- 1 **Running title:** Role of ROS in SA-induced acquired thermotolerance
- 2 **Corresponding author:**
- 3 Da Xing
- 4 MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, College of
- 5 Biophotonics, South China Normal University, Guangzhou 510631, China
- 6 Telephone: +86-20-8521-0089
- 7 Fax: +86-20-8521-6052
- 8 Email: xingda@scnu.edu.cn
- 9 **Research area most appropriate for the paper:** Signaling and Response

10 **A Potential Role for Mitochondrial Produced Reactive Oxygen**
11 **Species in Salicylic Acid-Mediated Plant Acquired**
12 **Thermotolerance**

13 Shengjun Nie, Haiyun Yue and Da Xing*
14 MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, College of
15 Biophotonics, South China Normal University, Guangzhou 510631, China

16 **One-sentence Summary**

17 SA functions and acts upstream of AtHsfA2 in acquired thermotolerance, which requires a
18 pathway with H₂O₂ production involved and is dependent on increased expression of *Hsp* genes.

19 **Footnotes**

20 This research is supported by the Program for Changjiang Scholars and Innovative Research Team
21 in University (IRT0829), the Key Program of NSFC-Guangdong Joint Funds of China (U0931005)
22 and the National High Technology Research and Development Program of China (863 Program)
23 (2007AA10Z204).

24 *Corresponding author: Da Xing

25 Email: xingda@scnu.edu.cn

27 **ABSTRACT**

28 To characterize the function of salicylic acid (SA) in acquired thermotolerance, the effects of heat
29 shock (HS) on wild-type and *sid2* (for SA induction deficient 2) was investigated. After HS
30 treatment, the survival ratio of *sid2* mutant was lower than that of wild-type. However,
31 pretreatment with hydrogen peroxide (H₂O₂) rescued the *sid2* heat sensitivity. HsfA2 is a key
32 component of acquired thermotolerance in Arabidopsis. The expression of *HsfA2* induced by SA
33 was highest among those of heat-inducible Hsfs (HsfA2, HsfA7a, HsfA3, HsfB1, and HsfB2) in
34 response to HS. Furthermore, the application of AsA, an H₂O₂ scavenger, significantly reduced
35 the expression level of *HsfA2* induced by SA. Although SA enhanced the survival of *sid2* mutant,
36 no significant effect on the *hsfA2* mutant was observed, suggesting that HsfA2 is responsible for
37 SA-induced acquired thermotolerance as a downstream factor. Further, real-time PCR analysis
38 revealed that after HS treatment, SA also up-regulated mRNA transcription of HS protein (*Hsp*)
39 genes through AtHsfA2. Time course experiments showed an increase in the fluorescence
40 intensity of DCF in the mitochondria occurred earlier than in other regions of the protoplasts in
41 response to SA. The cytochrome reductase activity analysis in isolated mitochondria
42 demonstrated that SA-induced mitochondrial ROS possibly originated from complex III in the
43 respiration chain. Collectively, our data suggest that SA functions and acts upstream of AtHsfA2
44 in acquired thermotolerance, which requires a pathway with H₂O₂ production involved and is
45 dependent on increased expression of *Hsp* genes.

47 **INTRODUCTION**

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

60 As a countermeasure to heat shock (HS), plants synthesize a set of highly conserved proteins
 61 known as heat shock proteins (HSPs), and expression of HSPs is governed by heat shock factors
 62 (Hsfs), which are transcription factors, binding to heat shock promoter elements (HSEs) in the
 63 promoter region of the *HSPs* genes (Nover et al., 2001; Akerfelt et al., 2010; Liu and Charng,
 64 2013). In the eukaryotic organisms, the number of Hsf genes differs greatly. The mammalian
 65 contains three Hsf isoforms, Hsf1, Hsf2, and Hsf4, with a distinct biological function (Fujimoto
 66 et al., 2004; Xing et al., 2005). *Drosophila melanogaster* and *Caenorhabditis elegans* each has
 67 a single Hsf gene playing an essential biological role in HSR (Nover et al., 1996; Hsu et al.,
 68 2003). However, in Arabidopsis, 21 Hsfs have been sorted into three classes (classes A, B, and
 69 C) according to their unique structural characteristics (Nover et al., 2001; Kotak et al., 2004).
 70 Interestingly, of the 21 Hsfs, the heat-inducible class A Hsfs, *HsfA2*, *HsfA7a*, and *HsfA3*, and
 71 class B Hsfs, *HsfB1*, *HsfB2a*, and *HsfB2b*, play an essential role in acquired thermotolerance
 72 (Busch et al., 2005; Charng et al., 2007; Larkindale and Vierling, 2008; Schramm et al., 2008;
 73 Meiri and Breiman, 2009). The heat ability of these Hsfs is a feature unique to plants, but is not
 74 found in animals and yeast (Larkindale and Vierling, 2008; Schramm et al., 2008). In the early

75 response to HS, HsfA1s are activators of genes for HSPs and promote the activities of other
76 HS-inducible Hsfs (Lohmann et al., 2004; Busch et al., 2005; Liu et al., 2011). Once these
77 HS-inducible Hsfs are induced, HsfA2 becomes the dominant Hsf and mediates the amplification
78 of a subset of HSR genes (Busch et al., 2005; Schramm et al., 2006; Liu and Charng, 2013). A
79 recent study by Charng et al. (2007) suggests that only the *hsfA2* mutant showed an obvious
80 phenotype among the tested mutants corresponding to 48 heat-induced genes. Following heat
81 acclimation at 37°C, the *hsfA2* mutant was more sensitive to severe heat stress than wild-type
82 after long recovery periods. The presence of HsfA2 exclusively in acquired thermotolerance
83 suggests its importance in recovery after HS (Baniwal et al., 2004; Charng et al., 2007).
84 However, whether these heat-inducible Hsfs function in SA-mediated plant acquired
85 thermotolerance has not been elucidated, if so, which one or even more could be involved in
86 acclimation to acquired thermotolerance.

87 Reactive oxygen species (ROS) are generated in response to diverse stresses. Hydrogen
88 peroxide (H₂O₂), a major and the most stable type of ROS, acts as a central signaling molecular
89 to regulate many processes, such as stomatal movement, plant-pathogen interactions and
90 different response to abiotic stress (Levine et al., 1994; Alvarez et al., 1998; Bais et al., 2003;
91 Apel and Hirt, 2004; Rhee, 2006). Several groups have reported the connection between plant
92 Hsfs and oxidative stress (Li et al., 2005; Miller and Mittler, 2006; Zhang et al., 2009). The
93 increased level of H₂O₂ in response to HS resulted in Hsfs induction and HSPs accumulation
94 (Zhong et al., 1998; Ahn and Thiele, 2003; Volkov et al., 2006; Banti et al., 2010). Conversely,
95 peroxide scavengers and inhibitors of H₂O₂ can reduce the expression of these HSPs (Volkov et
96 al., 2006). Panchuk et al. (2002) studied the transgenic plants over-expressing HsfA1b and
97 reported that Hsf is implicated in the induction of ascorbate peroxide (*Apx2*), which encodes the
98 H₂O₂ scavenging enzyme. The study by Schramm et al. (2006) reveals that the induction of
99 *Apx2* is totally abolished in the absence of HsfA2. Furthermore, disruption of HsfA2 displayed
100 reduced obtained oxidative stress tolerance, while transgenic plants over-expressing *HsfA2*
101 increased tolerance (Ogawa et al., 2007). Under anoxia, *HsfA2* is also strongly up-regulated by
102 H₂O₂ (Banti et al., 2010). These results confirm the involvement of H₂O₂ in HS signaling and
103 the role of plant Hsfs in the ROS signaling network.

104 This study was to elucidate the relationship among SA, an intermediate H₂O₂ and plant Hsfs in

105 acquired thermotolerance using the wild-type and *sid2* mutant, and to assess the behavior and
106 function of mitochondria in response to SA. The results presented demonstrate that SA-induced
107 mitochondrial H₂O₂ generation is implicated in the acquired thermotolerance, through induction
108 transcripts of *HsfA2* and *HSPs* genes. This work may contribute to the understanding of the
109 mitochondria-dependent mechanism of the SA-induced biological responses in plants, and
110 provide a new insight into the cellular signaling cascade in SA-mediated thermotolerance
111 response.

112

113 **RESULTS**

114 **SA Promotes Acquired Thermotolerance in Arabidopsis Plants**

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

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

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

135 **SA-induced Plant Acquired Thermotolerance Is Related with H₂O₂ Signaling**

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

140 Ascorbic acid (AsA), as an important reducing substrate, could remove H₂O₂ (Noctor and Foyer,
141 1998). Treatment of wild-type plants with AsA (1 mM) showed the reduced survival ratio. *vtc2*

Figure 1

Figure 1. Effects of HS on the endogenous level of free SA and survival ratio in wild-type and *sid2* mutant. A, Endogenous free SA level in aerial parts of wild-type and *sid2* mutant subjected to 37°C for 1 h and then recovered for the indicated time. Different letters indicate statistically significant differences among the samples ($P < 0.05$; Duncan's multiple range tests). B, Survival ratio of *sid2* mutant to HS. The seedlings grown at 24°C were subjected 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 at 1, 3 and 5 d later. C, Comparison of survival ratios between wild-type and *sid2* mutant in (B). Values are means of 100 plants. D, The necrosis on wild-type and *sid2* plant leaves. The necrotic lesions on WT and *sid2* plants after 37 °C pre-condition for 1 h, incubation at 24 °C for 2 d, and then exposed to HS at 44°C for 45 min and photographed 6 days later. Necrotic lesions were measured as percentage of total leaf area of each plant. Scale bars, 1 cm. E, Comparison of the necrotic lesions between wild-type and *sid2* mutant. Asterisk indicates significant differences to wild-type under HS (Student's t-test, $P < 0.05$). Data are means $\pm$ SE of at least three samples, each consisting of 15 plants.

142 exhibited a small (approximately 9%) decrease compared with wild-type, as an AsA-deficient
 143 mutant of Arabidopsis (for 10% to 30% of wild-type levels of AsA; Jander et al., 2002;
 144 Müller-Moulé et al., 2003). However, *sai1*, a SA-insensitive mutant (Shah et al., 1997), showed

Figure 2

Figure 2. Effect of H₂O₂ 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 H₂O₂, 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 of 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 in **A**. **C**, Necrotic lesions in wild-type and different mutants (*sid2*, *vtc2*, *sai1*). 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 H₂O₂. 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.

145 a more severe reduction (approximately 75%) in acquired thermotolerance (Fig. 2, A and B).
 146 Besides the survival ratio, the necrosis in *sai1* mutant was exhibited an obvious increase compared
 147 with that in wild-type (Fig. 2, C and D).

148 SA Induces ROS Production in Arabidopsis Protoplasts and Plants

149 Having established that H₂O₂ is required for SA-induced acquired thermotolerance (Figs.1 and
 150 2), we then examined whether external SA might cause increase generation of H₂O₂.

Figure 3

Figure 3. ROS generation in response to SA in Arabidopsis. **A**, Effects of SA on the DCF fluorescence during 1 h. At the indicated time, the protoplasts with or without 100 μM SA treatment were subjected to 5 μM H₂DCFDA 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 (*vtc2*, *sai1*, *sid2*, and *NahG*) protoplasts during 1 h. Scale bars, 100 μm. **D**, Changes in H₂O₂ 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.

151 Arabidopsis protoplasts were treated with 100 μM SA, and the fluorescent compound
 152 dichlorofluorescein (DCF), produced from the non-fluorescent compound H₂DCFDA in the
 153 presence of endogenous esterases and H₂O₂ (Jakubowski and Bartosz, 2000), was used. The

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 μ M SA, which reached a peak at 100 μ M 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 μ M 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 also increased staining of the wild-type and *vtc2* mutant, but had no effect on the *NahG* 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

Figure 4

Figure 4. Transcriptional analysis of the effects of SA on heat-inducible Hsfs between the *hsfA2* and wild-type plants. The wild-type and *hsfA2* mutant grown at 24°C were pretreated with 100 μ M SA or not, and then exposed to heat acclimation at 37°C for 1 h. A, Comparison of the expression of heat-inducible Hsfs (*HsfA2*, *HsfA3*, *HsfA7a*, *HsfB1*, *HsfB2a*, and *HsfB2b*) in response to SA, HS or SA + HS in wild-type plants. B, Comparison of the expression of heat-inducible Hsfs in response to SA, HS or SA + HS in *hsfA2* mutant. In (A) and (B), asterisk indicates significant differences to Control (Student's t-test, $P < 0.05$); #, $P < 0.05$ vs HS in *hsfA2* mutant; ##, $P < 0.01$ vs HS in WT. Data are means $\pm$ SE of three independent experiments.

184 37°C, which was far higher than the values for *HsfB1*, *HsfB2a*, *HsfA7a*, *HsfB2b*, *HsfA3* (183%,
 185 209%, 210%, 161%, and 150% of the level under HS, respectively; Fig. 4A). In the *hsfA2*
 186 mutant, there was reduced *HsfA2* mRNA level and no obvious difference in the other

187 heat-inducible Hsfs mRNA levels under 37°C compared with those in wild-type. Exogenous
188 pre-treatment with 100 µM SA failed to increase the expression of *HsfA2* gene (Fig. 4B).

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

191 To further confirm the interaction between SA and HsfA2 in plant acquired thermotolerance,
192 the effect of SA on the expression of *HsfA2* gene between wild-type and *sid2* mutant was
193 examined. 2.5-week-old plants were pre-treated with SA, and subjected to 37°C treatment for 1
194 h followed by different time recovery, and then exposed to HS stress at 44°C for 45 min. Under
195 control conditions (24°C), the level of *HsfA2* mRNA in the *sid2* mutant was about 55% lower
196 than that of wild-type (Fig. 5A). Upon HS treatment, the *HsfA2* level increased initially and
197 then decreased gradually in the wild-type, and it had a pronounced increase after 60 min recovery,
198 approximately 210% higher than the control (Fig. 5A: Recovery time 1 h versus control). In the
199 *sid2* mutant, the peak time occurred at a 60-min heat stress without SA treatment (Fig. 5A:
200 Recovery time 0 h versus control), but was still lower than that of the wild-type. However, in
201 the presence of SA, the dramatic rise in the expression of *HsfA2* was detected, with the value to
202 202% of that in the wild-type at the peak time (Fig. 5A: WT, Recovery time 1 h after HS + SA
203 versus Recovery time 1 h after HS). In the *sid2* mutant, the value of *HsfA2* mRNA was near the
204 wild-type level after SA + HS treatment (Fig. 5A: *sid2*, SA + HS versus WT, HS). The results
205 in the *vtc2* mutant were similar to those in wild-type. After 60 min recovery, the value of *HsfA2*
206 mRNA under SA + HS was about 280% higher than that under HS alone. Conversely, in the
207 *sai1* mutant, SA treatment had no effect on the expression of *HsfA2* gene under HS condition.

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

214 To elucidate further details of the above process, the effect of SA on the survival of wild-type
215 and *hsfA2* was compared. After 2 d of recovery, the survival of wild-type was 35% higher than
216 that of the *hsfA2* mutant. The application of SA increased the survival ratio of wild-type by

Figure 5

Figure 5. Effect of SA on acquired thermotolerance in *sid2* and *hsfA2* mutant seedlings. **A**, Analysis of the effects of SA on *HsfA2* mRNA level from Arabidopsis leaves by real-time PCR. The wild-type and different mutants (*sid2*, *vtc2*, *sai1*) were pre-treated with 100 μ M SA or not, then exposed to heat acclimation at 37°C for 1 h, followed by indicated time of recovery at 24°C, then exposed to HS at 44°C for 45 min. Statistical analysis was performed with Student's t-test: *, $P < 0.05$ vs control under SA+HS; **, $P < 0.01$ vs control under SA+HS; #, $P < 0.05$ vs control under HS. **B**, Effects of H_2O_2 on the expression of *HsfA2*. Seedlings of 2-week-old were treated with increasing concentrations of H_2O_2 (0-5 mM), and the expression of *HsfA2* was analyzed by real-time PCR. **C**, Analysis of the effect of H_2O_2 scavenger AsA on the *HsfA2* expression. Asterisks indicate significant differences to SA+HS (Student's t-test, $P < 0.05$). **D**, Comparison of survival ratios for acquired thermotolerance between wild-type and *hsfA2* mutant. The wild-type and different mutants (*sid2*, *vtc2*, *sai1*) grown at 24°C were pre-treated with 100 μ M SA, then 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. The survival ratio was examined 3 d later. Below are representatives of treated seedlings of the indicated genotypes. Statistical analysis was performed with Student's t-test: *, $P < 0.05$ vs corresponding control under HS; #, $P < 0.05$ vs WT under HS. **E**, Effects of H_2O_2 on the survival ratios for acquired thermotolerance among the wild-type, *sid2*, and *hsfA2* mutant. Below are representatives of treated seedlings of the indicated genotypes. Asterisks indicate significant differences to HS (Student's t-test, $P < 0.05$); #, $P < 0.05$ vs WT under HS. Data are means $\pm$ SE of three independent experiments.

217 about 21%, but had no effect on the *hsfA2* mutant. SA treatment increased the survival of *vtc2*
 218 mutant by 30% approximately. In the *sai1* mutant, no effect on the survival under SA + HS
 219 treatment was detected compared with that under HS treatment (Fig. 5D), which was similar to

220 the expression of *HsfA2* gene in this mutant.

221 Similarly, the effect of H₂O₂ on the survival of wild-type and *hsfA2* was obtained. H₂O₂ (1.5
222 mM) increased the survival ratio of wild-type and *sid2* by 19% and 25%, respectively, whereas
223 no significant effect on the *hsfA2* mutant was observed (Fig. 5E).

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

225 The hypocotyl elongation assay, which also reflected acquired thermotolerance (Charng et al.,
226 2007), was carried out. Without severe HS (44°C for 45 min), the level of tolerance of *hsfA2*
227 seedlings had no significant difference with the wild-type regardless of SA pretreatment (Fig. 6,
228 A and D). After severe HS (44°C for 45 min), the *hsfA2* seedlings showed a significant
229 decrease in hypocotyl elongation compared with wild-type without SA pretreatment. The *sid2*
230 and *sai1* mutants also exhibited an obvious decrease hypocotyl length. The *vtc2* mutant showed
231 no significant alteration compared with wild-type. However, the application of SA potentiated
232 the ratio of hypocotyl growth of wild-type, *sid2* and *vtc2* by about 35%, 50%, and 26%,
233 respectively, after challenge at 48 h recovery. Conversely, SA treatment failed to enhance the
234 ratio of hypocotyl growth in *hsfA2* and *sai1* seedlings (Fig. 6, B and D).

235 The enhanced tolerance to HS was exhibited in the wild-type, *sid2* and *vtc2* mutants after
236 H₂O₂ treatment. While in the *hsfA2* seedlings, H₂O₂ treatment failed to increase the hypocotyl
237 elongation and enhance HS tolerance (Fig. 6C). These results indicate that H₂O₂ accumulation
238 is not influenced by the lacking mutation of HsfA2, which contributes to the SA-induced plants
239 HS tolerance.

240 **Disruption of HsfA2 Lowered Expression Levels of HSP genes in SA-induced** 241 **Thermotolerance**

242 HSPs expression has been known to be an important component of acquired thermotolerance
243 (Charng et al., 2007; Banti et al., 2010). We examined the effects of SA and AtHsfA2 on the
244 transcriptional regulation of HSPs under HS. *AtHsp18.1-CI* and *AtHsp25.3* were chosen as
245 marker genes. As shown in Figure 7A, levels of *AtHsp18.1-CI* mRNA in the *hsfA2*, *sid2*, and
246 *sai1* mutants were reduced to 45%, 36% and 43% of levels in the wild-type, respectively.
247 Exogenous 1.5 mM H₂O₂ pre-treatment stimulated *AtHsp18.1-CI* transcriptional level in the *sid2*
248 mutant, whereas no clear effect on the *hsfA2* mutant was observed. Pre-treatment of wild-type
249 with AsA significantly decreased *AtHsp18.1-CI* level compared with the wild-type. However,

Figure 6

Figure 6. Role of HsfA2 in acquired thermotolerance. The wild-type, *hsfA2*, *vtc2*, *sid2*, and *sai1* seedlings were first conditioned at 37 °C for 1 h, recovered for the indicated times, and then without (A) or subjected to (B, C) severe HS at 44 °C for 45 min. The elongation of hypocotyl during 2.5 d of recovery in the dark was measured as indicated. The relative hypocotyl length in (B) was expressed as percentage of the numbers in (A). D, Hypocotyl elongation phenotype of seedlings between wild type and different mutants. After growth for 3.5 days in the dark at 24 °C with or without SA pretreatment, seedlings were either maintained at 24 °C; or treated at 37 °C for 1 h; or at 37 °C for 1 h followed by 48 h at 24 °C and then 44 °C for 45 min. Seedlings were returned to 24 °C for 2.5 days and then photographed. Groups of the seedlings that received the same treatment, as indicated above the panel, are shown. Bar = 1 mm. In (A), (B) and (C), asterisks indicate significant differences to the relevant control (Student's t-test, $P < 0.05$). Data are means $\pm$ SE of three different experiments, with similar results.

250 as for an AsA-deficient mutant *vtc2*, the level of *AtHsp18.1-CI* mRNA was not markedly affected
 251 compared with that in wild-type.

252 Similar results were obtained for the expression of *AtHsp25.3* in the mutant. After HS

Figure 7

Figure 7. Effect of SA and H₂O₂ through HsfA2 on *Hsp18.1-CI* and *Hsp25.3* expression. Real-time PCR analysis for expression of *Hsp18.1-CI* (A) and *HSP25.3* (B). The 2.5-week old seedlings grown at 24°C were pretreated with or without 1 mM AsA or 1.5 mM H₂O₂, and then exposed to heat acclimation at 37°C for 1 h, followed by 1 h of recovery at 24°C, and then exposed to 44°C for 45 min. *Actin2* was used as an internal control. Here, statistical analysis was performed with Student's t-test: *, *P* < 0.05 vs WT; #, *P* < 0.05 vs *sid2* mutant. Data are means ± SE from three biological replicates.

253 treatment, *AtHsp25.3* mRNA level in the *hsfA2*, *sid2* and *sai1* mutants were significantly lower
 254 than that in the wild-type (by 66%, 58%, and 62%, respectively). The *sid2* mutant pre-treated
 255 with H₂O₂ showed restored *AtHsp25.3* expression, however, there was no obvious different in the

256 *hsfA2* mutant. Similarly, AsA pretreatment decreased the level of the wild-type by about 52%
257 compared with its control (Fig. 7B).

258 **Subcellular Localization of ROS Accumulation Induced by SA**

259 As shown in Figure 3, application of SA enhanced the relative fluorescence intensity of DCF.
260 To verify which cellular compartments underwent increases in fluorescence intensity,
261 intracellular ROS production and localization were monitored by LCSM at the single-cell level
262 *in vivo*, through double staining with the mitochondria-specific marker MitoTracker Red
263 CMXRos and the ROS probe H₂DCFDA. As shown in Figure 8A, the wild-type protoplasts
264 double-stained with DCF and MitoTracker Red CMXRos showed obvious fluorescent overlap
265 that colocalized in the cytoplasmic areas in which mitochondria were present at 0.5 h. By 1.5 h
266 of SA treatment, a strong increase was found which was co-localized not only with mitochondria
267 but also with chloroplasts in the protoplasts. By contrast, during the whole assessment time, no
268 detectable DCF signal was observed in protoplasts without SA treatment. In addition,
269 Pretreatment of AsA dramatically depleted the increase in fluorescence from the organelles, and
270 the chloroplasts were more sensitive to AsA than mitochondria. In *sai1* mutant protoplasts, the
271 application of SA had no effect on the increased fluorescence (Fig. 8B), which is in agreement
272 with the result in Figure 3C. However, in *hsfA2* mutant, a strong increase in DCF fluorescence
273 co-localized with mitochondrial signal during the time, exhibiting no significant change in
274 fluorescence compared with that of wild-type (Fig.8C).

275 To clarify the results quantitatively, the kinetics of H₂O₂ accumulation was detected in
276 isolation mitochondria (Supplemental Fig. S4). At about 15 min after SA treatment, DCF
277 fluorescence intensity began to increase and was obviously boosted after 60 min. During 90
278 min, the level of ROS kept a slight increase.

279 **Changes of Mitochondrial Respiratory Complex Activity under SA Treatment**

280 Time course experiments established that mitochondria might be the main regions involved in
281 SA-induced ROS production (Fig. 8), possibly mitochondrial components that are important for
282 electron transport were implicated in this process. To test this hypothesis, SA-dependent H₂O₂
283 production in isolated mitochondria was assayed in the presence of inhibitors of the electron
284 transport chain (ETC). ROS generation is determined in the presence of complex I (malate +
285 glutamate) or II (succinate) substrates, which acts to feed electrons into the ETC at the level of

Figure 8

Figure 8. Subcellular localization of SA-induced ROS production. Protoplasts from WT (A), *sai1* (B), and *hsfA2* (C) mutants leaves were treated with or without 100 μ M SA for the indicated time, double-stained with H₂DCFDA and MitoTracker Red CMXRos, and detected using a LCSM. Protoplasts without SA were kept in dim light for 1.5 h (Control). Protoplasts were treated with 100 μ M SA and then kept in dim light for 0.5 h, 1 h, or 1.5 h. In (A), protoplasts were pre-incubated with AsA at 1 mM for 30 min in the dark and treated with 100 μ M SA for 1.5 h in dim light. Scale bars, 10 μ m.

286 complex I via NADH or directly to complex II, respectively. As shown in Figure 9 A and B,
 287 mitochondria stimulated by malate/glutamate or succinate under SA produced a significant
 288 increase in H₂O₂ generation, whereas exogenous AsA almost completely attenuated this process.

Figure 9

Figure 9. Effects of SA on the mitochondrial ROS and changes of respiratory complex activity. (A) and (B), Mitochondria were isolated and stimulated in the presence of complex I (malate + glutamate) or II (succinate) substrates, and H_2O_2 production was measured in the absence or presence of SA. H_2O_2 production in mitochondria stimulated with malate/glutamate or succinate as respiration substrates in the presence of SA was considered 100%. Asterisks indicate significant differences to control under SA treatment (Student's t-test, $P < 0.05$). (C) and (D), The activity of complex III in SA-treated isolated mitochondria with or without pretreatment of 1 mM AsA. Error bars are $\pm$ SE values for three replicates.

289 Rotenone and antimycin A, a specific inhibitor of complex I and III, respectively, produced a
 290 significant rate of H_2O_2 generation without the addition of SA. However, SA co-administration
 291 with antimycin A showed rates similar to those observed with antimycin A alone. Further, the
 292 decreases in rates of H_2O_2 production were not observed with complex I, II and IV inhibitors
 293 under SA treatment. Rotenone also decreased H_2O_2 generation in mitochondria via succinate,
 294 but to a lesser extent than antimycin A (Fig. 9B). These data indicate that complex III is

295 centrally involved in H₂O₂ generation by SA.

296 To confirm the role of complex III in modulating intracellular ROS generation, the activity of
297 complex III in mitochondrial ETC was determined under SA treatment. Results showed that
298 complex III activity exhibited a time- and concentration-dependent decrease (Fig. 9C). As
299 early as 10 min after SA treatment, the activity of complex III obviously began to decrease,
300 which is prior to the generation of mitochondrial ROS (Figs. 8, 9C, and Supplemental Fig. S4).
301 Moreover, pretreatment of AsA failed to inhibit the decline in the activity of complex III (Fig.
302 9D), indicating that complex III might be as a direct target for SA treatment.

303

304 **DISCUSSION**

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

309 **SA and Acquired Thermotolerance in Arabidopsis**

310 SA was produced in response to a variety of environmental stresses in plants, including HS (Dat
311 et al., 1998a). Mutations disrupting SA signaling decrease the ability of plants to acquired
312 thermotolerance (Larkindale et al., 2005). Thus, we examined the effects of HS on endogenous
313 SA content between wild-type and *sid2*, which shows impaired SA production (Wildermuth et al.,
314 2002). As expected, the endogenous concentrations of free SA and conjugated SA in wild-type
315 were about ninefold and 11-fold, respectively, those in the *sid2* mutant under the normal and HS
316 conditions (Fig. 1A and Supplemental Fig. S5). The reduced survival of the *sid2* mutant indicated
317 its sensitivity to HS. The SA level and survival of *NahG* mutant varied in a similar manner as
318 those of *sid2* plants (Supplemental Fig. S1). Therefore, the reduced SA level and survival ratios
319 synchronously in the *sid2* and *NahG* mutants compared with those of wild-type, suggesting the
320 function of SA on acquired thermotolerance.

321 **The Signaling Pathways for SA-Mediated Plant Acquired Thermotolerance**

322 An essential component of acquired thermotolerance in plants is the induction of Hsfs, which is
323 a conserved protein present and activates HSR in eukaryotic organisms (Nover et al., 2001; Banti
324 et al., 2010; Liu and Charny, 2013). Busch et al. (2005) and Larkindale and Vierling (2008)
325 reported that the heat-inducible class A Hsfs, namely *HsfA2*, *HsfA7a*, and *HsfA3*, and B-type Hsfs
326 (*HsfB1*, *HsfB2a*, and *HsfB2b*) are up-regulated by heat. To test which one or even more
327 heat-inducible Hsfs could involve(s) in acclimation to acquired thermotolerance, the expression of
328 these heat-inducible Hsfs under SA treatment was examined. SA significantly potentiated the
329 expression of *HsfA2* (about 3.3 times) under heat; other heat-inducible Hsfs were not significantly
330 responsive to SA and HS. In the *hsfA2* mutant, the expression of *HsfA7a*, *HsfA3*, *HsfB1*, *HsfB2a*,
331 and *HsfB2b* was revealed no substantial alteration. Further, the ratio of hypocotyl growth in
332 *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 H₂O₂ 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 H₂O₂ 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 H₂O₂ via AtHsfA2 on SA-mediated acquired thermotolerance, the effects of H₂O₂ 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 H₂O₂ (Figs. 3 and 5), indicating that H₂O₂ is closely associated with the induction of *HsfA2* (Miller and Mittler, 2006). (b) *HsfA2* mRNA expression increased as the H₂O₂ level increased, and the application of AsA reduced SA-induced *HsfA2* expression (Fig. 5). (c) The application of H₂O₂ improved the survival ratios of *sid2* mutant in response to HS (Fig. 2), which also indicates the effect of H₂O₂ 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, H₂O₂ 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

363 downstream of H₂O₂ in SA-induced HS signaling and accordingly, H₂O₂ accumulation is not
364 influenced by the lacking mutation of HsfA2 (Supplemental Fig. S7).

365 Downstream components of the HS signaling known as Hsfs contribute to thermotolerance by
366 controlling HSPs through Hsfs binding to HSEs (Baniwal et al., 2004; Snyman and Cronjé, 2008).
367 These HSPs are classed as Hsp60, Hsp70/Hsc70, Hsp90, Hsp100 and small Hsps according to
368 their molecular masses. The small HSPs, as an important type, play essential roles in survival
369 and development (Zhang et al., 2009). In this study, Hsp18.1-CI (Lohmann et al., 2004) and
370 Hsp25.3 (Osteryoung et al., 1993) were chosen to demonstrate how SA and H₂O₂ induce
371 thermotolerance via HsfA2. Lack of HsfA2 in mutant caused substantially reduced expression of
372 tested HSP genes. Under HS conditions, application of H₂O₂ restored expression of *Hsp18.1-CI*
373 and *Hsp25.3* genes in *sid2* mutant, but not in the *hsfA2* mutant, which resulted in the lack of
374 thermotolerance in H₂O₂-pretreated *hsfA2* mutant (Figs. 2, 5-7). As for the other heat-inducible
375 Hsfs, the levels of *AtHsp18.1-CI* and *AtHsp25.3* mRNA were not markedly affected compared
376 with these in wild-type (Supplemental Fig. S8), which was in agreement with the manifestation in
377 hypocotyl growth (Supplemental Fig. S6). Collectively, the mechanism through which SA
378 influences acquired thermotolerance via H₂O₂ involves the expression of *HsfA2* and *HSPs* and
379 establishes acquired thermotolerance.

380 **The function of Mitochondria in SA-induced H₂O₂ in Arabidopsis**

381 SA and ROS are essential signaling molecules involved in physiological response. Although,
382 in tobacco, SA can inhibit respiration in the mitochondria (Xie and Chen, 1999), an effect that
383 might increase ROS production within this organelle, it has not yet been elucidated how SA
384 affects mitochondrial functions. On one hand, our observation showed that SA induced the
385 accumulation of ROS using the Arabidopsis protoplasts, and this effect was dose dependent (Fig. 3;
386 Supplemental Fig. S3). However, this is different from the observation that the change of H₂O₂
387 accumulation was not substantially influenced in the *hsfA2* mutant compared with wild-type (Fig.
388 8C; Supplemental Fig. S7), for the reason that HsfA2 is a key component of the H₂O₂ pathway and
389 acts downstream of H₂O₂ in SA-induced signal transduction.

390 On the other hand, mitochondria are major sources of ROS (Puntarulo et al., 1988) and
391 participate in the ROS burst in plants (Braidot et al., 1999; Xie and Chen, 1999; Clarke et al.,
392 2000). On the basis of protoplasts double-stained with DCF and MitoTracker Red CMXRos, the

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

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

420 MATERIALS AND METHODS

421 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 *sail* (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 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) 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

452 For the Arabidopsis seedlings treatment, 1 mL of SA or H₂O₂ solution at a final concentration
453 of 0.1 mM or 1.5 mM, respectively, were sprayed onto the leaf surfaces of 10-d-old wild-type
454 and different mutants (*sid2*, *hsfA2*, *vtc2*, and *sail1*) grown at 24°C on MS-agar medium after filter
455 sterilization. The control seedlings were treated with water. After 1 h of pre-treatment, the
456 seedlings were exposed to heat stress treatment, and analyzed the seedlings survival ratio.

457 For the Arabidopsis plants treatment, 2.5-week-old plants were sprayed with SA solution or
458 H₂O₂ at the final concentration of 0.1 mM or the different concentrations (0-5 mM), respectively.
459 The control plants were treated with water. After 1 h of pre-treatment, the plants were exposed
460 to heat stress treatment (For SA treatment). After which, the total RNA was extracted from leaf
461 tissue, and the heat-inducible genes were analyzed by real-time RT-PCR.

462 **Extraction and Quantification of SA**

463 Plants were grown as described above and incubated at 24°C for a recovery time following a
464 60 min pre-treatment at 37°C. Plant leaves were collected and crumbled. Samples each 200
465 mg were further homogenized using liquid nitrogen and transferred to a 1.5 mL Eppendorf tube.
466 An aliquot (1 mL) of 90 % methanol was added to the homogenate, soaked for 20 min, and then
467 centrifuged for 3 min at 4,000 g. The supernatant was collected in a 2 mL Eppendorf tube.
468 The pellet was re-suspended in 1 mL 100% methanol, and the centrifugation steps repeated.
469 The supernatants were combined and centrifuged again, and the methanol:water mixtures were
470 evaporated in a SpeedVac concentrator at a high drying speed. After this step, the endogenous
471 free salicylic acid fraction was partitioned again with 1 mL acetic acid: sodium acetate buffer
472 (pH 5.5) and the samples were sonicated for 15 min. The free SA was quantified by
473 spectrofluorescence monitoring at 412 nm emission based on previously described (Meuwly and
474 Metraux, 1993). All data were corrected for recovery of the internal standard salicylic acid, and
475 the free SA was measured.

476 **Isolation and Treatment of Arabidopsis Mesophyll Protoplasts**

477 The isolation of protoplasts from 14-day-old plant leaves was carried out at room temperature
478 according to a modified procedure as described by He et al. (2006). The brief procedures are as
479 follows: healthy leaves from 15 to 20 were sliced into small leaf strips (0.5–1 mm), which were
480 vacuum-infiltrated with enzyme solution [1–1.5% (w/v) Cellulase R10 (Yakult Honsha, Tokyo,
481 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 or 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*, *sai1*, *NahG* and *sid2* leaves were vacuum infiltrated with 1 mg

512 mL⁻¹ DAB solution under 25°C. An Amplex Red Hydrogen Peroxide Assay Kit was used to
513 measure the concentration of H₂O₂ in leaves according to the manufacturer's recommendations.

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

515 Total RNA was extracted from approximately 100 mg of leaf tissue of 2.5-week-old plants
516 frozen in liquid nitrogen with TRIZOL reagent (Invitrogen) according to the manufacturer's
517 protocol. The heat-inducible genes were analyzed by real-time RT-PCR using RNA isolated
518 from the wild-type or *hsfA2* mutant. Real-time PCR was performed using the LightCycler
519 Quick System 350S (Roche Diagnostics K.K.) with SYBR Premix Ex Taq (Takara Bio, Inc.).
520 Each PCR reaction contained 1 × SYBR Premix Ex Taq, 0.2 μM of each primer, and 2 μL of a
521 1:10 dilution of the cDNA in a final volume of 20 μL. The PCR programme was used: initial
522 denaturation, 95°C, 10 s; PCR, 40 cycles of 95°C, 5 s, 60°C, 20 s with a temperature transition
523 rate of 20 °C s⁻¹. In melting curve analysis, PCR reactions were denatured at 95°C, annealed at
524 65 °C, then a monitored release of intercalator from PCR products or primer dimers by an
525 increase to 95 °C with a temperature transition rate of 0.1°C s⁻¹. Standard curves were created
526 using PCR products by 10-fold serial dilutions. The heat-inducible genes expression profiles
527 were normalized using *Actin2* mRNA as an internal control. The sequences of the primers for
528 real-time PCR analysis for each gene are described in Supplemental Table S1.

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

530 Mitochondria were isolated and purified by differential centrifugation as described by
531 Neuburger et al. (1982) and Tiwari et al. (2002). All steps were carried out at 4°C. Briefly,
532 4000 mg of leaves were disrupted with a tissue grinder in 10 mL of cold medium (0.5 M Sucrose,
533 50 mM Tris, 5 mM EDTA, 0.2% (w/v) BSA, 0.3% (v/v) β-Mercaptoethanol, 0.3% (w/v) PVP-40
534 and 5 mM Cysteine; pH 7.5). The homogenate was filtered through two layers of miracloth
535 (Calbiochem-Behring) using a syringe. The debris was spun down by centrifugation at 105 g
536 for 10 min and the supernatant was further spun at 1600 g for 10 min. Supernatants were then
537 collected and centrifuged at 15000 g for 20 min and the pellets resuspended in approximately 1.5
538 mL of cold medium. The resuspended pellets were centrifuged at 10500 g for 15 min with
539 three replicates, and suspended by acetone and recentrifuged at 15000 g for 10min. Finally, the
540 mitochondria fraction was suspended in assay buffer (300 mM sucrose, 2 mM HEPES, 0.1 mM
541 EDTA; pH 7.4). After isolation, the mitochondria were treated with or without SA in the

542 present or absent of inhibitors of the electron transport chain, and then H₂O₂ were measured with
543 a fluorescence spectrometer (PerkinElmer, LS55, UK).

544 The activity of cytochrome reductase was assayed as described by Tiwari et al. (2002). The
545 enzyme activity assay was carried out according to the instruction manual of the Tissue
546 Mitochondrial Complex III Assay Kit (Genmed Scientifics Inc., Arlington, MA). The assay
547 mixture of the complex III contained mitochondrial protein (10 µg), EDTA, potassium phosphate
548 buffer (pH 7.5), EDTA, bovine serum albumin, and substrates (decylubiquinol and oxidized
549 ferricytochrome c). After incubation for 5 min at 30°C, the activity was measured by the
550 increase in absorbance at 550 nm using a molar absorbtion coefficient of 21.84 mmol⁻¹ L cm⁻¹ for
551 the reduction of ferricytochrome c. The complex III antimycin A sensitive enzyme activity was
552 expressed as nmol min⁻¹ mg⁻¹ protein.

553 **Supplemental Data**

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

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

558 **Supplemental Figure S3.** *In situ* detection of leaf H₂O₂ in wild-type and different mutants (*vtc2*,
559 *sai1*, *sid2*, and *NahG*).

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

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

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

566 **Supplemental Figure S7.** Time course of H₂O₂ accumulation in wild-type and *hsfA2* mutant in
567 response to HS.

568 **Supplemental Figure S8.** The expression of *Hsp18.1-C1* and *Hsp25.3* genes in *hsfA3*, *hsfA7a*,
569 *hsfB1*, *hsfB2a*, and *hsfB2b* mutants.

570 **Supplemental Figure S9.** Subcellular localization of SA-induced ROS production in *hsfA3* and

571 *hsfA7a* mutants.

572 **Supplemental Figure S10.** Subcellular localization of SA-induced ROS production in *hsfB1*,

573 *hsfB2a*, and *hsfB2b* mutants.

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

575 **ACKNOWLEDGEMENTS**

576 We are thankful to Dr. Yee-yung Charng (Agricultural Biotechnology Research Center,

577 Academia Sinica, Taipei, Taiwan) for kindly providing us with the *hsfA2* seeds. We also thank

578 Mr. David Chen of the University of Colorado at Boulder for his help in English proofreading

579 the manuscript.

581

582

Parsed Citations

Ahn SG, Thiele DJ (2003) Redox regulation of mammalian heat shock factor 1 is essential for Hsp gene activation and protection from stress. Gene Dev 17: 516-528

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Akerfelt M, Morimoto RI, Sistonen L (2010) Heat shock factors: integrators of cell stress, development and lifespan. Nat Rev Mol Cell Biol 11: 545-555

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Alvarez ME, Pennell RI, Meijer PJ, Ishikawa A, Dixon RA, Lamb C (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell 92: 773-784

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55: 373-399

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Bais HP, Vepachedu R, Gilroy S, Callaway RM, Vivanco JM (2003) Allelopathy and exotic plant invasion: from molecules and genes to species interactions. Science 301: 1377-1380

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Baniwal SK, Bharti K, Chan KY, Fauth M, Ganguli A, Kotak S, Mishra SK, Nover L, Port M, Scharf KD, et al (2004) Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors. J Bioscience 29: 471-487

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Banti V, Mafessoni F, Loreti E, Alpi A, Perata P (2010) The heat-inducible transcription factor HsfA2 enhances anoxia tolerance in Arabidopsis. Plant Physiol 152: 1471-1483

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Borsani O, Valpuesta V, Botella MA (2001) Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in Arabidopsis seedlings. Plant Physiol 126: 1024-1030

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Braidot E, Petrusa E, Vianello A, Macri F (1999) Hydrogen peroxide generation by higher plant mitochondria oxidizing complex I or complex III substrates. FEBS letters 451: 347-350

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Busch W, Wunderlich M, Schoffl F (2005) Identification of novel heat shock factor-dependent genes and biochemical pathways in Arabidopsis thaliana. Plant J 41: 1-14

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Chang YY, Lin HC, Liu NY, Chi WT, Wang CN, Chang SH, Wang TT (2007) A heat-inducible transcription factor, HsfA2, is required for expression of acquired thermotolerance in Arabidopsis. Plant Physiol 143: 251-262

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Chang YY, Liu HC, Liu NY, Hsu FC, Ko SS (2006) Arabidopsis Hsa32, a novel heat-shock protein, is essential for acquired thermotolerance during a long recovery period after acclimation treatment. Plant Physiol 140: 1297-1305

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Clarke JD, Volko SM, Ledford H, Ausubel FM, Dong XN (2000) Roles of salicylic acid, jasmonic acid, and ethylene in cpr-induced resistance in Arabidopsis. Plant Cell 12: 2175-2190

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Dat JF, Foyer CH, Scott IM (1998a) Changes in salicylic acid and antioxidants during induced thermotolerance in mustard seedlings. Plant Physiol 118: 1455-1461

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Dat JF, Lopez-Delgado H, Foyer CH, Scott IM (1998b) Parallel changes in H₂O₂ and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. Plant Physiol 116: 1351-1357

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Dat JF, Lopez-Delgado H, Foyer CH, Scott IM (2000) Effects of salicylic acid on oxidative stress and thermotolerance in tobacco. J Plant Physiol 156: 659-665.

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Dempsey DA, Shah J, Klessig DF (1999) Salicylic acid and disease resistance in plants. Crit Rev Plant Sci 18: 547-575

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Diara C, Castagna A, Baldan B, Sodi AM, Sahr T, Langebartels C, Sebastiani L, Ranieri A (2005) Differences in the kinetics and scale of signaling molecule production modulate the ozone sensitivity of hybrid poplar clones: the roles of H₂O₂, ethylene and salicylic acid. New Phytol 168 (2): 351-364

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Fujimoto M, Izu H, Seki K, Fukuda K, Nishida T, Yamada S, Kato K, Yonemura S, Inouye S, Nakai A (2004) HSF4 is required for normal cell growth and differentiation during mouse lens development. EMBO J 23: 4297-4306

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261: 754-756

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Green DR, Reed JC (1998) Mitochondria and apoptosis. Science 281: 1309-1312.

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

He P, Shan L, Sheen J (2006) The use of protoplasts to study innate immune responses. Methods Mol Biol 354: 1-10

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Hong SW, Lee U, Vierling E (2003) Arabidopsis hot mutants define multiple functions required for acclimation to high temperatures. Plant Physiol 132: 757-767

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Hong SW, Vierling E (2000) Mutants of Arabidopsis thaliana defective in the acquisition of tolerance to high temperature stress. Proc Natl Acad Sci USA 97: 4392-4397

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Hsu A-L, Murphy CT, Kenyon C (2003) Regulation of aging and age-related disease by DAF-16 and heat-shock factor. Science 300: 1142-1145

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Ikeda M, Mitsuda N, Ohme-Takagi M (2011) Arabidopsis HsfB1 and HsfB2b act as repressors of the expression of heat-inducible Hsfs but positively regulate the acquired thermotolerance. Plant Physiol 157: 1243-1254

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Jakubowski W, Bartosz G (2000) 2, 7-Dichloro fluorescein oxidation and reactive oxygen species: what does it measure? Cell Biol Int 24: 757-760

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Jander G, Norris SR, Rounsley SD, Bush DF, Levin IM, Last RL (2002) Arabidopsis map-based cloning in the post-genome era. Plant Physiol 129: 440-450

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Klessig DF, Malamy J (1994) The salicylic acid signal in plants. Plant Mol Biol 26: 1439-1458

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Koch JR, Creelman RA, Eshita SM, Seskar M, Mullet JE, Davis KR (2000) Ozone sensitivity in hybrid poplar correlates with insensitivity to both salicylic acid and jasmonic acid. The role of programmed cell death in lesion formation. Plant Physiol 123: 487-496

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Kotak S, Port M, Ganguli A, Bicker F, Von Koskull-Döring P (2004) Characterization of C-terminal domains of Arabidopsis heat stress transcription factors (Hsfs) and identification of a new signature combination of plant class A Hsfs with AHA and NES motifs essential for activator function and intracellular localization. Plant J 39: 98-112

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Kunkel BN, Brooks DM (2002) Cross talk between signaling pathways in pathogen defense. Curr Opin Plant Biol 5: 325-331

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Larkindale J, Hall JD, Knight MR, Vierling E (2005) Heat stress phenotypes of Arabidopsis mutants implicate multiple signaling pathways in the acquisition of thermotolerance. Plant Physiol 138: 882-897

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Larkindale J, Vierling E (2008) Core genome responses involved in acclimation to high temperature. Plant Physiol 146: 748-761

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Levine A, Tenhaken R, Dixon RA, Lamb C (1994) H₂O₂ from the oxidative burst orchestrates the plant hypersensitive response. Cell 79: 583-593

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Li C, Chen Q, Gao X, Qi B, Chen N, Xu S, Chen J, Wang X (2005) AtHsfA2 modulates expression of stress responsive genes and enhances tolerance to heat and oxidative stress in arabidopsis. Sci China Ser C 48: 540-550

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Liu HC, Charng YY (2013) Common and distinct functions of arabidopsis class A1 and A2 heat shock factors in diverse abiotic stress responses and development. Plant Physiol 163: 276-290

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Liu HC, Liao HT, Charng YY (2011) The role of class A1 heat shock factors (HsfA1s) in response to heat and other stresses in Arabidopsis. Plant Cell Environ 34: 738-751

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Lohmann C, Eggers-Schumacher G, Wunderlich M, Schoffl F (2004) Two different heat shock transcription factors regulate immediate early expression of stress genes in Arabidopsis. Mol Genet Genomics 271: 11-21

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Lopez-Delgado H, Dat JF, Foyer CH, Scott IM (1998) Induction of thermotolerance in potato microplants by acetylsalicylic acid and H₂O₂. J Exp Bot 49: 713-730

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Maxwell DP, Nickels R, McIntosh L (2002) 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

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Meiri D, Breiman A (2009) Arabidopsis ROF1 (FKBP62) modulates thermotolerance by interacting with HSP90.1 and affecting the accumulation of HsfA2-regulated sHSPs. Plant J 59: 387-399

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Meuwly P, Metraux JP (1993) Ortho-anisic acid as internal standard for the simultaneous quantitation of salicylic acid and its putative biosynthetic precursors in cucumber leaves. Anal Biochem 214: 500-505

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Miller G, Mittler R (2006) Could heat shock factors function as hydrogen peroxide sensors in plants? Ann Bot-Lond 98: 279-288

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Møller IM (2001) Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. Annu Rev Plant Physiol Plant Mol Biol 52: 561-591

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Molina A, Bueno P, Marin MC, Rodriguez-Rosales MP, Belver A, Venema K, Donaire JP (2002) Involvement of endogenous salicylic acid content, lipoxygenase and antioxidant enzyme activities in the response of tomato cell suspension cultures to NaCl. New Phytol 156: 409-415

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Müller-Moulé P, Havaux M, Niyogi KK (2003) Zeaxanthin deficiency enhances the high light sensitivity of an ascorbate-deficient mutant of Arabidopsis. Plant Physiol 133: 748-760

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Murphy MP (2009) How mitochondria produce reactive oxygen species? Biochem J 417, 1-13

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Neuburger M, Journet EP, Bligny R, Carde JP, Douce R (1982) Purification of plant mitochondria by isopycnic centrifugation in density gradients of percoll. Arch Biochem Biophys 217: 312-23

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Noctor G, Foyer CH (1998) ASCORBATE AND GLUTATHIONE: keeping active oxygen under control. Ann Rev Plant Physiol Plant Mol Biol 49: 249-279

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Norman C, Howell KA, Millar AH, Whelan JM, Day DA (2004) Salicylic acid is an uncoupler and inhibitor of mitochondrial electron transport. Plant Physiol 134: 492-501

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Nover L, Bharti K, Doring P, Mishra SK, Ganguli A, Scharf KD (2001) Arabidopsis and the heat stress transcription factor world: how many heat stress transcription factors do we need? Cell Stress Chaperon 6: 177-189

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Nover L, Scharf KD, Gagliardi D, Verger P, Czarnecka-Verner E, Gurley WB (1996) The Hsf world: classification and properties of plant heat stress transcription factors. Cell Stress Chaperon 1: 215-223

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Ogawa D, Yamaguchi K, Nishiuchi T (2007) High-level overexpression of the Arabidopsis HsfA2 gene confers not only increased thermotolerance but also salt/osmotic stress tolerance and enhanced callus growth. J Exp Bot 58 (12): 3373-3383

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Orozco-Cárdenas ML, Ryan CA (2002) Nitric oxide negatively modulates wound signaling in tomato plants. Plant Physiol 130: 487-493

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Osteryoung Kw, Sundberg H, Veiling E (1993) Poly(a) tail length of a heat shock protein rna is increased by severe heat stress, but intron splicing is unaffected. Mol Gen Genet 239: 323-333

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Panchuk II, Volkov RA, Schoffl F (2002) Heat stress- and heat shock transcription factor-dependent expression and activity of ascorbate peroxidase in Arabidopsis. Plant Physiol 129: 838-853

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Puntarulo S, Sánchez RA, Boveris A (1988) Hydrogen peroxide metabolism in soybean embryonic axes at the onset of germination. Plant Physiol 86(2): 626-630

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Raha S, Robinson BH (2000) Mitochondria, oxygen free radicals, disease and ageing. Trends Biochem Sci 25: 502-508

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Rhee SG (2006) H₂O₂, a necessary evil for cell signaling. Science 312: 1882-1883

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Rhoads DM, Umbach AL, Subbaiah CC, Siedow JN (2006) Mitochondrial reactive oxygen species. Contribution to oxidative stress and interorganellar signaling. Plant Physiol 141: 357-366

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Schramm F, Ganguli A, Kiehlmann E, Englich G, Walch D, von Koskull-döring P. (2006) The heat stress transcription factor HsfA2 serves as a regulatory amplifier of a subset of genes in the heat stress response in Arabidopsis. Plant Mol Biol 60: 759-772

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Schramm F, Larkindale J, Kiehlmann E, Ganguli A, Englich G, Vierling E, von Koskull-Döring P (2008) A cascade of transcription factor DREB2A and heat stress transcription factor HsfA3 regulates the heat stress response of Arabidopsis. Plant J 53: 264-274

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Senaratna T, Touchell D, Bunn E, Dixon K (2000) Acetyl salicylic acid (Aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. Plant Growth Regul 30: 157-161

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Shah J, Tsui F, Klessig DF (1997) Characterization of a salicylic acid-insensitive mutant (sai1) of Arabidopsis thaliana, identified in a selective screen utilizing the SA-inducible expression of the tms2 gene. Mol Plant Microbe Interact 10: 69-78

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Snyman M, Cronjé MJ (2008) Modulation of heat shock factors accompanies salicylic acid-mediated potentiation of Hsp70 in tomato seedlings. J Exp Bot 59: 2125-2132

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Tiwari BS, Belenghi B, Levine A (2002) Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death. Plant Physiol 128: 1271-1281

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Volkov RA, Panchuk II, Mullineaux PM, Schöffl F (2006) Heat stress-induced H₂O₂ is required for effective expression of heat shock genes in Arabidopsis. Plant Mol Biol 61: 733-746

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2002) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature 30: 417-571

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Xie Z, Chen Z (1999) Salicylic acid induces rapid inhibition of mitochondrial electron transport and oxidative phosphorylation in tobacco cells. Plant Physiol 120: 217-226

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Xing H, Wilkerson DC, Mayhew CN, Lubert EJ, Skaggs HS, Goodson ML, Hong Y, Park-Sarge O-K, Sarge KD (2005) Mechanism of hsp70i gene bookmarking. Science 307: 421-423

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Yao N, Greenberg JT (2006) Arabidopsis ACCELERATED CELL DEATH2 modulates programmed cell death. Plant Cell 18: 397-411

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Zhang LR, Li YL, Xing D, Gao CJ (2009) Characterization of mitochondrial dynamics and subcellular localization of ROS reveal that HsfA2 alleviates oxidative damage caused by heat stress in Arabidopsis. J Exp Bot 60: 2073-2091

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Zhang W, Zhou RG, Gao YJ, Zheng SZ, Xu P, Zhang SQ, Sun DY (2009) Molecular and genetic evidence for the key role of atcam3 in heat-shock signal transduction in Arabidopsis. Plant Physiol 149: 1773-1784

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Zhong M, Orosz A, Wu C (1998) Direct sensing of heat and oxidation by Drosophila heat shock transcription factor. Mol Cell 2: 101-108

Pubmed: [Author and Title](#)
CrossRef: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)