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NO serves as a signaling intermediate downstream of H_2O_2 to modulate dynamic microtubule cytoskeleton during responses to VD-toxins in Arabidopsis

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Although hydrogen peroxide (H_2O_2) and nitric oxide (NO) can act as an upstream signaling molecule to modulate the dynamic microtubule cytoskeleton during the defense responses to *Verticillium dahliae* (VD) toxins in Arabidopsis, it is not known the relationship between these two signaling molecules. Here, we show that VD-toxin-induced NO accumulation was dependent on prior H_2O_2 production, NO is downstream of H_2O_2 in the signaling process, and that H_2O_2 acted synergistically with NO to modulate the dynamic microtubule cytoskeleton responses to VD-toxins in Arabidopsis.

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Reactive oxygen species including hydrogen peroxide (H_2O_2) and nitric oxide (NO) are well established as signaling molecules, mediating a wide range of cellular responses. H_2O_2 signals have been shown to induce large transcriptional changes and cellular reprogramming that can either protect the plant cell or induce programmed cell death.[1-4] Moreover, NO has emerged as an important signaling molecule that mediates many developmental and physiological processes.[5-8] It has been demonstrated that NO cooperates with H_2O_2 to activate the hypersensitive reaction in plants.[9-12] However, the interaction of NO and H_2O_2 is still far from being clearly elucidated.[13-17] We have recently demonstrated that NO and H_2O_2 can act as an upstream signaling molecule to modulate the dynamic microtubule cytoskeleton during the defense responses to *Verticillium dahliae* (VD) toxins in Arabidopsis.[18,19] Here, we provide evidence that NO serves as a signaling intermediate downstream of H_2O_2 to modulates the dynamic microtubule cytoskeleton during the responses to VD-toxins in Arabidopsis.

The Interaction of NO and H_2O_2 in VD-Toxins-Induced Responses in Arabidopsis

The levels of NO and H_2O_2 in wild-type Arabidopsis leaves were monitored by cell permeable fluorophores, DAF-2DA and H_2DCF-DA, respectively. The fluorescent intensity in leaves significantly increased after treatment with VD-toxins (**Fig. 1A, b and h, B and C**).

To investigate the interaction between the NO and H_2O_2 production, wild-type seedlings were co-treated with VD-toxins plus DPI (a potent inhibitor of NADPH oxidase), DMTU (a H_2O_2 scavenger), cPTIO (a NO scavenger) and sodium tungstate (a potent inhibitor of nitrate reductase).

The VD-toxin-induced H_2O_2 production was almost completely prevented by supplements of DPI and DMTU, but only partially restricted by supplements of cPTIO or sodium tungstate (**Fig. 1A and B**). In contrast, the VD-toxin-induced NO production almost completely blocked by supplement of DPI or DMTU, cPTIO or sodium tungstate (**Fig. 1A and C**). This result showed that NO and H_2O_2 were signaling molecules in VD-toxin-induced responses in Arabidopsis, and that H_2O_2 was located upstream of NO in this pathway. Thus, VD-toxin-induced NO accumulation was dependent on H_2O_2 production in Arabidopsis.

NO and H_2O_2 Modulates VD-Toxins-Induced Dynamic Microtubule Cytoskeleton

Previous experiments indicated that NO is produced mostly by the nitrate reductase (NR) pathway in response to VD-toxins in Arabidopsis leaves.[20] The wild-type and *nia1*, *nia2* NR-deficient mutants of Arabidopsis were used to visualize microtubules in living leaf cells. The results showed that VD-toxins induced a time-dependent microtubule depolymerization, and that microtubule depolymerization was more severe in WT than in *nia1*, *nia2* NR-deficient mutants, especially at the later stages (**Fig. 2**). The data

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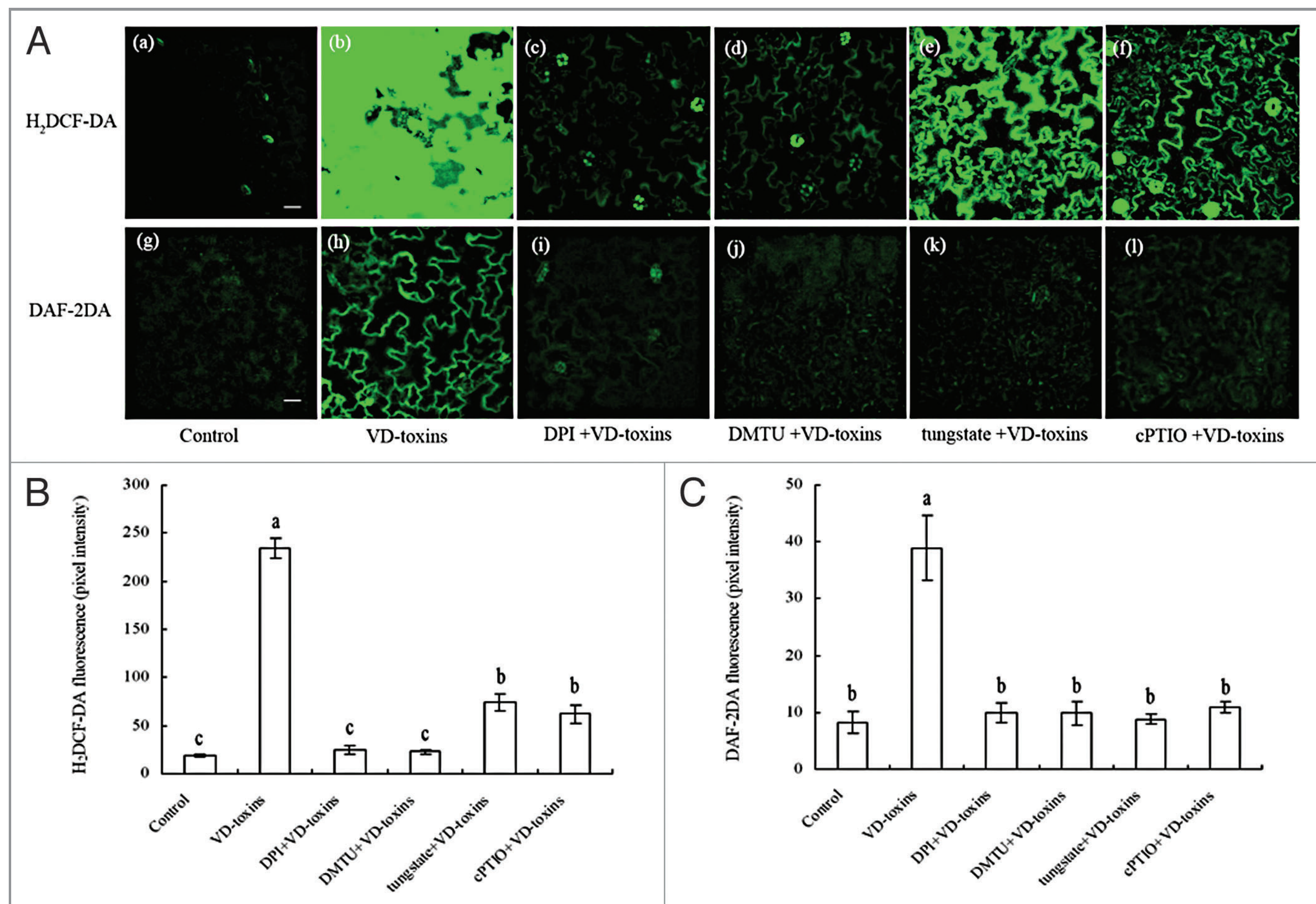

Figure 1. Effect of an inhibitor of NADPH oxidase (DPI), a H_2O_2 scavenger (DMTU), a NO scavenger (cPTIO) and an inhibitor of nitrate reductase (sodium tungstate) on VD-toxin-induced H_2O_2 and NO production in the leaves of wild-type Arabidopsis. (A) H_2O_2 and NO were detected by fluorescence resulting from H_2DCF-DA and DAF-2DA. Pictures were taken 60 min post-treatment. Bars = 20 μm. (B) The H_2DCF-DA fluorescence intensities in the leaves of wild-type Arabidopsis. (C) The DAF-2DA fluorescence intensities in the leaves of wild-type Arabidopsis. Confocal data are displayed as estimated mean pixel intensities and associated 95% confidence intervals. Error bars indicate standard deviations. Values of each group with the same letters were not significantly different ($p < 0.05$).

indicate that NO accumulation was involved in modulating VD-toxins-induced the dynamic microtubule cytoskeleton.

To further examine the role of H_2O_2 on NO modulation of VD-toxins-induced the dynamic microtubule cytoskeleton, we used different concentrations of exogenous H_2O_2 to treat the wild-type and *nia1*, *nia2* NR-deficient mutant seedlings. The depolymerization of cortical microtubules increased with increasing concentrations of exogenous H_2O_2; moreover, microtubule depolymerization was more severe in WT than in *nia1*, *nia2* mutants (**Fig. 3**). The results suggest that H_2O_2 modulated the dynamic microtubule cytoskeleton through the activity of NR. It is possible that VD-toxin-induced NO accumulation was dependent on prior H_2O_2 production, and that H_2O_2 acted synergistically with NO to modulate the dynamic microtubule cytoskeleton responses to VD-toxins in Arabidopsis.

Additionally, time course experiments with fluorescent probes showed that there was temporal separation of increases in H_2O_2 and NO, and NO production occurred after that of H_2O_2.[18,19] Taken together, these data suggest that NO serves as a signaling intermediate downstream of H_2O_2 to modulates the dynamic microtubule cytoskeleton during the responses to VD-toxins in Arabidopsis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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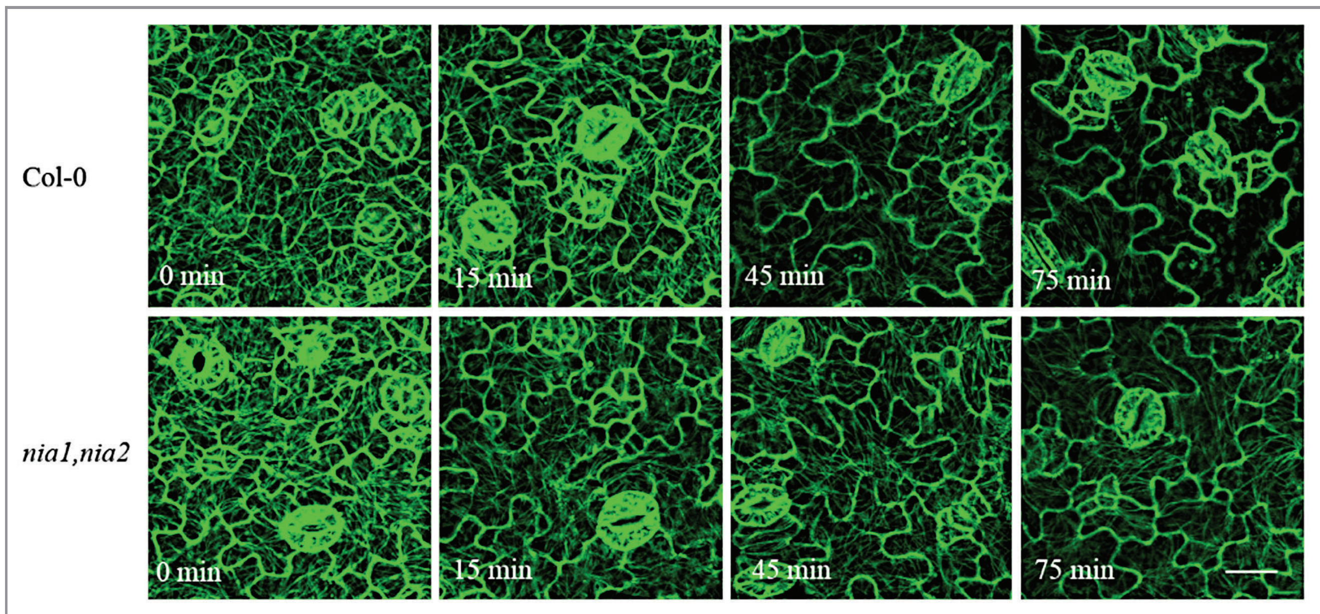

Figure 2. Sequential images of cortical microtubule alterations induced by VD-toxins (150 μg mL^{-1}) in the leaf pavement cells of the wild-type (Col-0) and *nia1*, *nia2* mutants expressing GFP–tubulin of Arabidopsis. Bars = 20 μm.

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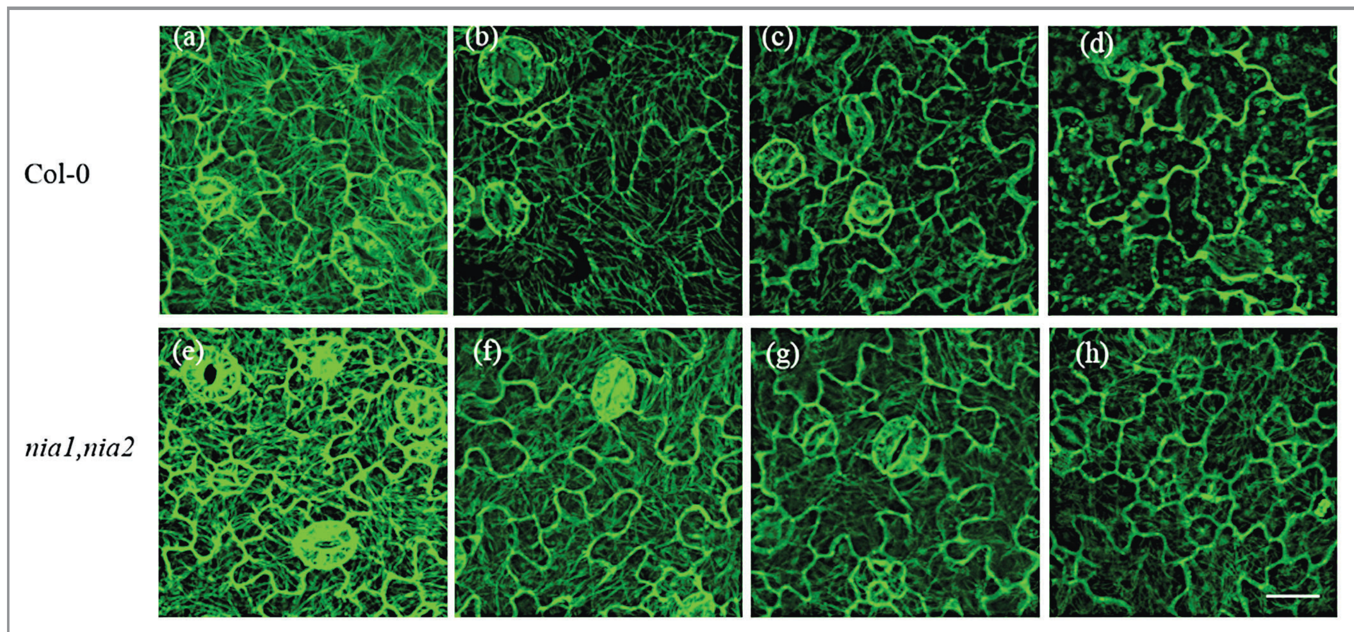

Figure 3. Disruption of the microtubule cytoskeleton induced by exogenous H_2O_2 in the leaf pavement cells of wild-type (a–d) and *nia1*, *nia2* mutants (e–h) Arabidopsis. (a) and (e) Control, leaves were untreated; (b) and (f) leaves were treated with 1 mM H_2O_2; (c) and (g) leaves were treated with 5 mM H_2O_2; and (d) and (h) leaves were treated with 10 mM H_2O_2. Pictures were taken 90 min post-treatment. Bar = 20 μm.

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