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Nitric oxide suppresses the inhibitory effect of abscisic acid on seed germination by S-nitrosylation of SnRK2 proteins

Pengcheng Wang[1], Jian-Kang Zhu[1,2,*], and Zhaobo Lang[2,*]

[1]Department of Horticulture and Landscape Architecture; Purdue University; West Lafayette, IN USA; [2]Shanghai Center for Plant Stress Biology; Shanghai Institutes for Biological Sciences; Chinese Academy of Sciences; Shanghai, China

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Nitric oxide (NO) plays important roles in plant development, and biotic and abiotic stress responses. In a recent study, we showed that endogenous NO negatively regulates abscisic acid (ABA) signaling in guard cells by inhibiting sucrose nonfermenting 1 (SNF1)-related protein kinase 2.6 (SnRK2.6)/open stomata 1(OST1) through S-nitrosylation. Application of NO breaks seed dormancy and alleviates the inhibitory effect of ABA on seed germination and early seedling growth, but it is unclear how NO functions at the stages of seed germination and early seedling development. Here, we show that like SnRK2.6, SnRK2.2 can be inactivated by S-nitrosoglutathione (GSNO) treatment through S-nitrosylation. SnRK2.2 and the closely related SnRK2.3 are known to play redundant roles in ABA inhibition of seed germination in Arabidopsis. We found that treatment with the NO donor SNP phenocopies the *snrk2.2snrk2.3* double mutant in conferring ABA insensitivity at the stages of seed germination and early seedling growth. Our results suggest that NO negatively regulates ABA signaling in germination and early seedling growth through S-nitrosylation of SnRK2.2 and SnRK2.3.

Nitric oxide (NO) is an important signaling molecule that regulates many physiological processes in plants, including immunity against pathogens, senescence, growth and development, and flowering.[1-5] NO also affects the signaling of phytohormones such as abscisic acid (ABA), cytokinin, auxin, gibberellins, and salicylic acid.[2,6-8] In ABA signaling, NO was considered as a second messenger and its generation can be enhanced by ABA.[2,9-11] ABA inhibits seed germination and early seedling growth. In contrast, application of exogenous NO breaks seed dormancy and alleviates the inhibitory effects of ABA on seed germination and early seedling growth.[12,13] The *nia1-nia2noa1* triple mutant that is deficient in NO generation shows delayed seed germination, and is hypersensitive to ABA at seed germination and early seedling development stages.[14] ABA induces stomatal closure, and the *nia1nia2noa1* triple mutant is hypersensitive to ABA in stomatal closure. These results suggest that endogenous NO has a negative role in ABA signaling in guard cells as well as in seed germination and early seedling establishment. In response to ABA, 3 SnRK2 family members, SnRK2.2, SnRK2.3 and SnRK2.6/OST1, are activated and phosphorylate many downstream effectors to cause stomatal closure, and inhibition of seed germination and seedling growth.[18-22] *SnRK2.6* is preferentially expressed in guard cells, and its dysfunction impairs ABA induction of stomatal closure.[21] *SnRK2.2* and *SnRK2.3* are mainly expressed in seeds and young seedlings, and they play redundant roles in ABA inhibition of seed germination and seedling growth.[10] In a recent study,[15] we discovered that endogenous NO negatively regulates ABA signaling in guard cells by S-nitrosylation of SnRK2.6. We showed that NO caused the S-nitrosylation of SnRK2.6 at cysteine 137 in vitro and in vivo, and the S-nitrosylation blocked the kinase activity of SnRK2.6. Dysfunction of the S-nitrosoglutathione (GSNO) reductase (GSNOR) gene in the *gsnor1* mutant causes NO over-accumulation, leading to impairment of ABA effect on stomatal movement.[15] Together with a previous study showing that NO inhibits the generation of reactive oxygen species by S-nitrosylation of NADPH oxidase,[16] the work revealed a novel mechanism of NO-mediated negative feedback regulation of ABA signaling in guard cells.[15]

The S-nitrosylation site in SnRK2.6, Cys-137, is conserved in all 10 members of the SnRK2 family in Arabidopsis (**Fig. 1A**), indicating that SnRK2s besides SnRK2.6 may also be inactivated by S-nitrosylation. To test whether NO regulates the activity of other SnRK2s in ABA signaling, the effect of GSNO on MBP-tagged SnRK2.2 was tested by an in vitro kinase assay. As shown in **Figure 1B**, GSNO inhibited SnRK2.2 activity in a dose-dependent manner, and application of DTT reversed this inhibitory effect of GSNO. The biotin-switch assay revealed that the GSNO treatment induced the S-nitrosylation of SnRK2.2, which was abolished by application of DTT (**Fig. 1C**). These results

*Correspondence to: Zhaobo Lang; Email: lang.katrina@gmail.com, Jian-Kang Zhu; Email: jkzhu@purdue.edu
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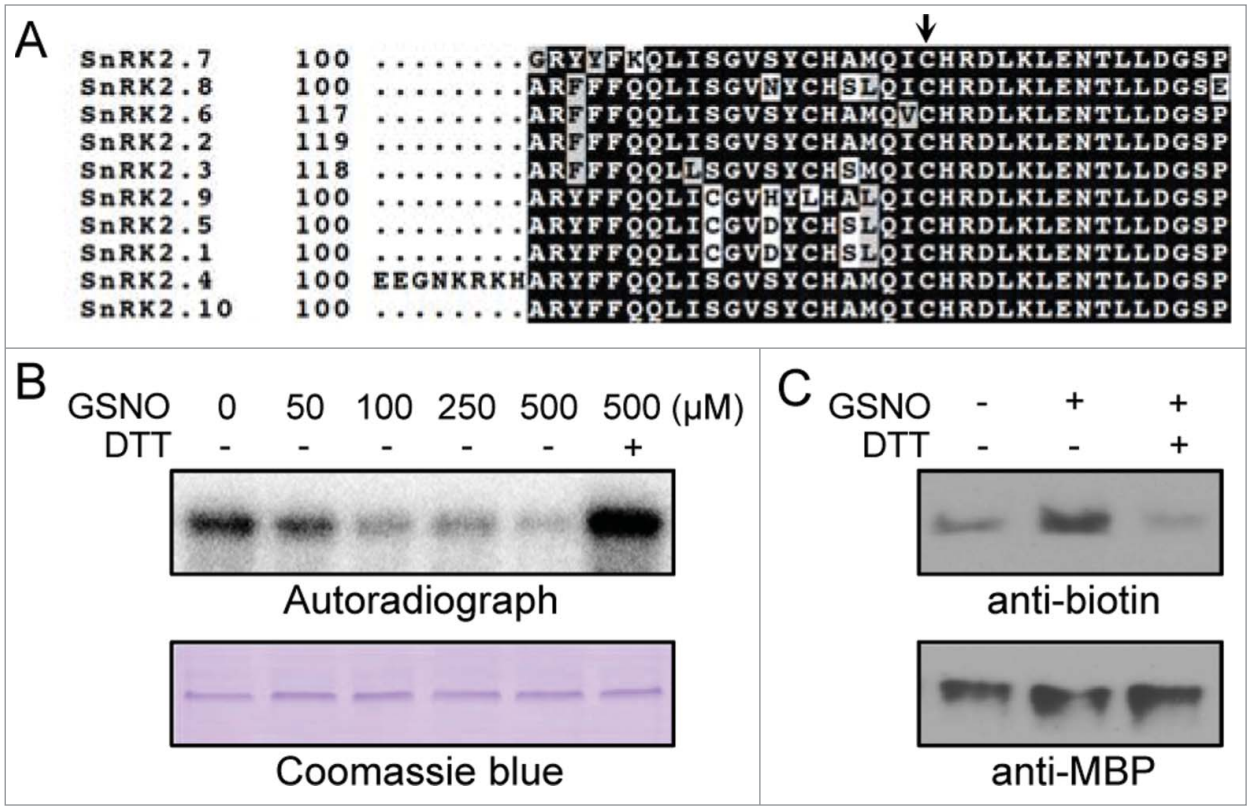

Figure 1. GSNO inhibits SnRK2.2 activity and induces the S-nitrosylation of SnRK2.2. (**A**) Cys137 of SnRK2.6 is conserved in all SnRK2s in Arabidopsis. The conserved cysteine is indicated by the arrow. (**B**) The nitric oxide donor GSNO inhibits the activity of SnRK2.2 in a dose-dependent manner. MBP–SnRK2.2 was incubated with the indicated concentration of GSNO for 10 min and then [$\gamma-^{32}$P]ATP was added to determine the autophosphorylation of SnRK2.2. In the rightmost lane (DTT+), 1 mM DTT was added to the reaction before adding [$\gamma-^{32}$P]ATP. (**C**) GSNO causes S-nitrosylation of SnRK2.2 as detected by the biotin-switch assay.

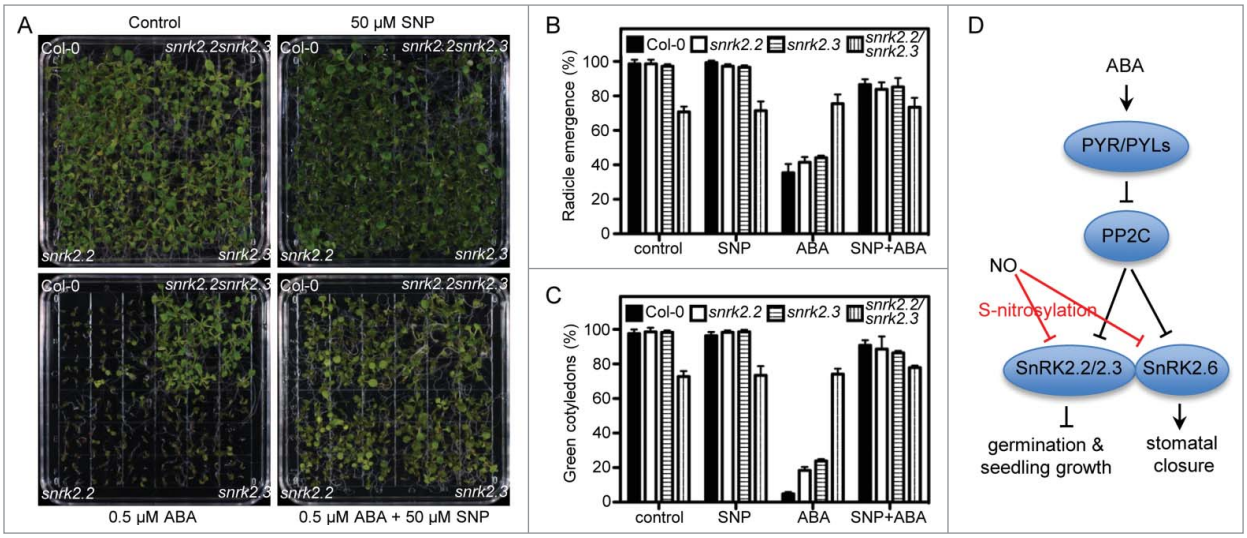

Figure 2. NO suppresses ABA inhibition of seed germination and early seedling growth. (**A**) Col-0 wild type, *snrk2.2, snrk2.3*, and *snrk2.2snrk2.3* seedlings grown on ½ MS medium (control) or ½ MS medium supplemented with 0.5 μM ABA, 50 μM SNP or 0.5 μM ABA and 50 μM SNP 10 days after imbibition. (**B**) Quantification of radicle emergence of each genotype 4 days after imbibition on ½ MS medium or ½ MS medium containing ABA and SNP. (**C**) Percentage of seedlings with green cotyledons 7 days after imbibition on ½ MS medium or ½ MS medium containing ABA and SNP. (**D**) Model showing NO negative regulates ABA signaling by S-nitrosylation of SnRK2.2, SnRK2.3 and SnRK2.6. Arrows and bars indicate positive and negative effects, respectively. The red bars indicate the S-nitrosylation mediated inhibition of SnRK2s by NO.

show that GSNO blocks the kinase activity of SnRK2.2 by S-nitrosylation. Since the sequences of SnRK2.2, SnRK2.3 and SnRK2.6 are very similar,[17] the inhibitory effect of GSNO on both SnRK2.2 and SnRK2.6 suggests that SnRK2.3 is likely also inhibited by S-nitrosylation. We were able to purify GST (glutathione S-transferase)-tagged but not MBP-tagged SnRK2.3 with detectable kinase activity. Because GST can be S-nitrosylated, we were unable to use GST-SnRK2.3 to test S-nitrosylation of SnRK2.3.

We tested the seed germination and seedling development of Col-0 wild type, the *snrk2.2* and *snrk2.3* single mutants, and the *snrk2.2snrk2.3* double mutant in the presence of ABA, NO donor sodium nitroprusside (SNP), or combination of ABA and SNP. As shown in **Fig. 2**, ABA treatment inhibited the germination and seedling development in wild type Col-0 and *snrk2.2* and *snrk2.3* mutants, but not in the *snrk2.2snrk2.3* double mutant. In the presence of SNP, the inhibitory effects of ABA on germination and cotyledon development were almost totally abolished, and there was no obvious difference among the wild type, *snrk2.2, snrk2.3* and *snrk2.2snrk2.3* mutants. The result is consistent with our notion that ABA activation of SnRK2.2 and SnRK2.3 is blocked by the application of exogenous NO. Our data here suggest that NO negatively regulates ABA signaling by S-nitrosylation of SnRK2s not only in stomatal closure but also in the inhibition of seed germination and seedling growth (**Fig. 2D**). In the presence of ABA, SnRK2.2 and SnRK2.3 are activated through the ABA-PYR/PYLs-PP2C core signaling pathway, and thereby inhibit seed germination by phosphorylating downstream effectors. Application of exogenous NO or accumulation of endogenous NO inhibits the kinase activities of SnRK2.2 and SnRK2.3 by S-nitrosylation and thus blocks ABA signaling. This explains how NO breaks seed dormancy and promotes seed germination, which have been observed in Arabidopsis, barley and many other plants.[12-14,23,24]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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