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Molecules and Cells

Overexpression of *Arabidopsis* Dehydration-Responsive Element-Binding Protein 2C Confers Tolerance to Oxidative Stress

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Dehydration-responsive element-binding proteins (DREBs) regulate plant responses to environmental stresses. In the current study, transcription of *DREB2C*, a class 2 *Arabidopsis DREB*, was induced by a superoxide anion propagator, methyl viologen (MV). The oxidative stress tolerance of *DREB2C*-overexpressing transgenic plants was significantly greater than that of wild-type plants, as measured by ion leakage and chlorophyll fluorescence under light conditions. The transcriptional activity of several ascorbate peroxidase (*APX*) genes as well as APX protein activity was induced in *DREB2C* overexpressors. Additionally, the level of H_2O_2 in the overexpressors was lower than in wt plants under similar oxidative stress conditions. An electrophoretic mobility shift assay and transient activator-reporter assay showed that *APX2* expression was regulated by heat shock factor A3 (HsfA3) and that *HsfA3* is regulated at the transcriptional level by DREB2C. These results suggest that *DREB2C* plays an important role in promoting oxidative stress tolerance in *Arabidopsis*.

INTRODUCTION

DREBs belong to the AP2/ERF family of transcription factors (Yamaguchi-Shinozaki and Shinozaki, 1994), which confer stress endurance in plants and are the largest and most diverse family of proteins involved in the regulation of plant responses (Agarwal et al., 2007). In *Arabidopsis*, there are two classes of *DREB* genes, class 1 and class 2 (*DREB1* and *DREB2*, respectively) (Sakuma et al., 2002). The deduced amino acid sequences of class 1 and class 2 genes show no significant level of sequence similarity except in their conserved DNA-binding domains (Jaglo-Ottosen et al., 1998). *DREB1* genes are transcriptionally induced by low temperature, and the corresponding gene products induce the expression of multiple target genes that impart tolerance to freezing and drought in transgenic plants (Liu et al., 1998; Oh et al., 2007). *DREB2* genes were initially identified as drought and high-salinity response genes (Liu et al., 1998), and were later shown to be induced in response to heat stress as well (Lim et al., 2007; Sakuma et al., 2006). Recent studies identified *heat shock transcription factor A3* (*HsfA3*) as a highly up-regulated heat-inducible gene in transgenic plants over-expressing DREB2s (Chen et al., 2010; Yoshida et al., 2008). Moreover, transient promoter reporter assays using mesophyll protoplasts demonstrated that *HsfA3* expression is directly regulated by DREB2s under conditions of heat stress (Chen et al., 2010; Schramm et al., 2008) and that *DREB2*-overexpressing transgenic plants have increased tolerance to heat stress (Almoguera et al., 2009; Lee et al., 2010; Lim et al., 2007; Matsukura et al., 2010). Recent work established that DREB2C physically interacts with ABF2, a bZIP protein regulating abscisic acid (ABA)-responsive gene expression, and its overexpression affected ABA sensitivity (Lee et al., 2010). Thus, the results suggesting that DREB2s may function as multi stimuli-response factor that interact with genes and/or proteins during different stress conditions (Lee et al., 2009). Despite the important roles played by DREB2s in abiotic stress responses, regulation with respect to oxidative stress has not been determined.

In the current study, oxidative damage triggered the expression of *DREB2C* at the mRNA level. The expression of *DREB2C* correlated with the up-regulation of *HsfA3* and transcriptional activation of the antioxidant gene *APX2* in *Arabidopsis*. The current results suggest a model in which DREB2C activates *HsfA3* expression, and HsfA3 in turn regulates the expression of numerous oxidative stress-inducible genes, including *APX2,* to impart tolerance to oxidative stress.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. ecotype Columbia (Col-0) plants or *DREB2C*-overexpressing transgenic *Arabidopsis* Col-0 plants (Lim et al., 2007) were grown on 1× MS medium without any phytohormones (MSO, pH 5.8) with 2% sucrose under a 16 h light cycle (cool-white fluorescent light, photon flux of 70 μM $m^{-2}s^{-1}$) at 22°C. To induce synchronous germination, seeds were vernalized at 4°C for three days in the dark and then transferred to a growth chamber. All external stress treatments were performed on 2-week-old plants.

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RNA extraction and reverse transcriptase (RT)-PCR
To analyze the expression of *DREB2C*, RNA was isolated from wt *Arabidopsis* Col-0 plants exposed to 10 μM MV. For RT-PCR analysis, cDNA was synthesized from 2 μg of total RNA. Each cDNA sample was diluted 1:10 and then 1 μl of the diluted cDNA was used as the template for PCR amplification, as described previously (Lim et al., 2007).

To examine the expression of *Arabidopsis APX* isoforms, total RNA was isolated from 2-week-old *DREB2C*-overexpressing transgenic lines grown under normal growth conditions. Complementary DNA synthesis and RT-PCR were performed as described above using *APX* gene primers (Supplementary Table S1). PCR products were sequenced directly to confirm that the amplified sequences were identical to the predicted sequences of the respective mRNAs based on *Arabidopsis* genomic data.

Ion leakage assay
Ion leakage was measured according to Scarpeci et al. (2008), with minor modifications. Two-week-old wt and *DREB2C*-overexpressing *Arabidopsis* plants grown on solid MSO were uprooted, thoroughly rinsed with de-ionized water, and then incubated in water (control) or in water supplemented with 0.5 μM MV for 1 to 4 days under dim light conditions at 22°C. After incubation, the conductivity of the suspension solution was measured with a conductance meter (Orion 3-Star Plus, Thermo Scientific, USA) before and after autoclaving at 121°C for 15 min to release all electrolytes. Relative ion leakage was expressed as a percentage of the total conductivity.

Chlorophyll fluorescence measurement
MV-treated *Arabidopsis* plants were dark-adapted for 15 min. *In vivo* chlorophyll fluorescence was measured at room temperature using a plant efficiency analyzer (Handy PEA, Hansatech Instruments, UK), according to the method described by Yu et al. (2002). Maximum quantum efficiency (Fv/Fm) was calculated as previously described (Baker and Rosenqvist, 2004).

Protein extraction and APX activity assay
To monitor the activity of APX, soluble protein fractions were extracted from 100 mg of 2-week-old wt or transgenic *Arabidopsis* plants as previously described (Panchuk et al., 2002). Protein concentrations were determined using a Bradford Protein Assay Kit (Bio-Rad, USA) and bovine serum albumin as a reference. The oxidation rate of AsA was measured by UV/Vis spectroscopy (Genesys 10 UV, Spectronic Unicam, USA) by monitoring the decrease in A_{290} 1 min after the addition of protein extract.

3,3′-Diaminobenzidine (DAB) staining and H_2O_2 measurement
DAB staining was performed according to Thordal-Christensen et al. (1997), with slight modifications. Two-week-old wt and *DREB2C*-transgenic plants grown in 1× MSO medium were transferred to water or 0.5 μM MV and then incubated for 2 days under a 16 h light/8 h dark cycle at 22°C. Following this, plants were incubated for 9 h in 1 mg ml^{-1} DAB-HCl (pH 3.8) under dim light. Chlorophyll was removed by soaking the plants in a graded ethanol series (90, 80, 70, 60, 50, 30, 10%) for several hours. H_2O_2 causes polymerization of DAB, which results in a brown color.

Quantitative analysis of H_2O_2 was performed as described by Xing et al. (2007). Wt and transgenic plants were transferred to water (control) or 0.5 μM MV and then incubated for 2 days under a 16 h light/8 h dark cycle at 22°C. H_2O_2 was quantified using the Amplex Red Assay Kit (Invitrogen, USA) and a fluorescence microplate reader (SpectraMax Gemini XPS Molecular Devices, USA). Excitation wavelength was set at 530 nm and fluorescence was measured at 590 nm.

Promoter sequence analysis
Promoter sequences were analyzed to identify putative *cis*-elements in the *APX2* (At3g09640) and *HsfA3* (At5g03720) gene promoters. The upstream sequences of *APX2* and *HsfA3* (711- and 1094-bp, upstream of the transcription start site, respectively), were retrieved from The Arabidopsis Information Resource (http://www.arabidopsis.org/tools/bulk/sequences/index.jsp). These sequences were analyzed for over-represented motifs using MEME (http://meme.sdsc.edu/meme/) (Bailey and Elkan, 1995).

Electrophoretic mobility shift assay (EMSA)
The EMSA was performed as described by Chen et al. (2010). The probes were as follows: an 85-bp [α-^{32}P]dATP-labeled fragment (*APX2-HSE-85*) containing the heat shock element (HSE) within the *APX2* promoter, and an 80-bp fragment (*HsfA3-DRE-80*) containing the dehydration responsive element /C-repeat (DRE/CRT) element within the *HsfA3* promoter (Supplementary Fig. S1). Recombinant glutathione-*S*-transferase (GST)-DREB2C$^{145-528}$ or GST-HsfA3 was expressed in *E. coli* BL21 (DE3) pLysE and purified using affinity chromatography as described by Lim et al. (2007). The specificity of *cis* element-binding was measured by competition assays using unlabeled *APX2-HSE-85* and *HsfA3-DRE-80* oligonucleotides (Chen et al., 2010).

Activator and reporter constructs used in the transient expression assays
To determine the levels of expression of *DREB2C* and *HsfA3* in mesophyll protoplasts, PCR was carried out using cDNA isolated from *Arabidopsis* seedlings as the template and gene-specific primers (Supplementary Table S2). Amplified sequences were sub-cloned into a pUC19-derived plasmid between the CaMV 35S promoter and nopaline synthase terminator (Kim et al., 2007).

For promoter-reporter plasmid construction, the putative promoter sequences of *APX2* (856-bp) and *HsfA3* (1094-bp) (Supplementary Fig. S1) were amplified by PCR from genomic DNA using gene-specific primers (Supplementary Table S2). Amplified sequences were fused in-frame to the *GUS* gene sequence in a pUC19-derived plasmid (Kim et al., 2007). Protoplasts isolated from *Arabidopsis* mesophyll cells were transiently transformed using polyethylene glycol-calcium fusion, according to Yoo et al. (2007). GUS reporter assays and analysis of protein expression in *Arabidopsis* protoplasts were performed as described previously (Kim et al., 2007).

RESULTS AND DISCUSSION

DREB2C transcription is induced by oxidative stress
To determine the effect of oxidative stress on *DREB2C* expression, 2-week-old wild-type (wt) *Arabidopsis* plants were exposed to exogenous MV. Figure 1A shows that *DREB2C* transcripts were elevated in 3 h after treatment with MV, reached a peak at 6 h and remained elevated. These results clearly demonstrate that *DREB2C* is an oxidative stress-responsive gene, and suggest that *DREB2C* may be involved in the plant response to oxidative stress.

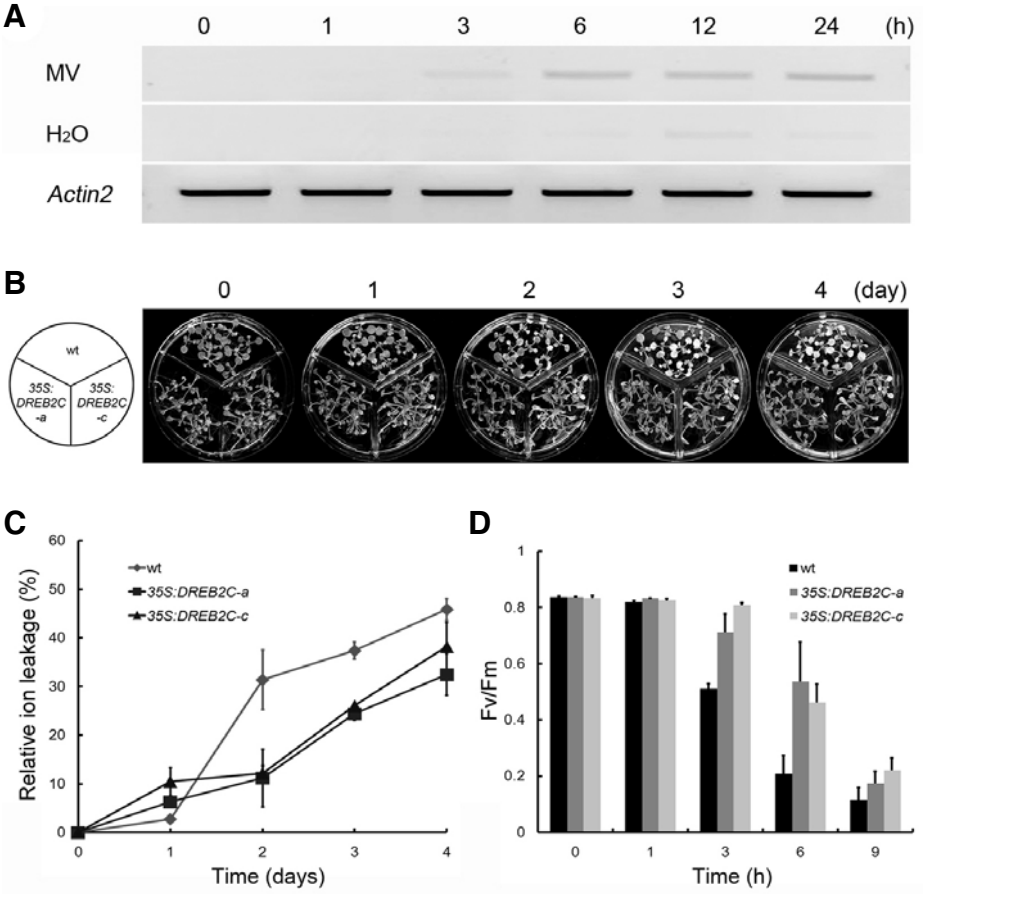

Fig. 1. Enhanced oxidative stress tolerance of *DREB2C* overexpressors. (A) Changes in *DREB2C* transcript levels in response to oxidative stresses. RT-PCR analysis was used to determine mRNA transcript levels. Two-week-old plants were exposed to 10 μM MV for the indicated periods of time. *Arabidopsis Actin2* (At3g18780) served as a control. (B) Two-week-old plants (wt and *DREB2C* overexpressors) grown at 22°C were exposed to 0.5 μM MV for the indicated time periods. Scale bar = 0.5 cm. (C) Ion leakage, an indicator of oxidative stress tolerance, was estimated as a percentage of total conductivity. Two-week-old plants grown at 22°C were exposed to 0.5 μM MV for the indicated periods of time. (D) PSII activity (Fv/Fm) was measured at 22°C after adaptation to the dark for 30 min. Two-week-old plants grown at 22°C were exposed to 0.5 μM MV for the indicated periods of time. Data represent the means ± SE of at least five independent experiments.

DREB2C-overexpressing plants exhibit increased tolerance to MV-induced oxidative stress

DREB2C transcription in wt plants was substantially elevated after treatment with MV (Fig. 1A). To investigate whether transgenic plants overexpressing *DREB2C* exhibited oxidative stress tolerance, *DREB2C* overexpressors (*35S:DREB2C-a* and *-c*) (Lim et al., 2007) and wt plants were placed in a solution of 0.5 μM MV under 16 h light/8 h dark conditions at 22°C. As shown Fig. 1B, wt plants exhibited more severe injury than *35S: DREB2C-a* and *-c* plants two days after exposure to MV. Ion leakage and photosynthesis efficiency, which reflect the level of cellular damage due to oxidative stress (Noriega et al., 2007), were also measured. As shown Fig. 1C, MV caused severe ion leakage in wt plants, whereas *DREB2C*-transgenic plants were much less affected. PSII activity (Fv/Fm) gradually decreased in both the wt and transgenic plants (Fig. 1D). There was no difference in PSII activity between wt and *DREB2C* overexpressors exposed the plants for 1 h in MV solution, whereas after 3 h, PSII activity in the *DREB2C* overexpressors was significantly higher than wt plants. This conditional phenotype suggested that DREB2C plays a substantial role in oxidative stress tolerance, perhaps at the level of regulation of cellular antioxidant gene expression.

APX genes were induced in DREB2C overexpressors

Since *DREB2C* expression was regulated by oxidative stress (Fig. 1A) and *DREB2C* overexpressors exhibited an increased tolerance to oxidative stress (Figs. 1B-1D), we hypothesized that DREB2C may regulate oxidative stress-inducible antioxidant genes. Among antioxidants, APXs play a pivotal role in removing reactive oxygen species by catalyzing the conversion of highly damaging H_2O_2 into H_2O (Giacomelli et al., 2007). Hence, we assessed the expression of nine *Arabidopsis APX* isoforms (Mittler et al., 2004) in *DREB2C* overexpressors by RT-PCR. As shown Fig. 2A, expression of the cytosolic (*APX2*) and mitochondrial (*APX7*) *APX* isoforms was induced in *DREB2C* overexpressors under non-stressed conditions, which suggested that DREB2C may activate the transcription of these antioxidant genes in *Arabidopsis*.

Additionally, to determine whether *DREB2C* overexpression resulted in increased APX protein activity, APX activity was measured by soluble enzymatic activity assay (Panchuk et al., 2002). As shown in Fig. 2B, there was no significant difference in APX activity between wt and *DREB2C* overexpressors under

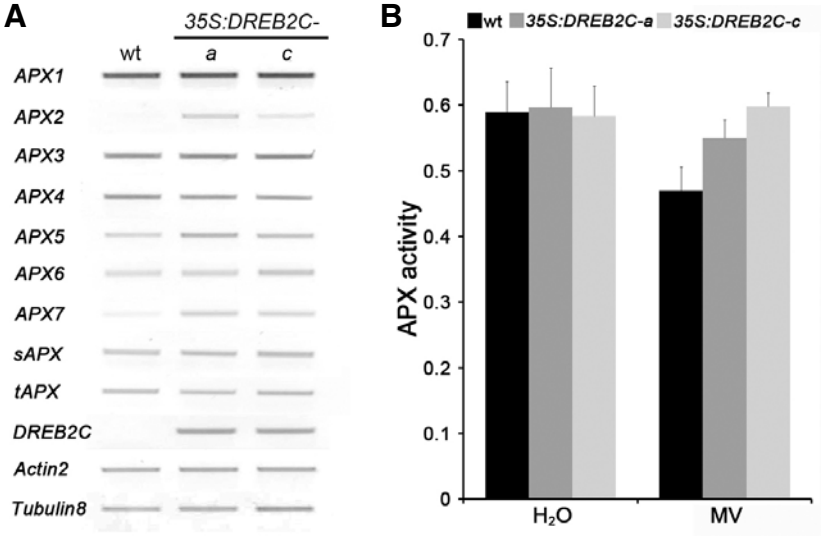

Fig. 2. Expression of *APX* genes is elevated in *DREB2C* overexpressors. (A) Expression of nine *Arabidopsis APX* genes in *DREB2C* overexpressors was analyzed by RT-PCR using gene-specific primers (Supplementary Table 1). *Arabidopsis Actin2* (At3g18780) and *Tublin8* (At5g23860) were used as constitutively-expressed controls. Total RNA was isolated from 2-week-old plants (wt, *35S:DREB2C-a*, and *35S:DREB2C-c*). (B) APX activity in *DREB2C* overexpressors. Total soluble APX activity in 2-week-old wt and transgenic plants (*35S:DREB2C-a*, *35S: DREB2C-c*) incubated in water (H_2O) or 0.5 μM MV for two days. Data represent the means ± SE of at least five independent experiments.

control conditions. However, APX activity persisted in *DREB2C* overexpressors grown for two days in MV solution, but not in the wt. These data revealed that the transcriptional activity of *APX*s and intracellular levels of APX was sustained in *DREB2C* overexpressors.

H_2O_2 accumulation is not increased in DREB2C overexpressors

H_2O_2 accumulation in *DREB2C* overexpressors was examined after exposure to 0.5 μM MV for 2 days. Under conditions of MV stress, more H_2O_2 accumulated in the leaves of wt plants than in *DREB2C* overexpressors (Fig. 3A). The pattern of H_2O_2 accumulation is similar to the appearance of bleaching in plants grown in MV solution (Fig. 1B), both in terms of timing and localization. H_2O_2 levels were also quantified (Fig. 3B). H_2O_2 production in wt leaves exposed to MV stress was higher than in *DREB2C* overexpressors; in fact, in the transgenic plants, similar levels of H_2O_2 were observed in water and MV. These data indicated that persisted levels of APX activity (Fig. 2) may lead to decreased accumulation of H_2O_2.

The APX2 promoter was activated by HsfA3

Based on the oxidative stress tolerance phenotype of *DREB2C* overexpressors (Fig. 1) and the fact that *APX* transcription and enzymatic activity were induced in these plants (Fig. 2), we were interested in the possibility that oxidative damage triggers the expression of *DREB2C*, and that DREB2C in turn induces the expression of *APX*s. To test this hypothesis, *APX2* was selected for further analysis, since the *APX2* promoter has been shown to be strongly induced by oxidative stresses (Karpinski et al., 1999). A 711-bp region of the *APX2* promoter upstream of the putative transcriptional initiation site did not contain a DRE sequence (5′-ACCGAC-3′) but did contain a reversed DRE core sequence (5′-CAGCCA-3′). To determine whether DREB2C was able to bind to this reversed DRE core sequence in the *APX2* promoter, *in vitro* EMSAs were carried out using a 72-bp ^{32}P-dATP-labeled *APX2* probe (*APX2-rDRE-72*, Supplementary Fig. S1A) and protein extracts from *E. coli* expressing recombinant DREB2C$^{145\text{-}528}$ (Lim et al., 2007). There was no detectable binding of the *APX2-rDRE-72* probe to DREB2C$^{145\text{-}528}$ (Supplementary Fig. S2). To further explore

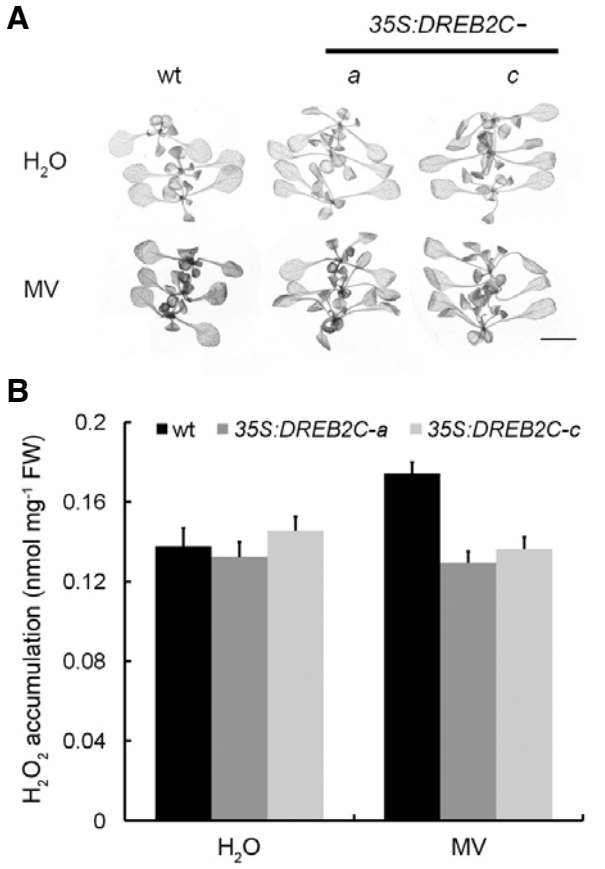

Fig. 3. Accumulation of H_2O_2 in *DREB2C* overexpressors. (A) Histochemical detection of H_2O_2 production by DAB staining. Two-week-old plants (wt, *35S:DREB2C-a*, and *35S:DREB2C-c*) were incubated in water (H_2O) or 0.5 μM MV for two days. Scale bar = 0.5 cm. (B) Hydrogen peroxide measurements were performed on plants grown under the conditions used in DAB staining. All experiments were repeated three times using samples from independent treatments.

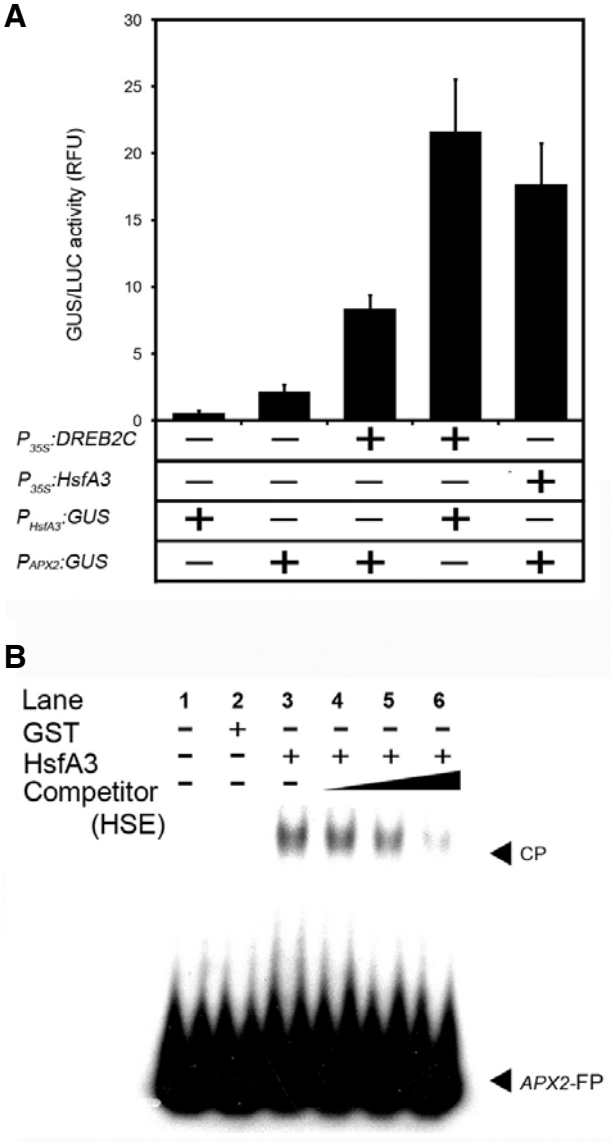

Fig. 4. HsfA3 regulates the expression of *APX2* in a transcriptional cascade downstream of DREB2C. (A) Transient activator-reporter assay in *Arabidopsis* mesophyll protoplasts. The effector constructs consisted of the CaMV 35S promoter fused to full-length *DREB2C* (P_{35S}*:DREB2C*) or *HsfA3* (P_{35S}*:HsfA3*) cDNA. The reporter constructs consisted of the *HsfA3* (P_{HsfA3}*:GUS*) or *APX2* (P_{APX2}*:GUS*) promoter fused to *GUS*. GUS activity (relative fluorescence units, RFU) represents the means ± SD of three independent replicates. (B) EMSAs were performed using GST alone or GST-HsfA3 fusion protein and an 85-bp ^{32}P-labeled fragment containing the HSE-like region from the *APX2* promoter as a probe. Lane 1, *APX2*-free probe; lane 2, 10 μg GST; lane 3, 10 μg GST-HsfA3. For competition assays, 10 μg of GST-HsfA3 was pre-incubated with a 500- (lane 4), 1500- (lane 5) or 3000- (lane 6) fold molar excess of unlabeled *APX2* probe prior to incubation with the ^{32}P-labeled probe. Free (*APX2*-FP) and HsfA3 protein-complexed *APX2* probes (CP) were separated on 5% polyacrylamide gels and visualized by autoradiography.

this interaction between DREB2C and *APX2 in vivo*, transient activator-reporter assays were performed in *Arabidopsis* mesophyll protoplasts transiently expressing full-length *DREB2C* under the control of the CaMV35S promoter (P_{35S}*:DREB2C*). The reporter construct consisted of an 856-bp fragment of the *APX2* promoter (Supplementary Fig. S1A) fused to *GUS* (P_{APX2}*:GUS*). As shown in Fig. 4A, co-transformation with the P_{35S}*:DREB2C* construct induced the expression of *APX2,* with an approximately 4-fold increase in GUS activity. These results supported the idea that there are other transcription factors downstream of DREB2C involved in the regulation of *APX2* promoter activity.

In previous efforts to identify transcription factors that interact with DREB2s in *Arabidopsis*, three research groups reported independently that *HsfA3* functions downstream of the DREB2A stress-regulatory system (Schramm et al., 2008; Yoshida et al., 2008) and DREB2C functions as a transcriptional activator of *HsfA3* during the heat stress response (Chen et al., 2010). Hence, we hypothesized that DREB2C play a role in the activation of *HsfA3*, and that activated HsfA3 may then induce *APX2* gene expression. To test this hypothesis, we explored this interaction between HsfA3 and *APX2 in vivo*, transient activator-reporter assays were performed in protoplasts expressing full-length *HsfA3* (P_{35S}*:HsfA3*) and a reporter construct containing an 856-bp region upstream of the *APX2* promoter fused to *GUS* (P_{APX2}*:GUS*, Supplementary Fig. S1A). As shown in Fig. 4A, co-expression of HsfA3 resulted in an approximately 8-fold increase in the transactivation of the *APX2* reporter gene (P_{APX2}*:GUS*) compared to protoplasts that did not express HsfA3. To further investigate whether the ability of recombinant HsfA3 to bind directly to the *APX2* promoter was assessed by EMSA using an 85-bp *APX2* probe (*APX2-HSE-85*, Supplementary Fig. S1A) containing an intact heat shock element (HSE; nGAAnnTTCn or nTTCnnGAAn). As shown in Fig. 4B, recombinant HsfA3 extracted from *E. coli* bound directly to the *APX2* promoter sequence. These results, based on both GUS reporter assays and EMSAs, indicated that HsfA3 may regulate the expression of *APX2* to promote oxidative stress tolerance in *Arabidopsis*.

In the current study, a novel interaction of HsfA3 with the *APX2* promoter leading to the tolerance of oxidative stress was described. This cascade of regulatory interactions resulted in sustained APX activity and decreased accumulation of H_2O_2, thereby enhancing plant tolerance to oxidative damage. In light of these results, a reasonable model emerges for the role of DREB2C in the *Arabidopsis* oxidative stress response (Supplementary Fig. S3). DREB2C binds to the *HsfA3* promoter and induces the expression of *HsfA3*. This in turn results in the activation of expression of other antioxidant genes, which leads to decreased levels of H_2O_2. This regulatory axis thereby functions to enhance the ability of the plant to adapt to oxidative stress.

The involvement of DREB2C and HsfA3 in the induction of *APX2* expression indicates a crosstalk between heat and oxidative stress signaling (Panchuck et al., 2002). Although further studies are required to fully elucidate the DREB2C network in *Arabidopsis*, DREB2C appears to be a key regulator of heat inducible oxidative stress signaling.

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

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