Arabidopsis scaffold protein RACK1A interacts with diverse environmental stress and photosynthesis related proteins

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Introduction

The widely conserved eukaryotic scaffold protein RACK1 (Receptor for Activating C Kinase1) regulates different signal transduction pathways ranging from cell division to environmental stress responses by interacting with diverse proteins.1-5 The protein was identified through its ability to function as a scaffold protein, stabilizing signaling complexes involving protein kinase C.6 RACK1 proteins with seven WD-40 repeats are highly conserved (70–80% at the protein level) in a wide range of species, including plants, humans, rats, chickens, flies, nematodes, algae and yeasts. Though plant RACK1 has been implicated in regulating diverse signaling pathways that include but are not limited to, drought and salt stress, growth hormones, innate immunity and rare sugars,4,7,8 compared with the metazoan domain, virtually no information is available about its interacting proteins. So far the Biomolecular Interaction Network Database9 reports that metazoan RACK1 interacts with 87 different proteins ranging from ion channels to diverse ribosomal proteins. However RACK1 has not been reported to be used as bait in any conventional yeast two hybrid (Y-2-H) based library screens where baits are required to transactivate reporter gene expression only after interacting with the prey. The reported RACK1 interacting proteins were identified, in most cases, when the respective protein was used as bait to detect RACK1 as the prey partner (BIND database). As Y-2-H based protein-protein interaction requires reconstitution of a split transcription factor in the yeast nucleus, frequently proteins with transactivation domains active in yeast are excluded as bait. The yeast split-ubiquitin system has been developed to overcome the limitations of the classical Y-2-H system.10,11 The split-ubiquitin method is based on the ability of Nub and Cub, the N- and C-terminal halves of ubiquitin fused to a bait or to a prey separately, to assemble into a quasi-native ubiquitin (Ub).10,11 Ub specific proteases recognize the reconstituted Ub, though not its halves, and cleave the Ub moiety off a reporter protein, which has been linked to the C-terminus of Cub. The release of the reporter serves as readout indicating interactions. As a consequence, the yeast cells lose their capability to grow in...
the absence of uracil, and thus permits negative selection in the presence of the otherwise toxic URA3p specific antimetabolite 5-fluoroorotic acid (5-FOA). This technique offers major advantages over classical yeast two-hybrid screen in that it can be modified to identify both cytosolic and membrane proteins, and to identify transient interactions.10,11

Due to the inability of using RACK1 as bait in a traditional yeast two hybrid screen, the repertoire of the RACK1 interacting proteins may actually be much larger than the subset currently identified. To ascertain for a potentially comprehensive functional role of RACK1 protein, it is imperative that we use a system where RACK1 could be used as bait to screen a random cDNA library. Here we report the use of Arabidopsis RACK1A as bait to screen for potential interacting proteins. As opposed to a single gene in metazoan, Arabidopsis genome maintains three different RACK1 genes—termed as RACK1A, RACK1B and RACK1C. RACK1A—the predominant member with an unequal redundancy effect has been extensively studied.4,7,8 We have employed the split-ubiquitin based system that does not require the nuclear transactivation process, a central impediment to use RACK1 as bait in the traditional Y-2-H library screens.10,11 The identification of more than 90 Arabidopsis RACK1A interacting proteins from the screen indicates the efficacy of the split-ubiquitin based library screen.

**Results and Discussion**

As a scaffold protein, RACK1 connects distinct signaling pathways to regulate important cellular activities including cell growth and proliferation, transcription and protein synthesis.5 Unlike animal RACK1, which is encoded by a single copy gene in the respective genomes, the presence of more than one copy of RACK1 in most plant species provides multiple opportunities for RACK1 based protein-protein interaction signaling modules. In this study, a split-ubiquitin based Arabidopsis inflorescence cDNA library was used to systematically screen for RACK1A interacting proteins. The split-ubiquitin based cDNA library screen allows to overcome problems associated with the auto-activation properties of RACK1 when used as bait in conventional Y-2-H screens as this screen does not depend on the transactivation capabilities of the bait.10 Any protein interacting with RACK1A would be recognized as the interaction mode would reconstitute a functional ubiquitin protein tagged with the URA3 reporter protein. Proteolytic degradation of the URA3 reporter protein renders the yeast cells URA- auxotroph and resistant to the counter selection agent 5-FOA induced toxicity. Non-interacting clones with the functional URA3 will not be able to survive the FOA toxicity. Following the scheme, several rounds of screens were performed using RACK1A as bait to identify potential interacting proteins. RACK1 Y248 residue phosphorylation has been implicated in many cellular functions (4). In order to discern whether RACK1A Y248 phosphorylation is required for any potential interaction, we have also used a site-directed mutagenesis construct Y248F-RACK1A, as bait. As can be seen from Figure 1, the RACK1A bait appeared to potentially interact with diverse proteins as FOA resistant colonies were found to grow on FOA selection plates (Fig. 1, upper panel right), whereas, when Y248F-RACK1A was used as bait, no FOA resistant colonies were found (Fig. 1, lower panel right) indicating that tyrosine phosphorylation on the Y248 residue may dictate any potential interaction under the conditions used. The successful yeast transformation process is evident from the growth of yeast colonies harboring both the bait (WT and mutant) and prey constructs on selection plates lacking both His and Trp (Fig. 1, upper and lower panel left). We are cognizant that bait stability is a concern for this kind of library screen. URA-protopotrophy was examined by allowing the bait clone to grow on plates lacking uracil (data not shown). As the yeast colonies harboring the baits were able to grow on plates lacking uracil, we used the baits in the respective screening process.

Like in other genetic and conventional Y-2-H screens, the split-ubiquitin system has a tendency to result in false positives that may arise from several distinct mechanisms during the initial growth selection process. Promoter mutations to activate the reporter gene even in the absence of any potential interaction have been cited to give rise to false positives.11 In addition, overexpression of certain proteins in yeast may non-specifically activate the reporter genes. However, these false positives can be partly eliminated using a set of genetic criteria that can be rapidly tested. Therefore, based on the published optimized procedure to screen a split ubiquitin library,11 a methodological scheme was undertaken to eliminate potential false positives. The FOA-resistant clones were re-streaked on minimal plate lacking His, Trp and Ura (M-HTU). Only the true interactors where the reporter URA3 clone is proteolytically degraded were not able to grow on the M-HTU plates (data not shown). The URA3 DNA sequence from a few of the selected clones was also checked for
any mutations that may render such clones FOA-resistant. The low rate of mutations in the URA3 provided confirmation for the elimination of certain false positives by this scheme.

To identify the interacting partners, specific PCR primers targeting the prey protein constructs were used to rescue the plasmid. A typical result from the PCR reactions to identify the interacting proteins is shown in Figure 2. The amplified PCR products were sequenced to reveal the identity of the encoded interacting proteins. Some of the FOA resistant colonies did not produce any PCR bands; most likely being either false positives or due to failure of the PCR reaction. However, out of almost 150 PCR bands, positive identifications of 97 interacting proteins were completed by DNA sequencing (Table 1; Table S1). Many of the key interacting proteins from the photosynthetic pathways were identified more than once, indicating that the library screen has been successful in eliminating potential false positives.

Nine of the potential interacting proteins representing different classes of the identified proteins were tested for their ability as prey to interact with the bait RACK1A in a one-to-one split ubiquitin based interaction assay. As the Y248F-RACK1A bait failed to show any potential interactions (Fig. 1B), we used the bait as a negative control in the co-transformation assay. Figure 3A–C show the results from the representative selection plates with seven of the nine tested interactions. Both the individual bait (WT RACK1A or mutant Y248F-RACK1A) and the prey constructs: Chlorophyll a/b binding protein1 (CAB1) (lane 1); Plantacyanin (lane 2); Copper/Zinc Superoxide Dismutase 1, CSD1 (lane 3); Plastocyanin (lane 4); Rubisco small subunit 1A (lane 5); Carbonic anhydrase1 (lane 6); and Prolyl cis-trans isomerase (CYP20-3) (lane 7), were being expressed on the M-HT selection plate (Fig. 3A). When tested on M-HT+FOA plate, only the WT-RACK1A bait and individual prey harboring yeast colonies were able to grow, confirming their interaction (Fig. 3B). However, no colony was able to grow to a significant level on the same plate, indicating lack of or very weak interaction with the mutant bait (Fig. 3B). To further confirm that the growth from the M-HT+FOA plates resulted from the interaction based degradation of the reporter protein URA3, the same colonies from the FOA plates were tested on the selection plate lacking any exogenously supplemented uracil (Fig. 3C). As expected, the non-interacting bait and prey harboring yeast colonies were able to grow on the uracil lacking plates-indicating the maintenance of a functional URA3 protein; whereas the FOA resistant colonies were not able to survive on the plate. Similar results from additional plates were obtained with the Photosystem I Light Harvesting Complex Gene 2 (LHCA2) and Photosystem I Light Harvesting Complex Gene 3 (LHCA3) proteins. By considering both the positive and negative selection results from the represented interactions, it can be concluded that the screen employed to identify RACK1A protein interactors has been quite successful.

Conforming to RACK1A’s role in the environmental stress response pathways, 26% of the interacting partners are found to be directly or indirectly involved with environmental stress signaling pathways (Fig. 4). Also 14% of interacting proteins were found to be ribosomal proteins, supporting the reported localization of RACK1 in the ribosome to regulate global translation. Moreover, 18% of RACK1A interacting proteins have coordinated roles in regulating photosynthesis and light regulated physiological processes (Table 1). In addition, 30% of interacting proteins were found from different cellular functional categories, including transcriptional regulation, protein folding and RNA splicing. Several interacting partners appear to be unknown or novel (8%) and 4% of the interacting proteins are related to pathogen defense.

Interestingly, one of the interacting partners found in the cDNA library screen using RACK1A as bait was a Cu-Zn SOD important enzyme to regulate the reactive oxygen species within the cell. In plants, Cu-Zn SOD is found in the cytosol and in the extracellular space as well as in the chloroplast. Cu-Zn SODs are important regulators of many of the environmental stress responses including drought, salt, flooding and pathogen infection pathways. It is conceivable that by potentially interacting with Cu-Zn SOD, RACK1A can negatively regulate the specific SOD function and, under stress conditions, this...
was peptidyl-prolyl cis-trans isomerase, which is also implicated in the potential interacting protein identified during the library screen. This enzyme plays a crucial role in several important plant metabolic processes that are activated significantly during drought and high temperature. Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) is a key enzyme of the photosynthetic process. The putative interaction of RACK1A with Rubisco subunits suggests a role for RACK1A in photosynthesis, thus indirectly implicated in the drought response. It is possible that inactivation of RACK1A may provide plant with avenues to channel its excess absorbed energy during drought/high light stress condition by channeling the excess energy load toward the photosynthesis pathway. The potential interaction with carbonic anhydrase 1 (CA1) also indicates a role of RACK1A in the transition between C3 and C4 types of photosynthetic processes. In a related study, we have shown that RACK1A homo- and hetero-dimerizes and the tyrosine phosphorylation at Y248 residue is an important post-translational modification that dictates the dimerization event.

Table 1. List of multifunctional interacting partners of RACK1A

| Locus name | Name of the protein | Function | Reference |
|------------|--------------------|----------|-----------|
| AT1G67090  | Rubisco small subunit 1A | Oxidative and drought stress, photosynthesis | 17 |
| AT3G62030  | Peptidyl-prolyl cis-trans isomerase CYP20-3 | Stress response | 25 |
| AT3G13520  | Arabinogalactan protein 21 | Salt stress | 15 |
| AT1G08830  | Superoxide dismutase [Cu-Zn], CSD1 | Oxidative stress response | 14 |
| AT2G02850  | Plantacyanin | Stress response, defense | 26 |
| AT3G01500  | Carbonic anhydrase 1,CA1 | Stress response | 27 |
| AT5G04140  | Fd-GOGAT | Photosynthesis | 18 |
| AT1G20340  | Plastocyanin major isoform, DRT12 | Photosynthesis | 28 |
| AT1G9930   | Chlorophyll a-b binding protein 1CAB1 | Photosynthesis | 29 |
| AT2G34420  | Light-harvesting complex II chlorophyll a/b binding protein 1 | Photosynthesis | 20 |
| AT5G20010  | GTP-binding nuclear protein Ran-1 | Protein import into the nucleus | 30 |
| AT1G06190  | Rho termination factor | Transcription termination | 31 |
| AT4G21660  | Splicing factor 3B subunit 4 | RNA splicing | 32 |

Selected proteins from the stress and photosynthetic pathways are presented. The functional categorization was ascertained by the entry of the references on the PubMed database. The complete list of identified interacting proteins is presented as a Supplemental file.

negative regulation can be relieved to allow the specific SOD enzyme to detoxify generated reactive oxygen species. Another important stress responsive protein found from the library screen was Arabinogalactan-protein9 (AGP9). AGPs play a crucial role in salt-stressed cells as massive upregulation of AGPs has been reported under such conditions. Phosphorespiration is one of the important plant metabolic processes that is activated significantly during drought and high temperature. Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) is a key enzyme of the photosynthetic process. The putative interaction of RACK1A with Rubisco subunits suggests a role for RACK1A in photosynthesis, thus indirectly implicated in the drought response. It is possible that inactivation of RACK1A may provide plant with avenues to channel its excess absorbed energy during drought/high light stress condition by channeling the excess energy load toward the photosynthesis pathway. The potential interaction with carbonic anhydrase 1 (CA1) also indicates a role of RACK1A in the transition between C3 and C4 types of photosynthetic processes. In a related study, we have shown that RACK1A homo- and hetero-dimerizes and the tyrosine phosphorylation at Y248 residue is an important post-translational modification that dictates the dimerization event.

Photosystem I Light Harvesting Complex Gene 2 (LHCA2) is linked with photosystem II (PS II). Both LHCA2 and LHCA3 (Photosystem I Light Harvesting Complex Gene 3) are potential interacting partners of RACK1A, suggesting a possible role of RACK1A in the switching of PSI and PSII. The identification of diverse photosynthesis related genes: LHCA2, LHCA3, chlorophyll a/b binding protein 1 (CA1); photosystem I reaction center subunit XI; photolyase/blue-light receptor 2, PHR2; photosystem I reaction center subunit III; photosystem I reaction center subunit N as potential interacting partner suggests a major role for RACK1A during photosynthesis (Table 1; Table S1). The potential interaction with carbonic anhydrase 1 (CA1) also indicates a role of RACK1A in the transition between C3 and C4 types of photosynthetic processes. In a related study, we have shown that RACK1A homo- and hetero-dimerizes and the tyrosine phosphorylation at Y248 residue is an important post-translational modification that dictates the dimerization event.

The promoter regions of the stress and photosynthetic genes were analyzed to uncover conserved transcription factor binding sites (Fig. S1). The Athena database that allows rapid visualization and systematic analysis of Arabidopsis promoter sequences reveals three distinct TF sites within the 27 analyzed gene promoters. The ABRE-like binding site motif (C/G/T)ACGTG(G/T)(A/C) known to occur in genes encoding proteins that respond to dehydration and low temperature was found in 15 promoters with a p-value of < 10⁻⁴. The Ibox promoter motif (GATAAAG) is found in the promoters of light regulated genes was found in all of the 16 RACK1A interacting photosynthetic genes. The low statistical p-value of < 0.0040 indicates the significance of this finding. The
MYB2AT site (TAACTG), known to be involved in the regulation of genes that are responsive to water-stress, is present in 11 of the stress related proteins that interact with RACK1A protein. Considering that the cDNA library was developed from inflorescence tissues, it is quite intriguing that many of the photosynthetic proteins appear to interact with RACK1A. Arabidopsis genome wide studies showed that the green tissues like sepals, carpels, siliques in the inflorescence tissue maintain many of the photosynthetic proteins.

Protein-protein interactions are the dominant modes to virtually regulate every cellular process ranging from DNA replication and translations to cellular stress responses. As a scaffold protein RACK1 brings other proteins together and is therefore directly and/or indirectly involved in many signaling pathways. Here we present a screen for the identification of Arabidopsis RACK1A interacting proteins. Conforming to its published role in the environmental stress response, translational control and developmental pathways, the screen revealed that the majority of RACK1A interacting proteins are related with these pathways. RACK1A was found to be a common interacting partner of many stress responsive proteins indicating a major role in crosstalk among these signaling pathways. Identification of the conserved TF sites within the promoters of genes encoding these interacting proteins should help to elucidation the regulatory mechanisms involved in the RACK1A-mediated environmental stress and photosynthetic pathways. Such knowledge will eventually help in developing biotechnological strategies to develop suitable environmental stress tolerant transgenic crops.

Materials and Methods

E. coli and yeast strains. All plasmid constructions and amplifications were performed using the E. coli strain XL1-Blue and DH5α (Stratagene). The genotype of wild type yeast strain used for the split-ubiquitin assays was JD53 (Ura-, Trp-, Leu-, Lys-).

Plasmid construct. The split-ubiquitin assay was performed in S. cerevisiae strain JD53. Gateway Entry clones were made by insertion of full length RACK1A cDNA into pCR8/GW/TOPO vector (Invitrogen). Entry clones were maintained in the E. coli strain DH5α in LB medium supplemented with spectinomycin (50 μg/ml). Following recombination, the RACK1A from the Entry clone was transferred to the destination bait vector pMKZ [pMet-KZ::GWY Cassette-Cub-URA3-CYC1 (His)]. Similarly, Arabidopsis cDNAs were maintained in the prey vector pCUP-CGK-pCup-NuI::AttB1-EcoRI-cDNA-polyA-XhoI-AttB2-CYC1 (Trp). The prey vector was selected with ampicillin (100 μg/ml).

cDNA Library construction. To construct the library, total RNA was isolated from frozen Col-0 floral material using the RNA extraction kit from RNAwiz (Ambion). PolyA+ RNA was purified with the Dynabead Kit and subsequently quantified. For the synthesis of the cDNA library, the cDNA synthesis kit from Stratagene was used. Seven mg of polyA+ RNA was used for synthesis following the protocol of the manufacturer. Based on analyzing a random number of individual clones, the average size of the inserts was 1.3 kb. The cDNA library was contained...
in the pCup-CGK vector. Modified prey vector containing two bacterial selection markers, AmpR, KanR were used. This vector was compatible with the Gateway system.

**Electroporation.** Isolated bait plasmid RACK1A DNA was electroporated into *Saccharomyces cerevisiae* strain JD53. Transformants were selected on minimal medium plate with yeast nitrogen base without amino acids (Sigma) and glucose, supplemented with lysine, leucine, uracil, tryptophan (M-H) plates was used to select transfected colonies, pMKZ-RACK1 expressing yeast cells were transfected with *Arabidopsis* cDNA library plasmid DNA (2 μg). All transformants were selected on 5-fluoroorotic acid (5-FOA) plates containing minimal medium with yeast nitrogen base without amino acids (Sigma) and glucose, supplemented with leucine, lysine, uracil (M-HT), copper chloride (100 μM) and 0.5 mg/ml 5-FOA.

**PCR amplification.** Yeast DNA from FOA-resistant colonies was isolated after treating the cells with 40 U of lyticase (Sigma) for 10 min at 37°C. PCR-based amplification of the isolated DNA was performed in a 50 μl reaction mixture. Amplification of the DNA was performed by using NULF: 5′-GAT TTT CGT CAA GAC TTT GAC CGG TA-3′ as forward and CYC.R: 5′-TTT CGG TTA GAG CGG ATG TG-3′ as reverse primers in a thermocycler with an initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, with a final extension at 72°C for 15 min. The amplified PCR products were gel purified and sequenced using the NULF as sequencing primer. The PCR products were sequenced by a commercial vendor (Genewiz Inc.). BLAST search was used to reveal the identity of the DNA sequences (GenBank).

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