlings (4 ½ days-old) were gently removed from the agar surface and incubated in liquid ½ MS medium with or without 20 µM ABA for different periods of time. They were then harvested and snap-frozen in liquid nitrogen. Total RNA was isolated using the Qiagen Plant Mini RNA isolation kit (Qiagen Inc.) and cDNA was synthesized with Omniscript RT kit (Qiagen Inc.). Quantitative real-time PCR was performed using the MJ MiniOpticon real-time PCR system (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada, http://www.bio-rad.com) and IQ SYBR Green Supermix (Bio-Rad Laboratories). The real-time PCR primers used for analyzing the transcript levels of *RACK1A, RACK1B, RACK1C* and *ACTIN2* (used for normalization) were the same as described previously (Guo and Chen, 2008). The experiments were repeated three times and data with similar trends were obtained.

For promoter::GUS assay, the *PRACK1::GUS* lines described previously (Guo et al., 2009a) were used. Seeds were germinated on ½ MS medium and plates were placed vertically. Seedlings (4 ½ days-old) were incubated in liquid ½ MS medium with or without ABA for 6 h and then subjected to GUS staining as described previously (Guo et al., 2009a). Pictures of seedlings were taken under a dissecting microscope.

Upon request, all novel materials described in this article will be made available in a timely manner for non-commercial research purposes.

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FIGURE LEGENDS

Figure 1. Analysis of DNA microarray data.
A. A Venn diagram shows the number of genes that are co-up-regulated \( \geq 2.0 \) fold by 50 \( \mu \)M ABA treatment and by \( \text{rack1} \) mutation. The number of genes that were co-regulated by ABA treatment and \( \text{rack1} \) mutation appears in the overlapped portion of the circles and the number of genes that were not co-regulated appears in the non-overlapping portions for the 2-fold up-regulated genes.
B. A Venn diagram shows the number of genes that are co-down-regulated \( \geq 2.0 \) fold by 50 \( \mu \)M ABA treatment and by \( \text{rack1} \) mutation.
C. Scatter plot shows the correlation of the genes that were regulated by ABA treatment and of genes that were regulated by \( \text{rack1a rack1b} \) mutation. The calculated Pearson correlation coefficient was 0.494, indicating a moderate correlation level.

Figure 2. Functional categorization of genes that were up-regulated two-fold or more in \( \text{rack1a rack1b} \) mutant background treated with ABA compared with Col treated with ABA. Functional categorization of genes was obtained through the TAIR Gene Ontology (GO) Annotations tool (http://www.arabidopsis.org/tools/bulk/go/index.jsp).

Figure 3. Complementation assay for failed pseudohyphal growth in the diploid \( S. \ cerevisiae \) \( \text{cpc2} \) mutant, using three Arabidopsis \( \text{RACK1} \) genes. Transformants were patched on nitrogen starvation plates and grown for 5 days before pictures were taken.
A. RH2656 (wild type) + \( p424\text{MET25} \) (empty vector), B. RH3246 (\( \text{cpc2} \)) + \( p424\text{MET25} \) (empty vector), C. RH3246 (\( \text{cpc2} \)) + \( p424\text{MET25-CPC2} \), D. RH3246 (\( \text{cpc2} \)) + \( p424\text{MET25-RACK1A} \), E. RH3246 (\( \text{cpc2} \)) + \( p424\text{MET25-RACK1B} \), F. RH3246 (\( \text{cpc2} \)) + \( p424\text{MET25-RACK1C} \).

Figure 4. Physical interaction between \( \text{RACK1} \) and eIF6 detected in yeast two-hybrid assays and in the BiFC system.
A. Interactions between \( \text{RACK1s} \) and eIF6s in the yeast two-hybrid assay. eIF6 genes were cloned into pDEST32 and \( \text{RACK1} \) genes were cloned into pDEST22. The interaction between
eIF6 and the empty prey vector was used as a negative control. The ability of yeast cells to grow on synthetic medium lacking leucine, tryptophan and histidine, and containing 10 mM 3-Amino-1,2,4-triazole (3-AT), is scored as a positive interaction.

**B.** Interactions between RACK1 and eIF6 in BiFC. RACK1 proteins were fused with the N-terminal half of YFP and eIF6 proteins were fused with C-terminal half of YFP. YFP, yellow fluorescent protein; CHE/DIC, overlay of mCherry images and DIC images of the same field. The interaction between AtOFP1 (Wang et al., 2007) and RACK1/eIF6 proteins was used as a negative control. The HY5-mCherry is included in each transfection to serve as a control for successful transfection as well as for nuclear localization. Image shown are the same transformants photographed under YFP fluorescence and DIC microscopic setups. Images were pseudo-colored with Image J for easy visualization.

**Figure 5.** Arabidopsis eIF6 homologues.

**A.** RT-PCR assay for expression of eIF6 genes in different Arabidopsis tissues and organs. PCR was performed with 30 cycles.

**B.** *In silico* analysis of the relative transcript levels of eIF6A (At3g55620) and eIF6B (At2g39820) in various tissue and organs in Arabidopsis. Data were imported from the Genevestigator *Arabidopsis thaliana* microarray database (https://www.genevestigator.ethz.ch/; Zimmermann et al., 2004).

**Figure 6.** eif6 mutant alleles.

**A.** T-DNA insertional mutant alleles of eIF6A and eIF6B in Arabidopsis. The exons are depicted by boxes and the intron and intergenic regions are depicted by lines. The T-DNA insertion sites are drawn as triangular boxes (not to scale). LB, T-DNA left border.

**B.** RT-PCR analysis of eif6b-1 and eif6b-2 alleles.

**C.** The eif6a mutants are embryo lethal. Each pair of pictures is representative of the green seeds (top) and white seed (bottom) from the same silique.

**D.** Three-week-old eif6b-1 and eif6b-2 mutant plants grown under 14/10 h photoperiod.
**Figure 7.** The synergistic effect of anisomycin treatment and *rack1* mutation on Arabidopsis seedling root growth.

A. Root growth of *rack1* single mutants in the presence of 10 μM anisomycin.

B. Root growth of *rack1* double mutants in the presence of 5 μM anisomycin.

C. The primary root length of Col and the *rack1* mutants in the absence of anisomycin treatment. The experiments were repeated three times and the same data trends were obtained. Data from one experiment are presented here with the standard error (n=20) indicated on the top of each column. * indicates significant difference from Col using Student’s t-test (p<0.05).

**Figure 8.** Ribosome profiling of *rack1a rack1b* mutant and ABA-treated Arabidopsis seedlings.

A. The overlay of the ribosome profiles of Col and *rack1a rack1b* mutant without ABA treatment.

B. The overlay of the ribosome profiles of Col with or without ABA treatment. The positions of 40S ribosomal subunits, 60S ribosomal subunits and 80S ribosomes were located based on the absorbance peaks at 260nm and are indicated with arrows. Profiles are average of four independent experiments with standard error bar. * indicates a significant difference using paired t-test (P<0.05). Shown in (B) is sucrose density gradient analysis of polysomes extracted from four and half-day old Col seedlings, with or without 50 μM ABA treatment for 8 h.

C. The overlay of the ribosome profiling of Col and *rack1a rack1b* mutant after ABA treatment. The position of 40S ribosomal subunits, 60S ribosomal subunits and 80S ribosomes was located based on the absorbance peaks at 260nm and is indicated with arrows. Profiles are average of four independent experiments with standard error bar. * indicates a significant difference using paired t-test (P<0.05). Shown is sucrose density gradient analysis of polysomes extracted from four and half-day old Col seedlings, with or without 50 μM ABA treatment for 8 h.

**Figure 9.** The regulation of *RACK1* and *eIF6* expression by ABA.

A. Quantitative RT-PCR analysis of *RACK1* and *eIF6* gene expression. The transcript levels of *RACK1* and *eIF6A* genes were normalized against the transcript level of *ACTIN2* for each sample.
Total RNA was extracted from four and half day old Arabidopsis seedlings and used for qRT-PCR analysis. Shown are the averages of three biological replicates ± standard error.

B. Promoter::GUS assay. Four and half-day old seedlings were incubated in ½ MS liquid medium with or without 50 µM ABA for 6 h and then subjected to GUS staining.
SUPPLEMENTARY MATERIAL

Figure S1. Gene ontology distribution of the genes that are misregulated in rack1a rack1b mutants.

Figure S2. Gene ontology distribution of the genes that were down-regulated in rack1a rack1b mutants and in Col after ABA treatment.

Figure S3. RACK1 co-expression analysis.

Figure S4. Subcellular localization of RACK1 and eIF6.

Figure S5. The effect of ABA on the interaction of RACK1 and eIF6 in BiFC system.

Figure S6. Arabidopsis eIF6 homologues.

Figure S7. Alignment of the amino acid sequences of three Arabidopsis RACK1 proteins.

Figure S8. A summary of the effect of ABA in protein translation and the role of RACK1 in this process.

Figure S9. The role of RACK1 gene in ABA-inhibited stomatal opening.

Table S1. The list of genes that were up- or down-regulated ≥2.0-fold in rack1a rack1b mutant compared to Col after ABA treatment.

Table S2. The list of genes that were up- or down-regulated ≥2.0-fold in both the rack1a rack1b mutant without ABA treatment and Col with ABA treatment.

Table S3. The list of genes that are co-expressed with RACK1 genes in the PRIME database.

Protocol S1. Supplementary materials and methods.
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Figure 1. Analysis of DNA microarray data.
A. A Venn diagram shows the number of genes that are co-up-regulated by 50 μM ABA treatment and by rack1 mutation for 2-fold or higher. The number of genes that were co-regulated by ABA treatment and rack1 mutation appears in the overlapped portion of the circles and the number of genes that were not co-regulated appears in the non-overlapping portions for the 2-fold up-regulated genes.
B. A Venn diagram shows the number of genes that are co-down-regulated by 50 μM ABA treatment and by rack1 mutation for half or lower.
C. Scatter plot shows the correlation of the genes that were regulated by ABA treatment and that regulated by rack1a rack1b mutation. The calculated pearson correlation coefficient was 0.494, indicating a moderate correlation level.
Figure 2. Functional categorization of genes that were up-regulated two-fold or more in *rack1a* *rack1b* mutant background treated with ABA compared with Col treated with ABA. Functional categorization of genes was obtained through the TAIR Gene Ontology (GO) Annotations tool (http://www.arabidopsis.org/tools/bulk/go/index.jsp).
Figure 3. Complementation assay for failed pseudohyphal growth in the diploid *S. cerevisiae* cpc2 mutant, using three Arabidopsis *RACK1* genes. Transformants were patched on nitrogen starvation plates and grown for 5 days before pictures were taken.

A. RH2656 (wild type) + *p424MET25* (empty vector), B. RH3246 (cpc2) + *p424MET25* (empty vector), C. RH3246 (cpc2) + *p424MET25-CPC2*, D. RH3246 (cpc2) + *p424MET25-RACK1A*, E. RH3246 (cpc2) + *p424MET25-RACK1B*, F. RH3246 (cpc2) + *p424MET25-RACK1C*. 
Figure 4
Figure 4. Physical interaction between RACK1 and eIF6 detected in yeast two-hybrid assays and in the BiFC system.

A. Interactions between RACK1s and eIF6s in the yeast two-hybrid assay. *eIF6* genes were cloned into pDEST32 and *RACK1s* were cloned into pDEST22. The interaction between eIF6s and the empty prey vector was used as a negative control. The ability of yeast cells to grow on synthetic medium lacking leucine, tryptophan and histidine, and containing 10 mM 3-AT, is scored as a positive interaction.

B. Interactions between RACK1s and eIF6s in BiFC. RACK1 proteins were fused with the N-terminal half of YFP and eIF6 proteins were fused with C-terminal half of YFP. YFP, yellow fluorescence protein; CHE/DIC, overlay of mCherry images and DIC images of the same field. The interaction between OFP1 (Wang et al., 2007) and RACK1/eIF6 proteins was used as a negative control. The HY5-mcherry is included in each transfection to serve as a control for successful transfection as well as for nuclear localization of RACK1s/eIF6s/OFP1. Image shown are the same transformants pictured under YFP fluorescent and DIC microscopic setups. Images were pseudo-coloured with Image J for easy visualization.
Figure 5. Arabidopsis eIF6 homologues.
A. RT-PCR assay for expression of eIF6 genes in different Arabidopsis tissues. PCR was performed with 30 cycles.
B. In silico analysis of the relative transcript levels of eIF6A (At3g55620) and eIF6B (At2g39820) in various tissue and organs in Arabidopsis. Data were imported from the Genevestigator Arabidopsis thaliana microarray database (https://www.genevestigator.ethz.ch/; Zimmermann et al., 2004).
Figure 6. *eif6* mutant alleles.

A. T-DNA insertion mutant alleles of *eIF6A* and *eIF6B* in Arabidopsis. The exons are depicted by boxes and the intron and intergenic regions are depicted by lines. The T-DNA insertion sites are drawn as triangular boxes (not to scale). LB, T-DNA left border.

B. RT-PCR analysis of *eif6b-1* and *eif6b-2* alleles.

C. The *eif6a* mutants are embryo lethal. Each pair of pictures is representative of the green seeds (top) and white seed (bottom) from the same silique.

D. Three-week-old *eif6b-1* and *eif6b-2* mutant plants grown under 14/10 h photoperiod.
Figure 7. The synergistic effect of anisomycin treatment and rack1 mutation on Arabidopsis seedling root growth.

A. Root growth of rack1 single mutants in the present of 10 µM anisomycin.

B. Root growth of rack1 double mutants in the present of 5 µM anisomycin.

C. The primary root length of all the rack1 mutants and Col.

The experiments were repeated three times and the same data trends were obtained. Data from one experiment are presented here with the standard error (n=20) indicated on the top of each column. * indicates significant difference from that of Col using Student’s t-test (p<0.05).
Figure 8

A

B

C

Fraction #

A$_{260}$

Polysomes

Col-0

rack1ab

Col-0

Col-50

rack1ab-50

40S

60S

80S

60S

80S

40S


Figure 8. Ribosome profiling of rack1a rack1b mutant and ABA-treated Arabidopsis seedlings.

A. The overlay of the ribosome profiles of Col and rack1a rack1b mutant without ABA treatment.
B. The overlay of the ribosome profiles of Col with or without ABA treatment.
C. The overlay of the ribosome profiling of Col and rack1a rack1b mutant after ABA treatment. The position of 40S ribosomal subunits, 60S ribosomal subunits and 80S ribosomes was located based on the absorbance peaks at 260nm and is indicated with arrows. Profiles are average of four independent experiments with standard error bar. * indicates a significant difference using paired t-test (P<0.05). Shown is sucrose density gradient analysis of polysomes extracted from four and half-day old Col seedlings, with or without 50 µM ABA treatment for 8 h.
**Figure 9.** The regulation of *RACK1* and *eIF6* expression by ABA.

**A.** Quantitative RT-PCR analysis of *RACK1* and *eIF6* gene expression. The transcript levels of *RACK1* and *eIF6A* genes were normalized against the transcript level of *ACTIN2* for each sample. Total RNA was extracted from four and half day old Arabidopsis seedlings and used for qRT-PCR analysis. The presented are the average of three biological replicates ± standard error.

**B.** Promoter::GUS assay. Four and half-day old seedlings were incubated in ½ MS liquid medium with or without 50 µM ABA for 6 h and then subjected to GUS staining.