A new role for an old enzyme: Nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in *Arabidopsis thaliana*

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The plant hormone abscisic acid (ABA), synthesized in response to water-deficit stress, induces stomatal closure via activation of complex signaling cascades. Recent work has established that nitric oxide (NO) is a key signaling molecule mediating ABA-induced stomatal closure. However, the biosynthetic origin of NO in guard cells has not yet been resolved. Here, we provide pharmacological, physiological, and genetic evidence that NO synthesis in *Arabidopsis* guard cells is mediated by the enzyme nitrate reductase (NR). Guard cells of wild-type *Arabidopsis* generate NO in response to treatment with ABA and nitrite, a substrate for NR. Moreover, NR-mediated NO synthesis is required for ABA-induced stomatal closure. However, in the NR double mutant, *nia1*, *nia2* that has diminished NR activity, guard cells do not synthesize NO nor do the stomata close in response to ABA or nitrite, although stomatal opening is still inhibited by ABA. Furthermore, by using the ABA-insensitive (ABI) *abi1–1* and *abi2–1* mutants, we show that the ABI1 and ABI2 protein phosphatases are downstream of NO in the ABA signal-transduction cascade. These data demonstrate a previously uncharacterized signaling role for NR, that of mediating ABA-induced NO synthesis in *Arabidopsis* guard cells.

Increased biosynthesis and subsequent action of the hormone abscisic acid (ABA) is a key plant response to water-deficit stress. ABA initiates several processes, including stomatal closure, thereby leading to water conservation. The intracellular signaling cascades by which ABA effects guard cell shrinkage resulting in stomatal closure are complex, with several new signaling intermediates having been identified recently (1, 2). One such molecule is nitric oxide (NO), a signal molecule of increasing importance in plants (3, 4). Recent work has demonstrated that NO is an essential signaling intermediate in ABA-induced stomatal closure in *Pisum sativum* and *Vicia faba* (5, 6). However, despite these emerging new roles for NO, its biosynthetic origins in plants have not yet been resolved. Elucidation of the biosynthetic route(s) for NO, particularly during stomatal responses to ABA, is an important research goal, because it may facilitate the production of plants with enhanced drought tolerance.

Two potential enzymatic sources of NO in plants are NO synthase (NOS) and nitrate reductase (NR). NOS is a family of well characterized enzymes in mammalian cells that catalyze the conversion of L-arginine to L-citrulline and NO. NOS-like activity has been demonstrated in various plant tissues by using biochemical and pharmacological approaches (7). However, in *Arabidopsis thaliana*, NOS inhibitors have variable effects (8–10), and no obvious NOS-like sequences have been located in the *Arabidopsis* genome (11).

NR is a central enzyme of nitrogen assimilation in plants, catalyzing the transfer of two electrons from nicotinamide-adenine dinucleotide phosphate [NAD(P)H] to nitrate to produce nitrite (12). NR also catalyzes the NAD(P)H-dependent reduction of nitrite to NO (13), and this NO-generating capacity of NR has been demonstrated both *in vitro* and *in vivo* (14–16). However, a physiological role for NR-mediated NO synthesis has not yet been established.

In this article, we provide genetic evidence that NR-mediated NO synthesis is required for ABA-induced stomatal closure in *Arabidopsis*. Guard cells in epidermal peels of wild-type *Arabidopsis* generate NO in response to ABA and nitrite, such synthesis being essential for stomatal closure. However, in the NR double mutant *nia1, nia2* that has greatly diminished NR activity (17), guard cells do not synthesize NO, nor do the stomata close in response to ABA or nitrite, although they still respond to exogenous NO. These data reveal a previously uncharacterized signaling role for NR in *Arabidopsis*, that of mediating ABA-induced stomatal closure.

Materials and Methods

Plant Material. Wild type and various mutants of the Landsberg *erecta* (Ler) and Columbia (Col-O) ecotypes of *A. thaliana* were sown in Levington's F2 compost and grown under a 16-h photoperiod (250–300 $\mu E \cdot m^{-2} \cdot s^{-1}$) and 80% humidity in plant growth chambers (Sanyo Gallenkamp, Loughborough, U.K.) for 3–4 weeks before being used. The *nia1, nia2* double mutant seeds (background Col-O) were obtained from the Nottingham *Arabidopsis* Stock Centre (Nottingham, U.K.); *abi1–1* seeds (background Ler) were obtained from Peter Morris (Heriot-Watt University, Edinburgh); and *abi2–1* seeds (background Ler) were obtained from Maarten Koornneef (Wageningen University and Research Centre, Wageningen, The Netherlands). *abi1–1* and *abi2–1* genotypes were confirmed by diagnostic PCR (18). For all experiments using mutants, the appropriate background was used for wild-type controls.

Stomatal Bioassays. Stomatal assays were performed with epidermal peels and leaves, as indicated in the figures. Stomatal bioassays using leaves and epidermal fragments were carried out essentially as described (1). For experiments using epidermal peels, leaves were fixed onto cellotape with the abaxial side stuck down. The mesophyll cells were subsequently peeled off by using another strip of cellotape, and peels left stuck to the cellotape were incubated in CO_2-free Mes/KCl buffer (5 mM KCl/10 mM Mes/50 μM $CaCl_2$, pH 6.15) for 3 h. Once the stomata were fully open, peels were treated with ABA or various compounds and incubated in the same buffer for a further 3 h. Stomatal apertures were measured by using a light microscope (20 stomata per

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: ABA, abscisic acid; ABI, ABA-insensitive; DAF-2DA, diaminofluorescein diacetate; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NOS, NO synthase; NR, nitrate reductase; SNP, sodium nitroprusside.

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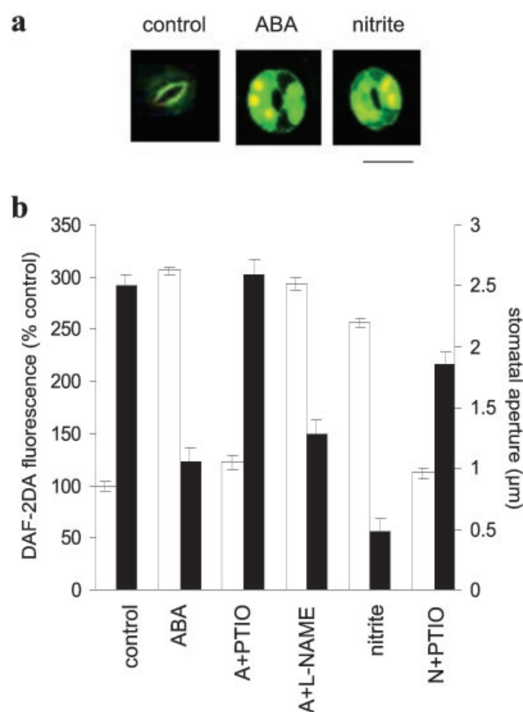

Fig. 1. ABA- and nitrite-induced NO generation correlate with stomatal closure. (*a*) ABA and nitrite induce NO synthesis in *Arabidopsis* guard cells. NO synthesis in wild-type epidermal fragments was monitored by DAF-2DA fluorescence (after 30 min) in the absence (control) or presence of ABA (50 μM) or sodium nitrite (1 mM). (Bar = 14 μm.) (*b*) NO generation (white bars) was determined by DAF-2DA fluorescence of wild-type guard cells either without (control) or with ABA (A, 50 μM) or sodium nitrite (N, 1 mM) in the presence or absence of PTIO (200 μM) or L-NAME (25 μM). Fluorescence intensities were determined 1 h after treatment (n = 43–252 for various treatments ± SE). Stomatal apertures (black bars) were measured for the same treatments in wild-type epidermal peels. Data are from three independent experiments (n = 60 stomata per treatment ± SE).

treatment) with a calibrated micrometer scale. Data are presented as the mean of three independent experiments.

Confocal Microscopy. NO measurement was performed by using the fluorescent NO indicator dye DAF-2DA (diaminofluorescein diacetate, Calbiochem). Epidermal strips were prepared by homogenizing leaves in a Waring blender for 20 s, and the strips were collected on a 100-μm nylon mesh (SpectraMesh, BDH-Merck) and incubated for 2–3 h in Mes/KCl buffer. After this step, the strips were loaded with 10 μM DAF-2DA for 10 min, followed by a wash step (with Mes/KCl buffer) for 20 min. The strips were subsequently incubated in buffer alone or treated with ABA, nitrite, or other compounds for various times as indicated in the text, before imaging with confocal microscopy (excitation 488 nm, emission 515–560 nm; Nikon PCM2000). Data acquired from the confocal microscope were analyzed by using SCION IMAGE software (Scion, Frederick, MD). Data are presented either as a percentage of fluorescence intensities of control-treated guard cells or as average intensities from several guard cells analyzed in different experiments.

Results

ABA and Nitrite Induce NO Synthesis in *Arabidopsis* Guard Cells. To determine the effects of ABA on NO synthesis in *Arabidopsis* guard cells, epidermal fragments prepared from *Arabidopsis* leaves were treated with 50 μM ABA, and NO synthesis was observed by using the fluorescent dye DAF-2DA and confocal microscopy (Fig. 1*a*). ABA induced a rapid increase in NO synthesis; a maximum response was reached within 10 min and persisted for at least 60 min (data not shown). Fluorescence was apparent throughout the cytoplasm and, in some cells, was particularly enhanced in and around the chloroplasts, in a manner similar to the ABA-induced DAF-2DA fluorescence previously observed in *P. sativum* and *V. faba* guard cells (5, 6). NO synthesis induced by ABA preceded stomatal closure (data not shown).

Nitrite is a substrate for NO synthesis mediated by NR both *in vitro* and *in vivo* (14, 15). To determine the potential role of NR in ABA-induced NO synthesis in *Arabidopsis* guard cells, epidermal fragments were incubated in sodium nitrite (1 mM) and DAF-2DA-fluorescence monitored. Nitrite induced NO synthesis in a manner similar to ABA (Fig. 1*a*), suggesting that guard cell NO synthesis might be mediated by NR. At this concentration, within the physiological range used *in vitro* (14) and measured *in vivo* (15), nitrite was not toxic to the cells (data not shown). Epidermal fragments also were loaded with 4AF-DA to serve as a negative control for NO-induced DAF-2DA fluorescence, as described (6). ABA treatment did not induce any increase in 4AF-DA fluorescence (data not shown).

NO Is Required for ABA- and Nitrite-Induced Stomatal Closure. Induction of NO synthesis is required for ABA-induced stomatal closure in pea and *V. faba* (5, 6). To determine the involvement of NO in the regulation of stomatal aperture in *Arabidopsis*, the effects of two NO donors, sodium nitroprusside (SNP) and *S*-nitrosoglutathione (GSNO), were assessed. Both SNP and GSNO induced stomatal closure (data not shown), similar to their effects in pea (5).

The relationship between NO synthesis and stomatal closure was investigated further by assessing these responses to the same treatments. A striking correlation between NO generation and stomatal closure was observed (Fig. 1*b*). Generation of NO after treatment of epidermal fragments with either ABA or nitrite correlated with stomatal closure. On the other hand, removal of NO by coincubation with the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) reduced both stomatal closure and DAF-2DA fluorescence induced by ABA or nitrite (Fig. 1*b*). Moreover, incubation in NaCl (as a negative control for sodium nitrite) did not induce closure (data not shown), indicating that the nitrite effects were not simply ionic. These data indicate that NO synthesis is required for stomatal closure in response to ABA or nitrite.

Various compounds have been used to inhibit NO synthesis in plants (7). The NOS inhibitors N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{G}-monomethyl-L-arginine monoacetate (L-NMMA) have been used to conclude that NOS is a potential source of NO in several plant species (5, 19, 20), including *Arabidopsis* (8, 10). However, L-NAME had little effect on ABA-induced stomatal closure or NO synthesis in *Arabidopsis* epidermal peels (Fig. 1*b*), implying that ABA-induced NO synthesis in *Arabidopsis* guard cells proceeds via a NOS-independent route. Taken together, the induction by nitrite of NO synthesis and stomatal closure and the lack of inhibition by L-NAME indicate NR as the likely source of NO in the ABA signaling cascade in *Arabidopsis* guard cells.

Nitrate Reductase Mediates ABA-Induced NO Synthesis and Stomatal Closure in Guard Cells. Currently, there are no specific inhibitors of NR available. Consequently, we adopted a genetic approach to provide evidence of a signaling role for NR in ABA-induced stomatal closure. Two genes in the *Arabidopsis* genome, *NIA1* and *NIA2*, encode nitrate reductase. The double mutant, *nia1, nia2*, that has <1% of the NR activity of the wild type (17) did not emit NO under conditions in which the wild type did (21), although wounding-induced NO production was still observed (10). It is not yet known which of the two *NIA* genes is expressed

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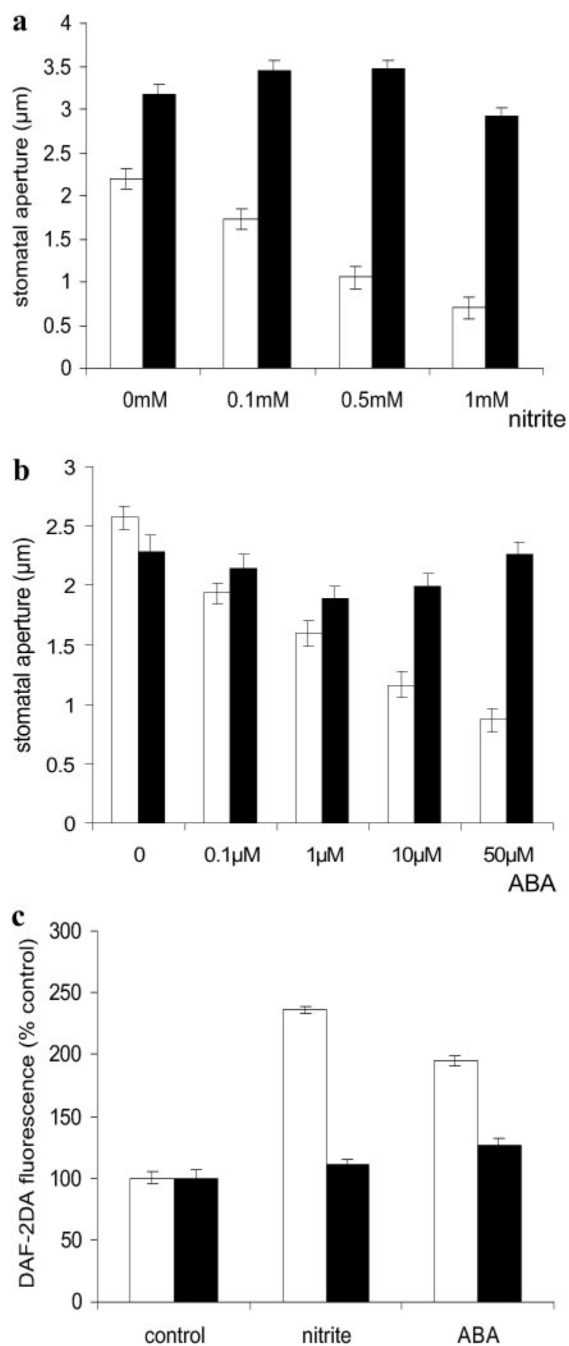

Fig. 2. Nitrite and ABA do not induce stomatal closure and NO synthesis in the *nia1, nia2* NR-deficient mutant. (*a*) Nitrite-induced stomatal closure. Epidermal peels from leaves of either wild type (white bars) or *nia1, nia2* (black bars) were treated with various concentrations of sodium nitrite, and stomatal apertures were measured after 3 h. (*b*) ABA-induced stomatal closure. Epidermal peels from leaves of either wild type (white bars) or *nia1, nia2* (black bars) were treated with various concentrations of ABA, and stomatal apertures were measured after 3 h. Data for *a* and *b* are from three independent experiments ($n = 60$ stomata per treatment ± SE). (*c*) ABA- and nitrite-induced NO synthesis. DAF-2DA fluorescence was determined (30 min) from control, nitrite-treated (1 mM), or ABA-treated (50 μM) guard cells of either wild type (white bars) or *nia1, nia2* (black bars; $n = 44$–124 ± SE).

in guard cells; therefore, the double mutant was used to determine the role of NR in ABA-mediated NO synthesis and stomatal closure.

Nitrite failed to induce stomatal closure in epidermal peels of the *nia1, nia2* mutant, whereas there was a clear dose–response in the wild type (Fig. 2*a*). Similarly, *nia1, nia2* stomata were far less sensitive to ABA (Fig. 2*b*). This insensitivity of *nia1, nia2* stomata to nitrite and ABA correlated with their inability to generate NO: both ABA and nitrite, when used at concentrations that induce NO synthesis in wild-type guard cells, did not induce NO synthesis in *nia1, nia2* guard cells (Fig. 2*c*). These data demonstrate that NR is required to generate NO that subsequently mediates stomatal responses to ABA.

To confirm that the insensitivity of *nia1, nia2* stomata to ABA and nitrite reflects their reduced NR activity, epidermal peels were exposed to NO via incubation in SNP. *nia1, nia2* stomata showed a response similar to wild-type stomata (Fig. 3*a*), indicating that NR lies upstream of NO in the signaling pathway leading to closure. Stomatal closure was also similarly induced in *nia1, nia2* as in the wild type by hydrogen peroxide (H_2O_2; Fig. 3*a*), another key signaling intermediate in the stomatal closure response activated by ABA (1), as well as by incubation in darkness (Fig. 3*b*), demonstrating that the failure of *nia1, nia2* stomata to close in response to ABA or nitrite is not caused by a general malfunction in the mechanisms of closure. The effect of ABA on inhibition of stomatal opening, a process differing from closure (22), was also determined. ABA inhibited stomatal opening in *nia1, nia2* as in the wild type (Fig. 3*b*), revealing that not all stomatal responses to ABA are reduced in *nia1, nia2.*

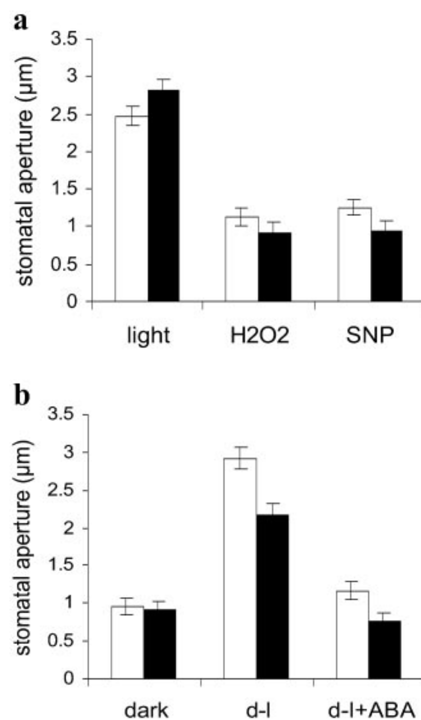

Fig. 3. Stomatal movements *per se* are not impaired in *nia1, nia2* plants. (*a*) Stomatal closure response. Epidermal peels of either wild type (white bars) or *nia1, nia2* (black bars) were kept in the light in the absence (control) or presence of H_2O_2 (100 μM) or SNP (50 μM), and stomatal apertures were measured after 3 h. (*b*) Stomatal opening response. Epidermal peels previously kept in the light to induce stomatal opening were transferred to the dark for 3 h (dark). Epidermal peels incubated in the dark to induce stomatal closure were transferred to the light and incubated for 3 h to induce stomatal opening in the absence (d-l) or presence of 50 μM ABA (d-l+ABA). Data from three independent experiments ($n = 60$ stomata per treatment ± SE).

Guard Cells of *abi1-1* and *abi2-1* Synthesize NO in Response to ABA but Do Not Respond to NO. Recently, the protein phosphatase 2C enzymes ABI1 and ABI2 have been placed in the H_2O_2 signaling

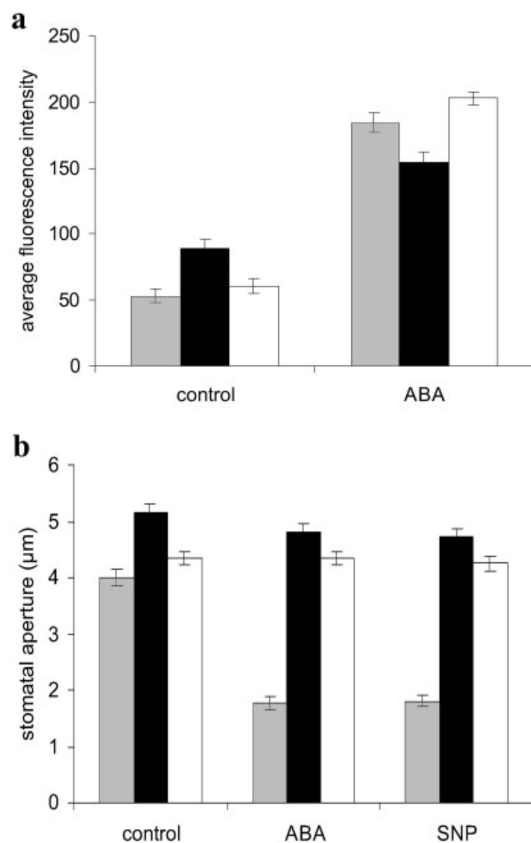

Fig. 4. ABA and NO signaling in ABA-insensitive mutants. (*a*) Guard cells of both *abi1-1* and *abi2-1* generate NO in response to ABA. DAF-2DA fluorescence was determined (30 min) from either control or ABA-treated (50 μM) guard cells of wild type (gray bars), *abi1-1* (black bars), or *abi2-1* (white bars) ($n = 16$–$41 \pm$ SE). (*b*) Stomata of *abi1-1* or *abi2-1* do not close in response to exogenous NO or ABA. *Arabidopsis* leaves of wild type (gray bars), *abi1-1* (black bars), or *abi2-1* (white bars) were treated either without (control) or with ABA (50 μM) or SNP (50 μM), and stomatal apertures were measured after 3 h ($n = 40$ stomata per treatment $\pm$ SE).

pathway induced by ABA that leads to stomatal closure (23). To establish their potential role in ABA-induced NO signaling during stomatal responses, we investigated NO synthesis and stomatal closure in *abi1-1* and *abi2-1* guard cells. Guard cells of both *abi1-1* and *abi2-1* synthesized NO in response to ABA (Fig. 4*a*), but their stomata did not close in response to treatment with exogenous ABA or NO, even at concentrations that induced nearly complete closure in wild-type plants (Fig. 4*b*, data not shown). These data position abi1-1p and abi2-1p downstream of NO synthesis (and hence NR) in the ABA signal-transduction pathway in guard cells.

Discussion

The data reported in this paper have considerable significance for both fundamental and applied plant biology. They demonstrate a previously uncharacterized signaling role for NR, that of mediating ABA-induced NO synthesis in *Arabidopsis* guard cells, required for ABA-induced stomatal closure. This conclusion is based on (*i*) pharmacological and physiological evidence showing that ABA and the NR substrate nitrite induce NO synthesis in guard cells and that removal of this NO inhibits ABA-induced stomatal closure, and on (*ii*) genetic evidence obtained through use of the NR-deficient *nia1, nia2* mutant. *nia1, nia*2 guard cells do not generate NO in response to ABA or nitrite, nor do *nia1, nia2* stomata close in response to these stimuli, although they still close in response to NO itself or to other closing stimuli. Other studies, including the use of NR-deficient mutants (13, 16, 21, 24), have shown that NR can generate NO in plant cells, but this article reports a physiological role for such NO generation. Whether or not induction of NR-mediated NO synthesis by ABA is a general response of all plant cells remains to be determined, although a recent report that ABA failed to induce NO synthesis in tobacco cell suspension cultures (25) suggests that it may be restricted to specialized ABA target cells such as guard cells. It is also possible that more than one route to NO production exists, and that different stimuli induce different biosynthetic pathways in different cells and species. For example, mechanical stress-induced NO formation in *Arabidopsis* was recently reported to be inhibited by NOS inhibitors but not reduced in the NR-deficient mutant (10).

It will be important to elucidate the mechanisms by which ABA rapidly activates the NO-generating capacity of NR in guard cells and how NO fits into the complex signaling web by which ABA influences stomatal movements (2, 22). NR activity is known to reflect the NR phosphorylation state; dephosphorylation, by a protein phosphatase (PP), is required to activate NR (13). Therefore, it may be that ABA activates NR via the activation of a PP enzyme. This possibility would accord with the known inhibition by okadaic acid of ABA-induced stomatal closure in *Arabidopsis* (22, 26).

The action of the two PP2C enzymes, ABI1-1 and ABI2-1, do not seem to be required for NO synthesis, because guard cells of both *abi1-1* and *abi2-1* were able to generate NO in response to ABA. However, stomata of neither mutant closed in response to NO, indicating that the action of these two enzymes is downstream of NO synthesis. Both ABI1 and ABI2 can act as direct targets for *in vitro* modification by H_2O_2 (27, 28). However, whether or not these enzymes are also modified by NO, either *in vitro* or *in vivo*, remains to be determined.

Drought stress is a major environmental constraint on crop production. Thus, the elucidation of previously uncharacterized signaling pathways mediating water stress tolerance provides new opportunities to enhance the water use efficiency of plants. However, under well watered conditions, the *nia1, nia2* mutant does not have a wilty phenotype, despite the ABA insensitivity of guard cells in epidermal peels. This observation might reflect the involvement of other ABA-signaling intermediates, such as H_2O_2 (1, 23), or complex cellular interactions in the whole plant. In fact, preliminary data indicate that *nia1, nia2* guard cells do synthesize H_2O_2 in response to ABA (data not shown). Given the complexity of ABA signaling in guard cells (2) and the interactions between H_2O_2 and NO (4), it is likely that H_2O_2 and NO interact to effect stomatal closure in response to ABA.

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