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Overexpression of Heat Shock Factor Gene *HsfA3* Increases Galactinol Levels and Oxidative Stress Tolerance in *Arabidopsis*

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Heat shock factors (Hsfs) are central regulators of abiotic stress responses, especially heat stress responses, in plants. In the current study, we characterized the activity of the Hsf gene *HsfA3* in *Arabidopsis* under oxidative stress conditions. *HsfA3* transcription in seedlings was induced by reactive oxygen species (ROS), exogenous hydrogen peroxide (H_2O_2), and an endogenous H_2O_2 propagator, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB). *HsfA3*-overexpressing transgenic plants exhibited increased oxidative stress tolerance compared to untransformed wild-type plants (WT), as revealed by changes in fresh weight, chlorophyll fluorescence, and ion leakage under light conditions. The expression of several genes encoding galactinol synthase (GolS), a key enzyme in the biosynthesis of raffinose family oligosaccharides (RFOs), which function as antioxidants in plant cells, was induced in *HsfA3* overexpressors. In addition, galactinol levels were higher in *HsfA3* overexpressors than in WT under unstressed conditions. In transient transactivation assays using *Arabidopsis* leaf protoplasts, HsfA3 activated the transcription of a reporter gene driven by the *GolS1* or *GolS2* promoter. Electrophoretic mobility shift assays showed that *GolS1* and *GolS2* are directly regulated by HsfA3. Taken together, these findings provide evidence that *GolS1* and *GolS2* are directly regulated by HsfA3 and that GolS enzymes play an important role in improving oxidative stress tolerance by increasing galactinol biosynthesis in *Arabidopsis*.

INTRODUCTION

High temperatures induce the generation of reactive oxygen species (ROS) in plants. ROS function in signaling and, in excessive amounts, cause oxidative damage to proteins, lipids, and nucleic acids, which impairs cellular and metabolic functions and leads to cell death (Miller et al., 2010). This universal phenomenon is known as heat stress (HS)-induced oxidative stress (Vallelian-Bindschedler et al., 1998). Understanding how plants cope with HS-induced oxidative stress during growth is crucial for improving HS endurance in crops to increase agricultural productivity (Hall, 2001).

The HS response (HSR) is primarily regulated at the transcriptional level by heat shock factors (Hsfs), which are activated by HS. Activated Hsfs specifically bind to palindromic heat shock elements (HSEs: 5′-nGAAnnTTCn-3′), leading to the transcription of these genes. This process functions during both basal and acquired HSRs in plants (Xuan et al., 2010). In the *Arabidopsis thaliana* genome, a total of 21 *Hsf*s genes have been identified, encoding Hsfs assigned to three major classes (A, B, and C) based on the characteristics of their oligomerization domains (Nover et al., 2001). *Arabidopsis* class A Hsfs function as positive regulators of environmental stress-responsive genes, which results in the constitutive expression of HS proteins (HSPs) and other proteins in biochemical pathways that increase thermotolerance levels (Panikulangara et al., 2004). By contrast, class B Hsfs may act as coactivators and/or repressors, while no clear activation or repression functions have been identified for class C Hsfs (Ikeda et al., 2011).

Arabidopsis contains 15 class A Hsfs, including HsfA1a and HsfA1b, which play important roles in the early phase of the HSR (Nover et al., 2001), as well as HsfA2, which maintains *HSP* expression during extended acquired thermotolerance responses (Charng et al., 2007). *HsfA3* transcript levels increase under HS and excess light, and the *hsfA3* T-DNA insertion mutant shows substantially reduced thermotolerance, while overexpression of *HsfA3* increases thermotolerance (Jung et al., 2013; Yoshida et al., 2008). *In vivo* and *in vitro* experiments have demonstrated that HsfA3 functions directly downstream of dehydration-responsive element-binding factor 2A (DREB2A) and/or DREB2C, important transcription factors involved in plant responses to heat and salt stress (Chen et al., 2010; Yoshida et al., 2008). In addition to regulating *HSP* genes, DREB2-induced HsfA3 directly regulates cytosolic *ascorbate peroxidase 2* (*APX2*), encoding a key enzyme in the ROS scavenging system of *Arabidopsis* (Hwang et al., 2012; Jung et al., 2013; Shin et al., 2013). Additionally, HsfA3 regulates the expression of many heat-inducible genes in the transcriptional cascade downstream of the K homology (KH) domain-containing nucleus-localized putative RNA-binding protein (RCF3)

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stress-regulatory system, an important upstream regulator of the HSR in *Arabidopsis* (Guan et al., 2013). These findings suggest that HsfA3 plays important roles in the regulation of HS-induced oxidative stress signaling; however, such roles remain to be demonstrated.

HS-induced oxidative stress brings about the accumulation of a number of metabolic intermediates in plant cells that act as antioxidants and/or signals (Nishizawa et al., 2008). *Arabidopsis* HsfA1b and HsfA2 directly regulate *galactinol synthase* (*GolS*) *1* and *GolS2*, encoding a key enzyme in the biosynthesis of raffinose family oligosaccharides (RFOs), which function as osmoprotectants and/or antioxidants in the abiotic stress tolerance response (ElSayed et al., 2014; Panikulangara et al., 2004). Galactinol is formed from UDP-galactose and *myo*-inositol *via* the activity of GolS, a key regulator of this pathway (Keller and Pharr 1996). As *GolS* genes are Hsf-dependent, overexpressing these genes increases tolerance to drought, high salinity and osmotic stresses in *Arabidopsis* (Busch et al., 2005; Sun et al., 2013; Taji et al., 2002). There is substantial evidence that galactinol and RFOs function as signals that mediate stress responses (Kim et al., 2008; Valluru and den Ende, 2011). However, such roles for galactinol and RFOs remain to be demonstrated (ElSayed et al., 2014).

In the current study, we determined that oxidative damage triggers the expression of *HsfA3*, which is correlated with the upregulation of *GolS* genes. These findings suggest that HsfA3 activates the expression of *GolS* genes, which in turn regulate the biosynthesis of galactinol to increase oxidative stress tolerance in plants.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana L. Heynh. ecotype Columbia (Col-0) plants (WT) and *HsfA3*-overexpressing transgenic *Arabidopsis* lines were grown on phytohormone-free MS medium (MSO) containing 2% sucrose and 0.25% Phyta-gel (pH 5.8) at 22°C under a 16 h light/8 h dark cycle (light intensity 100 μE m^{-2} s^{-1}). To induce synchronous germination, the seeds were primed at 4°C for 3 days in the dark, followed by transfer to a growth chamber (Hwang et al., 2012).

A T-DNA insertional mutant line containing a single T-DNA insertion in the *HsfA3* gene was identified in the SALK T-DNA collection (SALK_011107). To identify mutants homozygous for the T-DNA insertion, genomic DNA was obtained from seedlings and subjected to PCR genotyping using the following *HsfA3* primer sets: *HsfA3* forward (P1: 5′-ATGAGCCCAAAAAAAGATGC-3′) and reverse primer (P2: 5′-CTAAGGATCATTCATTGG-3′); and T-DNA right (P3: 5′-TGGGAAAACGGGCGTTACCCAACTTAAT-3′) and left border primer (P4: 5′-GTGATGGTTCACGTAGTGGGCCATCG-3′)(Supplementary Figs. 1A and 1C).

RNA extraction and reverse transcription (RT)-PCR

Total RNA was isolated from 10-day-old WT and *HsfA3* transgenic lines using TRIzol reagent (Invitrogen, USA). Complementary DNA synthesis and RT-PCR were performed as described by Hwang et al. (2012) using *HsfA3*- and *GolS*-specific primer pairs (Supplementary Table 1). PCR products were sequenced to confirm that the amplified sequences were identical to the predicted sequences of the respective mRNAs based on *Arabidopsis* genomic data. Relative levels of *HsfA3* and *GolS* expression were determined using ImageJ software (http://rsb. info.nih.gov/ij).

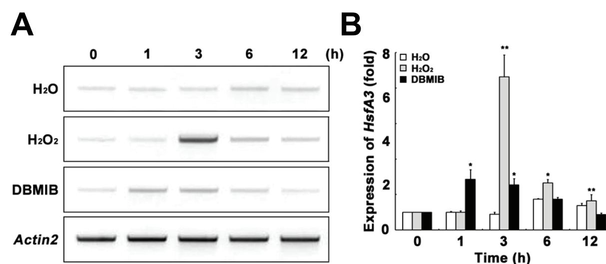


Fig. 1. Transcript levels of *HsfA3* in response to oxidative stress. RT-PCR analysis was performed to determine transcript levels in 10-day-old plants following exposure to 20 mM H_2O_2 or 25 μM DBMIB for the times indicated. *Arabidopsis Actin2* was amplified as a control for normalization. The expected sizes of amplicons and gene-specific primers used are described in Supplementary Table 1. (B) Quantitative RT-PCR analysis indicating relative *HsfA3* expression. Values represent mean ± SD; values prior to treatment (0 h) were set at 1. Statistically significant differences between water and stress treatments are indicated by asterisks (n = 3; * P < 0.05; ** P < 0.01 by Student's t-test).

Construction of transgenic plants

To amplify full-length *HsfA3* cDNA, RT-PCR was performed as described above (in Plant materials and growth conditions) using P1 forward and P2 reverse primers, and the RT-PCR product was cloned into pGEM-T Easy (Promega, USA) using TA-overhangs. The integrity of the construct was verified by sequencing. Subsequently, *HsfA3* cDNA was digested with *Xba*I and *Bam*HI, and the resulting 1,239 bp fragment was inserted between the cauliflower mosaic virus (*CaMV*) *35S* promoter and nopaline synthase terminator in pBI121 (Clontech, USA) (Supplementary Fig. 1B). The recombinant plasmid (*35SΩ:HsfA3*) was introduced into *Agrobacterium tumefaciens* LBA4404 and used to transform *Arabidopsis* plants *via* the floral dip method (Martinez-Trujillo et al., 2004). Homozygous T_3 lines containing a single T-DNA insertion were used for further analysis as described by Lim et al. (2007) (Supplementary Fig. 1D).

Stress treatments

To analyze the transcriptional expression of *HsfA3* in response to oxidative stress, primed WT seeds were germinated on MSO medium without or with 20 mM H_2O_2 (Sigma-Aldrich, USA) or 25 μM 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB; Santa Cruz Biotechnology, USA). The seeds of WT and transgenic *Arabidopsis* plants were harvested and stored as described by Je et al. (2014).

To analyze the oxidative stress tolerance of plants by phenotypic comparison, primed Col-0, transgenic *35SΩ:HsfA3* (L12 and L20), and *HsfA3* knockout mutant (*hsfa3*) seeds were germinated on MSO plates containing H_2O_2 (4, 5, and 6 mM) or methyl viologen (MV; 0.3, 0.5, and 0.7 μM). The resulting plants were allowed to grow for 10 days before analysis.

Fresh weight, chlorophyll fluorescence, and ion leakage assay

Untransformed WT, *35SΩ:HsfA3*, and *hsfa3* seeds were germinated and grown on MSO medium under normal growth conditions and exposed to oxidative stress treatments. After 10 days, the plants were weighed and their chlorophyll fluorescence was measured according to Yu et al. (2002). The plants

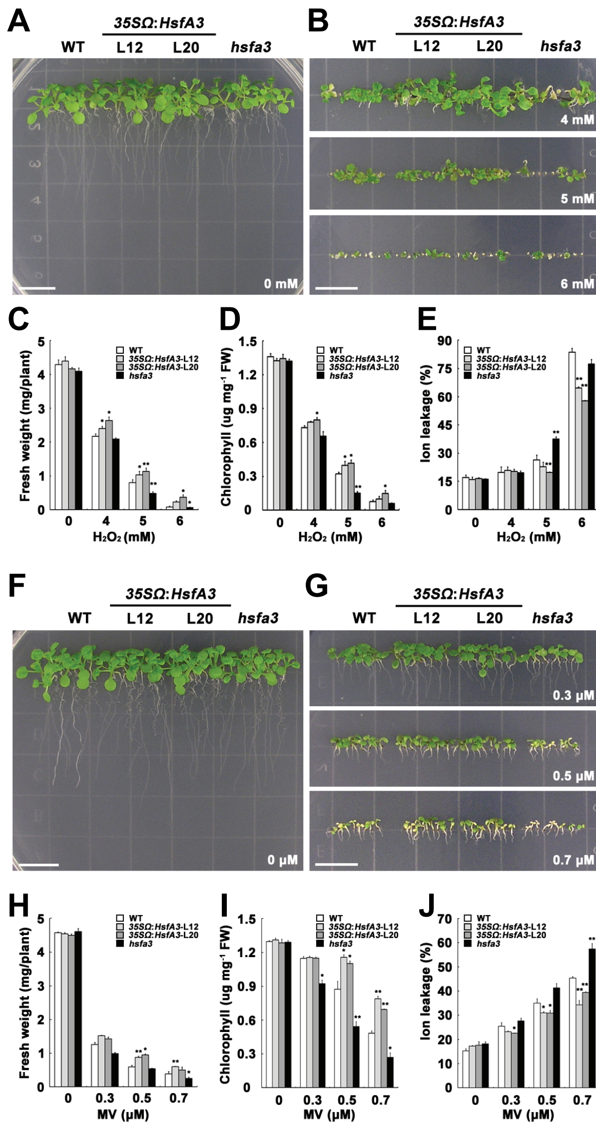


Fig. 2. Oxidative stress tolerance of transgenic *Arabidopsis* plants. Imbibed seeds of wild type (WT), two independent *35SΩ:HsfA3* transgenic lines (L12 and L20), and *HsfA3* knockout line (*hsfa3*) were incubated on 1/2MSO plates without (A) and with H_2O_2 (B). Plates were incubated vertically and photographed after 10 days. Scale bars: 1 cm. Fresh weight of upper plant parts (C), chlorophyll content of upper plant parts (D), and ion leakage of seedlings (E) were measured for the plants shown in (A) and (B). The data were obtained from an average of 100 seedlings in triplicate. Bars represent mean ± SD ($n = 3$; $^{*}P > 0.05$; $^{**}P > 0.01$ by Student's *t*-test). (F-J) are the same as (A-E), but plants were treated with different concentrations of methyl viologen (MV).

were dark-adapted for 15 min just before harvest, and chlorophyll fluorescence was measured at room temperature using a plant efficiency analyzer (Handy PEA, Hansatech Instruments, UK). Ion leakage was measured according to Scarpeci et al. (2008). Ten-day-old Col-0 and transgenic plants grown on solid MSO plates as above were uprooted, thoroughly rinsed with de-ionized water, and suspended in de-ionized water. The conductivity of the suspension solution was measured with a conductance meter (Orion 3-Star Plus, Thermo Scientific, USA) before and after autoclaving (121°C for 15 min) to release all electrolytes.

Transient transactivation assay

The effector construct contained *HsfA3* cDNA fused in-frame at its N-terminus to a Flag-tag sequence (F), which was expressed constitutively from a chimeric promoter consisting of the *CaMV35S* promoter and the TMV translation enhancing omega element (*Ω*). This construct was designated *35SΩ:F-HsfA3* (Fig. 4A). For reporter constructs, putative promoter sequences of the *GolS* genes were amplified by PCR from Col-0 genomic DNA using *GolS* gene-specific primer sets (Supplementary Table 2). An approximately 1.0 kb fragment of each of the *GolS* upstream regulatory regions was inserted upstream of the *β*-glucuronidase reporter gene (*GUS*) in a pUC19-derived plasmid vector (Kim et al., 2007) to yield reporter constructs $Prom_{GolS1}$*:GUS*, $Prom_{GolS2}$*:GUS*, $Prom_{GolS3}$*:GUS*, and $Prom_{GolS4}$*:GUS* (Fig. 4A).

Transient transactivation assays were performed as described by Song et al. (2014) using *Arabidopsis* leaf protoplasts (300 μl, 5×10^6/ml), 15 μg of effector construct, 15 μg of *GUS* reporter construct, and 5 μg of $Prom_{35S}$*:LUC* construct. Quantitative GUS activity assays were performed on protein extracts using the fluorogenic substrate 4-methy-lumbellyferyl-*β*-D-glucuronide (4-MUG; Sigma-Aldrich, USA), as described previously (Jefferson et al., 1987). Luciferase assays were conducted using the Luciferase Assay System (Promega, USA) and measured on a 20/20^n Luminometer (Turner BioSystems, USA) as described by Chen et al. (2010). Promoter activity was calculated as units of GUS activity per unit of luciferase activity.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assays were performed as described by Chen et al. (2010). Synthetic double-stranded (ds) oligonucleotides spanning the HSE in each of the native *GolS1*, *GolS2*, and *GolS4* promoters were designated *GolS1-wHSE*, *GolS2-wHSE*, and *GolS4-wHSE*, respectively (Fig. 4). One variant of each ds-oligonucleotide probe, containing mutations in the HSE, was also synthesized; these probes were designated *GolS1-mHSE* and *GolS2-mHSE* (Fig. 4A). ^{32}P-labeling of the probes and purification of recombinant glutathione-*S*-transferase (GST)-HsfA3 were carried out as described by Lim et al. (2007).

Measurement of galactinol levels

Galactinol was extracted and measured according to Nishizawa et al. (2008). The sixth and seventh leaves of 4-week-old plants were weighed to obtain fresh weight (FW), ground to a fine powder in liquid N_2, and homogenized in 10 ml of 80% ethanol at 80°C. The homogenate was boiled for 10 min at 90°C, centrifuged for 10 min at 7,000 × *g*, and extracted twice in 1 ml of 80% ethanol at 90°C. The extracts were dried and dissolved in water (0.5 mg/ml). Galactinol levels were analyzed by high performance anion exchange chromatography using a CarboPac PA100 column (3 × 250 mm) on a Dionex ICS-3000 gradient system coupled with pulsed amperometric detection system (Thermo Scientific). Fifteen microliter samples were injected onto the CarboPac PA100 column and separated in a NaOH gradient. The flow rate was 1 ml/min. Identification and quantification were performed against a galactinol chemical standard (Fluka, USA).

RESULTS

HsfA3 transcription is induced by oxidative stress treatment

To verify the effect of oxidative stress on *HsfA3* expression, we exposed 10-day-old WT plants to exogenous H_2O_2 or to the endogenous H_2O_2 propagator DBMIB (Šlesak et al., 2003) under low light conditions. As determined by RT-PCR, *HsfA3* transcript levels reached a peak at 3 h of H_2O_2 treatment, followed by a rapid decline. The peak *HsfA*3 transcript level was approximately 7-fold higher than that under untreated conditions (Fig. 1). Although its expression levels were lower than under H_2O_2 treatment, *HsfA3* expression rapidly increased within 1 h of DBMIB treatment, followed by a rapid return to baseline levels after 6 h of treatment. Other studies have also demonstrated that treatment with the superoxide anion propagator MV, or low light plus DBMIB, rapidly induces the expression of *HsfA3* (Guan et al., 2013; Jung et al., 2013). These findings demonstrate that *HsfA3* is an oxidative stress-responsive gene, and they suggest that the transcription factor HsfA3 regulates the HS-induced oxidative stress response (Chen et al., 2010).

Overexpressing *HsfA3* increases oxidative stress tolerance in *Arabidopsis*

Since the expression of *HsfA3* was triggered by oxidative stress, we investigated whether *HsfA3* overexpression is associated with increased oxidative stress tolerance. We compared a number of morphological phenotypes of WT and *HsfA3* transgenic *Arabidopsis* plants, including an *HsfA3* overexpressor (*35SΩ:HsfA3*) and knockout mutant (*hsfa3*), which were grown *in vitro* in the absence or presence of various concentrations of H_2O_2 or MV (Fig. 2). Under normal growth conditions (no H_2O_2 or MV), the *HsfA3* transgenic plants exhibited no differences in morphology or growth compared to WT (Figs. 2A and 2F). However, as the concentrations of H_2O_2 or MV increased, the growth inhibition and degree of leaf yellowing gradually became more severe in both the control and transgenic plants, but the growth inhibition was much more severe in WT and *hsfa3* than in the *HsfA3* overexpressors (Figs. 2B and 2G). For example, the FW of the overexpressors was reduced by approximately 45% in response to 4 mM H_2O_2 treatment, 75% in response to 5 mM H_2O_2 treatment, and 90% in response to 6 mM H_2O_2 treatment. In WT, the FW was reduced by 50%, 80%, and 98% in response to 4, 5, and 6 mM H_2O_2 treatment, respectively. The reduction in FW was much more severe in *hsfa3* than in WT, with a reduction of 90% in response to 5 mM H_2O_2 treatment (Fig. 2C). The chlorophyll contents were altered in a similar manner (Fig. 2D). Ion leakage measurements revealed that although 5 and 6 mM H_2O_2 treatment caused considerable ion leakage in all plants examined, ion leakage rates were approximately 10% lower in H_2O_2-treated *HsfA3* overexpressors compared to WT or *hsfa3* plants (Fig. 2E). When WT and *HsfA3* transgenic plants were grown on MSO agar medium supplemented with various concentrations of MV for 10 days, the FW of the *HsfA3* overexpressors was reduced by approximately 70% on 0.3 μM MV, 80% in 0.5 μM MV, and 90% on 0.7 μM MV. By contrast, the FW of WT plants was reduced by 80%, 90%, and 95% on 0.3 μM, 0.5 μM, and 0.7 μM MV, respectively (Fig. 2H). The response of *hsfa3* was more severe than that of WT. The chlorophyll contents in the *HsfA3* overexpressors after 10 d of MV treatment were higher than those of WT and *hsfa3* plants: the chlorophyll contents were almost twice as high as both WT and *hsfa3* levels after 0.7 μM MV treatment (Fig. 2I), suggesting that HsfA3 prevents chlorophyll degradation to maintain plant photosynthesis under oxidative stress conditions. While in MV-treated WT and *hsfa3* plants, ion leakage increased by 50-60% after 0.7 μM MV treatment, this value increased by only 30-40% in the overexpressors (Fig. 2J), indicating that there was a much lower degree of ROS-induced damage in the *HsfA3* overexpressors than in WT and knockout plants. These results indicate that *HsfA3* increases oxidative stress tolerance during seed germination and seedling growth.

Expression of *GolS* genes in *HsfA3* overexpressors

Oxidative stress is neutralized by ROS detoxifying enzymes and by the biosynthesis of compatible solutes, such as amino acids, quaternary ammonium compounds, amines, and several sugars, such as RFOs (Jung et al., 2013). HsfA3 can directly bind to the HSEs of genes encoding ROS detoxifying enzymes, especially the ascorbate peroxidase 2 (*APX2*) gene (Hwang et al., 2012; Jung et al., 2013). By contrast, little is known about how HsfA3 regulates the biosynthetic genes of compatible solutes. Therefore, we performed quantitative RT-PCR to compare the expression levels of galactinol synthase (GolS; EC 2.4.1.123) genes in WT and transgenic plants, as GolS catalyzes the first committed step in the biosynthesis of RFOs (Lahuta et al., 2014). *Arabidopsis* contains seven *GolS* genes (*GolS1* to *GolS7*) (Taji et al., 2002), including *GolS1* to *GolS4*, which were shown to be induced by several abiotic stress treatments; experimental proof of the protective role of RFOs came from the analysis of transgenic plants (Nishizawa et al., 2008). Therefore, we examined the expression of *GolS1* to *GolS4* in WT and *HsfA3* transgenic seedlings under normal growth conditions. As shown in Fig. 3A, *GolS1*, *2*, and *4*, but not *GolS3*, were induced in *HsfA3* transgenic seedlings. The levels of *GolS1*, *2*, and *4* transcripts were approximately 2.5-9.5-fold higher in the *HsfA3* overexpressors than in WT and *hsfa3* seedlings (Fig. 3B).

Since these *GolS* transcripts were upregulated in the *HsfA3* overexpressors, we investigated whether the upregulated *GolS* transcripts were associated with galactinol biosynthesis. Specifically, we examined galactinol levels in the leaves of WT, overexpressors, and *hsfa3* plants under normal growth conditions (Table 1). In the WT, the level of galactinol was 22.9 ± 2.8 nmol/g FW. Galactinol clearly accumulated in the *HsfA3* overexpressors (36.6 ± 0.8 and 102.1 ± 11.3 nmol/g FW), whereas *hsfa3* contained only 13.8 ± 1.8 nmol/g FW galactinol. These results suggest that HsfA3 activates the transcription of *GolS* genes and that increased *GolS* transcript levels lead to increased galactinol biosynthesis during seed germination and seedling growth.

HsfA3 *trans*-activates *GolS* gene expression

Based on the oxidative stress tolerance phenotype of the *HsfA3* overexpressors (Fig. 2) and the observation that *GolS* expression was induced in these plants (Fig. 3), we reasoned that oxidative stress triggers the expression of *HsfA3* and that HsfA3 in turn activates the expression of *GolS*s. To test this hypothesis, we carried out transient promoter activation assays. An approximately 1 kb region upstream of the putative transcriptional start site of *GolS1* contains a tandem inverted repeats of the HSE (5′-TTCCAGAACTTTC-3′; core HSE is underlined), and both *GolS2* (5′-GAAGCTTC-3′) and *GolS4* (5′-TTCATGAA-3′) contain a symmetrical HSE, but the *GolS3* promoter sequence does not (Fig. 4A). As shown in Fig. 4B, the GUS/LUS activity of the *GolS1* construct ($Prom_{GolS1}$:*GUS*) increased 26-fold in transformation reactions including the

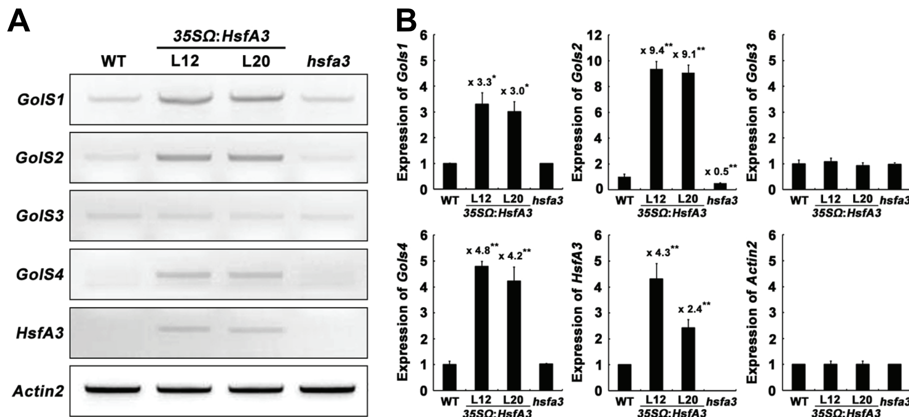
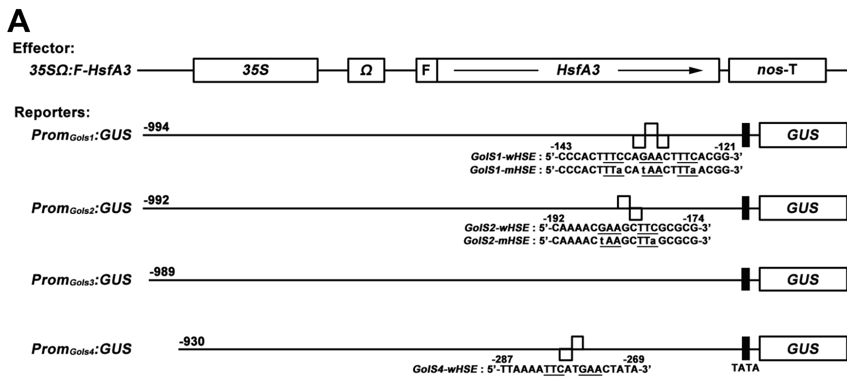
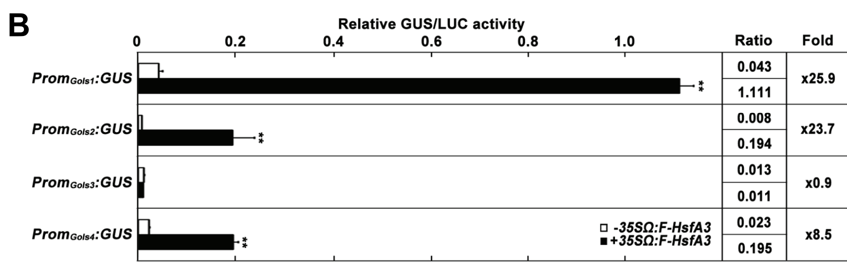


Fig. 3. Comparison of transcript levels of *GolS* genes in *HsfA3* overexpressors. (A) Expression of *GolS* genes was evaluated by RT-PCR from total RNA isolated from wild type (WT), *HsfA3*-overexpressing transgenic lines (*35SΩ:HsfA3*-L12 and *35SΩ:HsfA3*-L20), and *HsfA3* knockout mutant (*hsfa3*). Total RNA was extracted from 10-day-old plants grown under normal conditions. *Actin2* was used as an internal control. PCR was performed for 30 cycles for all targets. The expected sizes of amplicons and gene-specific primers used are described in Supplementary Table 1. (B) Relative expression level of each gene compared to its expression level in WT is indicated above the bars. Expression level of WT was set to 1. Bars represent mean ± SD ($n = 3$; * $P < 0.05$; ** $P < 0.01$ by Student's *t*-test).





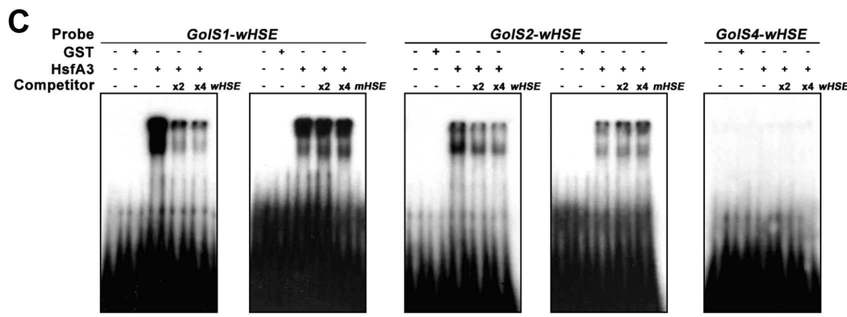


Fig. 4. HsfA3 binds to heat stress elements in the *GolS* promoters. (A) Transient promoter activation assays were performed by co-transformation of the effector construct (*35SΩ:F-HsfA3*) with reporter constructs containing the indicated *GolS* promoters fused to *GUS* and a construct containing *LUS* for normalization of transformation efficiency. The effector for constitutive overexpression of *HsfA3* contained N-terminal Flag (F)-tagged *HsfA3* under the control of the *CaMV35S* promoter (*35S*) and the TMV Ω enhancer (Ω). The nopaline synthase gene terminator (*nos*-T) was fused to the *HsfA3* ORF. The reporter constructs contained each of the native *GolS1-4* promoter fragments fused to *GUS* (*Prom$_{GolS1}$:GUS; Prom$_{GolS2}$:GUS; Prom$_{GolS3}$:GUS; Prom$_{GolS4}$:GUS*). Putative promoter sequences of *GolS* genes were amplified by PCR from WT genomic DNA using *GolS* gene-specific primer sets (Supplementary Table S2). The empty boxes above the line represent nGAAn in the *GolS* promoters, and those below the line represent nTTCn. Solid boxes on the line represent the TATA-box. Probes denote sequences of the oligonucleotides used in EMSA. Wild-type *GolS1* (*GolS1-wHSE*), *GolS2* (*GolS2-wHSE*), and *GolS4 HSE* (*GolS4-wHSE*) are oligonucleotides containing the conserved HSEs (underlined). Mutant *GolS1 HSE* (*GolS1-mHSE*) and *GolS2 HSE* (*GolS2-mHSE*) are oligonucleotides containing the mutations indicated in lower-case letters. (B) Histogram indicating GUS/LUS activity in transformants harboring the corresponding reporter construct with (or without) the effector construct, as indicated. The mean value depicted by each bar is indicated under the ratio. Fold represents the fold-increase in GUS/LUS activity in transformants harboring both reporter and effector compared to the GUS/LUS activity in transformants harboring reporter only. Bars represent mean ± SD ($n = 3$; ** $P < 0.01$ by Student's *t*-test). (C) EMSA of HSE-binding activity of HsfA3. Shown are the autoradiograms of gels used to analyze binding reactions of the indicated composition, where minus (-) indicates omission, plus (+) indicates addition, and "x" indicates double (x2) or 4-fold (x4) amount compared to (+) for the same component.

Table 1. Galactinol contents of the leaves of wild-type, *35SΩ:HsfA3* transgenic, and *hsfa3 Arabidopsis* plants

Line	Galactinol (nmol/g^{-1} FW[a])	Fold change
WT	22.9 ± 2.8[b]	1.0
35SΩ:HsfA3-L12	36.6 ± 0.8	1.6
35SΩ:HsfA3-L20	102.1 ± 11.3	4.5
hsfa3	13.8 ± 1.8	0.7

[a] FW, fresh weight.
[b] Values represent mean ± SD (*n = 3*).

35SΩ:F-HsfA3 effector compared to reactions lacking the effector construct, while that of the *GolS2* construct ($Prom_{GolS2}$*:GUS*) increased 23.7-fold and that of the *GolS4* construct ($Prom_{GolS4}$*:GUS*) increased 8.5-fold. These results indicate that HsfA3 *trans*-activates gene expression from the *GolS1*, *2*, and *4* promoters. The *GolS3* construct ($Prom_{GolS3}$*:GUS*) did not exhibit GUS/LUS activity, as it did not contain a HSE in the promoter sequence.

To directly assess the ability of HsfA3 to physically bind to the promoter of *GolS1*, *GolS2*, or *GolS4*, we purified bacterially expressed GST-HsfA3 fusion protein and characterized the DNA-binding ability of the recombinant protein to the HSE motifs in *GolS* promoters using EMSAs. As shown in Fig. 4C, radiolabeled bands with retarded mobility were observed when ^{32}P-*GolS1-wHSE* and *GolS2-wHSE* were used as probes with GST-HsfA3 but not with GST, indicating that HsfA3 specifically binds to the probe. The signal intensity of the retarded bands was reduced in a concentration-dependent manner by the addition of unlabeled wild-type *HSE*s (*wHSE*s), but not unlabeled mutated *HSE*s (*mHSE*s), to the binding reaction, indicating that the mutated bases are required for interaction with HsfA3. No retarded band was observed in reactions using ^{32}P-*GolS4-wHSE* as a probe, indicating that other factors are involved in the regulation of *GolS* promoter activity *in vivo*.

DISCUSSION

Oxidative stress arising from an imbalance in the generation and removal of ROS is a challenge faced by all aerobic organisms (Scarpeci et al., 2008). Hsfs play a central role in ROS sensing in plants (Davletova et al., 2005). However, little is known about the target genes of *Arabidopsis* Hsfs and their contribution to plant oxidative stress responses.

In the present study, we functionally characterized an *Arabidopsis* class A *Hsf* gene, *HsfA3*, under oxidative stress conditions. *HsfA3* expression was induced in response to exogenous H_2O_2 or DBMIB application in *Arabidopsis* seedlings (Fig. 1), and ectopic overexpression of *HsfA3* improved oxidative stress tolerance in these plants, as revealed by analysis of morphological and biochemical traits (Fig. 2). These findings suggest that HsfA3 is involved in the plant response to oxidative stress and plays a substantial role in oxidative stress signaling. We previously reported that the antioxidant gene *APX2* is upregulated by HsfA3 and that increased APX2 activity reduces the accumulation of H_2O_2, thereby increasing plant tolerance to oxidative damage (Hwang et al., 2012). Additionally, *GolS* and raffinose synthase 2 (*RS2*) genes, encoding committed enzymes in the RFO biosynthetic pathway (Peterbauer and Richter, 2001), are upregulated by HsfA1a, HsfA1b, and HsfA2 in *Hsf* class A-overexpressing transgenic *Arabidopsis* plants, thereby increasing plant tolerance to oxidative damage due to increased galactinol and raffinose levels (Busch et al., 2005; Nishizawa et al., 2006; 2008; Panikulangara et al., 2004). In this study, we also found that the expression of *GolS1*, *GolS2*, and *GolS4* was markedly induced in *HsfA3*-overexpressing transgenic *Arabidopsis* plants (Fig. 3), and intracellular galactinol levels were higher (Table 1) in these plants than in WT plants under control growth conditions. Furthermore, *GolS1* and *GolS2* transcript levels increased in response to salinity and drought stress, and *GolS2*-overexpressing *Arabidopsis* plants exhibited increased tolerance to drought stress (Taji et al., 2002). These findings indicate that in *HsfA1b*-, *HsfA2*-, and *HsfA3*-overexpressing transgenic *Arabidopsis* plants, *GolS* genes are upregulated, resulting in increased levels of galactinol and RFOs. Although it is unclear which products are formed by the reaction between ROS and RFOs, Nishizawa et al. (2008) reported that the enhanced levels of galactinol and raffinose in plants under stressful conditions may be closely related to the maintenance of ascorbate and glutathione levels in these plants. Hence, all of these findings suggest that several HsfAs regulate the expression of oxidative stress-responsive genes and that the target genes of HsfAs may contribute to increased biosynthesis of galactinol and RFOs in plants under stressful conditions. It is possible that galactinol and RFOs act as osmoprotectants and antioxidants, and that they play a role in protecting plants from oxidative damage caused by several abiotic stresses, especially HS.

In the current study, promoter transactivation assays and competitive EMSAs demonstrated that recombinant HsfA3 activates *GolS1*, *GolS2*, and *GolS4* transcription and directly binds to *GolS1* and *GolS2 via* the HSE motifs in their promoters (Fig. 4). *GolS3* was not induced by HsfA3 effector and failed to physically bind to HsfA3, as *GolS3* lacks a HSE in its promoter sequence. Similarly, Nishizawa et al. (2006) reported that HsfA2 can only activate HSE-dependent transcription of *GolS1* and *GolS2*. Thus, both HsfA2 and HsfA3 appear to activate *GolS1* and *GolS2* expression through interaction with the HSEs in their promoters. Interestingly, the *GolS1* promoter contains a tandem inverted repeat of the short consensus sequence nGAAn (*GolS1-wHSE*; 5′-nTTCnnGAAnnTTCn-3′, referred to as the "perfect" HSE) (Guo et al., 2008), whereas the *GolS2* promoter contains an adjacent motif (*GolS2-wHSE*; 5′-GAAnnTCC-3). The signal generated by binding of HsfA3 to *GolS1-wHSE* was stronger than that with *GolS2-wHSE*, providing additional evidence that HsfA3 binds more efficiently to tandem invert repeats of the HSE motif than to single adjacent HSE motifs. Interestingly, co-transformation with the *35SΩ:F-HsfA3* construct induced the expression of $Prom_{GolS4}$*:GUS* in promoter transactivation assays, with an approximately 8.5-fold increase in GUS activity (Fig. 4B), but HsfA3 did not physically bind to the *GolS4* promoter in EMSAs (Fig. 4C). This result indicates that *GolS4-wHSE* HSE sequences do not directly bind to HsfA3. This finding supports the notion that other transcription factors downstream of HsfA3 are involved in the regulation of *GolS4* promoter activity.

In light of these results, we propose a model for the role of HsfA3 in the oxidative stress response in *Arabidopsis*. HsfA3 binds to the promoters of *GolS* genes and induces their expression. This, in turn, results in an increase in galactinol and RFO biosynthesis, which leads to a decrease in ROS levels. This regulatory cascade enhances the ability of the plant to adapt to oxidative stress.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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