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SHORT COMMUNICATION

miR393s regulate salt stress response pathway in *Arabidopsis thaliana* through scaffold protein RACK1A mediated ABA signaling pathways

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ABSTRACT

Scaffold protein Receptor for Activated C Kinase 1 (RACK1) is a negative regulator of plant stress hormone – abscisic acid (ABA) mediated pathways. RACK1 has been reported to regulate global miRNA biogenesis pathway in *C. elegans*, humans, and in *Arabidopsis*. RACK1 regulates different steps of miRNA biogenesis and stability in response to different stimuli in plants. miR393s is implicated in salt stress response pathway through an antagonistic response between the stress hormone ABA-mediated salt stress and growth hormone auxin. Specifically, the known auxin receptor clade transcripts *TIR1/AFB2* are the target for the *miR393*s. By down-regulating the auxin signaling pathways, the *miR393*s inhibit the regulation of salt tolerance by auxin. Here we show that genetic loss of *RACK1A*- the predominant member of the three genes family of *RACK1* in *Arabidopsis*, results in the inhibition of miR393 level causing the same salt sensitivities as the individual *mir393a* or *mir393b* or the double mutant *mir393ab* phenotypes. We propose that down-regulation of auxin signaling through RACK1A induced miR393 biogenesis potentially regulates the *Arabidopsis* acclimation to salinity. Our findings fill up a molecular gap in our understanding of the role of miR393 mediated ABA and auxin-regulated salt stress responses.

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1. Introduction

1.1 Salt as a problem in cultivation

High salt concentrations in soil or water make cultivating crops of agronomic significance difficult, especially in arid, semi-arid regions and in irrigation areas. A recent study finds that every day for more than 20 years, an average of 2,000 hectares of irrigated land in arid and semi-arid areas across 75 countries have been degraded by salt.[1] The same study finds that the global annual cost of salt-induced land degradation in irrigated areas could be US$ 27.3 billion because of lost crop production.[1] Even trace amounts of salt in soil not only inhibits the plants growth, but also causes changes in the plants' physiology and metabolism from germination, seedling growth, bolting, flowering and fruit yields.[2,3] Salt stress affects plant physiology at both whole plant and cellular levels through osmotic and ionic stress.[4,5] The accumulation of toxic levels of ions has various effects on plant physiological processes such as increased respiration rate and ion toxicity, and decreased efficiency of photosynthesis.[4]

1.2 The role of RACK1

Receptor for Activated C Kinase 1 (RACK1) is a 37 kDa scaffold protein that is ubiquitously expressed in every eukaryotic organism and in few prokaryotes. The protein has seven tryptophan-aspartic acid amino acid repeats (WD-40 repeat) and it influences a variety of functions from signal transduction, regulation of transcription, growth hormones, control of conditions like drought and salt stress, innate immunity and senescence.[6] The structure and protein sequence of RACK1 is widely conserved in both plants and in the metazoan kingdom. The model plant, *Arabidopsis thaliana* (*A. thaliana*) genome contains three different *RACK1* genes: *RACK1A* (predominant form of the gene used in this study), *RACK1B* (87% sequence identity), and *RACK1C* (93% sequence identity).[7] Though use of genetic complementation studies in the individual loss of function mutant indicated that they are in principle functionally equivalent but based on gene activity levels, it is found that they operate on an unequal genetic redundant manner.[8] It is found that *RACK1A* is the ancestral gene which retains most functions of *RACK1* genes by inducing gene activity beyond the threshold level needed for RACK1 functions; whereas, *RACK1B* and *RACK1C* appear to be duplicated genes.[8] RACK1 mediates diverse environmental stress pathways by negatively regulating the stress hormone abscisic acid (ABA) signaling.[9] RACK1 has been found to interact with a plethora of proteins as a signal integrator for regulating diverse cellular and physiological process like translation, cell proliferation, hormone signaling, growth, development, photosynthesis and environmental stress.[6,10] This article presents the role of RACK1 on salt stress acclimation mediated by a cross-talk between ABA and auxin signaling pathways. This integrative role is exerted by regulating the biogenesis of a small RNAs (micro RNAs- *MIRNA*s), more specifically miR393s.

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1.3 miRNA/miR393, small RNAs and ABA

MicroRNAs (miRNAs) control many aspects of development and adaption in plants and animals by post-transcriptional control of mRNA stability and translatability.[11] Previously RACK1 has been implicated in the miRNA pathway of *C. elegans*, humans, and *Arabidopsis*.[11-13] It has been found that RACK1 mediates different steps of miRNA biogenesis, processing, and stability to regulate the miRNA abundance.[11] A combination of functions of RACK1 has been proposed in the miRNA pathway. They include *MIRNA* gene transcription/stabilization, pri-miRNA processing, and interaction with ARGONAUTE (AGO) effector protein to create RNA-induced silencing complex (RISC).[11] The proposed mechanism is supported by the findings of reduced miRNA level and increased accumulation of miRNA target mRNAs in *rack1* mutants.[11]

As a result of environmental stress, specific miRNAs may be induced to perform regulation of different genes and many have been implicated in salt, drought-like stress signaling pathways.[14–16] In this regard, miR393 has been found to be induced by salt and drought stresses.[14] During the experiment to identify global stress-related miRNAs in *Arabidopsis*, it was found that miR393 is strongly upregulated by cold, dehydration, NaCl, and ABA treatments.[14] The role of *miRNA393* in the ABA-mediated drought and salt stresses have been elucidated.[16] It is known that the target genes for the miR393 are F-box genes that encode auxin receptors TIR1, AFB2, and AFB3.[17] It is now proposed that *MIR393* exerts its ABA pathway function by an auxin antagonistic pathway; whereby upregulation of *miR393* by ABA results in the down-regulation of auxin-based signaling pathways. The evidence for this model comes from the use of an ABA hypersensitive mutant *fry1* mutants in *Arabidopsis*. The *fry1* mutants were found to be hypersensitive to the inhibition of lateral root development by ABA while displaying auxin-resistant like phenotypes.[16] The *fry1* mutants exhibited enhanced cleavage of auxin receptor transcripts as well as increased expression of the ABA-induced *miR393*.[16] Though it is now established that *MIR393* crosslinks to different hormone signaling pathways, the precise molecular mechanism of this antagonistic functions is not known.

Given the evidence that RACK1 is a negative regulator of the stress hormone ABA signaling, and a positive regulator of auxin signaling pathways,[19] it is not far-fetched to suggest that RACK1 may regulate these pathways possibly through miRNA regulation. The prominent role RACK1 plays in global miRNA biogenesis, processing, and stability indicates a role for RACK1 in the miRNA mediated ABA and auxin signaling pathways. Here we report that the known pathway of ABA-induced destabilization of the *TIR1/AFB* transcripts via *miR393*-guided cleavage potentially requires scaffold protein RACK1 function.

2. Materials and methods

2.1 Plant materials and growth conditions

A. thaliana ecotype Columbia (Col) was used in all experiments as the wild type (WT). Other *A. thaliana* plants used included the *rack1a-1* mutants, *miR393ab* mutants. *rack1a-1* (T-DNA is inserted into the first exon of the *RACK1A* gene) mutants[8] and *miR393* mutants seeds as reported in were obtained from Dr. Jarmolowski lab. All seeds were sterilized with 70% ethanol for 1 min, then for 10 min with bleach solution (30% bleach + 0.1% tween 20) and washed 3 times with DI H2O. Seeds were then planted on Murashige and Skoog growth media with basal salts and vitamins, Sucrose (30g/L) and Phytoblend (9g/L). MS plate grown two weeks old seedlings were transferred to the indicated plates and were allowed two additional weeks of growth. Growth chamber was set at 24–26°Celsius with 12 h of darkness and 12 h of light.

2.2 RNA isolation

All RNA was isolated using the trizol method following the manufacturer's recommended protocol (ThermoFisher Scientific, Waltham, MA). Briefly, 25 mg of whole plant tissue was homogenized with 0.5 ml of trizol and after a brief incubation of 5 min, 0.1 ml of chloroform was added and then centrifuged. The supernatant was used to precipitate RNA with 0.25 ml of isopropyl alcohol. The precipitated RNA was washed with 75% ethanol and air-dried pellet was dissolved in RNAse free water and quantified in a nanodrop equipment.

2.3 cDNA Synthesis and qPCR

cDNA synthesis was done from 500 ng of RNA using Superscript III First-Strand Synthesis Systems following the manufacturer's protocol. Note that the protocol calls for oligo (dT) primed cDNA synthesis that can amplify pri-miRNAs. Real-time quantitative PCR was done using standard protocols for the Bio-Rad CFX Manager 3.1 Detection System. For each reaction, 2µl of 1/25 dilution of cDNA was used with 2X Sybrgreen master mix (ThermoFisher Scientific, Waltham, MA) with 20 pmole of each primer (specific to the stem regions of miR393s) in 25 ul reaction volume. The reactions were amplified at 50° for 2 min, 95° for 10 min, then 39 cycles at 95° for 15 s and 55° for 1 min. Finally, 65° for 5 s and 95° for 5 s. All reactions had three technical replicates and two to five biological replicates. In-built BioRad software generated t-values that resulted in the pairwise significance level calculation.

2.4 PCR primers

Rack1a genomic DNA was amplified using primers: Forward: 5'-CTGAGGCTGAAAAGGCTGACAACAG-3′ Reverse: 5'-CTAG TAACGACCAATACCCCAAACTC-3' (~122bp). *MIR393a* genomic DNA was amplified using primers: miR393a-RT: Forward: AACCACATTGCTCTCAACTTTTAG and miR393a-RT: Reverse: GAGATAGCATGATCCAAAACCA (~162 bp). *MIR393b* genomic DNA was amplified using primers miR393b-RT: Forward: AAAACACCATTGCTCCCACCT and miR393b-RT: Reverse: CGCATGATCCGGAAAAGTAAG (~169bp). actin genomic DNA was amplified using primers: actin2-RT: Forward:

GGTAACATTGTGCTCAGTGGTGG and Actin2-RT: Reverse: AACGACCTTAATCTTCATGCTGC (~108bp).

3. Results and discussion

3.1 RACK1 is required for salt-induced *mir393* biogenesis

In addition, to be a negative regulator of ABA signaling pathways and a positive regulator of auxin-mediated lateral root development,[19] RACK1 protein has been implicated in miRNA biogenesis for several organisms and regulation of global miRNAs biogenesis process.[11] Among many other miRNAs, miR393 also specifically regulates ABA-mediated salt stress acclimatization process by targeting transcripts of auxin signaling pathways.[22] It is also known that double mutants of auxin receptors *tir1 afb2* is more tolerant to salt stress than wild-type plants and it has been proposed that down-regulation of auxin signaling might be part of *Arabidopsis* acclimation to salinity.[22] This salt acclimation is mediated by the salt-induced miR393 expression that resulted in the down-regulation of auxin receptors TIR1 and AFB2.[22] Several studies have also previously shown that *miR393* can be induced by different stresses including salt.[14,19-21,28] In order to investigate whether RACK1 has any role in the *MIR393* mediated pathways, salt-induced *miR393* expression level in WT and *rack1a-1* mutant plants were analyzed. Two-week-old *A. thaliana* seedlings were exposed to 100 mM NaCl for 14 days and gene expression level of *miR393a* and *miR393b* in WT and *rack1a-1* mutant plants were analyzed. As can be seen from Figure 1, NaCl induced the transcript level of both the *miR393a and miR393b* while in the *rack1a-1* mutant plants, neither of the *miR393* transcripts were expressed in the presence or in the absence of salt. This result indicates that the earlier findings of salt stress-induced upregulation of *miR393* transcripts[22] was based on the presence of a functional RACK1 protein. These results are consistent with the role of RACK1 in the biogenesis of the *miR393* transcripts. Though the study[22] found the *MIR393A* promoter, not *MIR393B* promoter, to be responsive to the salt treatment, our study shows that both of the *MIR393s* are induced by salt. The short treatment regimen of 2 h as opposed to our sustained 2-weeks long salt treatment may account for this discrepancy. It is possible that immediate response for salt acclimation is mediated by the *MIR393A* while a long sustained response may possibly be mediated by the *MIR393B*. It is interesting to note that salt induction of *MIR393B* was higher compared to that of *MIR393A* induction (Figure 1). A knowledge gap was present as to how the antagonistic effect of ABA and auxin during salt stress acclimation pathway operate and the findings of this result now show that scaffold protein RACK1A can potentially play the role of signal integration from these two apparently antagonistic pathways. RACK1 is known to integrate diverse signaling pathways ranging from the environmental stress to the growth and development of plants.[19]

3.2 Stress hormone ABA-induced mir393 expression requires RACK1A

MIR393 has been found to regulate many abiotic stresses like salt, cold, dehydration, and metal toxicity[15,16,22,23] Our hypothesis of RACK1 mediated miR393 biogenesis indicate that ABA-induced *miR393* transcript production[16] would be altered in the *rack1* mutant plants. Therefore, we investigated whether ABA-induced *miR393* expression requires the active presence of RACK1A protein. As seen in Figure 2(a,b), both *miR393a* and *miR393b* show significant ABA-induced expression in the WT plants but the expression is very attenuated in the *rack1a-1* mutant plant background indicating that an active RACK1A is required for the proper expression of the *miR393* to regulate ABA-induced stress signaling pathways which include salinity. Note that the attenuation is greater for the *miR393b* transcripts which correlates with our salt-induced *miR393* expression as well (Figure 1). Sustained

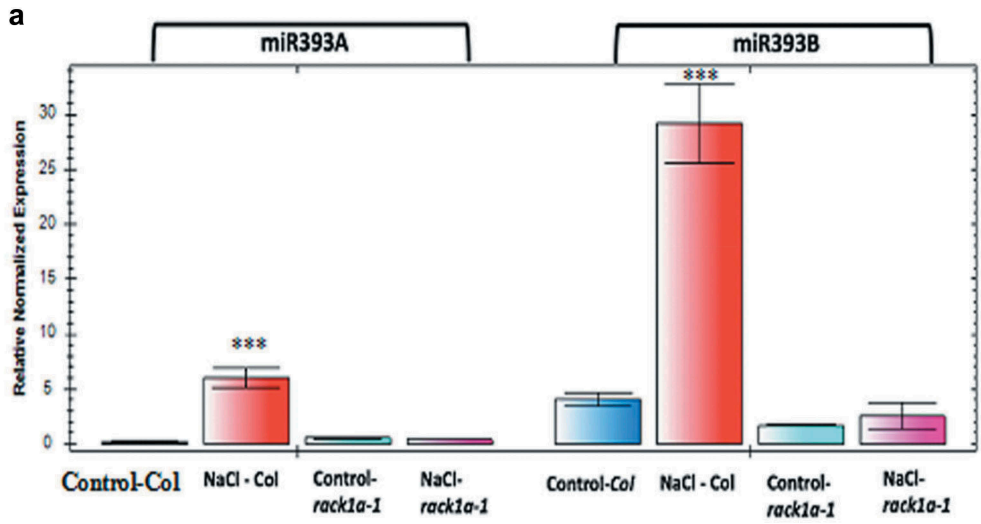

Figure 1. (a) RACK1A dependent *miR393* transcript expression in response to exogenous salt treatment. Quantifications were normalized to the expression of housekeeping gene Actin. The error bars represent standard deviation from three PCR replicate experiments. The WT (Col) and *rack1a-1* mutants were grown on MS plates with/without 100 mM NaCl for two weeks. Asterisks denote significant difference from the control plants (*** denotes $p < 0.01$, Student's t-test).

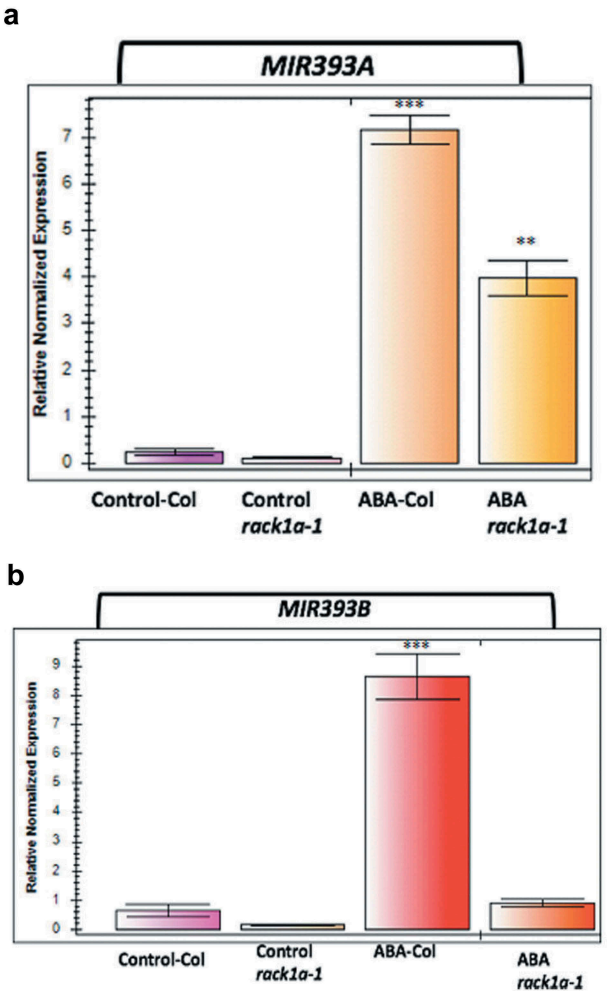

Figure 2. ABA treatment induced miR393 transcripts expression. qPCR analysis of miR393A (panel A) and miR393B (panel B) in response to exogenous ABA (0.5 mM). Quantifications were normalized to the expression of house-keeping gene Actin. The error bars represent standard deviation from three PCR replicate experiments. MS plate grown two-weeks old seedlings were transferred to MS plates containing ABA or methanol (vehicle control) and RNA were isolated after additional two weeks of growth. Asterisks denote significant difference from the control plants (** and *** denotes $p < 0.05$, $p < 0.01$, respectively, Student's t-test).

exposure to salt resulted in the higher induction of *MIR393B* indicating a role in the long-term salt stress response pathway. The *MIR393B* dominant function has been reported before[25], and it has been reported that the two *MIR393* genes have distinct, but partially overlapping expression profiles.[26] Our results also indicate a positive role of RACK1 in the ABA-induced *miR393* generation; however, it is known that RACK1 is a negative regulator of ABA signaling and short-term (2 h) ABA treatment resulted in the down-regulation of *RACK1* transcript expression.[9] However, it is quite possible that in the long-term (2 weeks) exposure to ABA-induced *miR393* biogenesis pathway, RACK1 has a distinct role through post-translational modification like phosphorylation of its key residues. RACK1 Y248 phosphorylation has been found to have a key role in mediating diverse stress responses.[27,28] ABA-induced RACK1 phosphorylation is quite possible in the RACK1 mediated *miR393* biogenesis. The results here clearly indicate that a functional RACK1 protein is required for the ABA-induced *miR393* biogenesis.

3.3 Auxin-induced *mir393* biogenesis

miR393-mediated posttranscriptional regulation of auxin receptors has been established as a crucial component of plant acclimation to salinity.[22] It has been proposed that through a feedback mechanism, auxin-induced receptors can positively regulate the *miR393* biogenesis.[25] However, when we assayed auxin-induced *miR393* biogenesis, we saw a modest increase (note the fold difference units on Y axis) in the *MIR393A* production but no significant induction was seen in the *MIR393B* biogenesis from auxin treatment for two weeks (Figure 3). While only *MIR393A* showed RACK1 dependency for its potential feedback induction, the *MIR393B* did not show any RACK1 regulated biogenesis (Figure 4). This indicates that possibly RACK1 regulates the *MIR393* accumulation by acting upstream of the biogenesis process and only *MIR393A* biogenesis through a feedback mechanism is regulated by RACK1. It is known that *MIR393A* is responsible to generate almost all the *miR393* in the root tissue[26] and auxin effect on lateral root development requires a functional RACK1 protein (Figure 4). Also, we cannot rule out an epistasis relationship between the MIR393A and MIR393B. In fact, when miRNA target sequence database is used, among the *MIR393A* target genes, *MIR393B* is listed among the targets (data not shown).

RACK1 has been found to have a role in MIRNA gene transcription and/or pri-miRNA stability.[11] Within the global pri-miRNA contents, some pri-miRNAs accumulated to a higher level and some pri-miRNA exhibited a reduction in the *rack1* triple mutants.[11] Notable among the upregulated transcripts was the *pri-miR393a* indicating RACK1 has a role in their transcription activation. However, those *pri-miR393a* transcripts were misprocessed and the mature level of *miR393a* was indeed lower in the *rack1* triple mutants[11] (Supplemental Fig.1 in reference 11) supporting the RACK1's positive role in the biogenesis of *miR393a* transcripts as we have reported here.

3.4 *MIR393* regulates lateral root abundance during salt stress

Chen et al.[16] demonstrated that *MIR393*-mediated attenuation of auxin signaling is essential for inhibition of lateral root growth by ABA or osmotic stress. *MIR393* was found to be involved in the repression of LR growth during salt stress.[22] As RACK1 has been found to be a regulator of *miR393* biogenesis, we investigated whether *rack1* mutant plants show similar inhibition of lateral root abundance during the salt stress. As can be seen in Figure 4, salt-induced reduction in the number of lateral roots is prominently observed in the WT plants while *mir393ab* double mutants showed insensitivity to the salt-induced reduction in the lateral root development. Likewise, *rack1a*-1 mutant plants also showed insensitivity in the same assay indicating that active *miR393* function is needed to attenuate lateral root development inhibition from salinity. Though the mutant plants started with a lower number of lateral roots, the percentage inhibition is much higher in the WT plants compared to that in both the mutant plants. This result, in conjunction with previous results,

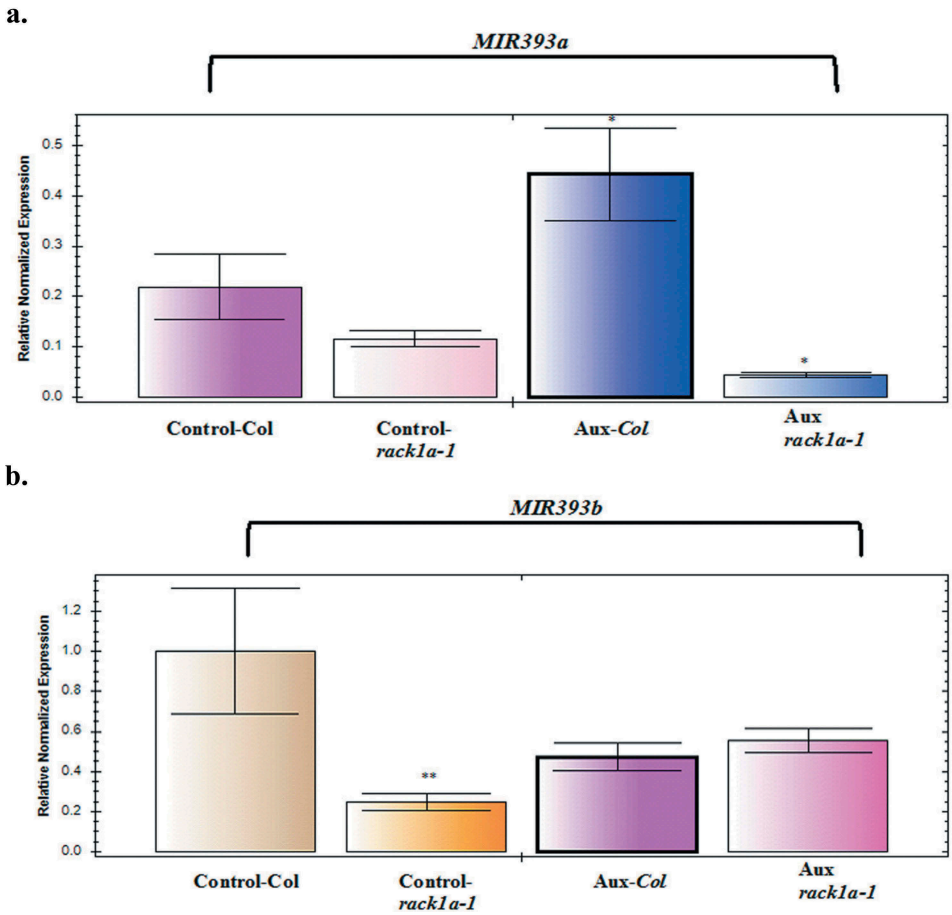

Figure 3. Auxin-induced miR393 transcript expression in WT and *rack1a-1* mutant plants. qPCR analysis of miR393A (panel A) and miR393B (panel B) in response to exogenous Auxin (10 mM). RNA was isolated from two weeks of treatment with 10 uM of auxin (IAA). The error bars represent standard deviation from three PCR replicate experiments. Quantifications were normalized to the expression of house-keeping gene Actin. Asterisks denote significant difference from the control plants (* and ** denotes $p < 0.1$, $p < 0.05$, respectively, Student's t-test).

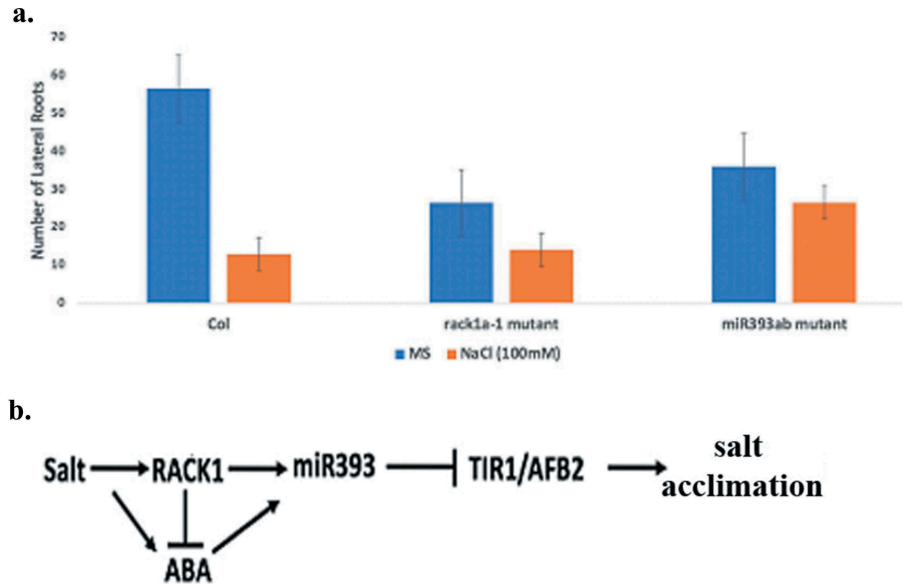

Figure 4. NaCl (100 mM) induced lateral root development in WT and *rack1a-1* and *mir393* mutant plants (a). Two weeks old seedlings were grown on NaCl plates for additional two weeks. While the WT plants showed statistically significant down-regulation in number of lateral roots the *rack1a-1* and *mir393ab* mutants did not show any significant difference in the lateral root numbers in response to salt treatment for two weeks. (b) proposed model. Signal Integration role of RACK1 crosslinks salt and/or ABA-induced RACK1 mediated auxin receptors targeting miRNA miR393. The down-regulation of auxin signaling affects the acclimation to salinity.

indicates that RACK1 mediated *miR393* generation may regulate the lateral root development during salt stress. The importance of root architecture during the stress is of paramount importance to plants and numerous genetic and physiological evidence indicate growth hormone auxin plays a dominant role in several developmental stages of LR development.[29] Our result now complements the earlier results that indicated a role of *miR393* in the inhibition of LR initiation and elongation under salinity and RACK1 as a regulator of *miR393* biogenesis plays an important role in this regard. In summary, it is quite possible that salt induces RACK1 that upregulates *miR393* biogenesis in the pericycle cells which subsequently down-regulate auxin signal to inhibit LR organogenesis. It is not far fetched to envision a role for stress hormone ABA to regulate LR growth under salt-stress as well.

Figure 4(b) presents a model that shows the relationship of RACK1, *miR393*, ABA and Auxin response genes on salt tolerance. Salt upregulates RACK1 and ABA, which leads to an increase of *miR393* that in turn inhibits auxin signaling and causes salt stress acclimation.

Conclusion: Survival of plants under salt stress requires rapid perception and adaptation to the stress condition. Though ABA has long been known to be a key regulator of stress responses, auxin is rapidly being implicated in regulating diverse environmental stresses including salinity stress. However, there is still many unknown as to how auxin and ABA can regulate a stress like salinity together. Here we fill a piece of the regulatory puzzle showing that scaffold protein RACK1 through the biogenesis of *miR393* can mediate the crosstalk between ABA and auxin in salt stress signaling pathway. We propose that RACK1A, which has previously been implicated in both the auxin and ABA pathway, functions as an integrator of *miR393* mediated ABA and auxin-regulated salinity stress responses. In this work, we showed that NaCl or ABA-mediated stress induced *miR393* expression and the expression is abolished in the *rack1a-1* mutant background. As the *mir393ab* double mutant plants show insensitivity to salt stress induced inhibition of lateral root development, we establish that it is a manifestation of *miR393* mediated attenuation of auxin signaling pathways that results in salinity acclimation. By combining the results, we proposed the model (Figure 4) where RACK1A occupies a major position to integrate the *miR393* mediated salt stress responses that combine opposing effects of two key plant hormones, ABA and auxin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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