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To cite this article: Chenchen Zhao, Shengguan Cai, Yizhou Wang & Zhong-Hua Chen (2016) Loss of nitrate reductases NIA1 and NIA2 impairs stomatal closure by altering genes of core ABA signaling components in Arabidopsis, Plant Signaling & Behavior, 11:6, e1183088, DOI: 10.1080/15592324.2016.1183088

To link to this article: http://dx.doi.org/10.1080/15592324.2016.1183088
ARTICLE ADDENDUM

Loss of nitrate reductases NIA1 and NIA2 impairs stomatal closure by altering genes of core ABA signaling components in Arabidopsis

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ABSTRACT
Nitrates reductases NIA1 and NIA2 determine NO production in plants and are critical to abscisic acid (ABA)-induced stomatal closure. However, the role for NIA1 and NIA2 in ABA signaling has not been paid much attention in nitrate reductase loss-of-function mutant nia1nia2. Recently, we have demonstrated that ABA-inhibited K+ current and ABA-enhanced slow anion current were absent in nia1nia2. Exogenous NO restored regulation of these channels for stomatal closure in nia1nia2. In this study, we found that mutating NIA1 and NIA2 impaired nearly all the key components of guard cell ABA signaling pathway in Arabidopsis. We also propose a simplified model for ABA signaling in the nia1nia2 mutant.

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 Ca2+, hydrogen peroxide (H2O2), 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 A TP 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 H2O2 production down-regulate the activity of the PP2Cs and activate Ca2+-permeable channels and anion channels. Elevated cytosolic Ca2+ activates Ca2+-Dependent Protein Kinases (CDPKs) that function in ABA-induced stomatal closing, and anion and Ca2+ 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 nia1nia2 were downregulated in response to ABA, while RCAR1, RCAR11, and RCAR12 were upregulated 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 were 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 It was 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-responsible 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 nia1nia2 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.

**Acknowledgments**

We thank Dr Anya Salih and Linda Westmoreland for technical assistance at Western Sydney University.

**Funding**

This work was supported by a Discovery Early Career Researcher Award (DECRA) from the Australian Research Council (DE140101143) and a Chinese Young 1000-Plan project to Z.H.C.Y.W. was a recipient of a Chinese Scholarship Council award.

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