Running title: Involvement of NO in \( \text{H}_2\text{O}_2 \) signaling in thermotolerance

Name and address of corresponding author:

Liqun Zhao
Institute of Molecular Cell Biology, School of Life Sciences, Hebei Normal University, Shijiazhuang 050024, China

Phone: +86-311-80787530
Fax: +86-311-80787549
E-mail address: zhaolq70@163.com

Research area: Signaling and Response
Hydrogen Peroxide Acts Upstream of Nitric Oxide in the Heat Shock Pathway in *Arabidopsis* Seedlings

Lei Wang\(^2\), Yunjing Guo\(^2\), Lixiu Jia, Hongye Chu, Shuo Zhou, Kunming Chen, Dan Wu, and Liqun Zhao*

Institute of Molecular Cell Biology, School of Life Sciences, Hebei Normal University, Shijiazhuang 050024, China (L.W., Y.G., L.J., H.C., S.Z., D.W., L.Z.)
Hebei Key Laboratory of Molecular and Cellular Biology, Hebei Normal University, Shijiazhuang 050024, China (L.W., Y.G., L.J., H.C., S.Z., D.W., L.Z.)
State Key Laboratory of Crop Stress Biology in Arid Areas, College of Life Sciences, Northwest A & F University, Yangling 712100, China (K.C.)

**One-Sentence Summaries:**
An elevated internal NO level rescues the plant’s heat sensitivity due to a deficiency of H\(_2\)O\(_2\).
Footnotes:

1 This work was financially supported by grants from the Natural Science Foundation of China (No. 31270299) for the preparation of transgenic plants, and from the Natural Science Foundation of China (No. 31370301) for the heat stress-dependent phenotype analyses, and from the Program for New Century Excellent Talents in Universities (NCET-11-0440) for the pharmacological experiments, and from the Natural Science Foundation of Hebei Province (C2012205005) for the heat shock factors and heat shock protein analyses.

2 These authors contributed equal to this article.

*Address correspondence to zhaolq70@163.com

The author(s) responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Liqun Zhao (zhaolq70@163.com)
ABSTRACT

We previously reported that nitric oxide (NO) functions as a signal in thermotolerance. To illustrate its relationship with hydrogen peroxide (H$_2$O$_2$) in the tolerance of Arabidopsis (*Arabidopsis thaliana*) to heat shock (HS), we investigated the effects of heat on Arabidopsis seedlings of the following types: wild type; three NADPH oxidase-defective mutants that exhibit reduced endogenous H$_2$O$_2$ levels (*atrbohB*, *atrbohD*, and *atrbohB/D*); and a mutant that is resistant to inhibition by fosmidomycin (*noa1*, for nitric oxide-associated protein 1). 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$_2$O$_2$ signaling as a downstream factor. This point was verified by phenotypic analyses and thermotolerance testing of transgenic seedlings that overexpressed *NIA2* and *NOA1*, respectively, in an *atrbohB/D* background. Electrophoretic mobility shift assays, Western blot and real time RT-PCR analyses demonstrated that NO stimulated the DNA-binding activity of HS factors (HSFs) and the accumulation of heat shock proteins (HSPs) through H$_2$O$_2$. These data indicate that H$_2$O$_2$ acts upstream of NO in thermotolerance, which requires increased HSFs DNA-binding activity and HSP accumulation.
INTRODUCTION

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). Though 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, HSPs induction is dependent on heat shock factors (HSFs), which are transcription factors that bind to HS 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 Arginine-dependent NO synthase (NOS) and nitrite (NO$_2$)-dependent nitrate reductase (NR) pathways. Though 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 NOS gene in Arabidopsis thaliana (hereafter, Arabidopsis). However, critical questions have been raised about the role of AtNOS1 in NO biosynthesis, leading to the suggestion that NOA1 be renamed NO-associated 1 (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). Though 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., 2010a; Xuan et al., 2010; Mantal et al., 2012). NR, the other enzyme that catalyzes NO production, is encoded by two genes in Arabidopsis: NIA1 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 (ABA)-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 kinase 6 (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 HS signaling molecule, the interaction of NO with other molecules remains elusive. 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 (H₂O₂), 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 H₂O₂ (Gechev and Hille, 2005). In Arabidopsis, NADPH oxidase is encoded by ten genes, named AtrbohA-J, 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 ABA-induced stomatal closure (Kwak et al., 2003). In addition, atrbohB and atrbohD mutants reportedly showed a significant sensitive phenotype after heat treatment, implying atrbohB and atrbohD’s possible roles in thermotolerance (Larkindale et al., 2005). It has also been reported that the level of H₂O₂ increases following exposure to elevated temperatures, leading to HSF activation and HSP accumulation in Arabidopsis, tobacco (Nicotiana tabacum), and bacteria (Clostridium acetobutylicum), respectively (Volkov et al., 2006; Königshofer et al., 2008; Banti et al., 2010). In contrast, peroxide scavengers and inhibitors of H₂O₂ 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 H₂O₂ in the HS signaling pathway.

As signaling molecules, NO and H₂O₂ 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 as to which is upstream of the other. Some studies have shown that they cooperate in physiological and biochemical reactions. For example, both NO and H₂O₂ 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 crosstalk between NO and H₂O₂ in citrus plants (Tanou et al., 2010). Other studies indicate that H₂O₂ 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 H₂O₂ accumulation). For example, pretreating broad bean guard cells with an NO donor increased the H₂O₂ content (He et al., 2005). NO mediates antioxidant enzyme activity so as to influence the H₂O₂ level (Zhang et al., 2007, 2009). As yet, the relationship between NO and H₂O₂ under HS conditions is obscure (See Fig. 1 in Saidi et al.,
In this study, we used noa1, atrbohB, atrbohD, and atrbohB/D as loss-of-function mutants to examine the connection between NO and H$_2$O$_2$ in HS signaling, and we investigated the role of H$_2$O$_2$ in the induction of adaptive responses. Our results demonstrate the involvement of NO in H$_2$O$_2$ signaling as a downstream factor by stimulating HSF DNA-binding activity and HSP expression.

RESULTS

Effects of HS on H$_2$O$_2$ Accumulation in Wild-type Seedlings

H$_2$O$_2$ 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$_2$O$_2$ accumulation in wild-type seedlings. Intracellular H$_2$O$_2$ formation was detected using the fluorescent probe CM-H$_2$DCFDA, 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 Fig. S1 showed that pretreatment of 100 μM H$_2$O$_2$ increased the fluorescence density, whereas H$_2$O$_2$ scavenger 125 units CAT decreased it. However, NO donors, 20 μM SNP and SNAP, and NO scavenger 50 μM cPTIO had no clear effect on it, indicating that CM-H$_2$DCFDA was the special probe for H$_2$O$_2$, not for NO. Thereafter, fluorescence analysis showed that under HS treatment at 45°C, the H$_2$O$_2$ level in 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$_2$O$_2$ level remained higher than that in the control at 60 min (Fig. 1). Based on these results, 30 min was used as the length of the HS period to induce H$_2$O$_2$ accumulation in our subsequent experiments.
Effects of HS on H$_2$O$_2$ Accumulation and Survival in Wild-type, noa1, atrbohB, atrbohD, and atrbohB/D Seedlings

Next, the H$_2$O$_2$ levels were examined in the seedlings of wild type, noa1, atrbohB, atrbohD, and the atrbohB/D double mutant, which was deficient in the transcription of both AtrbohB and AtrbohD (Supplement Fig. S2). Under normal growth conditions, no obvious difference existed among the H$_2$O$_2$ levels in these seedlings. After HS treatment, the H$_2$O$_2$ 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 days of recovery at 22°C (Mittler, 2006). Under normal growth conditions, the noa1 seedlings exhibited chlorosis and were extremely small in size compared to 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).

DAF-FM DA was selected for use as a fluorescent probe for NO because it’s highly specific for NO and does not react with other ROS. DAF-FM DA permeated the membrane and was transformed by intracellular esterases into 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 level and survival ratio compared to 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 on thermotolerance.
Our upon data indicate that in response to HS treatment, the *atrbohB, atrbohD,* and *atrbohB/D* plants showed lower H$_2$O$_2$ levels and survival ratios than the wild-type plants, with the lowest values observed for the *atrbohB/D* double mutant. However, *noa1,* exhibited heat sensitivity due to reduced NO production (See Fig. 1 in Xuan et al., 2010; Fig. 3), maintained a higher H$_2$O$_2$ level and the lowest survival ratio, suggesting an intimate relationship between NO and H$_2$O$_2$ 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$_2$O$_2$ 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 Fig. 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 Fig. S3.

**Effects of Internal H$_2$O$_2$ Levels on the Survival of Wild-type and**
**noa1 Seedlings**

Next, a solution containing varying concentrations of H$_2$O$_2$ was applied to pretreat the wild-type and noa1 seedlings. This increased the internal H$_2$O$_2$ level in both types of seedlings in a concentration-dependent manner, reaching a maximum value at 50-100 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$_2$O$_2$ levels, reaching a maximum value at 100 μM (29% higher than the control) (Fig. 5, C and D), which was thoroughly reversed by treatment of 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, 4 and 5; Supplemental Fig. S4) showed that an elevated internal NO level increased the thermotolerance of the H$_2$O$_2$-deficient mutants atrbohB, atrbohD, and atrbohB/D, however, an elevated internal H$_2$O$_2$ level had no clear effect on the NO-deficient noa1 mutant, indicating NO involvement in H$_2$O$_2$ 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$_2$O$_2$ in thermotolerance, we obtained the triple mutant atrbohB/D/noa1, which was shown to be deficient in AtrbohB, AtrbohD, and NOA1 transcription by RT-PCR analysis (Fig. 6A). Under normal conditions, 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. 20 μM of SNP or SNAP increased the survival of these seedlings (Supplemental Fig. S5), implicating the effect of NO on thermotolerance even in the absence of H$_2$O$_2$. 
AtNIA2 and AtNOA1 Overexpression in an atrbohB/D Background Improves Thermotolerance

NIA2 is a major component of NR, which converts NO₂ 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 H₂O₂-deficient mutants under HS stress. The elevated expression of AtNIA2 and AtNOA1 was confirmed by RT-PCR, respectively (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, respectively, though 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 them of atrbohB/D/35S::NOA1-1 and atrbohB/D/35S::NOA1-2 by 46 and 61 %, respectively, compared to their background atrbohB/D under HS conditions (Fig. 8, D and E). This intriguing phenomenon might be due to the negative effect of excessive NO on thermotolerance as our previous report (see Figs. 2 and S2 in Xuan et al., 2010).

These results (Figs. 7 and 8) showed that the overexpression of AtNIA2 or AtNOA1 restored thermotolerance in the H₂O₂-deficient atrbohB/D mutant, providing genetic proof of the relationship between NO and H₂O₂ in thermotolerance.
Effects of H$_2$O$_2$ on the DNA-binding Activity of HSFs and AtHSP17.7 and AtHSP21 Expression through NO

To examine the underlying mechanism of NO- and H$_2$O$_2$-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, HSFs 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 to 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). Hsfs (Hsf2A, HsfA7a and HsfB2b) mRNA levels also showed the same changing pattern as the bindings 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$_2$O$_2$ 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 accumulation was lower in the mutants than in 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 to 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.
DISCUSSION

H$_2$O$_2$ and Thermotolerance in Arabidopsis Seedlings

In this work, we obtained evidence for the involvement of NO in H$_2$O$_2$ signaling in thermotolerance. H$_2$O$_2$, 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$_2$O$_2$ promotes thermotolerance in Arabidopsis.

H$_2$O$_2$ is regarded as a signaling molecule that contributes to thermotolerance; therefore, we first examined H$_2$O$_2$ production with its fluorescent probe CM-H$_2$DCFDA (Supplemental Fig. S1) under HS conditions. As shown in Fig. 1, the H$_2$O$_2$ level first increased then decreased gradually with the increased HS time. This may be attributed to an adaptive cellular mechanism inhibiting excessive H$_2$O$_2$ 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$_2$O$_2$ production, showed weaker defects under heat stress. Thus, we obtained these mutants to study the role of H$_2$O$_2$ in thermotolerance. Under HS stress, the survival ratios of atrbohB, atrbohD, and atrbohB/D seedlings were lower than that of wild-type seedlings, in accordance with the H$_2$O$_2$ levels (Fig. 2). These data suggest that AtrbohB and AtrbohD are related to thermotolerance as the implication of Larkindale et al. (2005). We also found that NO functions as a signal in thermotolerance using noa1 and nia1nia2 seedlings (Xuan et al., 2010). The NH$_4^+$, which was supplied in the culture medium for nia1nia2 due to its deficiency of NR, might induce abnormal nutrient status of the co-cultured wild-type and H$_2$O$_2$-deficient mutant seedlings so as to influence their natural thermotolerance. Thus, we utilized NO-deficient noa1 seedlings to examine the H$_2$O$_2$ level under HS conditions.
The higher H$_2$O$_2$ level and the lowest survival ratio in the noa1 mutant (Fig. 2) suggested a relationship between NO and H$_2$O$_2$ in HS signaling.

**Relationship between NO and H$_2$O$_2$ 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$_2$O$_2$-deficient mutants (Fig. 4, A and B), in accordance with the H$_2$O$_2$ 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 of H$_2$O$_2$ had no clear effect on that of noa1 seedlings (Fig. 5). A plausible explanation for these results is that NO is a key component of the H$_2$O$_2$ pathway in HS signaling, and, therefore, a deficiency in NO did not affect H$_2$O$_2$ accumulation (Fig. 2, A and B), whereas a deficiency in H$_2$O$_2$ inhibited NO production (Fig. 4, A and B). Also, supplementation with NO, a downstream molecule, restored the heat-sensitive status of the H$_2$O$_2$-deficient mutants *atrbohB*, *atrbohD*, and *atrbohB/D* seedlings (Fig. 4, C and D); however, supplementation with H$_2$O$_2$, an upstream molecule, had no effect on the appearance of noa1 seedlings, which were deficient in NO (Fig. 5). For the same reason, NO scavenger cPTIO inhibited the effect of H$_2$O$_2$ on the survival of wild type under HS (Fig. 5; Supplemental Fig. S4).

We also found that the application of a high concentration of H$_2$O$_2$ (200 µM) did not induce a higher internal H$_2$O$_2$ 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$_2$O$_2$ inhibits the accumulation of too much H$_2$O$_2$ through activation of antioxidant enzymes, e.g., catalase (CAT) (Supplemental Fig. S7).
**Effects of NO and H₂O₂ on Thermotolerance in Arabidopsis**

To clarify the effects of NO and H₂O₂ 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 H₂O₂ 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 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 H₂O₂.

Collectively, our data provide pharmacological proof of the existence of a novel signaling pathway in which H₂O₂ production is stimulated by HS to regulate NO accumulation so as to confer thermotolerance.

**The Mechanism Underlying the Effect of H₂O₂ through NO on Thermotolerance**

To determine the mechanism through which H₂O₂ affects thermotolerance via NO, we examined the effects of H₂O₂ and NO on HSFs 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 Ca²⁺ and calmodulin 3 (CaM3) are believed 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 HSFs DNA-binding activity and HSP accumulation (Xuan et al., 2010). The current data indicate that a decrease in the level of H₂O₂ 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$_2$O$_2$ effect, thereby influencing 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$_2$O$_2$ induces thermotolerance through NO. Western blot analysis indicated that under HS conditions, the decreased H$_2$O$_2$ 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$_2$O$_2$ activation of HSP expression through NO. H$_2$O$_2$ 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$_2$O$_2$ influences thermotolerance via NO involves alterations in HSFs DNA-binding activity and HSP gene expression.

To our knowledge, the present data provide the first evidence that H$_2$O$_2$ functions as a second messenger in the induction of thermotolerance through NO, which is dependent on the enhancement of HSFs 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$_2$O$_2$ 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$_2$O$_2$ 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. [www.plantphysiol.org](http://www.plantphysiol.org))
Indeed, two heat-activated Ca\textsuperscript{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\textsuperscript{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 kinase 3 is an important AtCaM downstream component of the HS signal transduction pathway (Liu et al., 2008). A CaM protein phosphatase, PP7, has been also 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 crosstalk among H\textsubscript{2}O\textsubscript{2}, NO, Ca\textsuperscript{2+} channels, and the activation of Ca\textsuperscript{2+}-CaM-dependent protein phosphatase or other factors to mediate HSFs 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* (ecotype Columbia [Col-0]) were surface-sterilized in 2% (v/v) sodium hypochlorite for 1 min then washed thoroughly with water. The sterilized seeds were plated on MS medium containing 3% sucrose and 0.7% agar, and kept at 4°C in the dark for 3 days. The plants were then transferred to a growth chamber set at 22°C and 120 µmol/m\textsuperscript{2}/s on a 16-h day/night cycle.

*noa1* seeds were obtained from Dr. N.M. Crawford (University of California, San Diego, CA, USA). *atrbohB* and *atrbohD* mutant seeds were obtained from Dr. M.A. Torres (Universidad Politécnica de Madrid, Madrid, Spain). *35S::NIA2-1* and *35S::NIA2-3* seeds were obtained from Dr. C.P. Song (Henan University, Henan, China). 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 sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP), 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (cPTIO), hydrogen peroxide (H$_2$O$_2$), and catalase (CAT) (all Sigma, St. Louis, MO, USA) at various concentrations were sprayed onto the leaf surfaces of 6-day-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 days. Those seedlings that were still green and continued to produce new leaves were scored as survivors. For Western blot analysis, 10-day-old seedlings were kept at 37°C for 2 h and analyzed for HSP accumulation (Liu et al., 2005).

**Fluorescence Microscopy**

NO and H$_2$O$_2$ were visualized using the NO-specific fluorescent probe 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) (Sigma) and H$_2$O$_2$ fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA) (Invitrogen Corp., Carlsbad, CA, USA), 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$_2$DCFDA 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 (ELIPE TE2000-U; Nikon Corp., Tokyo, Japan). The signal intensities were quantified using MetaMorph (Molecular Devices Corp., Sunnyvale, CA, USA).

**Construction of the Transgenic Lines**
To generate *AtNOA1-GFP* for the production of plants overexpressing *AtNOA1* in an *atrbohB/D* background, *AtNOA1* cDNA was amplified by RT-PCR using the primers NOA1F1 (5'-CACCATGGCGCTACGAACACTCTCAA-3') and NOA1F2 (5'-AAAG TACCATTTGGGTCTTACT-3'). The product was cloned in the sense orientation into pENTR/SD/D-TOPO and then into pMDC32 using Gateway LR Recombinase (Invitrogen Corp.) 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 hygromycin. The number of T-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 ten-day-old seedlings using the PrimeScript™ RT reagent kit (Takara) for first-stand cDNA synthesis. RT-PCR analyses of *AtrbohB* (*At1g09090*), *AtrbohD* (*At5g47910*), *NOA1* (*At3g47450*) and *NIA2* (*At1g37130*) transcription were performed using the Takara RNA PCR (AMV) 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 DTT, 0.1 mM EDTA, 5% glycerol, and
0.5 mM phenylmethylsulfonyl fluoride), and the extracts were clarified by centrifugation at 14,000g for 20 min at 4°C. The supematants 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-chloroindolyl phosphate/nitroblue tetrazolium (Amresco, Solon, OH, USA) was used for immunodetection.

EMSAs

The heat shock element (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-32P] ATP and T4 polynucleotide kinase (Takara Bio Inc.). 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). EMSAs were 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.
Supplementary Data

Supplemental Figure S1. Effects of H₂O₂ and NO on CM-H₂DCFDA 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 H₂O₂ 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 H₂O₂-induced HSFs and HSP gene expression by real-time RT-PCR.

Supplemental Figure S7. Effects of NO and H₂O₂ 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.

ACKNOWLEDGEMENTS

We thank Drs. N.M. Crawford, M.A. Torres and C.P. Song for providing the seeds used in this research, and Drs. E. Brown and J.F. Harper (University of Nevada, Reno) for their generous help.

LITERATURE CITED


Asai S, Ohta K, Yoshioka H (2008) MAPK signaling regulates nitric oxide and


Flores-Pérez U, Sauret-Gueto S, Gas E, Jarvis P, Rodriguez-Concepcion M


Huang XS, Liu JH, Chen XJ (2010) Overexpression of *PtrABF* gene, a bZIP


**Figure Legends**

**Figure 1.** Effects of HS on endogenous H$_2$O$_2$ levels in wild-type seedlings. (A) Six-day-old wild-type seedlings grown at 22°C were exposed to 45°C for 0-60 min, then examined for H$_2$O$_2$ by fluorescence microscopy in roots stained with CM-H$_2$DCFDA. Bar = 100 μm.
(B) Relative DCF fluorescence densities in the roots. The data presented are the mean ± SE of measurements taken from at least 10 roots for each treatment. *, P < 0.05; **, P < 0.01 versus 0 μM H₂O₂ (Student’s t test).

**Figure 2.** Effects of HS on the internal H₂O₂ levels and survival in wild-type, noa1, atrbohB, atrbohD, and atrbohB/D seedlings.

(A) Six-day-old wild-type, 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 H₂O₂ levels were then assessed by fluorescence microscopy in roots stained with CM-H₂DCFDA. Bar = 100 μm.

(B) Relative DCF fluorescence densities in the roots. The data presented are the mean ± 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 days later. 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 the mean ± SE of at least 5 independent experiments, with 50 seedlings per experiment. *, P < 0.05; **, P < 0.01 (Student’s t test). WT, wild type.

**Figure 3.** Effects of NO on the thermotolerance of noa1 seedlings.

(A) Six-day-old wild-type and noa1 seedlings grown at 22°C were pretreated with 2 mL of ultrapure water, 20 μM SNP or SNAP for 8 h, 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 the mean ± 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 days later. 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 the mean ± SE of at least 5 independent experiments, with 50 seedlings per experiment. *, P < 0.05; **, P < 0.01. WT, wild type.

Figure 4. Effects of NO on the thermotolerance of wild-type, atrbohB, atrbohD, and atrbohB/D seedlings.

(A) Six-day-old wild-type, atrbohB, atrbohD, and atrbohB/D seedlings grown at 22°C were pretreated with 2 mL of ultrapure water, 20 μM SNP or SNAP for 8 h, 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 the mean ± 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 days later. 1, wild-type; 2, atrbohB; 3, atrbohD; and 4, atrbohB/D.

(D) Survival ratios of the seedlings after HS treatment. The data presented are the mean ± SE of at least 5 independent experiments, with 50 seedlings per experiment. *, P < 0.05; **, P < 0.01 versus HS (Student’s t test). WT, wild type.

Figure 5. Effects of H2O2 on the thermotolerance of wild-type and noa1 seedlings.

(A) Six-day-old wild-type and noa1 seedlings grown at 22°C were pretreated with 2 mL of 0, 25, 50, 100, or 200 μM H2O2 for 8 h, then exposed to 45°C (HS) or maintained at 22°C (Control) for 30 min. The H2O2 levels were then assessed by fluorescence microscopy in roots stained with CM-H2DCFDA. Bar = 100 μm.

(B) Relative DCF fluorescence densities in the roots. The data presented are the mean ± SE of measurements taken from at least 10 roots for each treatment. *, P
< 0.05; **, P < 0.01 versus 0 \mu M 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 days later.

(D) Survival ratios of the seedlings after HS treatment. The data presented are the mean ± SE of at least 5 independent experiments, with 50 seedlings per experiment. *, P < 0.05 versus 0 \mu M H_2O_2 (Student’s t test). WT, wild type.

**Figure 6.** Survival status of the *atrbohB/D/noa1* triple mutant.

(A) RT-PCR analysis of *AtrbohB, AtrbohD,* and *AtNOA1* transcription in wild-type, *atrbohB, atrbohD, noa1,* and *atrbohB/D/noa1* seedlings. *ACT7* 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 days later. 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 the mean ± SE of at least 5 independent experiments, with 50 seedlings per experiment. *, P < 0.05; **, P < 0.01 (Student’s t test). WT, wild type.

**Figure 7.** Improved thermotolerance through *AtNIA2* overexpression in an *atrbohB/D* background.

(A) RT-PCR analysis of *AtNIA2* transcription in wild-type, *atrbohB/D, atrbohB/D/35S::NIA2-1,* and *atrbohB/D/35S::NIA2-3* seedlings. *ACT7* 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 \mu m.

(C) Relative DAF-FM fluorescence densities in the roots. The data presented are the mean ± SE of measurements taken from at least 10 roots for each treatment. *,

Copyright © 2014 American Society of Plant Biologists. All rights reserved.
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 days later. 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 the mean ± SE of at least 5 independent experiments after HS, with 50 seedlings per experiment. *, P < 0.05 versus *atrbohB/D* seedlings (Student’s *t* test). WT, wild type.

**Figure 8.** Improved thermotolerance through *AtNOA1* overexpression in an *atrbohB/D* background.

A) RT-PCR analysis of *AtNOA1* transcription in wild-type, *atrbohB/D*, *atrbohB/D/35S::NOA1-1* and *atrbohB/D/35S::NOA1-2* seedlings. *ACT7* 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 the mean ± 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 days later. 1, wild-type; 2, *noa1*; 3 and 4 represent *atrbohB/D/35S::NOA1-1* and *atrbohB/D/35S::NOA1-2*, respectively.

(E) Survival ratios of the seedlings. The data presented are the mean ± SE of at least 5 independent experiments after HS, with 50 seedlings per experiment. *, P < 0.05 versus *atrbohB/D* seedlings (Student’s *t* test). WT, wild type.

**Figure 9.** Effects of H$_2$O$_2$ through NO on HSFs DNA-binding activity and *AtHSP17.7* and *AtHSP21* expression.

(A) The titration experiment utilizing electrophoretic mobility shift binding assay
(EMSA) confirmed the binding of HSFs and HSEs in whole-cell extracts prepared from 10-day-old seedlings incubated at 37°C for 1 h. Unlabeled DNA probe in 30-fold (++) or 60-fold (+++) molar excess relative to the labeled one 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, 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-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-G) Wild-type, atrbohB, atrbohD, atrbohB/D, atrbohB/D+20 μM SNP, and atrbohB/D+20 μM SNAP seedlings. (H-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. WT, wild type.

**Figure 10.** Model for the involvement of H2O2 in NO pathway in HS signal transduction.

Black arrows indicate pathways supported by evidence and dotted arrows show hypothetical processes. Question mark indicates the unknown player.
A

Control

HS

WT noa1 atrbohB atrbohD atrbohB/D

B

Relative DCF Fluorescence density

Control

HS

WT noa1 atrbohB atrbohD atrbohB/D

C

Control

HS

D

Survival ratio (%)

WT noa1 atrbohB atrbohD atrbohB/D
### Table 1: AtHSP Expression Levels

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>HS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AtHSP17.7</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubulin</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb17b</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb17d</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb17b/D</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb17b/D+SNP</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb17b/D+SNAP</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

| **AtHSP21** |
| Tubulin     | 1.00    | 1.00        |
| Athb21      | 1.00    | 1.00        |
| Athb21/D    | 1.00    | 1.00        |
| Athb21/D+SNP | 1.00   | 1.00        |
| Athb21/D+SNAP | 1.00  | 1.00        |

---

### Figure Legends

**A** Labeled Probe + + + + +
Unlabeled probe − − + + +
Protein − + + + +

**B** Control WT WT atrb17b atrb17d/atb17b D+SNP atrb17b/D+SNAP
HS 0.00 1.00 1.00 0.87 0.71 0.30 0.83 0.67 0.21 0.23 0.88 0.62

**C** Control WT WT atrb17b D+SNP atrb17b/D+SNAP
HS 0.00 1.00 2.00 1.00 1.00 0.23 0.88 0.62

**D** AtHSP17.7

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubulin</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb17b</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb17d</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb17b/D</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb17b/D+SNP</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb17b/D+SNAP</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**E** AtHSP17.7

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubulin</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb17b</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb17d</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb17b/D</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb17b/D+SNP</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb17b/D+SNAP</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**F** AtHSP21

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubulin</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb21</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb21/D</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb21/D+SNP</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb21/D+SNAP</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**G** AtHSP21

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubulin</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb21</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb21/D</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb21/D+SNP</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Athb21/D+SNAP</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>