Retraction


The above article is being retracted by *Plant Physiology*, which issues the following statement:

After publication of the article on October 15, 2015, as a *Plant Physiology* Preview article, we were alerted to the fact that Figure 9 and a considerable amount of text in the Results and Discussion sections were identical (or nearly identical) to material in an article previously published by the same authors in *PLOS ONE* (Mitochondrial-Derived Reactive Oxygen Species Play a Vital Role in the Salicylic Acid Signaling Pathway in *Arabidopsis thaliana*; DOI 10.1371/journal.pone.0119853). The authors did not cite the *PLOS ONE* article in the *Plant Physiology* article, nor did they indicate that Figure 9 had been duplicated from this earlier publication.

ASPB invoked its ethics in publishing policies to evaluate this instance of self-plagiarism. On the basis of available submission and publication dates of the two articles, ASPB concluded that because the *PLOS ONE* paper was accepted for publication on January 16, 2015, more than four months before the *Plant Physiology* article was submitted on May 26, 2015, it is necessary to retract the *Plant Physiology* article in its entirety.
Running title: Role of ROS in SA-induced acquired thermotolerance

Corresponding author:

Da Xing

MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, College of Biophotonics, South China Normal University, Guangzhou 510631, China

Telephone: +86-20-8521-0089
Fax: +86-20-8521-6052
Email: xingda@scnu.edu.cn

Research area most appropriate for the paper: Signaling and Response
A Potential Role for Mitochondrial Produced Reactive Oxygen Species in Salicylic Acid-Mediated Plant Acquired Thermotolerance

Shengjun Nie, Haiyun Yue and Da Xing*

MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, College of Biophotonics, South China Normal University, Guangzhou 510631, China

One-sentence Summary
SA functions and acts upstream of AtHsfA2 in acquired thermotolerance, which requires a pathway with H$_2$O$_2$ production involved and is dependent on increased expression of $Hsp$ genes.
Footnotes

This research is supported by the Program for Changjiang Scholars and Innovative Research Team in University (IRT0829), the Key Program of NSFC-Guangdong Joint Funds of China (U0931005) and the National High Technology Research and Development Program of China (863 Program) (2007AA10Z204).

*Corresponding author: Da Xing

Email: xingda@scnu.edu.cn
ABSTRACT

To characterize the function of salicylic acid (SA) in acquired thermotolerance, the effects of heat shock (HS) on wild-type and *sid2* (for SA induction deficient 2) was investigated. After HS treatment, the survival ratio of *sid2* mutant was lower than that of wild-type. However, pretreatment with hydrogen peroxide (H$_2$O$_2$) rescued the *sid2* heat sensitivity. HsfA2 is a key component of acquired thermotolerance in Arabidopsis. The expression of *HsfA2* induced by SA was highest among those of heat-inducible Hsfs (HsfA2, HsfA7a, HsfA3, HsfB1, and HsfB2) in response to HS. Furthermore, the application of AsA, an H$_2$O$_2$ scavenger, significantly reduced the expression level of *HsfA2* induced by SA. Although SA enhanced the survival of *sid2* mutant, no significant effect on the *hsfA2* mutant was observed, suggesting that HsfA2 is responsible for SA-induced acquired thermotolerance as a downstream factor. Further, real-time PCR analysis revealed that after HS treatment, SA also up-regulated mRNA transcription of HS protein (*Hsp*) genes through AtHsfA2. Time course experiments showed an increase in the fluorescence intensity of DCF in the mitochondria occurred earlier than in other regions of the protoplasts in response to SA. The cytochrome reductase activity analysis in isolated mitochondria demonstrated that SA-induced mitochondrial ROS possibly originated from complex III in the respiration chain. Collectively, our data suggest that SA functions and acts upstream of AtHsfA2 in acquired thermotolerance, which requires a pathway with H$_2$O$_2$ production involved and is dependent on increased expression of *Hsp* genes.
INTRODUCTION

Plants are confronted with a multitude of biotic and abiotic stresses and have evolved a remarkable repertoire of survival mechanisms, including antipathogenic responses as well as their ability to acquire tolerance to lethal heat stress. Salicylic acid (SA), as an essential phenolic compound (Klessig and Malamy, 1994), has been found to act effectively in hypersensitive response (HR) (Kunkel and Brooks, 2002) and systemic acquired resistance (SAR) (Dempsey et al., 1999). In addition, SA participates in responses to abiotic stresses such as ozone (Koch et al., 2000; Diara et al., 2005), salt and osmotic stress (Borsani et al., 2001; Molina et al., 2002) and heat stress (Senaratna et al., 2000). In particular, recent studies describe potential effects of SA on both basal and acquired thermotolerance in mustard, tomato and potato (Dat et al., 1998a, 1998b, 2000; Lopez-Delgado et al., 1998; Hong and Vierling, 2000; Hong et al., 2003; Larkindale et al., 2005; Snyman and Cronje, 2008). In this work, we focused on the mechanisms of SA-mediated acquired thermotolerance in Arabidopsis.

As a countermeasure to heat shock (HS), plants synthesize a set of highly conserved proteins known as heat shock proteins (HSPs), and expression of HSPs is governed by heat shock factors (Hsfs), which are transcription factors binding to heat shock promoter elements (HSEs) in the promoter region of the HSPs genes (Nover et al., 2001; Akerfelt et al., 2010; Liu and Charng, 2013). In the eukaryotic organisms, the number of Hsf genes differs greatly. The mammalian contains three Hsf isoforms, Hsf1, Hsf2, and Hsf4, with a distinct biological function (Fujimoto et al., 2004; Xing et al., 2005). *Drosophila melanogaster* and *Caenorhabditis elegans* each has a single Hsf gene playing an essential biological role in HSR (Nover et al., 1996; Hsu et al., 2003). However, in Arabidopsis, 21 Hsfs have been sorted into three classes (classes A, B, and C) according to their unique structural characteristics (Nover et al., 2001; Kotak et al., 2004). Interestingly, of the 21 Hsfs, the heat-inducible class A Hsfs, *HsfA2*, *HsfA7a*, and *HsfA3*, and class B Hsfs, *HsfB1*, *HsfB2a*, and *HsfB2b*, play an essential role in acquired thermotolerance (Busch et al., 2005; Charng et al., 2007; Larkindale and Vierling, 2008; Schramm et al., 2008; Meiri and Breiman, 2009). The heat ability of these Hsfs is a feature unique to plants, but is not found in animals and yeast (Larkindale and Vierling, 2008; Schramm et al., 2008). In the early
response to HS, HsfA1s are activators of genes for HSPs and promote the activities of other HS-inducible Hsfs (Lohmann et al., 2004; Busch et al., 2005; Liu et al., 2011). Once these HS-inducible Hsfs are induced, HsfA2 becomes the dominant Hsf and mediates the amplification of a subset of HSR genes (Busch et al., 2005; Schramm et al., 2006; Liu and Charng, 2013). A recent study by Charng et al. (2007) suggests that only the hsfA2 mutant showed an obvious phenotype among the tested mutants corresponding to 48 heat-induced genes. Following heat acclimation at 37°C, the hsfA2 mutant was more sensitive to severe heat stress than wild-type after long recovery periods. The presence of HsfA2 exclusively in acquired thermotolerance suggests its importance in recovery after HS (Baniwal et al., 2004; Charng et al., 2007). However, whether these heat-inducible Hsfs function in SA-mediated plant acquired thermotolerance has not been elucidated, if so, which one or even how could be involved in acclimation to acquired thermotolerance.

Reactive oxygen species (ROS) are generated in response to diverse stresses. Hydrogen peroxide (H$_2$O$_2$), a major and the most stable type of ROS, acts as a central signaling molecular to regulate many processes, such as stomatal movement, plant-pathogen interactions and different response to abiotic stress (Severine et al., 1994; Alvarez et al., 1998; Bais et al., 2003; Apel and Hirt, 2004; Rhee, 2006). Several groups have reported the connection between plant Hsfs and oxidative stress (Li et al., 2005; Miller and Mittler, 2006; Zhang et al., 2009). The increased level of H$_2$O$_2$ in response to HS resulted in Hsfs induction and HSPs accumulation (Zhong et al., 1998; Ahn and Thiele, 2003; Volkov et al., 2006; Banti et al., 2010). Conversely, peroxide scavengers and inhibitors of H$_2$O$_2$ can reduce the expression of these HSPs (Volkov et al., 2006). Panchuk et al. (2002) studied the transgenic plants over-expressing HsfA1b and reported that Hsf is implicated in the induction of ascorbate peroxide (Apx2), which encodes the H$_2$O$_2$ scavenging enzyme. The study by Schramm et al. (2006) reveals that the induction of Apx2 is totally abolished in the absence of HsfA2. Furthermore, disruption of HsfA2 displayed reduced obtained oxidative stress tolerance, while transgenic plants over-expressing HsfA2 increased tolerance (Ogawa et al., 2007). Under anoxia, HsfA2 is also strongly up-regulated by H$_2$O$_2$ (Banti et al., 2010). These results confirm the involvement of H$_2$O$_2$ in HS signaling and the role of plant Hsfs in the ROS signaling network.

This study was to elucidate the relationship among SA, an intermediate H$_2$O$_2$ and plant Hsfs in
acquired thermotolerance using the wild-type and *sid2* mutant, and to assess the behavior and function of mitochondria in response to SA. The results presented demonstrate that SA-induced mitochondrial H$_2$O$_2$ generation is implicated in the acquired thermotolerance, through induction transcripts of *HsfA2* and *HSPs* genes. This work may contribute to the understanding of the mitochondria-dependent mechanism of the SA-induced biological responses in plants, and provide a new insight into the cellular signaling cascade in SA-mediated thermotolerance response.
RESULTS

SA Promotes Acquired Thermotolerance in Arabidopsis Plants

To establish the relationship between SA and acquired thermotolerance, the endogenous free SA level in wild-type and *sid2* (SA induction deficient 2) mutant was tested. Under normal growth condition (24°C), the free SA level in wild-type was about 9-fold that in *sid2* mutant. After HS treatment, changes in free SA in wild-type were dramatic during recovery time. At 1 h recovery, the level of SA increased by 70% relative to the control, and then declined to the control level during the following 6 h. However, in *sid2* mutant, the level of SA was consistently lower (Fig. 1A).

To examine physiological adaptability to acquired thermotolerance, the survival ratio of plants was investigated following heat acclimation at 37°C for 1 h, 2 d of recovery at 24°C, and then exposed to HS at 44°C for 45 min. The *sid2* lines were more sensitive than wild-type. The maximum difference was significant at 3 d after HS, with survival ratio of 65% and 21% for the wild-type and *sid2* mutant, respectively (Fig. 1, B and C). In addition, the necrotic lesions were observed on *sid2* plants, and image analysis demonstrated 9-fold more necrosis compared with the wild-type (Fig. 1, D and E).

To further determine whether the suppression of the SA level in the *sid2* mutant was responsible for the impaired thermotolerance, *sid2* mutant was pre-treated with 100 µM SA. The survival ratio was greatly increased to the control (Fig. 1C). The acquired thermotolerance of *NahG* plants, which over-express the *NahG* encoding an SA hydroxylase that converts SA to catechol (Gaffney et al., 1993), was also compared. The *NahG* showed lower survival ratio and SA level compared with wild-type (Supplemental Fig. S1).

SA-induced Plant Acquired Thermotolerance Is Related with H2O2 Signaling

To investigate the involvement of H2O2 in HS tolerance, the effect of H2O2 on the acquired thermotolerance of wild-type and *sid2* mutant was examined. The survival ratio of wild-type and *sid2* mutant was significantly increased by exogenous H2O2 (Fig. 2, A and B). Also, H2O2 pre-treatment interdicted the increased necrosis in *sid2* line (Fig. 2, C and D).

Ascorbic acid (AsA), as an important reducing substrate, could remove H2O2 (Noctor and Foyer, 1998). Treatment of wild-type plants with AsA (1 mM) showed the reduced survival ratio.
exhibited a small (approximately 9%) decrease compared with wild-type, as an AsA-deficient mutant of Arabidopsis (for 10% to 30% of wild-type levels of AsA; Jander et al., 2002; Müller-Moulé et al., 2003). However, *sail*, a SA-insensitive mutant (Shah et al., 1997), showed
a more severe reduction (approximately 75%) in acquired thermotolerance (Fig. 2, A and B).

Besides the survival ratio, the necrosis in sai1 mutant was exhibited an obvious increase compared with that in wild-type (Fig. 2, C and D).

**SA Induces ROS Production in Arabidopsis Protoplasts and Plants**

Having established that H$_2$O$_2$ is required for SA-induced acquired thermotolerance (Figs. 1 and 2), we then examined whether external SA might cause increase generation of H$_2$O$_2$. 

**Figure 2.** Effect of H$_2$O$_2$ on acquired thermotolerance in sid2 mutant. A, Survival ratios of wild-type and sid2 mutant after HS treatment. 10-d-old wild-type and different mutants (sid2, vtc2, sai1) grown at 24°C were pretreated with 1 mM ASA or 1.5 mM H$_2$O$_2$, then exposed to heat acclimation at 37°C for 1 h, followed by 2d of recovery at 24°C, and then exposed to HS at 44°C for 45 min. The survival ratio was examined 3 d later. Here, the survival of vtc2 and sai1 mutants were shown as positive and negative control, respectively, to show the effect at ASA level. Statistical analysis was performed with Student’s t-test; *: P < 0.05 vs WT; **: P < 0.05 vs sid2. B, Representatives of treated seedlings of the indicated genotypes. A 2.5-week-old wild-type and mutants grown at 24°C were exposed to heat acclimation at 37°C for 1 h, followed by 2d of recovery at 24°C, then exposed to HS at 44°C for 45 min and photographed 6 days later. What is more, the sid2 mutant was pretreated with 1.5mM H$_2$O$_2$. Asterisks indicate significant differences to WT (Student’s t-test, P < 0.05). D, Representatives of treated seedlings of the indicated genotypes in C. Scale bars, 1 cm. Data are means ± SE of three different experiments, with similar results.
Arabidopsis protoplasts were treated with 100 µM SA, and the fluorescent compound dichlorofluorescein (DCF), produced from the non-fluorescent compound H$_2$DCFDA in the presence of endogenous esterases and H$_2$O$_2$ (Jakubowski and Bartosz, 2000), was used. The

![Graph A: Effect of SA on DCF fluorescence in Arabidopsis protoplasts.](image)

![Graph B: Dose dependence of SA concentration on DCF fluorescence.](image)

![Graph C: Representative fluorescence images of SA-treated WT and mutants.](image)

![Graph D: Changes in H$_2$O$_2$ content in 100 µM SA-treated plants.](image)

**Figure 3**

**Figure 3** ROS generation in response to SA in Arabidopsis. A, Effects of SA on the DCF fluorescence intensity after 1 h. At the indicated time, the protoplasts with or without 100 µM SA treatment were subjected to 5 µM H$_2$DCFDA for 20 min in the dark, and the relative fluorescence intensity was quantified by a fluorescence spectrometer. B, Dose dependence for SA induced average relative fluorescence. The protoplasts were treated with different concentrations of SA for 1 h, and detected by a fluorescence spectrometer. In (A) and (B), different letters indicate statistically significant differences among the samples (P <0.05; Duncan’s multiple range tests). C, Representative fluorescence images of SA-treated WT and different mutants (vte2, sal1, sid2, and NahG) protoplasts during 1 h. Scale bars, 100 µm. D, Changes in H$_2$O$_2$ content in 100 µM SA-treated plants. 2.5-week-old Plants were treated with 100 µM SA. Leaves were harvested at indicated times (h). FW, Fresh Weight. Data are means ± SE of three different experiments, with similar results.
DCF fluorescence intensity was visible within 1 h, and application of SA significantly induced increase in the relative fluorescence intensity of DCF as early as 15 min, which was twice of the increase in the control, and continued to elevate during the following 45 min. Conversely, no increase in fluorescence was observed in the control protoplasts (Fig. 3A and Supplemental Fig. S2A). Different concentrations of SA were applied and an increase in DCF fluorescence intensity was observed within 150 μM SA, which reached a peak at 100 μM SA, about 4 times as much as the control (Fig. 3B).

The fluorescence among the vtc2, sid2, sai1 and NahG protoplasts was compared with that of wild-type protoplasts under 100 μM SA. Treatment for 30 min after SA led to a significant increase in DCF fluorescence in the wild-type protoplasts. The vtc2 protoplasts showed increased fluorescence. In the sid2 protoplasts, the value of the fluorescence was near the wild-type level after SA treatment. However, the fluorescence was not significantly increased at 30 min or 60 min in the sai1 and NahG protoplasts (Fig. 3C and Supplemental Fig. S2B).

The in situ accumulation of H2O2 using 3,3’-diaminobenzidine (DAB) staining procedure was also detected. The foliar application of SA increased staining of the wild-type and vtc2 mutant, but had no effect on the side and sai1 mutants. However, SA treatment increased H2O2 level of the sid2 by about 50% compared with its control (Fig. 3D and Supplemental Fig. S3).

**The Heat-inducible HsfA2 is strongly Up-regulated by SA in Arabidopsis**

According to the transcriptome studies of Busch et al. (2005), six Hsf genes, including class A Hsfs, namely HsfA2, HsfA7a, and HsfA3, and B-type Hsfs, HsfB1, HsfB2a, and HsfB2b, are heat-inducible genes and play an important role in acquired thermotolerance (Charng et al., 2007; Larkindale and Vierling, 2008; Schramm et al., 2008; Ikeda et al., 2011). To examine the interaction between SA and these heat-inducible Hsfs, the effect of SA on these heat-inducible Hsfs expression was examined.

Under control (24°C), the levels of these heat-inducible Hsfs were very low. These genes mRNA levels increased in the wild-type upon heat acclimation treatment at 37°C, suggesting that these heat-inducible Hsfs are responsive to heat. In the non-heat-acclimation samples, SA alone had no significant effect on these heat-inducible Hsfs gene expression. However, application of SA plus heat acclimation significantly potentiated the expression of HsfA2, to 330% of that under
37°C, which was far higher than the values for HsfB1, HsfB2a, HsfA7a, HsfB2b, HsfA3 (183%, 209%, 210%, 161%, and 150% of the level under HS, respectively; Fig. 4A). In the hsfA2 mutant, there was reduced HsfA2 mRNA level and no obvious difference in the other
heat-inducible Hsf mRNA levels under 37°C compared with those in wild-type. Exogenous pre-treatment with 100 µM SA failed to increase the expression of HsfA2 gene (Fig. 4B).

**SA Exerts HsfA2 Expression and Subsequent Thermotolerance through the ROS-dependent Signaling Pathway**

To further confirm the interaction between SA and HsfA2 in plant acquired thermotolerance, the effect of SA on the expression of HsfA2 gene between wild-type and sid2 mutant was examined. 2.5-week-old plants were pre-treated with SA, and subjected to 37°C treatment for 1 h followed by different time recovery, and then exposed to HS stress at 44°C for 45 min. Under control conditions (24°C), the level of HsfA2 mRNA in the sid2 mutant was about 55% lower than that of wild-type (Fig. 5A). Upon HS treatment, the HsfA2 level increased initially and then decreased gradually in the wild-type, and it had a pronounced increase after 50 min recovery, approximately 210% higher than the control (Fig. 5A: Recovery time 1 h versus control). In the sid2 mutant, the peak time occurred at a 60-min heat stress without SA treatment (Fig. 5A: Recovery time 0 h versus control), but was still lower than that of the wild-type. However, in the presence of SA, the dramatic rise in the expression of HsfA2 was detected, with the value to 202% of that in the wild-type at the peak time (Fig. 5A: WT, Recovery time 1 h after HS + SA versus Recovery time 1 h after HS). In the sid2 mutant, the value of HsfA2 mRNA was near the wild-type level after SA + HS treatment (Fig. 5A: sid2, SA + HS versus WT, HS). The results in the vtc2 mutant were similar to those in wild-type. After 60 min recovery, the value of HsfA2 mRNA under SA + HS was about 280% higher than that under HS alone. Conversely, in the sail mutant, SA treatment had no effect on the expression of HsfA2 gene under HS condition.

Next, the interaction of H2O2 and HsfA2 in SA-induced acquired thermotolerance was evaluated. As speculated, the level of HsfA2 was increased in response to H2O2 in a concentration-dependent manner in vitro (Fig. 5B). HsfA2 was activated at concentrations of H2O2 as low as 0.1 mM, and significantly enhanced as H2O2 dose increased. At 5 mM, H2O2 clearly promoted HsfA2 expression compared with the control. However, pre-treatment with AsA reduced the SA-induced HsfA2 expression under HS (Fig. 5C).

To elucidate further details of the above process, the effect of SA on the survival of wild-type and hsfA2 was compared. After 2 d of recovery, the survival of wild-type was 35% higher than that of the hsfA2 mutant. The application of SA increased the survival ratio of wild-type by...
about 21%, but had no effect on the hsfA2 mutant. SA treatment increased the survival of vtc2 mutant by 30% approximately. In the sail mutant, no effect on the survival under SA + HS treatment was detected compared with that under HS treatment (Fig. 5D), which was similar to
the expression of HsfA2 gene in this mutant.

Similarly, the effect of H$_2$O$_2$ on the survival of wild-type and hsfA2 was obtained. H$_2$O$_2$ (1.5 mM) increased the survival ratio of wild-type and sid2 by 19% and 25%, respectively, whereas no significant effect on the hsfA2 mutant was observed (Fig. 5E).

**Lack of HsfA2 Alleviates SA-induced Acquired Thermotolerance**

The hypocotyl elongation assay, which also reflected acquired thermotolerance (Charng et al., 2007), was carried out. Without severe HS (44°C for 45 min), the level of tolerance of hsfA2 seedlings had no significant difference with the wild-type regardless of SA pretreatment (Fig. 6, A and D). After severe HS (44°C for 45 min), the hsfA2 seedlings showed a significant decrease in hypocotyl elongation compared with wild-type without SA pretreatment. The sid2 and sai1 mutants also exhibited an obvious decrease hypocotyl length. The vtc2 mutant showed no significant alteration compared with wild-type. However, the application of SA potentiated the ratio of hypocotyl growth of wild-type, sid2 and vtc2 by about 35%, 50%, and 26%, respectively, after challenge at 48 h recovery. Conversely, SA treatment failed to enhance the ratio of hypocotyl growth in hsfA2 and sai1 seedlings (Fig. 6, B and D).

The enhanced tolerance to HS was exhibited in the wild-type, sid2 and vtc2 mutants after H$_2$O$_2$ treatment. While in the hsfA2 mutant, H$_2$O$_2$ treatment failed to increase the hypocotyl elongation and enhance HS tolerance (Fig. 6C). These results indicate that H$_2$O$_2$ accumulation is not influenced by the lacking mutation of HsfA2, which contributes to the SA-induced plants HS tolerance.

**Disruption of HsfA2 Lowered Expression Levels of HSP genes in SA-induced Thermotolerance**

HSPs expression has been known to be an important component of acquired thermotolerance (Charng et al., 2007; Banti et al., 2010). We examined the effects of SA and AtHsfA2 on the transcriptional regulation of HSPs under HS. *AtHsp18.1-CI* and *AtHsp25.3* were chosen as marker genes. As shown in Figure 7A, levels of *AtHsp18.1-CI* mRNA in the hsfA2, sid2, and sai1 mutants were reduced to 45%, 36% and 43% of levels in the wild-type, respectively. Exogenous 1.5 mM H$_2$O$_2$ pre-treatment stimulated *AtHsp18.1-CI* transcriptional level in the sid2 mutant, whereas no clear effect on the hsfA2 mutant was observed. Pre-treatment of wild-type with AsA significantly decreased *AtHsp18.1-CI* level compared with the wild-type. However,
as for an AsA-deficient mutant vtc2, the level of *AtHsp18.1-CI* mRNA was not markedly affected compared with that in wild-type.

Similar results were obtained for the expression of *AtHsp25.3* in the mutant. After HS
AtHsp25.3 mRNA level in the hsfA2, sid2, and sail mutants were significantly lower than that in the wild-type (by 66%, 58%, and 62%, respectively). The sid2 mutant pre-treated with \( \text{H}_2\text{O}_2 \) showed restored \( \text{AtHsp25.3} \) expression, however, there was no obvious difference in the Hsp25.3 expression level.
Subcellular Localization of ROS Accumulation Induced by SA

As shown in Figure 3, application of SA enhanced the relative fluorescence intensity of DCF. To verify which cellular compartments underwent increases in fluorescence intensity, intracellular ROS production and localization were monitored by LCSM at the single-cell level in vivo, through double staining with the mitochondria-specific marker MitoTracker Red CMXRos and the ROS probe H$_2$DCFDA. As shown in Figure 8A, the wild-type protoplasts double-stained with DCF and MitoTracker Red CMXRos showed obvious fluorescent overlap that colocalized in the cytoplasmic areas in which mitochondria were present at 0.5 h. By 1.5 h of SA treatment, a strong increase was found which was co-localized not only with mitochondria but also with chloroplasts in the protoplasts. By contrast, during the whole assessment time, no detectable DCF signal was observed in protoplasts without SA treatment. In addition, Pretreatment of AsA dramatically depleted the increase in fluorescence from the organelles, and the chloroplasts were more sensitive to AsA than mitochondria. In sail mutant protoplasts, the application of SA had no effect on the increase in fluorescence (Fig. 8B), which is in agreement with the result in Figure 3C. However, in hsfA2 mutant, a strong increase in DCF fluorescence co-localized with mitochondrial signal during the time, exhibiting no significant change in fluorescence compared with that of wild-type (Fig. 8C).

To clarify the results quantitatively, the kinetics of H$_2$O$_2$ accumulation was detected in isolation mitochondria (Supplemental Fig. S4). At about 15 min after SA treatment, DCF fluorescent intensity began to increase and was obviously boosted after 60 min. During 90 min, the level of ROS kept a slight increase.

Changes of Mitochondrial Respiratory Complex Activity under SA Treatment

Time course experiments established that mitochondria might be the main regions involved in SA-induced ROS production (Fig. 8), possibly mitochondrial components that are important for electron transport were implicated in this process. To test this hypothesis, SA-dependent H$_2$O$_2$ production in isolated mitochondria was assayed in the presence of inhibitors of the electron transport chain (ETC). ROS generation is determined in the presence of complex I (malate + glutamate) or II (succinate) substrates, which acts to feed electrons into the ETC at the level of
complex I via NADH or directly to complex II, respectively. As shown in Figure 9 A and B, mitochondria stimulated by malate/glutamate or succinate under SA produced a significant increase in H₂O₂ generation, whereas exogenous AsA almost completely attenuated this process.
Rotenone and antimycin A, a specific inhibitor of complex I and III, respectively, produced a significant rate of H$_2$O$_2$ generation without the addition of SA. However, SA co-administration with antimycin A showed rates similar to those observed with antimycin A alone. Further, the decreases in rates of H$_2$O$_2$ production were not observed with complex I, II and IV inhibitors under SA treatment. Rotenone also decreased H$_2$O$_2$ generation in mitochondria via succinate, but to a lesser extent than antimycin A (Fig. 9B). These data indicate that complex III is
centrally involved in $\text{H}_2\text{O}_2$ generation by SA.

To confirm the role of complex III in modulating intracellular ROS generation, the activity of complex III in mitochondrial ETC was determined under SA treatment. Results showed that complex III activity exhibited a time- and concentration-dependent decrease (Fig. 9C). As early as 10 min after SA treatment, the activity of complex III obviously began to decrease, which is prior to the generation of mitochondrial ROS (Figs. 8, 9C, and Supplemental Fig. S4). Moreover, pretreatment of AsA failed to inhibit the decline in the activity of complex III (Fig. 9D), indicating that complex III might be as a direct target for SA treatment.
DISCUSSION

In this work, we provided evidence for the role of ROS in SA-mediated plant acquired thermotolerance. SA, induced by HS, markedly enhanced the expression of HsfA2 and HSP genes in Arabidopsis; thus, it contributed positively to acquired thermotolerance. In this process, ROS functioned as an essential intermediate.

SA and Acquired Thermotolerance in Arabidopsis

SA was produced in response to a variety of environmental stresses in plants, including HS (Dat et al., 1998a). Mutations disrupting SA signaling decrease the ability of plants to acquired thermotolerance (Larkindale et al., 2005). Thus, we examined the effects of HS on endogenous SA content between wild-type and sid2, which shows impaired SA production (Wildermuth et al., 2002). As expected, the endogenous concentrations of free SA and conjugated SA in wild-type were about ninefold and 11-fold, respectively, those in the sid2 mutant under the normal and HS conditions (Fig.1A and Supplemental Fig. S5). The reduced survival of the sid2 mutant indicated its sensitivity to HS. The SA level and survival of NahG mutant varied in a similar manner as those of sid2 plants (Supplemental Fig. S1). Therefore, the reduced SA level and survival ratios synchronously in the sid2 and NahG mutants compared with those of wild-type, suggesting the function of SA on acquired thermotolerance.

The Signaling Pathways for SA-Mediated Plant Acquired Thermotolerance

An essential component of acquired thermotolerance in plants is the induction of Hsfs, which is a conserved protein present and activates HSR in eukaryotic organisms (Nover et al., 2001; Banti et al., 2010; Lin and Chang, 2013). Busch et al. (2005) and Larkindale and Vierling (2008) reported that the heat-inducible class A Hsfs, namely HsfA2, HsfA7a, and HsfA3, and B-type Hsfs (HsfB1, HsfB2a, and HsfB2b) are up-regulated by heat. To test which one or even more heat-inducible Hsfs could involve(s) in acclimation to acquired thermotolerance, the expression of these heat-inducible Hsfs under SA treatment was examined. SA significantly potentiated the expression of HsfA2 (about 3.3 times) under heat; other heat-inducible Hsfs were not significantly responsive to SA and HS. In the hsfA2 mutant, the expression of HsfA7a, HsfA3, HsfB1, HsfB2a, and HsfB2b was revealed no substantial alteration. Further, the ratio of hypocotyl growth in hsfA2 mutant declined faster compared with wild-type; whereas there was no substantial change in
hypocotyl growth in other heat-inducible Hsfs (Supplemental Fig. S6). The loss of HsfA2 in mutant apparently could not be compensated by the presence of other heat-inducible Hsfs. Because the structures of HsfA2 and other Hsfs are different, they may not be functionally redundant under HS treatment (Nover et al., 2001), and the dominant role for this gene (HsfA2) in acquired thermotolerance may be another possibility (Baniwal et al., 2004).

Subsequently, we are aware that the application of SA improved the survival ratios of wild-type and sid2 mutant, whereas no obvious effect was observed in the hsfA2 mutant. Similarly, SA treatment also failed to obviously enhance the ratio of hypocotyl growth in hsfA2 seedlings, although SA induced H$_2$O$_2$ accumulation in this mutant (Fig. 8C; Supplemental Fig. S7). A plausible explanation for these results is that HsfA2 is a key component of the SA pathway in response to HS, and therefore SA treatment, an upstream molecule, had no effect on the heat-sensitive status of the hsfA2 mutant due to the loss of the downstream element HsfA2 in Arabidopsis.

To prove this supposition, the effect of SA on HsfA2 transcription under HS was investigated. The HsfA2 mRNA level was up-regulated by SA in wild-type under HS. This trend was strongly inhibited in the sid2 seedlings, but was successfully increased by treatment with SA (Fig. 5), confirming that HsfA2 acts downstream of SA in response to HS.

Hsfs, as direct sensors of H$_2$O$_2$ in cells, play important roles in the ROS signaling network (Zhong et al., 1998; Panchuk et al., 2002; Ahn and Thiele, 2003). To determine the mechanism of the effect of H$_2$O$_2$ on AtHsfA2 on SA-mediated acquired thermotolerance, the effects of H$_2$O$_2$ on the expression of HsfA2 and subsequent thermotolerance were examined. The following results support this conclusion: (a) SA-induced the transcriptional induction of HsfA2 gene was preceded by accumulation of H$_2$O$_2$ (Figs. 3 and 5), indicating that H$_2$O$_2$ is closely associated with the induction of HsfA2 (Miller and Mittler, 2006). (b) HsfA2 mRNA expression increased as the H$_2$O$_2$ level increased, and the application of AsA reduced SA-induced HsfA2 expression (Fig. 5). (c) The application of H$_2$O$_2$ improved the survival ratios of sid2 mutant in response to HS (Fig. 2), which also indicates the effect of H$_2$O$_2$ on acquired thermotolerance in the absent of SA. (d) In AsA-pretreated wild-type plants, the reduced survival was observed after HS (Fig. 2). In addition, in hsfA2 seedlings, H$_2$O$_2$ treatment did not markedly increase the survival and hypocotyl elongation, which is in a similar manner as SA treatment (Figs. 5 and 6), indicating that HsfA2 acts
downstream of H$_2$O$_2$ in SA-induced HS signaling and accordingly, H$_2$O$_2$ accumulation is not
influenced by the lacking mutation of HsfA2 (Supplemental Fig. S7).

Downstream components of the HS signaling known as Hsfs contribute to thermotolerance by
controlling HSPs through Hsfs binding to HSEs (Baniwal et al., 2004; Snyman and Cronjé, 2008).
These HSPs are classed as Hsp60, Hsp70/Hsc70, Hsp90, Hsp100 and small Hsps according to
their molecular masses. The small HSPs, as an important type, play essential roles in survival
and development (Zhang et al., 2009). In this study, Hsp18.1-CI (Lohmann et al., 2004) and
Hsp25.3 (Osteryoung et al., 1993) were chosen to demonstrate how SA and H$_2$O$_2$ induce
thermotolerance via HsfA2. Lack of HsfA2 in mutant caused substantially reduced expression of
tested HSP genes. Under HS conditions, application of H$_2$O$_2$ restored expression of $Hsp18.1$-$CI$
and $Hsp25.3$ genes in $sid2$ mutant, but not in the $hsfA2$ mutant, which resulted in the lack of
thermotolerance in H$_2$O$_2$-pretreated $hsfA2$ mutant (Figs. 2, 5-7). As for the other heat-inducible
Hsfs, the levels of $AtHsp18.1$-$CI$ and $AtHsp25.3$ mRNA were not markedly affected compared
with these in wild-type (Supplemental Fig. S8), which was in agreement with the manifestation in
hypocotyl growth (Supplemental Fig. S6). Collectively, the mechanism through which SA
influences acquired thermotolerance via H$_2$O$_2$ involves the expression of HsfA2 and HSPs and
establishes acquired thermotolerance.

The function of Mitochondria in SA-induced H$_2$O$_2$ in Arabidopsis

SA and ROS are essential signaling molecules involved in physiological response. Although,
in tobacco, SA can inhibit respiration in the mitochondria (Xie and Chen, 1999), an effect that
might increase ROS production within this organelle, it has not yet been elucidated how SA
affects mitochondrial functions. On one hand, our observation showed that SA induced the
accumulation of ROS using the Arabidopsis protoplasts, and this effect was dose dependent (Fig. 3;
Supplemental Fig. S3). However, this is different from the observation that the change of H$_2$O$_2$
accumulation was not substantially influenced in the $hsfA2$ mutant compared with wild-type (Fig.
8C; Supplemental Fig. S7), for the reason that HsfA2 is a key component of the H$_2$O$_2$ pathway and
acts downstream of H$_2$O$_2$ in SA-induced signal transduction.

On the other hand, mitochondria are major sources of ROS (Pantarulo et al., 1988) and
participate in the ROS burst in plants (Braidot et al., 1999; Xie and Chen, 1999; Clarke et al.,
2000). On the basis of protoplasts double-stained with DCF and MitoTracker Red CMXRos, the
results showed obvious fluorescent overlap that colocalized in the cytoplasmic areas in which mitochondria were present (Fig. 8). The similar results were also obtained in other heat-inducible hsfA3, hsfA7a mutants (Supplemental Fig. S9), hsfB1, hsfB2a, and hsfB2b mutants (Supplemental Fig. S10). This phenomenon pointed to the question how the production of mtROS occurred under SA treatment. Previous study has showed that the mitochondrial electron transport system generates oxygen radicals through electron leaks as substrates are metabolized (Green and Reed, 1998), and Complex I and III are recognized as the major sites for ROS generation (Murphy, 2009). SA caused mitochondrial dysfunction, which mimicked the specific inhibition of mitochondrial electron transport caused by antimycin A (Maxwell et al., 2002). The most conspicuous effect is the rapid inhibition of electron flow and respiratory rate under SA treatment (Xie and Chen, 1999; Norman et al., 2004; Raha and Robinson, 2000). So it is of be interest to identify the target of SA action among the mitochondrial complexes. Our results pinpointed cytochrome c reductase (complex III) possible as the main target for SA in the mitochondrial ETC. Under normal conditions, the respiratory I or II substrates can feed electrons directly and flow into the ubiquinone cycle and transfer them to complex III (Rhoads et al., 2006). Under SA treatment, the activity of complex III was affected by SA in a time- and concentration-dependent manner, which could not be alleviated by eliminating ROS (Fig. 9). Therefore, the mtROS are generated through electron leaks, depending on inhibition of specific sites in the ETC or the reduction state of the ETC components, as substrates are metabolized (Møller, 2001), indicating that SA might act directly on the complex.

Taken together, the existence of a signaling pathway was tested in which ROS production was stimulated by SA to regulate the HsfA2 and HSPs genes transcription expression so as to influence acquired thermotolerance. Time course experiments and the generation of H2O2 in the mitochondria provide a possible explanation for the function and role of mitochondria in SA signaling pathway. Our study contributed to a more detailed understanding of the mitochondria-dependent mechanism of SA-induced biological response in acquired thermotolerance network.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**
Plants of Arabidopsis wild-type (ecotype Col-0), *vtc2* (SALK_076245), *hsfA3* (SAIL_661_B06), *hsfB2a* (SALK_012418), *sid2* (N16438, At1g74710) and *sai1* (SAIL-708_F09) were purchased from NASC. Seeds of the Arabidopsis T-DNA insertion (*hsfA2*, At2g26150), *hsfA7a* (SALK_080138), *hsfB1* (SALK_012292), and *hsfB2b* (SALK_047291) were provided by Dr Yee-yung Charng (Charng et al., 2007). All the seeds were sterilized and sown on Murashige and Skoog (MS) medium containing 0.8% agar and 1% sucrose in petri dishes, and allowed to stratify for 3 d at 4°C. Petri dishes were placed in a growth chamber (Conviron, model E7/2, Canada) with a 16 h lighting photoperiod (100 μmol photons m⁻² s⁻¹) and 82% relative humidity at 22-25°C.

2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) and MitoTracker Red CMXRos were obtained from Molecular Probes (Eugene, OR, USA). SA, ascorbic acid (AsA), rotenone (5 μM), TTFA (4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione; 10 μM), antimycin A (5 μM) and potassium cyanide (1 mM) were purchased from Sigma-Aldrich China (Shanghai, China).

**Heat Stress Treatments**

For seedling survival ratio assay, 10-d-old seedlings were exposed to a 45 min 44°C HS with a pre-treatment. The pretreatment consisted of 60 min at 37°C followed by a 2-d recovery period at 24°C before the HS. Following the 44°C HS, these plants were returned to 24°C. The surviving plants were counted or photographed for the indicated time.

For the necrosis measurements, 2.5-week-old plants were treated with 37°C pre-condition for 60 min, incubation at 24°C for 2 d, and then exposed to HS at 44°C for 45 min. After 6 days, all rosette leaves from each plant were detached and photographed. The percentage of total plant leaf area showing necrosis was measured by analysis of these images using PC_IMAGE software.

For the hypocotyls elongation assay, the vernalized seeds were surface sterilized and sown in rows on MS medium, and then placed vertically in dark for 3.5 d at 24°C covered with foil, during which time the plants were heated in a water bath at 37°C for 1 h followed by various times at 24°C and exposed to 44°C for 45 min, after which the hypocotyl positions were labeled. After an additional 2.5 d at 24°C in the dark, the hypocotyl lengths were measured as described by Charng et al. (2006).

**SA or H₂O₂ Application**
For the Arabidopsis seedlings treatment, 1 mL of SA or H$_2$O$_2$ solution at a final concentration of 0.1 mM or 1.5 mM, respectively, were sprayed onto the leaf surfaces of 10-d-old wild-type and different mutants (sid2, hsfA2, vtc2, and sai1) grown at 24°C on MS-agar medium after filter sterilization. The control seedlings were treated with water. After 1 h of pre-treatment, the seedlings were exposed to heat stress treatment, and analyzed the seedlings survival ratio.

For the Arabidopsis plants treatment, 2.5-week-old plants were sprayed with SA solution or H$_2$O$_2$ at the final concentration of 0.1 mM or the different concentrations (0-5 mM), respectively. The control plants were treated with water. After 1 h of pre-treatment, the plants were exposed to heat stress treatment (For SA treatment). After which, the total RNA was extracted from leaf tissue, and the heat-inducible genes were analyzed by real-time RT-PCR.

**Extraction and Quantification of SA**

Plants were grown as described above and incubated at 24°C for a recovery time following a 60 min pre-treatment at 37°C. Plant leaves were dissected and crumbled. Samples each 200 mg were further homogenized using liquid nitrogen and transferred to a 1.5 mL Eppendorf tube. An aliquot (1 mL) of 90 % methanol was added to the homogenate, soaked for 20 min, and then centrifuged for 3 min at 4,000 g. The supernatant was collected in a 2 mL Eppendorf tube. The pellet was re-suspended in 1 mL 100% methanol, and the centrifugation steps repeated. The supernatants were combined and centrifuged again, and the methanol:water mixtures were evaporated in a SpeedVac concentrator at a high drying speed. After this step, the endogenous free salicylic acid fraction was partitioned again with 1 mL acetic acid: sodium acetate buffer (pH 5.5) and the samples were sonicated for 15 min. The free SA was quantified by spectrofluorescence monitoring at 412 nm emission based on previously described (Meuwly and Metraux, 1993). All data were corrected for recovery of the internal standard salicylic acid, and the free SA was measured.

**Isolation and Treatment of Arabidopsis Mesophyll Protoplasts**

The isolation of protoplasts from 14-day-old plant leaves was carried out at room temperature according to a modified procedure as described by He et al. (2006). The brief procedures are as follows: healthy leaves from 15 to 20 were sliced into small leaf strips (0.5–1 mm), which were vacuum-infiltrated with enzyme solution [1–1.5% (w/v) Cellulase R10 (Yakult Honsha, Tokyo, Japan), 0.2–0.4% (w/v) Macerozyme R10 (Yakult Honsha), 0.4 M mannitol, 20 mM MES pH 5.7,
20 mM KCl, 10 mM CaCl₂] for 10–20 min, and then incubated in the dark at room temperature for 3 h. Protoplasts were isolated by filtration through 75 μm nylon mesh sieves and collected by centrifugation at 100 g for 3 min. Finally, the purified protoplasts were washed three times in W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 5 mM Glucose, and 1.5 mM MES-KOH, pH 5.6) and the concentration adjusted to between 10⁵ and 10⁶ protoplasts mL⁻¹ with additional W5 solution.

For the Arabidopsis protoplasts treatment, SA dissolved in water at the indicated concentration was added to 100 μL of protoplast suspension in 96-well plates and incubated for the indicated time at room temperature.

**Laser Confocal Scanning Microscopy (LCSM)**

All microscopic observations were performed using a Zeiss LSM 510 laser confocal scanning microscope (LSM 510/ConfoCor 2, Carl-Zeiss, Jena, Germany). H₂DCFDA signals were visualized with excitation at 488 nm and emission at 500–550 nm, and the chloroplast autofluorescence (excitation at 488 nm) was visualized at 650 nm. MitoTracker Red CMXRos signals were visualized in another detection channel with a 543 nm excitation light and a 565–615 nm bandpass filter. All images were taken with the 20 × water and 40 × oil-immersion objectives on the Zeiss LSM 510 and analyzed with Zeiss Rel3.2 image processing software (Zeiss, Germany).

**Detection of ROS Production and Distribution**

After SA treatment, the Arabidopsis protoplasts were incubated with H₂DCFDA at a final concentration of 5 μM. They were double-stained with H₂DCFDA and Mito-Tracker Red CMXRos (100 μM) in dark, as described by Yao and Greenberg (2006). The ROS production and distribution, as well as the chloroplast autofluorescence and the MitoTracker fluorescence, were visualized under the Zeiss LSM 510. In addition, the fluorescence intensity of DCF was also measured with a fluorescence spectrometer (PerkinElmer, LS55, UK) at 24°C (excitation 488 nm, emission 500–600 nm, slit width 5 nm) and the fluorescence intensity at 525 nm was used to determine the relative ROS production.

**Histochemical Staining of H₂O₂ and Quantitation in Leaf Extracts**

The histochemical staining of H₂O₂ was performed as described by Orozco-Cardenas and Ryan (2002). Wild-type, vtc2, sail, NahG and sid2 leaves were vacuum infiltrated with 1 mg
mL⁻¹ DAB solution under 25°C. An Amplex Red Hydrogen Peroxide Assay Kit was used to measure the concentration of H₂O₂ in leaves according to the manufacturer’s recommendations.

**Total RNA Extraction and Real-time qRT-PCR**

Total RNA was extracted from approximately 100 mg of leaf tissue of 2.5-week-old plants frozen in liquid nitrogen with TRIZOL reagent (Invitrogen) according to the manufacturer’s protocol. The heat-inducible genes were analyzed by real-time RT-PCR using RNA isolated from the wild-type or *hsfA2* mutant. Real-time PCR was performed using the LightCycler Quick System 350S (Roche Diagnostics K.K.) with SYBR Premix Ex Taq (Takara Bio, Inc.). Each PCR reaction contained 1 × SYBR Premix Ex Taq, 0.2 μM of each primer, and 2 μL of a 1:10 dilution of the cDNA in a final volume of 20 μL. The PCR programme was used: initial denaturation, 95°C, 10 s; PCR, 40 cycles of 95°C, 5 s, 60°C, 20 s with a temperature transition rate of 20 °C s⁻¹. In melting curve analysis, PCR reactions were denatured at 95°C, annealed at 65 °C, then a monitored release of intercalator from PCR products or primer dimers by an increase to 95 °C with a temperature transition rate of 0.1°C s⁻¹. Standard curves were created using PCR products by 10-fold serial dilutions. The heat-inducible genes expression profiles were normalized using *Actin2* mRNA as an internal control. The sequences of the primers for real-time PCR analysis for each gene are described in Supplemental Table S1.

**Preparation of Mitochondria and Assays of Activity of Respiratory Chain Complex III**

Mitochondria were isolated and purified by differential centrifugation as described by Neuburger et al. (1982) and Tiwari et al. (2002). All steps were carried out at 4°C. Briefly, 4000 mg of leaves were disrupted with a tissue grinder in 10 mL of cold medium (0.5 M Sucrose, 50 mM Tris, 5 mM EDTA, 0.2% (w/v) BSA, 0.3% (v/v) β-Mercaptoethanol, 0.3% (w/v) PVP-40 and 5 mM Cysteine; pH 7.5). The homogenate was filtered through two layers of miracloth (Calbiochem-Behring) using a syringe. The debris was spun down by centrifugation at 105 g for 10 min and the supernatant was further spun at 1600 g for 10 min. Supernatants were then collected and centrifuged at 15000 g for 20 min and the pellets resuspended in approximately 1.5 mL of cold medium. The resuspended pellets were centrifuged at 10500 g for 15 min with three replicates, and suspended by acetone and recentrifuged at 15000 g for 10 min. Finally, the mitochondria fraction was suspended in assay buffer (300 mM sucrose, 2 mM HEPES, 0.1 mM EDTA; pH 7.4). After isolation, the mitochondria were treated with or without SA in the
present or absent of inhibitors of the electron transport chain, and then H$_2$O$_2$ were measured with a fluorescence spectrometer (PerkinElmer, LS55, UK).

The activity of cytochrome reductase was assayed as described by Tiwari et al. (2002). The enzyme activity assay was carried out according to the instruction manual of the Tissue Mitochondrial Complex III Assay Kit (Genmed Scientifics Inc., Arlington, MA). The assay mixture of the complex III contained mitochondrial protein (10 µg), EDTA, potassium phosphate buffer (pH 7.5), EDTA, bovine serum albumin, and substrates (decylubiquinol and oxidized ferricytochrome c). After incubation for 5 min at 30°C, the activity was measured by the increase in absorbance at 550 nm using a molar absorption coefficient of 21.8 mmol$^{-1}$ L cm$^{-1}$ for the reduction of ferricytochrome c. The complex III antimycin A sensitive enzyme activity was expressed as nmol min$^{-1}$ mg$^{-1}$ protein.

**Supplemental Data**

**Supplemental Figure S1.** Effects of HS on the survival ratio between wild-type and NahG mutant.

**Supplemental Figure S2.** Effects of SA on the DCF fluorescence in wild-type and the mutants (vtc2, sai1, NahG, sid2) protoplasts.

**Supplemental Figure S3.** In situ detection of leaf H$_2$O$_2$ in wild-type and different mutants (vtc2, sai1, sid2, and NahG).

**Supplemental Figure S4.** Kinetics graphs of DCF signal intensity of SA-treated isolated mitochondria.

**Supplemental Figure S5.** Effects of HS on the endogenous concentration of conjugated SA in wild-type and sid2 mutant.

**Supplemental Figure S6.** Hypocotyl elongation phenotype of seedlings in wild-type, hsfA3, hsfA7a, hsfB1, hsfB2a, and hsfB2b mutants.

**Supplemental Figure S7.** Time course of H$_2$O$_2$ accumulation in wild-type and hsfA2 mutant in response to HS.

**Supplemental Figure S8.** The expression of Hsp18.1-CI and Hsp25.3 genes in hsfA3, hsfA7a, hsfB1, hsfB2a, and hsfB2b mutants.

**Supplemental Figure S9.** Subcellular localization of SA-induced ROS production in hsfA3 and
Supplemental Figure S10. Subcellular localization of SA-induced ROS production in \textit{hsfB1}, \textit{hsfB2a}, and \textit{hsfB2b} mutants.

Supplemental Table 1. Primers used for real-time qRT-PCR assays.

ACKNOWLEDGEMENTS

We are thankful to Dr. Yee-yung Charng (Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan) for kindly providing us with the \textit{hsfA2} seeds. We also thank Mr. David Chen of the University of Colorado at Boulder for his help in English proofreading the manuscript.


Maxwell DP, Nickels R, McInnes L (2009) Evidence of mitochondrial involvement in the transduction of signals required for the...
induction of genes associated with pathogen attack and senescence. Plant J 29(3): 269-279


Retraction


The above article is being retracted by Plant Physiology, which issues the following statement:

After publication of the article on October 15, 2015, as a Plant Physiology Preview article, we were alerted to the fact that Figure 9 and a considerable amount of text in the Results and Discussion sections were identical (or nearly identical) to material in an article previously published by the same authors in PLOS ONE (Mitochondrial-Derived Reactive Oxygen Species Play a Vital Role in the Salicylic Acid Signaling Pathway in Arabidopsis thaliana; DOI 10.1371/journal.pone.0119853). The authors did not cite the PLOS ONE article in the Plant Physiology article, nor did they indicate that Figure 9 had been duplicated from this earlier publication.

ASPB invoked its ethics in publishing policies to evaluate this instance of self-plagiarism. On the basis of available submission and publication dates of the two articles, ASPB concluded that because the PLOS ONE paper was accepted for publication on January 16, 2015, more than four months before the Plant Physiology article was submitted on May 26, 2015, it is necessary to retract the Plant Physiology article in its entirety.
Retraction


The above article is being retracted by *Plant Physiology*, which issues the following statement:

After publication of the article on October 15, 2015, as a *Plant Physiology Preview* article, we were alerted to the fact that Figure 9 and a considerable amount of text in the Results and Discussion sections were identical (or nearly identical) to material in an article previously published by the same authors in *PLOS ONE* (Mitochondrial-Derived Reactive Oxygen Species Play a Vital Role in the Salicylic Acid Signaling Pathway in *Arabidopsis thaliana*; DOI 10.1371/journal.pone.0119853). The authors did not cite the *PLOS ONE* article in the *Plant Physiology* article, nor did they indicate that Figure 9 had been duplicated from this earlier publication.

ASPB invoked its ethics in publishing policies to evaluate this instance of self-plagiarism. On the basis of available submission and publication dates of the two articles, ASPB concluded that because the *PLOS ONE* paper was accepted for publication on January 16, 2015, more than four months before the *Plant Physiology* article was submitted on May 26, 2015, it is necessary to retract the *Plant Physiology* article in its entirety.
RETRACTION

Plant Physiology Preview, 2015


The above article is being retracted by Plant Physiology, which issues the following statement:

After publication of the article on October 15, 2015, as a Plant Physiology Preview article, we were alerted to the fact that Figure 9 and a considerable amount of text in the “Results” and “Discussion” sections were identical (or nearly identical) to material in an article previously published by the same authors in PLoS ONE (Mitochondrial-Derived Reactive Oxygen Species Play a Vital Role in the Salicylic Acid Signaling Pathway in Arabidopsis thaliana; http://dx.doi.org/10.1371/journal.pone.0119853). The authors did not cite the PLoS ONE article in the Plant Physiology article, nor did they indicate that Figure 9 had been duplicated from this earlier publication.

The American Society of Plant Biologists (ASPB) invoked its ethics in publishing policies to evaluate this instance of self-plagiarism. On the basis of available submission and publication dates for the two articles, ASPB concluded that because the PLoS ONE paper was accepted for publication on January 16, 2015, more than 4 months before the Plant Physiology article was submitted on May 26, 2015, it was necessary to retract the Plant Physiology article in its entirety.