Hydrogen Peroxide Acts Upstream of Nitric Oxide in the Heat Shock Pathway in Arabidopsis Seedlings[1][C][W]

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We previously reported that nitric oxide (NO) functions as a signal in thermotolerance. To illustrate its relationship with hydrogen peroxide (H_2O_2) in the tolerance of Arabidopsis (*Arabidopsis thaliana*) to heat shock (HS), we investigated the effects of heat on Arabidopsis seedlings of the following types: the wild type; three NADPH oxidase-defective mutants that exhibit reduced endogenous H_2O_2 levels (*atrbohB*, *atrbohD*, and *atrbohB/D*); and a mutant that is resistant to inhibition by fosmidomycin (*noa1*, for *nitric oxide-associated protein1*). After HS, the NO levels in *atrbohB*, *atrbohD*, and *atrbohB/D* seedlings were lower than that in wild-type seedlings. Treatment of the seedlings with sodium nitroprusside or *S*-nitroso-*N*-acetylpenicillamine partially rescued their heat sensitivity, suggesting that NO is involved in H_2O_2 signaling as a downstream factor. This point was verified by phenotypic analyses and thermotolerance testing of transgenic seedlings that overexpressed *Nitrate reductase2* and *NOA1*, respectively, in an *atrbohB/D* background. Electrophoretic mobility shift assays, western blotting, and real-time reverse transcription-polymerase chain reaction demonstrated that NO stimulated the DNA-binding activity of HS factors and the accumulation of HS proteins through H_2O_2. These data indicate that H_2O_2 acts upstream of NO in thermotolerance, which requires increased HS factor DNA-binding activity and HS protein accumulation.

The recent warming of the world's climate system has become a limiting factor in plant growth and development (Battisti and Naylor, 2009; Perez et al., 2009). Although plants, as sessile organisms, cannot escape from heat stress, they possess methods and morphological adaptations to avoid being wounded by it. For example, as a countermeasure to heat stress, plants synthesize a set of proteins known as heat shock proteins (HSPs). In eukaryotes, HSP induction is dependent on heat shock factors (HSFs), which are transcription factors that bind to heat shock promoter elements (HSEs) in the promoter regions of HSP genes (Nielsen et al., 2005; Akerfelt et al., 2010). The regulation of HSP synthesis is considered to be a particularly important issue in thermotolerance.

Nitric oxide (NO) is a bioactive molecule that is extensively involved in plant defenses against environmental stimuli, including drought, salt, cold, heat, and disease (Zhao et al., 2004; Guo and Crawford, 2005; Neill et al., 2008; Wilson et al., 2008; Xuan et al., 2010). NO is mainly produced via two pathways: the Arg-dependent nitric oxide synthase (NOS) and nitrite-dependent nitrate reductase (NR) pathways. Although experiments utilizing mammalian NOS inhibitors suggest the existence of a plant NOS (Ninnemann and Maier, 1996; Zhao et al., 2004), a mammalian NOS-like enzyme has not been confirmed in plants. Guo et al. (2003) reported and isolated a NOS gene in Arabidopsis (*Arabidopsis thaliana*). However, critical questions have been raised about the role of AtNOS1 in NO biosynthesis, leading to the suggestion that NOA1 be renamed *nitric oxide-associated protein1* (*noa1*; Crawford et al., 2006). Flores-Pérez et al. (2008) demonstrated that the accumulation of plastid-targeted enzymes of the methylerythritol pathway conferring resistance to fosmidomycin in an isolated *noa1* allele named *rif1* (for *resistant to inhibition by fosmidomycin1*) is insensitive to NO donor, thus suggesting that the loss of NOA1/RIF1 function affects physiological processes unrelated to NO synthesis. Interestingly, NOA1/RIF1 contains a GTP-binding domain and has been suggested to be a member of the circularly permuted GTPase family of RNA/ribosome-binding proteins involved in ribosome assembly (Flores-Pérez et al., 2008; Moreau et al., 2008). Although it is uncertain how NOA1 affects NO accumulation, *noa1*, which exhibits reduced endogenous NO levels, is still a valuable tool for studies of NO function (Guo and Crawford, 2005; Zhao et al., 2007, 2009; Asai et al., 2008; Wang et al.,

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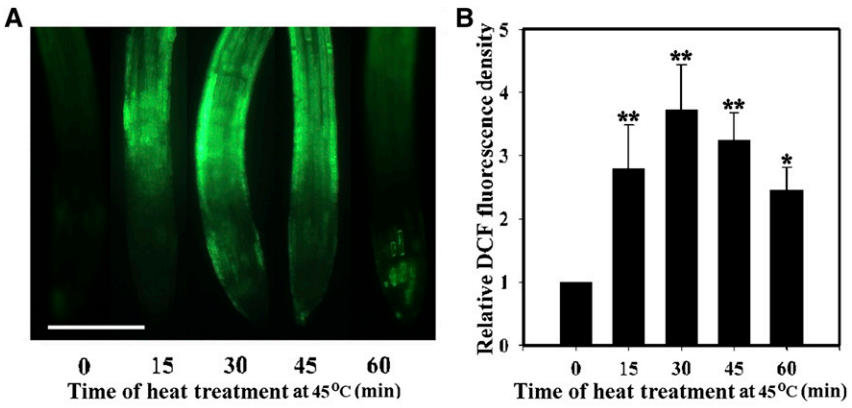

Figure 1. Effects of HS on endogenous H_2O_2 levels in wild-type seedlings. A, Six-day-old wild-type seedlings grown at 22°C were exposed to 45°C for 0 to 60 min and then examined for H_2O_2 by fluorescence microscopy in roots stained with CM-H_2DCFDA. Bar = 100 μm. B, Relative DCF fluorescence densities in the roots. The data presented are means ± SE of measurements taken from at least 10 roots for each treatment. $^*P < 0.05$, $^{**}P < 0.01$ versus 0 μM H_2O_2 (Student's *t* test). [See online article for color version of this figure.]

2010a; Xuan et al., 2010; Mandal et al., 2012). NR, the other enzyme that catalyzes NO production, is encoded by two genes in Arabidopsis: *Nitrate reductase2* (*NIA2*) and *NIA2*. *NIA2* accounts for 90% of the total NR activity in Arabidopsis; the rest is accounted for by *NIA1* (Wilkinson and Crawford, 1993). In guard cells, NIA1 deficiency inhibits abscisic acid-induced NO synthesis (Bright et al., 2006); however, in the process of lateral root development, NIA2 is thought to mediate NO production stimulated by mitogen-activated protein kinase6 (Wang et al., 2010a), suggesting that NIA1 and NIA2 play major roles in NO production in different situations and tissues, respectively. We previously found that NO functions as a signal in thermotolerance using the mutants *noa1* and *nia1nia2*, which show heat sensitivity due to a deficiency in NO (Xuan et al., 2010). As a novel heat shock (HS) signaling molecule, the interaction of NO with other molecules remains elusive.

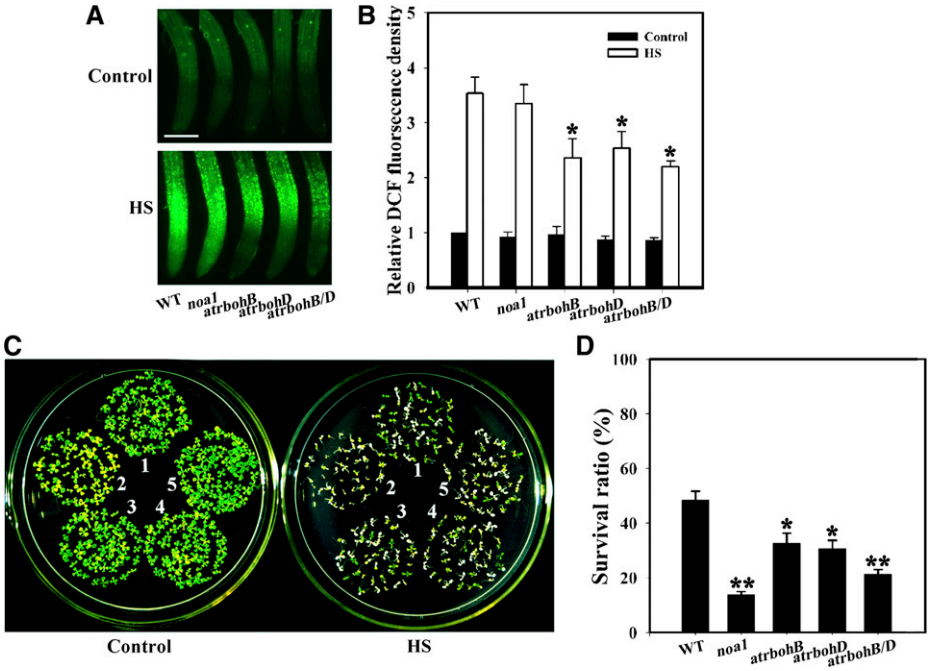

Figure 2. Effects of HS on the internal H_2O_2 levels and survival in wild-type, *noa1*, *atrbohB*, *atrbohD*, and *atrbohB/D* seedlings. A, Six-day-old wild-type (WT), *atrbohB*, *atrbohD*, and *atrbohB/D* seedlings grown at 22°C were exposed to 45°C (HS) or maintained at 22°C (Control) for 30 min. The H_2O_2 levels were then assessed by fluorescence microscopy in roots stained with CM-H_2DCFDA. Bar = 100 μm. B, Relative DCF fluorescence densities in the roots. The data presented are means ± SE of measurements taken from at least 10 roots for each treatment. $^*P < 0.05$ versus wild-type seedlings (Student's *t* test). C, The seedlings were exposed to 45°C (HS) or maintained at 22°C (Control) for 60 min, then returned to 22°C and photographed 5 d later. Clusters are as follows: 1, wild type; 2, *noa1*; 3, *atrbohB*; 4, *atrbohD*; and 5, *atrbohB/D*. D, Survival ratios of the seedlings after HS treatment. The data presented are means ± SE of at least five independent experiments with 50 seedlings per experiment. $^*P < 0.05$, $^{**}P < 0.01$ (Student's *t* test). [See online article for color version of this figure.]

Further study of this aspect of HS signaling will enrich our knowledge about thermotolerance.

Reactive oxygen species (ROS) are released rapidly in plants exposed to stressful conditions. Hydrogen peroxide (H_2O_2), the major and most stable type of ROS, plays a role in many important resistance mechanisms in plants. ROS production occurs at multiple locations in plant cells, including NADPH oxidase in the plasma membrane, the photosynthetic electron transport chain in chloroplasts, and peroxidase in the cell wall. Among these, NADPH oxidase is a crucial source of H_2O_2 (Gechev and Hille, 2005). In Arabidopsis, NADPH oxidase is encoded by 10 genes, named *AtrbohA* to *AtrbohJ*, which have both distinct and collaborative biological activities (Torres et al., 2002). For example, *AtrbohC* and *AtrbohF* exert powerful effects on wall strength and programmed cell death, respectively (Torres et al., 2002; Macpherson et al., 2008), whereas the double mutant *atrbohD/F* displays impaired abscisic acid-induced stomatal closure (Kwak et al., 2003). In addition, *atrbohB* and *atrbohD* mutants reportedly showed a significant sensitive phenotype after heat treatment, implying *atrbohB*'s and *atrbohD*'s possible roles in thermotolerance (Larkindale et al., 2005). It has also been reported that the level of H_2O_2 increases following exposure to elevated temperatures, leading to HSF activation and HSP accumulation in Arabidopsis, tobacco (*Nicotiana tabacum*), and bacteria (*Clostridium acetobutylicum*; Volkov et al., 2006; Königshofer et al., 2008; Banti et al., 2010). In contrast, peroxide scavengers and inhibitors of H_2O_2 generation have been shown to reduce HSP expression in HS-exposed Arabidopsis and tobacco, respectively (Volkov et al., 2006; Königshofer et al., 2008). These results confirm the involvement of H_2O_2 in the HS signaling pathway.

As signaling molecules, NO and H_2O_2 play important roles in eliciting plant resistance reactions. Studies in plants and yeast have shown significant overlap in their individual pathways; however, it remains controversial which is upstream of the other. Some studies have shown that they cooperate in physiological and biochemical reactions. For example, both NO and H_2O_2 are essential in the hypersensitive response (Delledonne et al., 1998; Murgia et al., 2004; Zago et al., 2006; Wilkins et al., 2011), antioxidant reactions (Li et al., 2009), heavy metal resistance (González et al., 2012), and adventitious root formation (Bai et al., 2012). Proteomic analysis uncovered cross talk between NO and H_2O_2 in citrus plants (Tanou et al., 2010). Other studies indicate that H_2O_2 acts upstream of NO in lateral root growth (Wang et al., 2010a), nitrogen-fixing nodule formation (Horchani et al., 2011), drought tolerance (Lu et al., 2009), and stomatal movement (Bright et al., 2006). Interestingly, some experiments have drawn the reverse conclusion (i.e. that NO influences H_2O_2 accumulation). For example, pretreating broad bean (*Vicia faba*) guard cells with an NO donor increased the H_2O_2 content (He et al., 2005). NO mediates antioxidant enzyme activity so as to influence the H_2O_2 level (Zhang et al., 2007, 2009). As yet, the relationship between NO

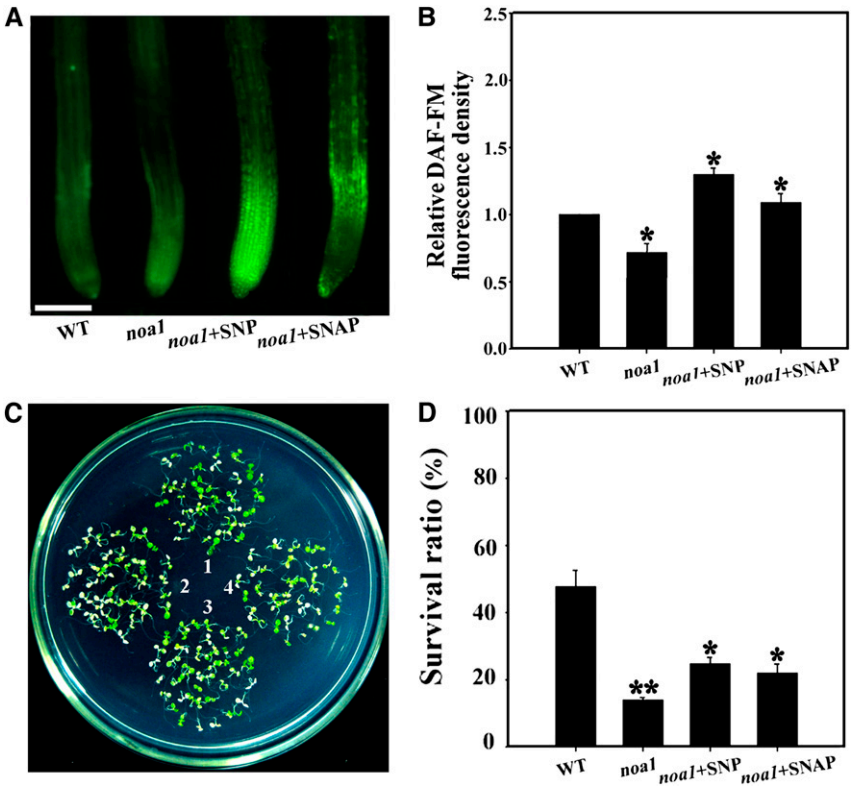

Figure 3. Effects of NO on the thermotolerance of *noa1* seedlings. A, Six-day-old wild-type (WT) and *noa1* seedlings grown at 22°C were pretreated with 2 mL of ultrapure water, treated with 20 μM SNP or SNAP for 8 h, and then exposed to 45°C for 60 min. The NO levels were then assessed by fluorescence microscopy in roots stained with DAF-FM DA. Bar = 100 μm. B, Relative DAF-FM fluorescence densities in the roots. The data presented are means ± SE of measurements taken from at least 10 roots for each treatment. $^*P < 0.05$. C, Seedlings were exposed to 45°C for 60 min, then returned to 22°C and photographed 5 d later. Clusters are as follows: 1, wild type; 2, *noa1*; 3, *noa1* + SNP; and 4, *noa1* + SNAP. D, Survival ratios of the seedlings after HS treatment. The data presented are means ± SE of at least five independent experiments with 50 seedlings per experiment. $^*P < 0.05$, $^{**}P < 0.01$. [See online article for color version of this figure.]

and H_2O_2 under HS conditions is obscure (see Figure 1 in Saidi et al., 2011).

In this study, we used *noa1*, *atrbohB*, *atrbohD*, and *atrbohB/D* as loss-of-function mutants to examine the connection between NO and H_2O_2 in HS signaling, and we investigated the role of H_2O_2 in the induction of adaptive responses. Our results demonstrate the involvement of NO in H_2O_2 signaling as a downstream factor by stimulating HSF DNA-binding activity and HSP expression.

RESULTS

Effects of HS on H_2O_2 Accumulation in Wild-Type Seedlings

H_2O_2 as a plant signaling molecule is involved in a wide range of environmental stresses, including HS (Königshofer et al., 2008; Banti et al., 2010). Thus, we first examined endogenous H_2O_2 accumulation in wild-type seedlings.

Intracellular H_2O_2 formation was detected using the fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), which is passively transported into cells, where its acetate groups are cleaved by intracellular esterases, yielding the fluorescent compound dichlorodihydrofluorescein (DCF; Kolbert et al., 2012). Supplemental Figure S1 showed that pretreatment with 100 μM H_2O_2 increased the fluorescence density, whereas the H_2O_2 scavenger catalase (CAT; 125 units) decreased it. However, the NO donors sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine (SNAP; both 20 μM) and the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (cPTIO; 50 μM) had no clear effect on it, indicating that CM-H_2DCFDA was the special probe for H_2O_2, not for NO. Thereafter, fluorescence analysis showed that under HS treatment at 45°C, the H_2O_2 level in the wild type increased swiftly, reaching its maximum (272% higher than in the control [normal growth conditions, 22°C]) at 30 min, then decreasing gradually; however, the H_2O_2 level remained higher than in the control at 60 min (Fig. 1). Based on these

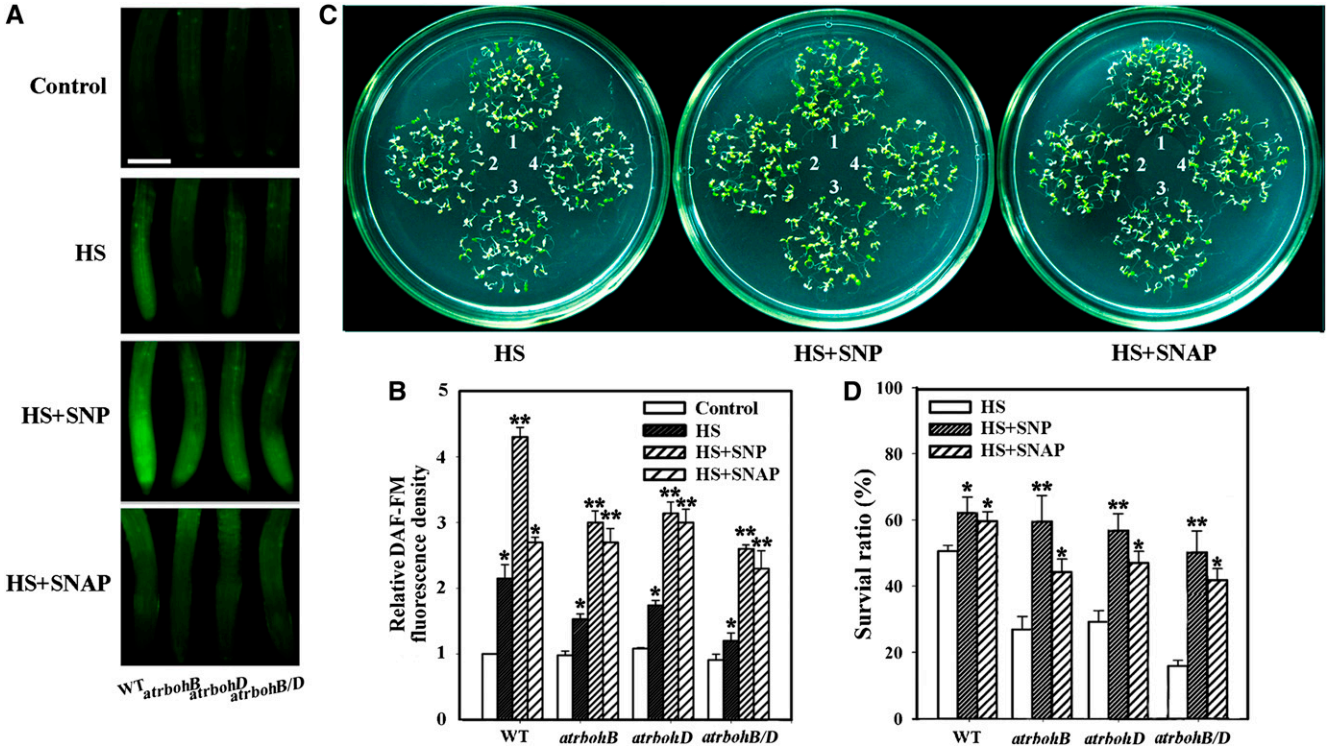

Figure 4. Effects of NO on the thermotolerance of wild-type, *atrbohB*, *atrbohD*, and *atrbohB/D* seedlings. A, Six-day-old wild-type (WT), *atrbohB*, *atrbohD*, and *atrbohB/D* seedlings grown at 22°C were pretreated with 2 mL of ultrapure water, treated with 20 μM SNP or SNAP for 8 h, and then exposed to 45°C (HS, HS+SNP, and HS+SNAP) or maintained at 22°C (Control) for 60 min. The NO levels were then assessed by fluorescence microscopy in roots stained with DAF-FM DA. Bar = 100 μm. B, Relative DAF-FM fluorescence densities in the roots. The data presented are means ± SE of measurements taken from at least 10 roots for each treatment. $^*P < 0.05$, $^{**}P < 0.01$ (Student's *t* test). C, Seedlings were exposed to 45°C for 60 min, then returned to 22°C and photographed 5 d later. Clusters are as follows: 1, wild type; 2, *atrbohB*; 3, *atrbohD*; and 4, *atrbohB/D*. D, Survival ratios of the seedlings after HS treatment. The data presented are means ± SE of at least five independent experiments with 50 seedlings per experiment. $^*P < 0.05$, $^{**}P < 0.01$ versus HS (Student's *t* test). [See online article for color version of this figure.]

results, 30 min was used as the length of the HS period to induce H_2O_2 accumulation in our subsequent experiments.

Effects of HS on H_2O_2 Accumulation and Survival in Wild-Type, *noa1*, *atrbohB*, *atrbohD*, and *atrbohB/D* Seedlings

Next, the H_2O_2 levels were examined in the seedlings of the wild type, *noa1*, *atrbohB*, *atrbohD*, and the *atrbohB/D* double mutant, which was deficient in the transcription of both *AtrbohB* and *AtrbohD* (Supplemental Fig. S2). Under normal growth conditions, no obvious difference existed among the H_2O_2 levels in these seedlings. After HS treatment, the H_2O_2 level increased by 253% in the wild-type seedlings, similar to that in the *noa1* seedlings; however, it only increased by 136%, 153%, and 119% in the *atrbohB*, *atrbohD*, and *atrbohB/D* seedlings, respectively (Fig. 2, A and B).

To measure physiological adaptability to heat stress, survival ratios were calculated for plants following HS treatment at 45°C for 60 min and 5 d of recovery at 22°C (Mittler, 2006). Under normal growth conditions, the *noa1* seedlings exhibited chlorosis and were extremely small compared with the other seedlings (Fig. 2C, control). After HS treatment, the survival ratio of the wild-type seedlings (53%) was higher than those of the *noa1*, *atrbohB*, *atrbohD*, and *atrbohB/D* mutant seedlings (14%, 33%, 31%, and 21%, respectively; Fig. 2, C and D).

4-Amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) was selected for use as a fluorescent probe for NO because it is highly specific for NO and does not react with other ROS. DAF-FM DA permeated the membrane and was transformed by intracellular esterases into 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM), which reacts with NO to create a highly fluorescent triazole compound (Kolbert et al., 2012).

Fluorescence analysis and thermotolerance experiment revealed that *noa1* seedlings displayed lower internal NO levels and survival ratios compared with wild-type seedlings under HS conditions, whereas exogenous pretreatment with 20 μM SNP or SNAP increased them greatly, particularly for SNP (Fig. 3), indicating the role of NO in thermotolerance.

Our data indicate that, in response to HS treatment, the *atrbohB*, *atrbohD*, and *atrbohB/D* plants showed lower H_2O_2 levels and survival ratios than the wild-

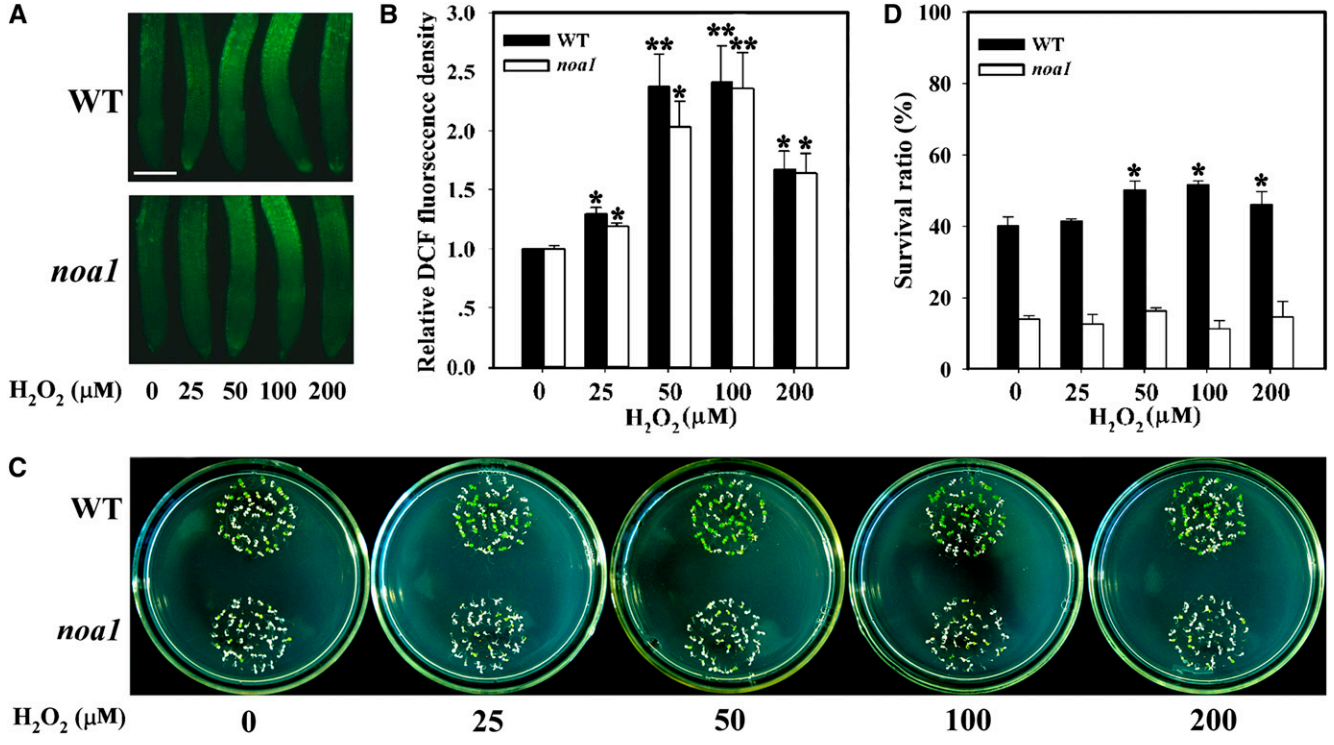

Figure 5. Effects of H_2O_2 on the thermotolerance of wild-type and *noa1* seedlings. A, Six-day-old wild-type (WT) and *noa1* seedlings grown at 22°C were pretreated with 2 mL of 0, 25, 50, 100, or 200 μM H_2O_2 for 8 h and then exposed to 45°C (HS) or maintained at 22°C (control) for 30 min. The H_2O_2 levels were then assessed by fluorescence microscopy in roots stained with CM-H_2DCFDA. Bar = 100 μm. B, Relative DCF fluorescence densities in the roots. The data presented are means ± SE of measurements taken from at least 10 roots for each treatment. *$P < 0.05$, **$P < 0.01$ versus 0 μM H_2O_2 (Student's t test). C, Seedlings were exposed to 45°C for 60 min, then returned to 22°C and photographed 5 d later. D, Survival ratios of the seedlings after HS treatment. The data presented are means ± SE of at least five independent experiments with 50 seedlings per experiment. *$P < 0.05$ versus 0 μM H_2O_2 (Student's t test). [See online article for color version of this figure.]

type plants, with the lowest values observed for the *atrbohB/D* double mutant. However, *noa1* exhibited heat sensitivity due to reduced NO production (see Figure 1 in Xuan et al., 2010; Fig. 3), maintaining a higher H_2O_2 level and the lowest survival ratio, suggesting an intimate relationship between NO and H_2O_2 in thermotolerance.

Effects of Internal NO Levels on the Survival of *atrbohB*, *atrbohD*, and *atrbohB/D* Seedlings

To investigate the relationship between NO and H_2O_2 in HS signaling, we examined NO production in *atrbohB*, *atrbohD*, and *atrbohB/D* seedlings. Our fluorescence analysis results indicate that at a normal temperature, no obvious difference existed among the NO levels in these seedlings (Fig. 4, A and B). Under HS conditions, the NO level in the wild-type seedlings increased remarkably, to 211% of the control level, which was far higher than the values obtained for *atrbohB*, *atrbohD*, and *atrbohB/D* seedlings (151%, 176%, and 118% of the control level, respectively; Fig. 4, A and B).

Exogenous pretreatment with 20 μM SNP or SNAP greatly increased internal NO levels in the seedlings under HS conditions, especially for SNP (Fig. 4, A and B). Synchronously, the survival ratios of the seedlings were increased, particularly for the mutant plants (Fig. 4, C and D).

Our previous work showed that NO increased obviously after HS conditions (see Figure 1 in Xuan et al., 2010), inconsistent with the findings of Lee et al. (2008), which might be due to light, age, and temperature effects on NO production, as shown in Supplemental Figure S3.

Effects of Internal H_2O_2 Levels on the Survival of Wild-Type and *noa1* Seedlings

Next, a solution containing varying concentrations of H_2O_2 was applied to pretreat the wild-type and *noa1* seedlings. This increased the internal H_2O_2 level in both types of seedlings in a concentration-dependent manner, reaching a maximum value at 50 to 100 μM and decreasing slightly at 200 μM (Fig. 5, A and B). However, the effect on the survival of wild-type and *noa1* seedlings was different. The change in survival ratio for the wild-type seedlings mirrored the trend seen in their H_2O_2 levels, reaching a maximum value at 100 μM (29% higher than the control; Fig. 5, C and D), which was thoroughly reversed by treatment with 50 μM cPTIO (Supplemental Fig. S4). However, no obvious effect was observed for the *noa1* seedlings (Fig. 5, C and D).

Taken together, these results (Figs. 3–5; Supplemental Fig. S4) showed that an elevated internal NO level increased the thermotolerance of the H_2O_2-deficient mutants *atrbohB*, *atrbohD*, and *atrbohB/D*; however, an elevated internal H_2O_2 level had no clear effect on the NO-deficient *noa1* mutant, indicating NO involvement in H_2O_2 signaling as a downstream factor.

Effects of HS on Thermotolerance in *atrbohB/D/noa1* Triple Mutant Seedlings

To examine the actions of NO and H_2O_2 in thermotolerance, we obtained the triple mutant *atrbohB/D/noa1*, which was shown to be deficient in *AtrbohB*, *AtrbohD*, and *NOA1* transcription by reverse transcription (RT)-PCR analysis (Fig. 6A). Under normal conditions,

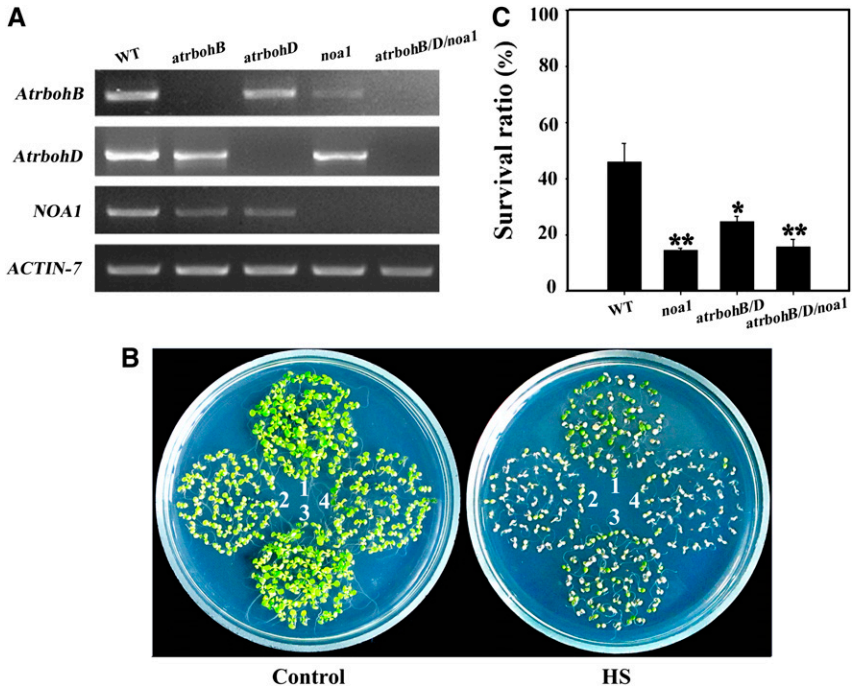

Figure 6. Survival status of the *atrbohB/D/noa1* triple mutant. A, RT-PCR analysis of *AtrbohB*, *AtrbohD*, and *AtNOA1* transcription in wild-type (WT), *atrbohB*, *atrbohD*, *noa1*, and *atrbohB/D/noa1* seedlings. *ACTIN7* was used as an internal control. B, Six-day-old seedlings grown at 22°C were exposed to 45°C (HS) or maintained at 22°C (Control) for 60 min, then returned to 22°C and photographed 5 d later. Clusters are as follows: 1, wild type; 2, *noa1*; 3, *atrbohB/D*; and 4, *atrbohB/D/noa1*. C, Survival ratios of the seedlings after HS treatment. The data presented are means ± SE of at least five independent experiments with 50 seedlings per experiment. $*P < 0.05$, $**P < 0.01$ (Student's *t* test). [See online article for color version of this figure.]

atrbohB/D/noa1 seedlings appeared chlorotic and small, similar to *noa1* seedlings (Fig. 6B, control). Under HS conditions, the survival ratio of the *atrbohB/D/noa1* seedlings was close to that of the *noa1* seedlings (Fig. 6, B and C), indicating that the absence of *AtrbohB* and *AtrbohD* did not exacerbate the heat sensitivity of *noa1* seedlings. SNP or SNAP at 20 μM increased the survival of these seedlings (Supplemental Fig. S5), indicating the effect of NO on thermotolerance even in the absence of H_2O_2.

AtNIA2 and *AtNOA1* Overexpression in an *atrbohB/D* Background Improves Thermotolerance

NIA2 is a major component of NR, which converts nitrite to NO. Therefore, we obtained two *AtNIA2*-overexpressing transgenic lines, *atrbohB/D/35S*::*NIA2-1* and *atrbohB/D/35S*::*NIA2-3* (with increased NR activity; data not shown), by crossing *atrbohB/D* with two transgenic lines, *35S*::*NIA2-1* and *35S*::*NIA2-3* (Wang et al., 2010a), and two *AtNOA1*-overexpressing transgenic lines, *atrbohB/D/35S*::*NOA1-1* and *atrbohB/D/35S*::*NOA1-2*, by the floral dip method, to examine the effects of excess internal NO on H_2O_2-deficient mutants under HS stress. The elevated expression of *AtNIA2* and *AtNOA1* was confirmed by RT-PCR (Figs. 7A and 8A).

DAF-FM fluorescence analysis revealed that the overexpression of *AtNIA2* and *AtNOA1* enhanced the internal NO levels in their transgenic lines, although the effect was obviously greater for *AtNIA2* overexpression under normal growth and high temperatures (Figs. 7 and 8). Conversely, *AtNIA2* overexpression only slightly increased the survival ratios of *atrbohB/D/35S*::*NIA2-1* and *atrbohB/D/35S*::*NIA2-3* by 17% and 28%, respectively (Fig. 7, D and E), whereas *AtNOA1* overexpression greatly increased the survival ratios of *atrbohB/D/35S*::*NOA1-1* and *atrbohB/D/35S*::*NOA1-2* by 46% and 61%, respectively, compared with their background *atrbohB/D* under HS conditions (Fig. 8, D and E). This intriguing phenomenon might be due to the

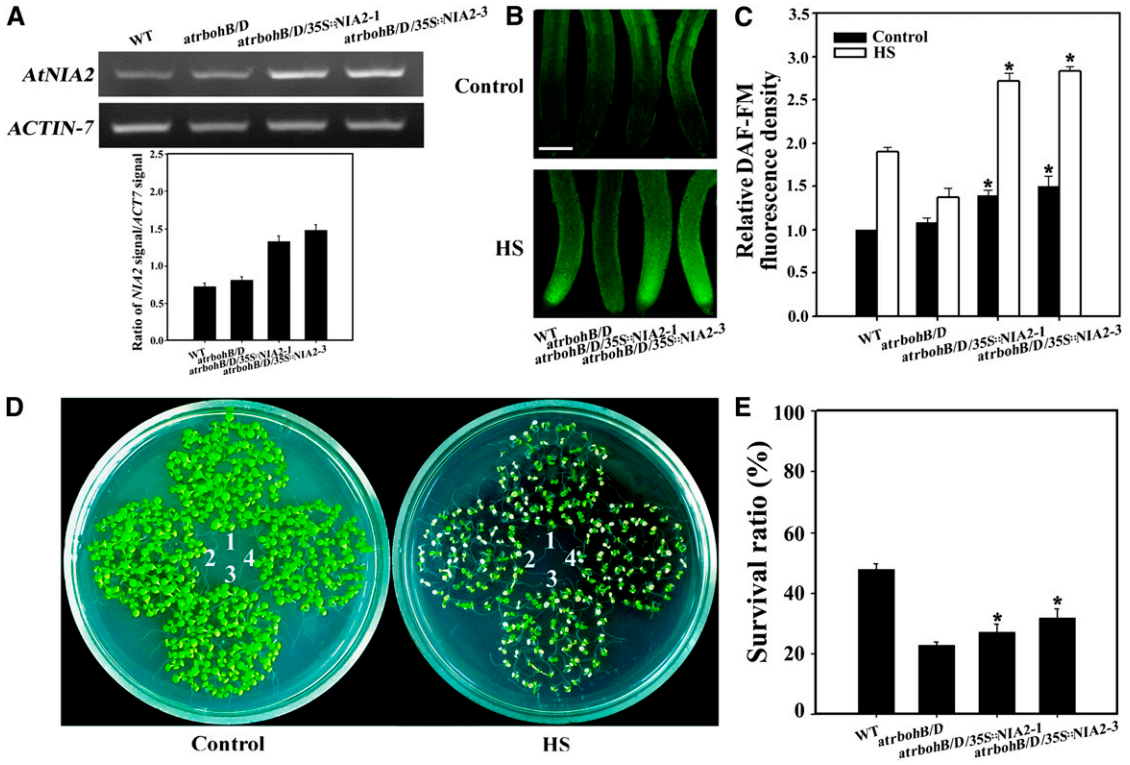

Figure 7. Improved thermotolerance through *AtNIA2* overexpression in an *atrbohB/D* background. A, RT-PCR analysis of *AtNIA2* transcription in wild-type (WT), *atrbohB/D*, *atrbohB/D/35S*::*NIA2-1*, and *atrbohB/D/35S*::*NIA2-3* seedlings. *ACTIN7* was used as an internal control. B, Six-day-old wild-type, *atrbohB/D*, *atrbohB/D/35S*::*NIA2-1*, and *atrbohB/D/35S*::*NIA2-3* seedlings grown at 22°C were exposed to 45°C (HS) or maintained at 22°C (Control) for 60 min. The NO levels were then assessed by fluorescence microscopy in roots stained with DAF-FM DA. Bar = 100 μm. C, Relative DAF-FM fluorescence densities in the roots. The data presented are means ± SE of measurements taken from at least 10 roots for each treatment. *$P < 0.05$ versus *atrbohB/D* seedlings (Student's *t* test). D, Seedlings were exposed to 45°C (HS) or maintained at 22°C (Control) for 60 min, then returned to 22°C and photographed 5 d later. Clusters are as follows: 1, wild type; 2, *atrbohB/D*; 3, *atrbohB/D/35S-NIA2-1*; and 4, *atrbohB/D/35S-NIA2-3*. E, Survival ratios of the seedlings. The data presented are means ± SE of at least five independent experiments after HS with 50 seedlings per experiment. *$P < 0.05$ versus *atrbohB/D* seedlings (Student's *t* test). [See online article for color version of this figure.]

negative effect of excessive NO on thermotolerance seen in our previous report (see Figure 2 and Supplemental Figure 2 in Xuan et al., 2010).

These results (Figs. 7 and 8) showed that the overexpression of *AtNIA2* or *AtNOA1* restored thermotolerance in the H_2O_2-deficient *atrbohB/D* mutant, providing genetic proof of the relationship between NO and H_2O_2 in thermotolerance.

Effects of H_2O_2 on the DNA-Binding Activity of HSFs and AtHSP17.7 and AtHSP21 Expression through NO

To examine the underlying mechanism of NO- and H_2O_2-induced thermotolerance in Arabidopsis, the binding of HSFs to HSEs in wild-type, *atrbohB*, *atrbohD*, *atrbohB/D*, and *atrbohB/D/noa1* seedlings as well as in two *AtNIA2*-overexpressing transgenic lines (*atrbohB/D/35S*::*NIA2-1* and *atrbohB/D/35S*::*NIA2-3*) was analyzed using an electrophoretic mobility shift assay (EMSA). Competition assays with 30- and 60-fold molar excess of unlabeled DNA probe indicated this special binding (Fig. 9A). The subsequent results showed that after HS treatment, HSF binding to HSEs in *atrbohB*, *atrbohD*, *atrbohB/D*, and *atrbohB/D/noa1* seedlings was weaker than that in wild-type seedlings (and weakest for *atrbohB/D* and *atrbohB/D/noa1*) but was significantly stimulated by 20 μM SNP and SNAP and was activated in the two transgenic lines compared with *atrbohB/D*. No binding was observed in wild-type plants that were not heated, suggesting that the band shift was specifically induced by heat (Fig. 9, B and C). *Hsf* (*Hsf2A*, *HsfA7a*, and *HsfB2b*) mRNA levels also showed the same changing pattern as the binding under HS conditions (Supplemental Fig. S6, A–C).

Because HSP expression is induced at moderately high temperature and leads to enhanced tolerance to severely high temperature, we next examined the effects of NO and H_2O_2 on the accumulation of AtHSP17.7 and AtHSP21 by western-blot analysis. Both of them were not detected at 22°C (Fig. 9, D, F, H, and J); however, their accumulation was observed at 37°C. The level of

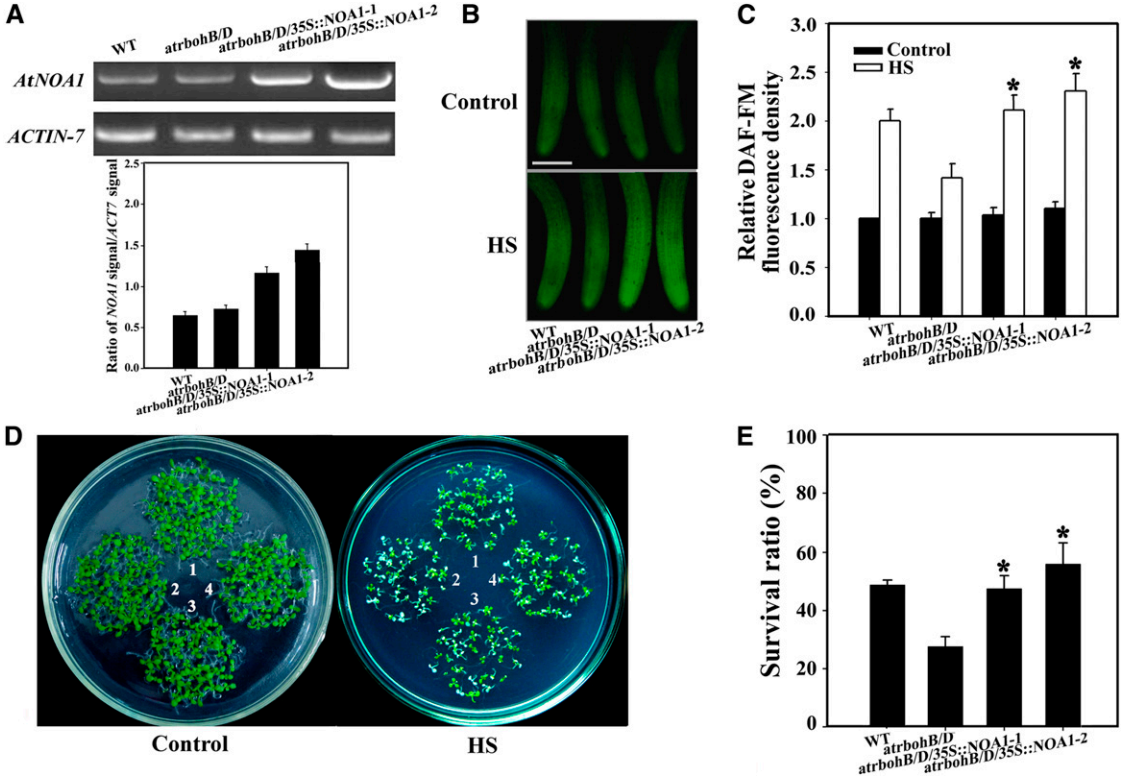

Figure 8. Improved thermotolerance through *AtNOA1* overexpression in an *atrbohB/D* background. A, RT-PCR analysis of *AtNOA1* transcription in wild-type (WT), *atrbohB/D*, *atrbohB/D/35S*::*NOA1-1*, and *atrbohB/D/35S*::*NOA1-2* seedlings. *ACTIN7* was used as an internal control. B, Six-day-old seedlings grown at 22°C were exposed to 45°C (HS) or maintained at 22°C (Control) for 60 min. The NO levels were then examined by fluorescence microscopy in roots stained with DAF-FM DA. Bar = 100 μm. C, Relative DAF-FM fluorescence densities in the roots. The data presented are means ± SE of measurements taken from at least 10 roots for each treatment. *$P < 0.05$ versus *atrbohB/D/* seedlings (Student's *t* test). D, Six-day-old seedlings were exposed to 45°C (HS) or maintained at 22°C (Control) for 60 min, then returned to 22°C and photographed 5 d later. Clusters are as follows: 1, wild type; 2, *noa1*; 3, *atrbohB/D/35S*::*NOA1-1*; and 4, *atrbohB/D/35S*::*NOA1-2*. E, Survival ratios of the seedlings. The data presented are means ± SE of at least five independent experiments after HS with 50 seedlings per experiment. *$P < 0.05$ versus *atrbohB/D* seedlings (Student's *t* test). [See online article for color version of this figure.]

accumulation was lower in the mutants than in the wild type (and lowest for *atrbohB/D* and *atrbohB/D/noa1*) and was partially restored by 20 μM SNP and SNAP; in addition, it was activated in *atrbohB/D/35S*::*NIA2-1* and *atrbohB/D/35S*::*NIA2-3* compared with *atrbohB/D* (Fig. 9, E, G, I, and K). In each of these experiments, tubulin was used to ensure equal sample loading. *HSP17.4* and *HSP22* mRNA levels also showed the same changing pattern as AtHSP17.7 and AtHSP21 expression under HS conditions (Supplemental Fig. S6, D and E).

DISCUSSION

H_2O_2 and Thermotolerance in Arabidopsis Seedlings

In this work, we obtained evidence for the involvement of NO in H_2O_2 signaling in thermotolerance. H_2O_2, induced by HS treatment, acted as a second messenger in the induction of NO production to regulate the DNA-binding activity of HSFs and the accumulation of HSPs. Thus, H_2O_2 promotes thermotolerance in Arabidopsis.

H_2O_2 is regarded as a signaling molecule that contributes to thermotolerance; therefore, we first examined H_2O_2 production with its fluorescent probe CM-H_2DCFDA (Supplemental Fig. S1) under HS conditions. As shown in Figure 1, the H_2O_2 level first increased and then decreased gradually with the increased HS time. This may be attributed to an adaptive cellular mechanism inhibiting excessive H_2O_2 levels to protect plants from oxidative damage.

Larkindale et al. (2005) reported that mutations in *atrbohB* and *atrbohD*, two isoforms of NADPH oxidase, which contributes to H_2O_2 production, showed weaker defects under heat stress. Thus, we obtained these mutants to study the role of H_2O_2 in thermotolerance. Under HS stress, the survival ratios of *atrbohB*, *atrbohD*,

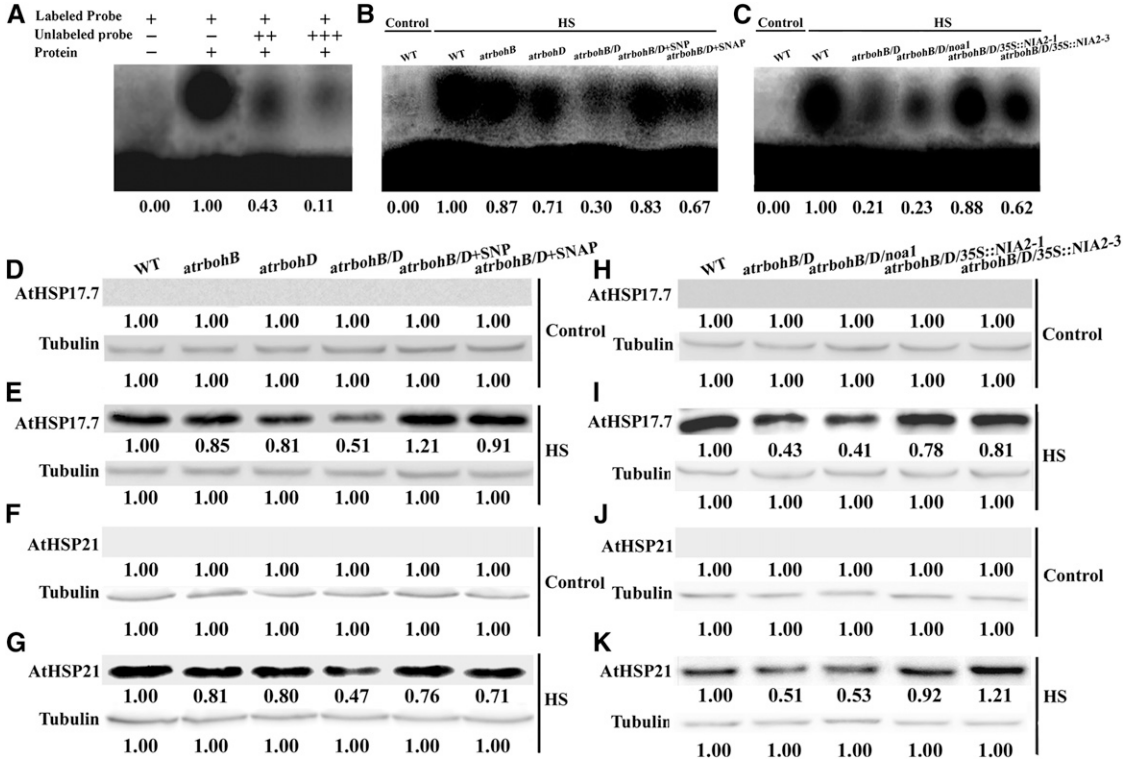

Figure 9. Effects of H_2O_2 through NO on HSF DNA-binding activity and AtHSP17.7 and AtHSP21 expression. A, The titration experiment utilizing EMSA confirmed the binding of HSFs and HSEs in whole-cell extracts prepared from 10-d-old seedlings incubated at 37°C for 1 h. Unlabeled DNA probe in 30-fold (++) or 60-fold (+++) molar excess relative to the labeled probe was used as the specific competitor. B and C, Results of an EMSA using the extracts incubated at 22°C (Control) or 37°C (HS) for 1 h. Equal amounts (50 μg each) of protein extracts were used in all lines. B, Wild-type (WT), *atrbohB*, *atrbohD*, *atrbohB/D*, *atrbohB/D* + 20 μM SNP, and *atrbohB/D* + 20 μM SNAP seedlings. C, Wild-type, *atrbohB/D*, *atrbohB/D/noa1*, *atrbohB/D/35S*::*NIA2-1*, and *atrbohB/D/35S*::*NIA2-3* seedlings. Three independent experiments were carried out; the results indicate similar trends in binding activity. The numbers below each lane represent the relative intensity of each signal. D to K, Seedlings grown at 22°C were exposed to 37°C (HS) or kept at 22°C (Control) for 2 h. Total protein was then extracted, separated by SDS-PAGE, and analyzed by western blotting. Tubulin was used as an internal control. D to G, Wild-type, *atrbohB*, *atrbohD*, *atrbohB/D*, *atrbohB/D* + 20 μM SNP, and *atrbohB/D* + 20 μM SNAP seedlings. H to K, Wild-type, *atrbohB/D*, *atrbohB/D/noa1*, *atrbohB/D/35S*::*NIA2-1*, and *atrbohB/D/35S*::*NIA2-3* seedlings. Three independent experiments were carried out; the results indicate similar trends in protein accumulation. The numbers below each lane represent the relative intensity of each signal.

and *atrbohB/D* seedlings were lower than that of wild-type seedlings, in accordance with the H_2O_2 levels (Fig. 2). These data suggest that *AtrbohB* and *AtrbohD* are related to thermotolerance, as indicated by Larkindale et al. (2005). We also found that NO functions as a signal in thermotolerance using *noa1* and *nia1nia2* seedlings (Xuan et al., 2010). NH_4^+, which was supplied in the culture medium for *nia1nia2* due to its deficiency of NR, might induce an abnormal nutrient status of the cocultured wild-type and H_2O_2-deficient mutant seedlings so as to influence their natural thermotolerance. Thus, we utilized NO-deficient *noa1* seedlings to examine the H_2O_2 level under HS conditions. The higher H_2O_2 level and the lowest survival ratio in the *noa1* mutant (Fig. 2) suggested a relationship between NO and H_2O_2 in HS signaling.

The Relationship between NO and H_2O_2 under HS Conditions in Arabidopsis

To test this hypothesis, we examined the NO levels in *atrbohB*, *atrbohD*, and *atrbohB/D* seedlings. The NO levels varied among the three H_2O_2-deficient mutants (Fig. 4, A and B), in accordance with the H_2O_2 levels under HS conditions (Fig. 2, A and B). A moderate concentration (20 μM) of two NO donors, SNP and SNAP, elevated the survival ratio of all three types of mutant seedlings (Fig. 4), whereas treatment with H_2O_2 had no clear effect on the survival ratio of *noa1* seedlings (Fig. 5). A plausible explanation for these results is that NO is a key component of the H_2O_2 pathway in HS signaling and, therefore, a deficiency in NO did not affect H_2O_2 accumulation (Fig. 2, A and B), whereas a deficiency in H_2O_2 inhibited NO production (Fig. 4, A and B). Also, supplementation with NO, a downstream molecule, restored the heat-sensitive status of the H_2O_2-deficient mutant *atrbohB*, *atrbohD*, and *atrbohB/D* seedlings (Fig. 4, C and D); however, supplementation with H_2O_2, an upstream molecule, had no effect on the appearance of *noa1* seedlings, which were deficient in NO (Fig. 5). For the same reason, the NO scavenger cPTIO inhibited the effect of H_2O_2 on the survival of the wild type under HS (Fig. 5; Supplemental Fig. S4).

We also found that the application of a high concentration of H_2O_2 (200 μM) did not induce a higher internal H_2O_2 level under HS conditions (Fig. 5, A and B), which might be due to plant self-protective behaviors against oxidative damage. NO has been reported to stimulate the activities of antioxidant enzymes under conditions of stress (Zhang et al., 2007, 2009); thus, there may be a self-regulatory mechanism under HS conditions in which NO stimulated by H_2O_2 inhibits the accumulation of too much H_2O_2 through the activation of antioxidant enzymes (e.g. CAT; Supplemental Fig. S7).

Effects of NO and H_2O_2 on Thermotolerance in Arabidopsis

To clarify the effects of NO and H_2O_2 on thermotolerance, we obtained the triple mutant *atrbohB/D/noa1*, which exhibited a phenotype similar to that of *noa1* under normal growth or HS conditions (Fig. 6, B and C), indicating that deficiencies in both NO and H_2O_2 do not potentiate the heat susceptibility caused by a deficiency in NO alone.

NO donors enhanced the thermotolerance of *atrbohB/D* and *atrbohB/D/noa1* seedlings (Supplemental Fig. S5). The overexpression of *NIA2* and *NOA1* enhanced both of the internal NO level and the survival ratio in their transgenic lines, respectively, in an *atrbohB/D* background under HS conditions (Figs. 7 and 8). These results indicate that enhancement of the NO level rescues the heat susceptibility of the plants due to the removal of H_2O_2.

Collectively, our data provide pharmacological proof of the existence of a novel signaling pathway in which H_2O_2 production is stimulated by HS to regulate NO accumulation so as to confer thermotolerance.

The Mechanism Underlying the Effect of H_2O_2 through NO on Thermotolerance

To determine the mechanism through which H_2O_2 affects thermotolerance via NO, we examined the effects of H_2O_2 and NO on HSF DNA-binding activity and HSP expression under HS conditions.

Downstream components of the HS signal transduction pathway known as HSFs contribute to thermotolerance by controlling HSP gene expression in response to phosphorylation (Kotak et al., 2007). Our group even reported that Ca^{2+} and calmodulin3 (CaM3) are believed

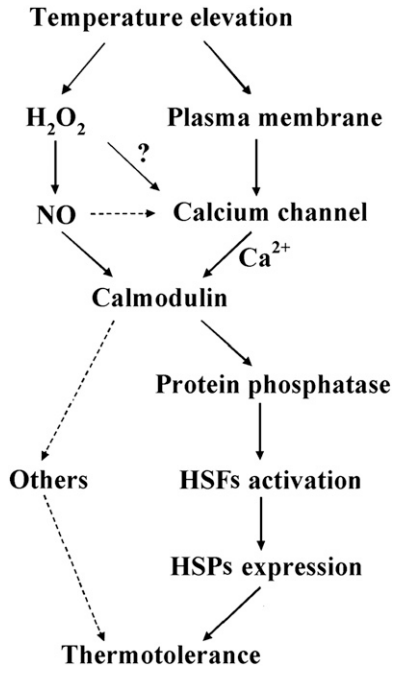

Figure 10. Model for the involvement of H_2O_2 in the NO pathway in HS signal transduction. Black arrows indicate pathways supported by evidence, and dotted arrows show hypothetical processes. The question mark indicates an unknown player.

to be involved in HSP expression in Arabidopsis (Liu et al., 2005). Furthermore, we found that NO acts upstream of AtCaM3 to induce thermotolerance by enhancing HSF DNA-binding activity and HSP accumulation (Xuan et al., 2010). Our data here indicate that a decrease in the level of H_2O_2 prohibits the DNA-binding activity of HSFs, whereas NO enhances the binding of HSFs to HSEs in *atrbohB/D* plants (Fig. 9, B and C). Therefore, NO appears to restore the H_2O_2 effect, thereby influencing the DNA-binding activity of HSFs by increasing the expression of AtCaM3, leading to thermotolerance.

HSP genes, which are activated by HSFs binding to HSEs, are classified according to their molecular masses as HSP100, HSP90, HSP70, HSP60, and small HSPs. In this study, we used HSP17.7 and HSP21, two small HSPs, to explore how H_2O_2 induces thermotolerance through NO. Western-blot analysis indicated that under HS conditions, the decreased H_2O_2 level reduced AtHSP17.7 and AtHSP21 expression, whereas NO donors and the overexpression of *NIA2* in *atrbohB/D* plants enhanced the accumulation of AtHSP17.7 and AtHSP21 (Fig. 9, D–K), indicating H_2O_2 activation of HSP expression through NO. H_2O_2 was also verified to play a key role in the transcriptional up-regulation of small HSPs and several HSFs (Supplemental Fig. S6). Collectively, the mechanism through which H_2O_2 influences thermotolerance via NO involves alterations in HSF DNA-binding activity and HSP gene expression.

To our knowledge, our data here provide the first evidence that H_2O_2 functions as a second messenger in the induction of thermotolerance through NO, which is dependent on the enhancement of HSF DNA-binding activity and HSP accumulation. We previously proposed a model for HS signaling in which the HS signal was identified by an unknown receptor, leading to an increased NO level, which directly activated AtCaM3 to initiate plant adaptations to heat stress (Xuan et al., 2010). In this study, H_2O_2 was found to act upstream of NO in the response to HS. NO is believed to mediate Ca^{2+} channel functioning in the plasma membrane (Delledonne et al., 1998). H_2O_2 application was shown to increase the intracellular level of free Ca^{2+} (Rentel and Knight, 2004). Ca^{2+} entry has been shown to be essential to the specific heat activation of a mitogen-activated protein kinase (Sangwan et al., 2002). Several plasma membrane cyclic nucleotide-gated Ca^{2+} channels (CNGCs) were believed to control land plant thermal sensing and acquired thermotolerance (Finka et al., 2012; Mittler et al., 2012). Indeed, two heat-activated Ca^{2+}-permeable channels, CNGC6 and CNGC12, were recently found to be involved in HS responses (Gao et al., 2012; Tunc-Ozdemir et al., 2013). Ca^{2+}-CaM can bind to target proteins to alter their function, acting as part of a calcium signal transduction pathway (Reddy et al., 2011). We even reported that CaM-binding protein kinase3 is an important AtCaM downstream component of the HS signal transduction pathway (Liu et al., 2008). A CaM protein phosphatase, PP7, also has been reported to interact with AtCaM3, suggesting a possible role in the activation of HSFs (Liu et al., 2007). Thus, it is likely to suggest a cross talk among H_2O_2, NO, Ca^{2+} channels, and the activation of Ca^{2+}-CaM-dependent protein phosphatase or other factors to mediate HSF DNA-binding activity and HSP accumulation in thermotolerance (Fig. 10).

MATERIALS AND METHODS

Plant Growth and Chemical Treatments

Seeds of wild-type and mutant Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia were surface sterilized in 2% (v/v) sodium hypochlorite for 1 min and then washed thoroughly with water. The sterilized seeds were plated on Murashige and Skoog (MS) medium containing 3% Suc and 0.7% agar and kept at 4°C in the dark for 3 d. The plants were then transferred to a growth chamber set at 22°C and 120 μmol m^{-2} s^{-1} on a 16-h day/night cycle.

noa1 seeds were obtained from Dr. Nigel M. Crawford. *atrbohB* and *atrbohD* mutant seeds were obtained from Dr. Miguel Angel Torres. *35S::NIA2-1* and *35S::NIA2-3* seeds were obtained from Dr. Chun-Peng Song. The *atrbohB/D* double mutant, *atrbohB/D/noa1* triple mutant, and transgenic lines *atrbohB/D/35S::NIA2-1* and *atrbohB/D/35S::NIA2-3* were obtained by crossing.

For chemical treatment, 2 mL of SNP, SNAP, cPTIO, H_2O_2, and CAT (all Sigma) at various concentrations were sprayed onto the leaf surfaces of 6-d-old seedlings after filter sterilization. Control seedlings were treated with ultrapure water. After 8 h of pretreatment, the seedlings were subjected to HS conditions.

Thermotolerance Testing

Six-day-old seedlings were exposed to 45°C and then allowed to recover at 22°C for 5 d. Those seedlings that were still green and continued to produce new leaves were scored as survivors. For western-blot analysis, 10-d-old seedlings were kept at 37°C for 2 h and analyzed for HSP accumulation (Liu et al., 2005).

Fluorescence Microscopy

NO and H_2O_2 were visualized using the NO-specific fluorescent probe DAF-FM DA (Sigma) and the H_2O_2 fluorescent probe CM-H_2DCFDA (Invitrogen), respectively, according to the method of Wang et al. (2010b) with some modifications. Wild-type and mutant seedlings were incubated in 1 mL of liquid MS medium (pH 5.8) with 10 μM DAF-FM DA or CM-H_2DCFDA for 20 min. Thereafter, the roots were washed three times for 15 min each in liquid MS medium prior to visualization using a fluorescence microscope (ELLIPE TE2000-U; Nikon). The signal intensities were quantified using MetaMorph (Molecular Devices).

Construction of the Transgenic Lines

To generate *AtNOA1-GFP* for the production of plants overexpressing *AtNOA1* in an *atrbohB/D* background, *AtNOA1* complementary DNA was amplified by RT-PCR using the primers NOA1F1 (5′-CACCATGGCGCTACGAACACTCTCAA-3′) and NOA1F2 (5′-AAAGTACCATTTGGGTCTTACT-3′). (The underlined sequence in NOA1F1 is used to link to pENTR/SD/D-TOPO within this reaction chain.) The product was cloned in the sense orientation into pENTR/SD/D-TOPO and then into pMDC32 using Gateway LR Recombinase (Invitrogen) to generate *35S::AtNOA1-GFP*, which rescued the phenotype of the *noa1* mutant (data not shown).

Transformation of this construct into Arabidopsis (*atrbohB/D*) was carried out by the floral dip method (Clough and Bent, 1998). Transformants were selected on plates containing 25 mg L^{-1} hygromycin. The number of transfer DNA insertions was determined at the T2 generation based on the segregation ratio of hygromycin resistance. After three rounds of selection, homozygous transgenic lines were identified for use in our experiments.

RT-PCR and Real-Time RT-PCR

Total RNA (500 ng) was isolated from 10-d-old seedlings using the PrimeScript RT reagent kit (Takara) for first-stand complementary DNA synthesis. RT-PCR analyses of *AtrbohB* (*At1g09090*), *AtrbohD* (*At5g47910*), *NOA1* (*At3g47450*), and *NIA2* (*At1g37130*) transcription were performed using the Takara RNA PCR

(Avian Myeloblastosis Virus) kit version 3.0 (Takara) with gene-specific primers (Supplemental Table S1). Real-time RT-PCR analyses of gene expression were done using an ABI 7000 sequence detection system (Applied Biosystems) with SYBR Premix Ex Taq (Takara) and gene-specific primers (Supplemental Table S2). *ACTIN* was used as an internal control to normalize all data.

Western-Blot Analysis

Ten-day-old seedlings were kept at 37°C for 2 h and then ground in liquid nitrogen. Total protein was extracted using an extraction buffer (10 mM HEPES, pH 7.9, containing 0.4 M NaCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol, and 0.5 mM phenylmethanesulfonyl fluoride), and the extracts were clarified by centrifugation at 14,000*g* for 20 min at 4°C. The supernatants were transferred to fresh tubes, and the protein content was determined using the method of Bradford (1976). Total proteins (50 μg) were separated by SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were blocked for at least 3 h and then probed with rabbit antiserum against AtHSP17.7 or AtHSP21 and mouse antiserum against the loading control, tubulin (Sigma). After extensive washing, the membranes were incubated with the appropriate secondary antibodies conjugated to alkaline phosphatase. Bromo-chloro-indolyl phosphate/nitroblue tetrazolium (Amresco) was used for immunodetection.

EMSA

The HSE (Hübel and Schöffl, 1994; Li et al., 2004; Xuan et al., 2010) oligonucleotides (5′-TCGAGGATCCTAGAAGCTTCCAGAAGCTTCTAGAAGCAGATC-3′ and 5′-TCGAGATCTGCTTCTAGAAGCTTCTGGAAGCTTCTAGGATCC-3′) were end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Takara). Ten-day-old seedlings were ground in liquid nitrogen and mixed with extraction buffer (10 mM Tris, pH 8.0, containing 1 mM EDTA, 10 mM boric acid, and 0.1 mM phenylmethylsulfonyl fluoride). After centrifugation, the supernatants were used as whole-cell extracts for HS treatment (37°C for 1 h). EMSA was carried out according to the method of Li et al. (2004).

CAT Activity Assay

CAT was spectrophotometrically measured at 240 nm as described by Huang et al. (2010). One unit of CAT activity was defined as the decrease of absorbance by 0.01 per min.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Effects of H_2O_2 and NO on CM-H_2DCFDA fluorescence densities in the roots after HS treatment.

Supplemental Figure S2. *AtrbohB* and *AtrbohD* transcription in wild-type, *atrbohB*, *atrbohD*, and *atrbohB/D* seedlings.

Supplemental Figure S3. Effects of light, age, and temperature on NO production in wild-type seedlings.

Supplemental Figure S4. Effects of cPTIO and H_2O_2 on the thermotolerance of wild-type seedlings.

Supplemental Figure S5. Effects of NO on the thermotolerance of wild-type, *noa1*, *atrbohB/D*, and *atrbohB/D/noa1* seedlings.

Supplemental Figure S6. Analysis of the effects of NO on H_2O_2-induced HSFs and HSP gene expression by real-time RT-PCR.

Supplemental Figure S7. Effects of NO and H_2O_2 on CAT activity in wild-type seedlings after HS treatment.

Supplemental Table S1. Primers used for RT-PCR.

Supplemental Table S2. Primers used for real-time RT-PCR.

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