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FASCICLIN LIKE ARABINOGALACTAN PROTEIN 4 and RESPIRATORY BURST OXIDASE HOMOLOG D and F independently modulate abscisic acid signaling

Hui Xue and Georg J Seifert*

Department of Applied Genetics and Cell Biology; University of Natural Resources and Life Science; Vienna, Austria

We previously suggested that *At-FLA4* and ABA signaling act in synergy. Reactive oxygen species generated from the NADPH oxidases *At-RBOHD* and *At-RBOHF* play an important role in cell wall integrity control and ABA signaling and here we investigate their role for the *At-FLA4* pathway. We find that in the *At-fla4 At-rbohD At-rbohF* triple mutant the root phenotype of *At-fla4* is enhanced. Moreover, the abnormally high level of reactive oxygen species in *At-fla4* mutant does not depend on *AtRBOHD* and *-F*. Likewise, suppression of the *At-fla4* phenotype by ABA does not depend on the 2 oxidases. Consistent with their lack of effect on ROS level in *At-fla4*, transcript level of *AtRBOHD* and *-F* is reduced in the *At-fla4* mutant background. Taken together, our findings suggest that neither *At-RBOHD* nor *At-RBOHF* is involved in the synergism between ABA and *At-FLA4*. Consistently, the oxidases and *At-FLA4* act independently of each other in ROS control.

Keywords: ABA, *At-FLA4*, *At-RBOHD*, At-rbohF, ROS, qRT-PCR

Abbreviations: ROS, reactive oxygen species; NADPH. nicotinamide adenine dinucleotide phosphate-oxidase; RBOH, respiratory burst oxidase homolog; FLAs, fasciclin-like arabinogalactan-proteins; AGP, arabinogalactan protein; ABA, abscisic acid.

*Correspondence to: Georg J Seifert; Email: georg.seifert@boku.ac.at

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Fasciclin-like arabinogalactan-proteins (FLAs) form a sub-group of arabinogalactan proteins (AGP), [1] which were previously implicated in cell wall polymer biosynthesis, cell wall remodeling, and signaling.[2] The *At-FLA4* locus of *A. thaliana* plays a non-redundant role for root growth and salt tolerance.[3-5] The root of *At-fla4* shows a short and fat phenotype, which is caused by abnormal expansion of epidermal, cortical, and endodermal cells.[5] Externally applied ABA suppresses the *At-fla4* phenotype, both its salt-oversensitivity and its root elongation defect under salt-free conditions. However, the mechanistic role of *At-FLA4* in ABA response is unclear.[3]

Arabidopsis contains 10 *RESPIRATORY BURST OXIDASE HOMOLOG* (*RBOH*) genes and RBOH-dependent Reactive oxygen species (ROS) have now been established as an important second messenger that regulates expression of hundreds of genes in response to stress.[6] Reactive oxygen species generated by the partially redundant RBOH isoforms D and F play an important role in abscisic acid (ABA) signaling in stomatal guard cells and roots.[7] ROS derived from *At-RBOHF* are involved in the regulation of osmosensitive metabolic changes[8] and both *At-RBOHD* and *At-RBOHF* play crucial roles in modulating ABA-inhibited root growth via production of ROS.[9] On the other hand, *At-RBOHD* and *At-RBOHF* are required for lignin deposition caused by cellulose biosynthesis inhibition in Arabidopsis roots implicating these loci with cell wall integrity control.[10,11]

Because of the involvement of *At-RBOHD* and *-F* in ABA signaling, salt tolerance and cell wall integrity control and the proposed role of *At-FLA4* as a link between cell walls and ABA signaling we tested the possibility that *At-FLA4* and *At-RBOHD* and *-F* might act in the same genetic pathway by isolating A*t-fla4 At-rbohD At-rbohF* triple mutants.[5,7] The *At-rbohD AtrbohF* and the *At-fla4 At-rbohD At-rbohF* mutant combinations are hitherto referred to as double and triple mutant, respectively.

On NaCl-free medium (MS0) the *At-fla4* mutant and the double mutant show

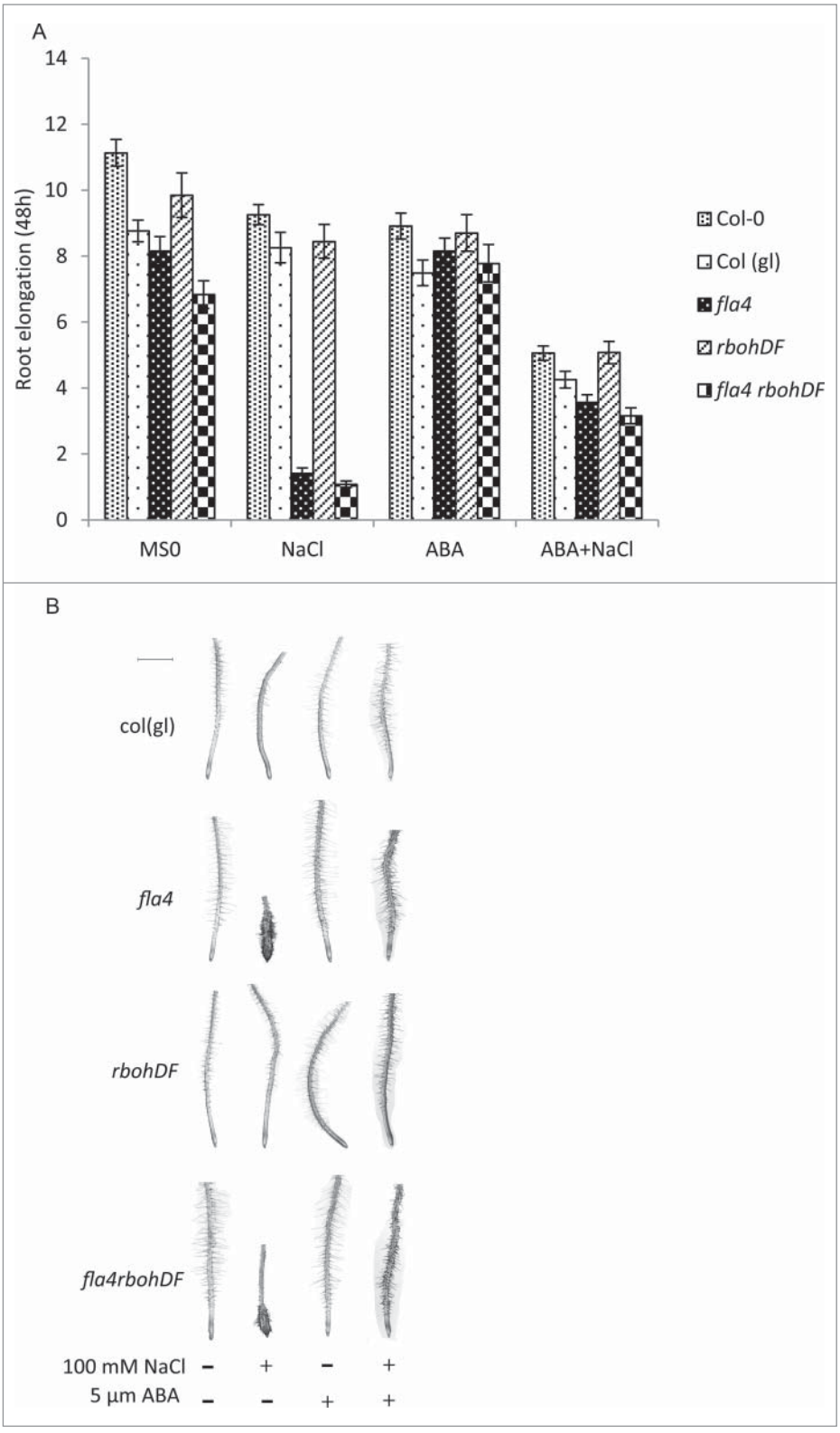

Figure 1. Interaction between *At-FLA4* and ABA is independent on *At-RBOHD* and *At-RBOHF*. (**A**) The effect of ABA on root length requires *At-FLA4*. Six-day-old WT, *At-fla4,* double and triple mutant seedlings were transferred to MS medium, or MS medium supplemented with 100 mM NaCl (NaCl), 5 μm ABA (ABA), or 100 mM NaCl +5 μm ABA (NaCl+ABA), respectively. Root length was measured after 48 h. Data are mean ± confidence interval ($n \geq 50$, $\alpha = 0.05$). (**B**) Salt tolerance in mutant in presence and absence of ABA. Six-day-old seedlings were transferred to different medium as described previously. Pictures were taken by a dissecting microscope (Leica EZ4 HD). Scale bar = 1 mm.

a significant reduction of root length (**Fig. 1A**), as previously reported.[3,7] Moreover, the triple mutant shows shorter root length compared to the *At-fla4* mutant and the double mutant (**Fig. 1A**). Transfer to and growth on 100 mM NaCl for 48 hrs leads to a moderate suppression of root growth in the wild type and the double mutant. The *At-fla4* single mutant and the triple mutant are both dramatically shorter ($P < 0.001$) than the wild type (**Fig. 1A**) and show root swelling to a comparable degree (**Fig. 1B**). The data indicate an additive effect of *At-FLA4* and the 2 oxidases on root growth.

As previously reported, the *At-fla4* phenotype is suppressed by 5 μM ABA both on salt free medium and on 100 mM NaCl (**Fig. 1**).[3] We also confirmed the recent observation that the double mutant is shorter and less responsive to ABA than wild type (**Fig. 1A**).[7] However, triple mutant roots are fully responsive to 5 μM ABA with respect to the suppression of the *At-fla4* mutant phenotype (**Fig. 1**). Taken together, this means that the ABA effect on *At-fla4* does not depend on the function of *At-RBOHD* and *At-RBOHF*.

Next, we analyzed salt stress-induced ROS in roots with 3, 3'-diaminobenzidine tetrahydrochloride (DAB).[12,13] Salt-stimulated ROS production show a biphasic profile with an elevation after 1 h NaCl treatment followed by a drop after 5 hrs and a second maximum at 24 hrs (**Fig. 2A**). Surprisingly, the double mutant shows the same profile and no significant differences to wild type. By contrast, in *At-fla4* and triple mutant roots the initial control level and final level of ROS are significantly ($P < 0.001$) higher compared to the wild type and the double mutant (**Fig. 2B**). This

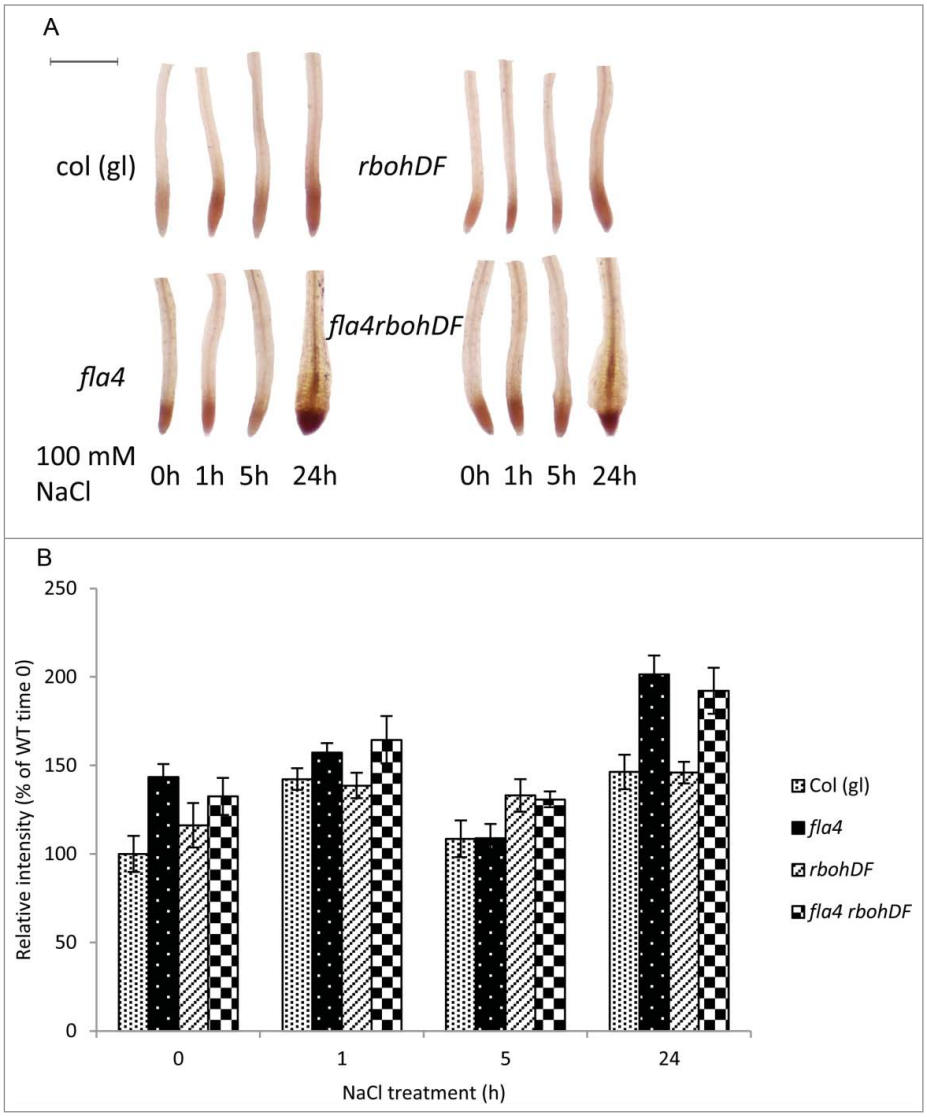

Figure 2. Effect of *At-FLA4*, *At-RBOHD*, *-F* and NaCl treatment on ROS levels. (**A**) DAB staining for ROS (brown) of seedlings grow on NaCl medium. The six-day-old seedlings were transferred to 100 mM NaCl medium for different time period. ROS was detected by DAB (Sigma) staining according to manufacturers' instructions. Pictures were taken by a dissecting microscope (Leica EZ4 HD). Scale bar = 0.5 mm. (**B**) Quantification of ROS after transfer to NaCl medium. The data showed is the inverted gray value in the meristem minus background measured by Image J ($n \geq 8$, $\pm$ confidence interval, $\alpha = 0.05$). Normalized to Col at time 0.

experiment suggests that loss of *At-FLA4* function triggers a signaling events leading to increased ROS production. However, the effect of *At-RBOHD* and *-F* in this process might either be negligible or too subtle to detect with DAB staining.

It was previously shown that NaCl medium leads to increased transcript levels of At-RBOHD and *-F*.[14] Consistently, transfer to and growth on 100 mM NaCl for 40 min leads to an increase of *At-RBOHF* mRNA level in both wild type and mutant (**Fig. 3**). However, despite the increase in DAB staining, RNA levels of both *At-RBOHD* and *-F* are significantly ($P < 0.05$) lower in *At-fla4* compared to wild type.

In this study, all the evidence suggests that the interaction between A*t-fla4* and ABA signaling is independent of *At-RBOHD* and *At-RBOHF*. ABA suppresses *At-fla4* and the triple mutant, both the salt-oversensitive phenotype and its root elongation phenotype, under salt-free conditions. ROS generated by *At-RBOHD* and *At-RBOHF* are not required for the *At-fla4* phenotype. The unchanged level of ROS in the double mutant compared to the wild type and in the triple mutant compared to the *At-fla4* single mutants in the absence and presence of NaCl, indicates that other cellular mechanism may contribute to ROS generation in Arabidopsis roots.[15] Our results do not exclude the possibility that other NADPH oxidase isoforms may be involved in the *At-FLA4* pathway. Apart from elevated ROS levels, the increase of DAB staining in *At-fla4* might also be due to altered peroxidase

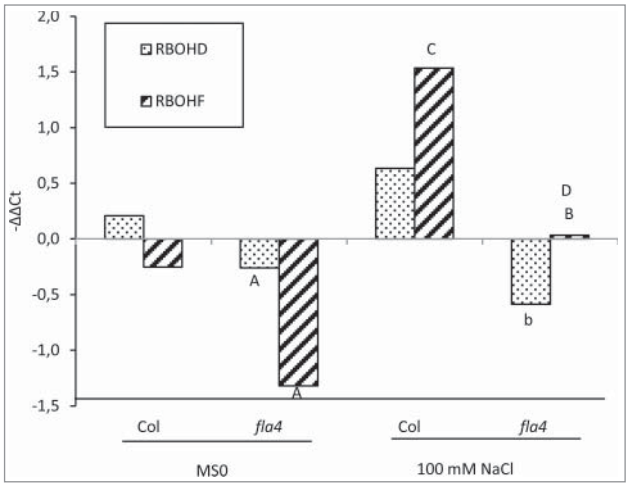

Figure 3. QRT-PCR-based analysis of the effects of NaCl treatment on *At-RBOHD* and *At-RBOHF* gene expression. 150 seedlings of Col-0 and *At-fla4* were grown on a nylon mesh (20 mm mesh size; Prosep, Belgium) for 5 d and were transferred to standard medium with or without 100 mM NaCl and incubated for 40 min. Roots were removed from the seedlings for RNA extraction. Samples were treated in biological triplicates. Detailed data analysis was described previously.[3] The indicated pairs were tested for statistically significant differences, and t-test values $\leq$0.05 and values $\leq$0.01 are indicated with lower case and uppercase letters, respectively, in the figures. (**A**) Col vs. *At-fla4* on standard medium (MS0), (**B**) Col vs. *At-fla4* on 100 mM NaCl, C: Col MS0 vs. Col NaCl, D: *At-fla4* MS0 vs. *At-fla4* NaCl.

levels in mutant roots. The mechanism behind ABA suppressing *At-fla4* phenotype is presently unknown, however our data indicate that this process acts independently of At-*RBOHD* and *-F*.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

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