Addendum to: Chen ZH, Wang Y, Wang JW, Babla M, Zhao C, Garcia-Mata C, Sani E, Differ C, Mak M, Hills A, Amtmann A, Blatt M.R. Nitrate reductase mutation alters potassium-nitrate-oxidase-mediated control of guard cell ion channels in Arabidopsis. New Phytol 2016; 209:1456–69, DOI: 10.1111/nph.13714.

Abscisic acid (ABA) is one of the most important phytohormones regulating plant tolerance to various environmental stresses especially drought. Drought-induced ABA accumulation triggers stomatal closure, reducing transpirational water loss from plants. Significance of ABA signaling pathways for stomatal closure has been well documented. ABA signaling pathway is composed by many elements including receptors, protein kinases and phosphatases, transcription factors, secondary messengers such as calcium (Ca^{2+}), hydrogen peroxide (H_{2}O_{2}), and nitric oxide (NO) and ion transporters. Therefore, a robust and complete ABA signaling pathway is significant for stomatal closure and plant drought tolerance. In Arabidopsis, ABA signal starts from ABA synthesis and its long-distance transport via ATP binding cassette transporters and nitrate transporters. Cytosolic ABA perception and signal transduction consists of the Pyrabactin Resistance (PYR)/Regulatory Component of the ABA Receptor (RCAR) ABA receptors. Protein Phosphatase 2Cs (PP2Cs) and Snf1-Related Protein Kinase 2 (SnRK2), PYR/RCAR-PP2C complex formation leads to inhibition of PP2C activity, thereby allowing activation of SnRK2 which targets major ion channels. ABA-induced H_{2}O_{2} and NO production down-regulate the activity of the PP2Cs and activate Ca^{2+}-permeable channels and anion channels. Elevated cytosolic Ca^{2+} activates Ca^{2+}-Dependent Protein Kinases (CDPKs) that function in ABA-induced stomatal closing, and anion and Ca^{2+} channel activation and directly phosphorylate PP2Cs and targets of SnRK2. Nitrate reductases NIA1 and NIA2 determine NO production in plants and are critical to ABA-induced stomatal closure. However, the exact role for NIA1 and NIA2 in ABA signaling has not been investigated in nitrate reductase loss-of-function mutant nia1nia2 as the two proteins are mainly studied for nitrogen metabolism.

**NIA mutation disrupts core components of the ABA signaling pathway**

It was unexpected that mutation in the NIA1 and NIA2 has led to dramatic changes in gene expression in Arabidopsis leaves in the control, ABA and NO treatments (Fig. 1). ABA is sensed by ABA receptors RCARs that interact with PP2Cs to release SnRK2s for downstream ABA signaling. ABA slightly upregulated the expression of RCAR1, RCAR11 and RCAR12, but reduced the transcript levels of RCAR10 and RCAR14 in wild type Col-0. However, except RCAR10 the other four key RCAR genes were upregulated in the nia1nia2 mutant in the control as compared to the WT. Interestingly, all five RCARs in nia1-nia2 were down regulated in response to ABA, while RCAR1, RCAR11, and RCAR12 were up-regulated by NO in nia1nia2 (Fig. 1). Both ABI1 and ABI2 were highly expressed in ABA and NO treatments in both genotypes with higher expression in nia1nia2. This indicates that the mutant may have high PP2C activities that may decrease the ABA signal transduction. All the tested protein kinases are upregulated by ABA in Col-0. Recently, it was reported that NO negatively regulates ABA signaling in guard cells by S-nitrosylation of OST1/SnRK2.6.19 We also found that NO downregulates the expression of OST1/SnRK2.6 in Col-0. However, except CBL-interacting protein kinase CIPK11 all the protein kinases in nia1nia2 were downregulated in all three conditions as compared to the control of Col-0 (Fig. 1). It again shows the ABA signal transduction
events are reduced in the nia1nia2 mutant. We further tested the key transcription factors (TFs) for their response to ABA and NO in Col-0 and nia1nia2. In response to ABA, all eight TFs showed some extent of upregulations, but four were downregulated in ABA treatment in nia1nia2. In the ABA signaling pathway, upregulation of ABA-responsive kinase substrate (AKS) and MYB domain proteins are usually key to the proper function and regulation of downstream components, but these genes are apparently disrupted in the nia1nia2 mutant (Fig. 1).

Moreover, the transcripts of NIA1 and NIA2 were downregulated, which may be compensated by the highly-induced GLU1 and GLN1 for normal nitrogen metabolism (Fig. 1). The nia1nia2 mutants showed a largely disrupted membrane transporters reflected by the high upregulation of KAT1 and AKT1 and downregulation of GORK and NRT2.1 (Fig. 1). In summary, all these abnormally expressed genes in the ABA signaling pathway demonstrate that NIA1 and NIA2 are required for ABA-induced stomatal closure.

A signaling model for stomatal response to ABA in nia1nia2

Based on our recent publication and the current data (Fig. 1), a simplified model (Fig. 2) is proposed for the dramatically disrupted ABA signaling transduction in the nia1nia1 mutant. In the guard cells of nia1nia2, ABA signaling is not properly...
transduced due to following mechanisms. 1.) The downregulation of ABA receptor genes RCARs may reduce the chance of ABA binding; 2.) The upregulation of protein phosphatase genes ABIs may inhibit the activity of protein kinase OST1/SnRK2.6; 3.) The downregulation of ABA-responsive protein kinase genes and unchanged OST1/SnRK2.6 may reduce their capacity to activate SLAC1 anion channel; 4.) The downregulation of SLAC1 may also lead to reduced stomatal closure; 5.) ABA responsive TFs downregulated in mutant may further decrease the chance of ABA signal transduction; 6.) The constitutively upregulated major K+ channel genes KAT1 and AKT1 will lead to continuous K+ uptake for stomatal opening even in ABA treatment; 7.) The downregulation of K+ release channel GORK may block K+ loss for ABA-induced stomatal closure. Taken together, the loss of NIA1 and NIA2 function renders the mutant unable to relay ABA signal for stomatal closure. However, further research is required to validate these signaling events at levels of protein and signal interactions in guard cells.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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