Reactive oxygen species (ROS) have been characterized as both important signaling molecules and universal stressors that mediate many developmental and physiological responses. So far, details of the transcriptional mechanism of ROS-responsive genes are largely unknown. In the study reported here, we identified seven potential ROS-responsive cis-acting elements (ROSEs) from the promoters of genes up-regulated by ROS in Arabidopsis (Arabidopsis thaliana). We also found that the APETALA2/ethylene-responsive element binding factor6 (ERF6) could bind specifically to the ROSE7/GCC box. Coexpression of ERF6 enhanced luciferase activity driven by ROSE7. The deficient mutants of ERF6 showed growth retardation and higher sensitivity to photodamage. ERF6 interacted physically with mitogen-activated protein kinase6 (MPK6) and also served as a substrate of MPK6. MPK6-mediated ERF6 phosphorylation at both serine-266 and serine-269 affected the dynamic alternation of the ERF6 protein, which resulted in changes in ROS-responsive gene transcription. These data might provide new insight into the mechanisms that regulate ROS-responsive gene transcription via a complex of MPK6, ERF6, and the ROSE7/GCC box under oxidative stress or a fluctuating light environment.

The MPK6-ERF6-ROS-Responsive cis-Acting Element7/GCC Box Complex Modulates Oxidative Gene Transcription and the Oxidative Response in Arabidopsis

Pengcheng Wang, Yanyan Du, Xiaoliang Zhao, Yuchen Miao, and Chun-Peng Song*

Institute of Plant Stress Biology, State Key Laboratory of Cotton Biology, Department of Biology, Henan University, Kaifeng 475001, China

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* Corresponding author; e-mail songcp@henu.edu.cn.

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regulate transcription are highly complicated, and the transcriptional regulator is usually separate from the sensor or transducer of ROS signaling. For example, the yeast (Saccharomyces cerevisiae) glutathione peroxidase Gpx3 acts as a sensor of hydroperoxide and activates the transcription factor Yap1 by promoting the formation of an intramolecular disulfide bond within Yap1 or an intermolecular bond with Gpx3 (Delaunay et al., 2002). This activation of Yap1 in response to oxidative results in its accumulation in the nucleus. Therefore, in this case, the enzyme Gpx3 acts as a receptor or sensor of H$_2$O$_2$ and also functions as a redox transducer to transduce the oxidative signal to the transcription factor (Delaunay et al., 2000, 2002).

In plants, several families of transcription factors, such as MYB, WRKY, zinc transporter (ZAT), heat shock transcription factor, and basic region-leucine zipper (bZIP), have been found to be involved in the regulation of gene transcription in response to oxidative stress (Desikan et al., 2001; Vranová et al., 2002; Pnueli et al., 2003; Rizhsky et al., 2004). For example, the expression levels of Zat12, Zat7, and WRKY25 are elevated simultaneously in cells in response to oxidative stress (i.e. application of H$_2$O$_2$ or paraquat), heat shock, or wounding (Rizhsky et al., 2004). Alterations in gene expression during oxidative stress in plants are often modulated by the protein nonexpressor of pathogenesis-related genes 1 (NPR1) and the TGACG motif-binding proteins (TGAs; Després et al., 2000; Mou et al., 2003). The latter belong to the family of bZIP-type transcription factors. Increasing evidence indicates that ethylene-responsive element-binding factor (ERF) proteins bind to the cis-acting element known as the GCC box to activate the expression of functional genes involved in responses to both abiotic and biotic stress (Ohme-Takagi and Shinshi, 1995; Stockinger et al., 1997; Liu et al., 1998; Riechmann et al., 2000; Chakravarthy et al., 2003; Bethke et al., 2009; Moffat et al., 2012). Tobacco (Nicotiana tabacum) ERF1 to ERF4, Arabidopsis ERF1 to ERF5 and ERF7, and tomato (Solanum lycopersicum) Pto-interacting 4 to Pto-interacting 6 and LeERF1 to LeERF4 appear to be involved in the regulation of many drought- and cold-inducible genes, including rd29A, kin1, cor6.6, rd17, and edr10, as well as the expression of PATHOGENESIS-RELATED (PR) genes and plant resistance against pathogen infection (Ohme-Takagi and Shinshi, 1995; Zhou et al., 1997; Fujimoto et al., 2000; Liu et al., 2000; Wu et al., 2002; Tournier et al., 2003; Song et al., 2005; Song and Galbraith, 2006).

The TGACG motif, which is known as the acs or as1 element, is the only well-characterized ROS-related cis-acting element in plants (Chen and Singh, 1999; Garretón et al., 2002). The bZIP TGA transcription factors bind to TGACG motifs and mediate the transcription of PR genes. However, only a few gene promoters, such as those of NtNPR1a, NtParA, Nt103, Gmhnsp26-A, AtGST, and AtPR-1, contain TGA motifs (Krawczyk et al., 2002). Another cis-acting element, CORE (for coordinate regulatory element for antioxidant defense), has been identified in three antioxidant defense genes in rice induced by oxidative stress and has been found to be involved in the expression of ROS-responsive genes, although its cognate binding protein has not been defined (Tsukamoto et al., 2005). Therefore, the DNA sequences that are regulated by H$_2$O$_2$ and their associations with transcription factors need to be defined and characterized further.

Many findings suggest that the transcription of ROS-responsive genes is regulated by the mitogen-activated protein kinase (MAPK) signaling cascade, which is modulated by redox status (Ulm et al., 2002; Kroj et al., 2003; Lee et al., 2004; Nakagami et al., 2006). This regulation involves two stages. In the first stage, several types of MAPK module, such as mitogen-activated protein kinase 1 (MPK1) to MPK3, MPK6 to MPK9, and MPK12 and MKK4/MKK5, MKK7/MKK9, and MKK1/MKK2, are activated in the presence of H$_2$O$_2$ (Kovtun et al., 2000; Ren et al., 2002; Moon et al., 2003; Nakagami et al., 2004; Rentel et al., 2004; Jammes et al., 2009; Wang et al., 2010; Takahashi et al., 2011). In the second stage, the activation of MAPKs amplifies ROS signals by directly regulating the activity of NADPH oxidase or by activating transcription factors that enhance the expression of NADPH oxidase genes (Asai et al., 2008). Using Arabidopsis protoplasts, these workers found that H$_2$O$_2$ activated two 42- and 44-kD MAPKs. H$_2$O$_2$ also activated stress-responsive promoters such as the oxidative-stress-responsive promoter of GST6 and the heat shock-responsive promoter of HSP18.2 but not the abscisic acid (ABA)-responsive promoter of RD29A (Kovtun et al., 2000). Although MAPK cascades activated by oxidative stress have been shown to regulate transcription in higher plants (Kovtun et al., 2000; Samuel and Ellis, 2002), the details of the regulatory mechanisms of oxidative signaling remain unclear.

In the study reported here, we identified seven ROS-responsive elements, designated ROSEs, by using bioinformatic analysis to search for promoters that were up-regulated in response to oxidative stress. We also found that the APETALA2/ethylene-responsive element binding protein (AP2/EREBP) transcription factor ERF6 could bind specifically to the ROSE7/GCC box and enhance the expression of a reporter gene under the control of the ROSE7/GCC box motif. ERF6 can interact physically with, and is phosphorylated by, MPK6. Mutation of Ser-266 and Ser-269 of ERF6 affected its intracellular dynamic changes, and the expression of ROS-responsive genes was strongly increased in 35S-ERF6 WT and 35S-ERF6 DD transgenic plants. These data suggest that an H$_2$O$_2$-activated MAPK cascade modulates the interaction between ERF6 and the ROSE7/GCC box and regulates oxidative gene transcription in Arabidopsis.

RESULTS
Identification of Putative ROS-Responsive Elements
To identify ROS-responsive elements, a bioinformatic strategy was used to search for motifs that were conserved in the promoters of ROS-responsive genes. On the basis of our microarray data and the results of other recent similar studies (Desikan et al., 2001; op den

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Promoters from the \textit{hilh} and \textit{fom} clusters were selected for analysis with the program MEME to identify potential transcription factor-binding sites. Then the present frequencies of each motif in \textit{hilh} and \textit{fom} cluster pools were calculated against a whole-genome set by the MAST program (http://meme.nbcr.net/meme/cgi-bin/mast.cgi). Only those motifs with significantly higher enrichment in the test pool than the whole-genome set were selected as potential ROSEs (Supplemental Table S2). As a result, a total of seven ROSEs were identified in the \textit{hilh} and \textit{fom} clusters (Fig. 1A). Table I provides detailed information about the seven ROSEs, including homologous sequences, examples of ROS-responsive genes in whose promoters they were found, et cetera. Several ROSE motifs matched known cis-acting elements, for example, the ABA-responsive element (ABRE; ROSE1), brassinosteroid-responsive element (ROSE2), W box (ROSE4), and GCC box (ROSE7). However, ROSE3, ROSE5, and ROSE6 were found to be novel putative ROS-responsive elements because they did not match any known cis-acting elements exactly.

To determine whether the putative ROSEs were sufficient to regulate the transcription of ROS-responsive genes, transient expression assays were carried out using the luciferase reporter system following treatment with different ROS reagents, which included H$_2$O$_2$, MV, and 3-AT. For the reporter constructs, four repeats of the core ROSE sequences were cloned upstream of the minimal promoter region of the cauliflower mosaic virus (CaMV) 35S promoter (Fujimoto et al., 2000; Song et al., 2005). The 4xROSE reporter genes were delivered into Arabidopsis leaves by particle bombardment. Under the control of ROSE7, luciferase activity in the presence of H$_2$O$_2$ increased 3.2-fold relative to control luciferase activity in the absence of H$_2$O$_2$ (Fig. 1B). In response to MV, 3- and 2.2-fold increases in luciferase activity were observed with the reporter constructs that contained ROSE4 and ROSE6, respectively. Meanwhile, 3-AT only activated ROSE5-LUC (for firefly luciferase) transcription significantly.

**ERF6 Binds to the ROSE7/GCC Box Motif and Acts as an Activator of Transcription under Oxidative Stress**

As mentioned above, the well-characterized GCC box cis-acting element (ROSE7) was found to be a novel putative ROS-responsive element (Table I). The application of exogenous H$_2$O$_2$ induced the expression of 12 genes of the AP2/EREBP family, which included ERF6 and ERF11, in Arabidopsis seedlings (Wang et al., 2006). Members of the AP2/EREBP family are known to be involved in the regulation of ROS-responsive gene expression.

**Figure 1.** Summary of putative ROSEs. A, Sequence logos of ROSE motifs conserved in the promoters of ROS-induced genes. The relative height of each letter indicates the relative abundance of the corresponding nucleotide at the given position in each ROSE. B, Effects of exogenous H$_2$O$_2$, MV, and 3-AT on luciferase activity under the control of ROSE motifs. To normalize the values obtained for each independent transcription, the luciferase gene from \textit{Renilla} spp. was used as an internal control. Luciferase activity is expressed in arbitrary units relative to \textit{Renilla} spp. luciferase activity. Each value represents the mean ± so of six independent experiments. Asterisks indicate statistically significant differences in luciferase activity ($P < 0.05$ under Student’s $t$ test) between the control and treatment conditions.
to ERF6 nor compete with the binding of the wild-type GCC box (Fig. 2C), which indicated that ERF6 bound specifically to the GCC box.

To see whether ERF6 also binds these promoters in planta and that this binding is affected by ROS stress in vivo, we generated transgenic plants expressing 35S-FLAG-ERF6 for the chromatin immunoprecipitation (ChIP) assay. Western-blot analysis showed that the FLAG-ERF6 abundance was significantly increased in 14-d-old seedlings treated with HL (2,000 μmol m⁻² s⁻¹ for 2 h), ROS, or 1-aminocyclopropane-1-carboxylic acid (ACC), whereas the amount of ERF6 protein was relatively low or undetectable under normal growth conditions (Fig. 2D). Thus, the FLAG-ERF6 transgenic plants treated for 2 h by HL were selected for further analysis. The ChIP assay was performed for the FLAG-ERF6 protein using a FLAG-specific monoclonal antibody. After immunoprecipitation of DNA/protein complexes, the DNA was recovered and analyzed by PCR. Previous reports show that significant changes of gene expression occur in response to oxidative stress, including RbohD, ZAT12, peroxidase, and GST, which are well-known oxidative signaling components (Rizhsky et al., 2004; Davletova et al., 2005). We attempted to identify ROSE7/GCC box in the upstream regions of these genes by performing a scan with MAST. The bioinformatics data indicated that 19 out of 37 genes contain typical ROSE7/GCC box in the promoters (Fig. 2E). The expression of several genes (PDF1.2a, At1g49450, At4g33720, At1g67810, At2g15480, and At2g37130) induced by ROS was also examined by qRT-PCR. The results show that the expression of these genes was indeed induced by HL (Supplemental Fig. S1). Thus, primer combinations were chosen that amplify fragments of approximately 150 bp within the promoter of the ROS-responsive genes that encompass the ROSE7/GCC box. The promoters of 11 ROS-responsive genes tested were all enriched in FLAG-ERF6 seedlings (Fig. 2E). Representative qRT-PCR results of ROS-responsive genes are shown in Figure 2F. Promoters from PDF1.2a, PDF1.2b, At2g37130, and At5g18470 exhibited significant enrichments of PCR products after HL treatment, whereas the negative control from the UBQ10 (UBQ10) promoter sequence was not enriched by ChIP in the same samples. These results confirmed the in vivo binding of ERF6 to the promoters of ROS-responsive genes containing the ROSE7/GCC cis-element.

To determine further whether ERF6 mediated the ROS-induced transcriptional activity of the ROSE7/GCC box motif, transient expression experiments were performed in which 4xGCC-LUC as the reporter and 35S-ERF6 as the effector (Fig. 2G) were cotransformed into Arabidopsis leaves. The data obtained indicated that the expression of the reporter construct increased significantly in the presence of ERF6 (approximately 4.2- ± 1.2-fold, P < 0.05 by Student’s t test; Fig. 2H), which suggests that ERF6 is an activator of GCC box-mediated transcription. Furthermore, when H₂O₂ was applied, the luciferase activity was increased 8.1-fold in the presence of ERF6 (P < 0.05 by Student’s t test; Fig. 2H) as compared with the control, which indicates that H₂O₂ could also activate ERF6 via an unknown, transcription-independent mechanism.

### The Effects of ERF6 Mutations on the Oxidative Stress Responses

To further investigate the role of the ERF6 gene in the transcription of ROS-responsive genes, we identified transfer DNA insertion mutants erf6-1 and erf6-2 from the Arabidopsis Biological Resource Center. The positions of the transfer DNA insertions for the alleles are nucleotides 840 and 47, respectively. The qRT-PCR results showed that erf6-1 (Salk_087356) is a null mutant and erf6-2 (Salk_030723) is a knockdown allele (Supplemental Fig. S2). Under our normal growth conditions in soil (see “Materials and Methods”), adult erf6 mutant plants were smaller than wild-type plants, displaying an approximately 12.3% reduction of fresh weight compared with the wild type (n = 60, P < 0.05 by Student’s t test; Fig. 3, A and B). The young leaves (indicated by arrows in Fig. 3C) turned a darker brown in erf6-1 than that in the wild type when treated with diaminobenzidine (DAB), suggesting that there is more accumulation of H₂O₂ in erf6 mutants.

Since we found that ERF6 accumulation was stimulated by HL and ROS in cells, it was reasonable to assume that the ERF6 gene functions in photooxidative

### Table 1. Similar cis-acting elements and putative functions of ROSEs

<table>
<thead>
<tr>
<th>ROSE</th>
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<th>hom</th>
<th>Motif Hits</th>
<th>Core Sequence</th>
<th>Function</th>
<th>Reference</th>
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<tr>
<td>ROSE1</td>
<td>+</td>
<td>+</td>
<td>ABRE</td>
<td>CACGTCG</td>
<td>ABA</td>
<td>Guiltinan et al. (1990); Hobo et al. (1999)</td>
</tr>
<tr>
<td>ROSE2</td>
<td>+</td>
<td>+</td>
<td>CE3</td>
<td>CGTTGC</td>
<td>ABA</td>
<td>Hobo et al. (1999)</td>
</tr>
<tr>
<td>ROSE3</td>
<td>+</td>
<td>+</td>
<td>BR</td>
<td>CGTTGC</td>
<td>BR</td>
<td>He et al. (2005)</td>
</tr>
<tr>
<td>ROSE4</td>
<td>-</td>
<td>+</td>
<td>W box</td>
<td>(T)TGAC</td>
<td>MV, wounding</td>
<td>Kim and Zhang (2004); Scarpeci et al. (2008)</td>
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<tr>
<td>ROSE5</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>ROSE6</td>
<td>-</td>
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<tr>
<td>ROSE7</td>
<td>+</td>
<td>-</td>
<td>GCC box</td>
<td>GCCGCCC</td>
<td>Ethylene, ABA, jasmonate</td>
<td>Büttner and Singh (1997); Brown et al. (2003); Song et al. (2005)</td>
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damage. Thus, we measured the chlorophyll fluorescence parameter $F_{v}/F_{m}$, the maximal quantum yield of PSII, in wild-type and mutant plants exposed to HL. The treatment protocol and times at which fluorescence parameters were imaged are shown in Figure 3, D and E (see “Materials and Methods”). Before exposure to HL treatment, wild-type and erf6-1 plants showed no difference in adapted $F_{v}/F_{m}$ under light inside the growth chamber (see “Materials and Methods”; Fig. 3E). However, after treatment with HL within 24, 36, and 48 h, leaves of erf6-1 had a lower $F_{v}/F_{m}$ value than the corresponding wild-type leaves ($n = 9$, $P < 0.05$ by Student’s $t$ test; Fig. 3E). Consistent with this finding, ROSE7-type genes lost their activation under HL treatment in erf6-1 mutants (Supplemental Fig. S1). However, some of these genes (e.g. At4g33720 and At1g67810) did not lose their differential activation under HL, which might be due to the ERF family’s redundant role (Moffat et al. 2010).
et al., 2012). These results suggest that the mutation of ERF6 renders mutants more sensitive to photoinhibition, possibly as a result of a decreased ability to regulate the expression of oxidative stress-responsive genes.

**MPK6 Interacts with and Phosphorylates ERF6**

It is well documented that application of ROS can activate MPK3 and MPK6 (Moon et al., 2003; Wang et al., 2010). Amino acid sequence analysis has also indicated that ERF6 contains two putative MAPK phosphorylation sites at its C terminus and a typical MAPK docking sequence (Fujimoto et al., 2000; Kiegerl et al., 2000; Sharrocks et al., 2000; Nakano et al., 2006). Thus, we anticipated that ERF6 might interact with MPK6 or MPK3 in the oxidative stress response in Arabidopsis.

To test this hypothesis, yeast two-hybrid experiments were performed using Arabidopsis MPK6, MPK3, and MPK4 as baits and ERF6 as prey. *Saccharomyces cerevisiae* Y190 cells that had been transformed with pACT2-ERF6 together with pAS2-MPK3, pAS2-MPK6, or pAS2-MPK4 were grown on synthetic dextrose medium. The combination of pACT2-ERF6 with pAS2-MPK3 or pAS2-MPK6 yielded strong β-galactosidase expression (Fig. 4A). In contrast, the control combination (i.e. pAS2-MPK4 and pACT2-ERF6) did not result in detectable β-galactosidase activity. These results show that MPK6 and MPK3 interacted physically with ERF6 in the yeast two-hybrid system (Fig. 4A).

To confirm the interaction between MPK3/MPK6 and ERF6, we monitored the association of transiently expressed MPK3/MPK6 and ERF6 in protoplasts of Arabidopsis leaves using bimolecular fluorescence complementation (BiFC; Walter et al., 2004). In our BiFC system, MPK3 and MPK6 were cloned into pSPYCE and ERF6 was cloned into pSPYNE to give constructs that encoded the fusion proteins MPK3-YCE, MPK6-YCE, and ERF6-YNE, respectively. When MPK6-YCE and ERF6-YNE were coexpressed in protoplasts of Arabidopsis leaves, fluorescence from reconstituted yellow fluorescent protein (YFP) was observed in the nucleus (Fig. 4B, a). However, coexpression of MPK3-YCE and ERF6-YNE did not result in fluorescence (Fig. 4B, b). In addition, no YFP signal was detected with the combinations of MPK3- YCE and ERF6-YNE or MPK3-YNE and ERF6-YCE (Fig. 4B, c–e). Consistent with the localization of the interaction signal, confocal microscopy of protoplasts transformed with MPK3, MPK6, or ERF6 fused with GFP showed that all three fusion proteins were localized in both the cytoplasm and the nucleus (Fig. 4B, f–h). These results confirmed that ERF6 and MPK6 could interact in planta but ERF6 and MPK3 could not.

The observations that the GFP fluorescence of ERF6 and MPK6 proteins was emitted from both the cytoplasm and the nucleus, and that the ERF6-MPK6 association only occurred in the nucleus, prompted us to carefully examine compartments of the protein interactions...
by coimmunoprecipitation. To this end, we isolated the nucleus from a 35S-ERF6 plant and investigated ERF6 and MPK6 localization and interaction in planta. Under untreated conditions, both ERF6 and MPK6 proteins could be detected in total cell extracts but not in the nucleus (Fig. 4C, input). Interestingly, after exposure of Arabidopsis seedlings to HL for 2 h, they could be detected by immunoblotting from nuclear extracts (Fig. 4C, output). The coimmunoprecipitation assays also indicated that the interaction between ERF6 and MPK6 mainly occurred in the nucleus after HL treatment. As shown in Figure 4C (output), MPK6 protein could indeed be detected in the complex that contained FLAG-tagged ERF6 protein, both in the whole cell extracts and in nuclear extracts of seedlings treated with HL but not in the nucleus from control experiments. Consistent with this observation, we performed immunoprecipitation experiments in the cytoplasm or nucleus of FLAG-ERF6 transgenic plants treated with 2 mM H2O2. As expected, in total cell extracts, FLAG-ERF6 was detected in the cytoplasm and nucleus. However, FLAG-ERF6 was almost not present in the nucleus of 35S-ERF6 transgenic plants without H2O2 treatment (Supplemental Fig. S3). These results suggest that H2O2 or HL treatment triggers the shuttling of ERF6 protein from the cytoplasm to the nucleus.

To investigate further whether ERF6 is a substrate of MPK6, a phosphorylation assay was performed with recombinant ERF6. Unfortunately, the recombinant ERF6 protein purified from E. coli was not suitable for the phosphorylation assay, because the majority of the recombinant protein was present in inclusion bodies. Therefore, transgenic plants that expressed recombinant FLAG-tagged ERF6WT were generated. Native ERF6 protein from these transgenic Arabidopsis seedlings was immunoprecipitated and used in an in vitro phosphorylation assay with MPK6. As shown in Figure 4D, activated MPK6 could phosphorylate ERF6WT. No FLAG-tagged protein was immunoprecipitated from nontransgenic plants, and no phosphorylation band was observed (Fig. 4D). These results indicate that ERF6 is a substrate of MPK6.

**Figure 4.** ERF6 interacts with MPK6 in vitro and in vivo and as a substrate of MPK6. A, MPK3 and MPK6 interacted with ERF6 in the yeast two-hybrid system. The GAL4 DNA-binding domain was fused with MPK3 or MPK6. Yeast cells were transformed with pAS2-MPK3, pAS2-MPK4, or pAS2-MPK6 as bait and pACT2-ERF6 as prey. These strains were subjected to growth on synthetic dextrose (SD) medium (left) and to β-galactosidase (β-gal) assay (right). B, Interaction between ERF6 and MPK6 on the basis of the analysis of BiFC: a, the YFP signal in the nucleus indicated an interaction between MPK6 and ERF6; b, no YFP signal was detected in protoplasts cotransformed with MPK6 and ERF6; c and d, no YFP signal was detected in protoplasts cotransformed with MPK3-YCE or MPK6-YCE and SPYNE; e, no YFP signal was detected in protoplasts cotransformed with ERF6-SPYNE and SPYCE; f to h, localization of GFP signals from MPK6, MPK3, and ERF6 fused with GFP. Left, Fluorescence images under confocal microscopy. Right, Bright-field images of the cells. Bars = 5 μm. C, HL treatment triggered the sorting and interaction of ERF6 and MPK6. Total cell and nucleus extracts from control and HL-treated FLAG-ERF6 transgenic plants were used for immunoprecipitation. ERF6 and MPK6 protein were detected by immunoblotting using the anti-FLAG and anti-MPK6 antibody, respectively. D, The phosphorylation assay showed that ERF6 was a substrate of MPK6. FLAG-tagged native ERF6 protein was immunoprecipitated from transgenic plants and used for a phosphorylation assay with preactivated recombinant MPK6 protein in the presence of [γ-32P]ATP. Phosphorylation was visualized by autoradiography, and the loading of FLAG-tagged ERF6 was determined by immunoblotting with an anti-FLAG antibody. WT, Wild type.
Both Ser-266 and Ser-269 of ERF6 Are Putative Phosphorylation Sites, and Their Modification Affects ERF6 Dynamic Changes in Cells

As mentioned above, ERF6 contains two putative MAPK phosphorylation sites. These sites are located within the nuclear export signal (NES; Ser-266 and Ser-269) at the C terminus (Fujimoto et al., 2000; Nakano et al., 2006). To test whether both Ser residues are MPK6 phosphorylation sites, the recombinant glutathione S-transferase (GST)-tagged C-terminal peptides of ERF6 (amino acids 261–282; pepS266A, pepS269A, and pepS266AS269A) were generated by mutating the Ser residues to Ala by site-directed mutagenesis (Fig. 5A). MPK6 was able to phosphorylate the pepS266A (pepA) and pepS269A (pepA) but not the pepS266AS269A (pepAA) mutant proteins (Fig. 5B). The single mutation pepA or pepA showed reduced phosphorylation as compared with that of pepWT. This suggests that both Ser-266 and Ser-269 of ERF6 are independent phosphorylation sites for MPK6.

Recently, it has been reported that the MAPK cascade activates transcription factors by evoking a change in the stability of the transcription factors ERF104 and EIN3 (Betheke et al., 2009; Yoo et al., 2009). We first assayed the MPK6/MPK3 kinase activity induced by H2O2 or HL by measuring the levels of phosphorylation in wild-type plants that were exposed to 2 mM H2O2 or HL. As shown in Figure 5C, exogenous application of H2O2 or HL promoted the kinase activity of MPK6 in a time-dependent manner. In contrast to that of MPK6, the MPK3 activity was relatively weak and remained nearly unaltered by H2O2 or HL under the same experimental conditions. Importantly, in the conditional gain-of-function (dexamethasone [DEX]-inducible) MKK5DD transgenic plants, DEX treatment caused the constitutive accumulation of the activation form MKK5 and increased the activities of MPK3 and MPK6 in a time-dependent manner (Fig. 5D, top panel). Interestingly, ERF6 protein accumulated rapidly after DEX treatment, with the maximum level of accumulation occurring 3 h after the addition of DEX, and then decreased to its basal level within 9 h (Fig. 5D, top panel). MKK5DD, on the other hand, remained quite high and continued to rise. Alternatively, we determined the dynamic changes of ERF6 in MKK5DD/mpk6-3 transgenic plants. As expected, an increase in the activities of MPK6 was not observed after DEX treatment, and ERF6 accumulation was not found in the null allele of mpk6-3 (Fig. 5D, bottom panel).

To confirm whether the dynamic changes of ERF6 are related to the phosphorylation of ERF6, we expressed FLAG-tagged ERF6 (35S-ERF6WT) and its mutants, 35S-ERF6AA and 35S-ERF6DD, in Arabidopsis and then analyzed the levels of ERF6 protein in the nonphosphorylated form (ERF6AA) and a phosphor-mimicking mutation of ERF6 (ERF6DD). Under HL treatment, the ERF6 protein levels were increased in both ERF6WT and ERF6AA transgenic plants. Consistent with the above observation, 35S-ERF6DD plants accumulated a much lower amount of ERF6 protein than plants that expressed 35S-ERF6WT or 35S-ERF6AA (Supplemental Fig. S4). These data imply that there might be a feedback-control mechanism to ensure that the expression of ROS-responsive genes or protein levels is tightly and transiently controlled.

Next, we employed immunoprecipitation to analyze the stability of ERF6 and the MPK6-ERF6 complex. FLAG-tagged ERF6 proteins were immunoprecipitated from 35S-ERF6WT, 35S-ERF6AA, and 35S-ERF6DD transgenic plants, and the resultant protein-agarose complexes were immunoblotted with an anti-MPK6 antibody. Consistent with the above analysis (Fig. 4C), MPK6 protein could be detected in the complex that contained ERF6WT protein. Importantly, the binding affinity of MPK6 for ERF6WT was much stronger when the complexes were isolated from cells that had been treated with H2O2 as compared with untreated cells (Fig. 5E, right panel, lane 1 versus lane 2), which was probably due to the phosphorylation of ERF6 by H2O2-activated MPK6. In accordance with this observation, the binding of MPK6 to ERF6DD, which mimicked the ERF6 phosphorylated at Ser-266 and Ser-269, was found to be stronger than the binding of MPK6 to ERF6WT (Fig. 5E, right panel, lane 4 versus lane 1). However, no clear band was observed for ERF6AA, which mimicked the unphosphorylated form of ERF6 (Fig. 5E, right panel, lane 3).

Amino acid sequence analysis of ERF6 indicated that the nuclear localization signal of ERF6 overlapped with the MAPK docking sequence and the Leu-rich NES (suppressor of cytokinesis) (S-C) (Fig. 5F). MPK6 controlled both the localization and stability of the ERF6 feedback-control mechanism that results in its dynamic changes. Taken together, these results showed that MPK6 controlled both the localization and activation of ERF6 by phosphorylation.

**Phosphorylation of ERF6 by H2O2-Activated MPK6 Enhances ROSE7/GCC Box Transcriptional Activity and the Expression of Downstream Genes**

Next, we used a transient expression assay to examine whether the phosphorylation of ERF6 by MPK6 affected the transcriptional activity of target genes. As shown in Figure 6, A and B, coexpression of ERF6WT, ERF6AA, and ERF6DD with the 4×GCC-LUC reporter dramatically increased the luciferase activity produced. Expression of ERF6DD, the constitutively active form of ERF6, increased LUC expression by 2.4- and 3.2-fold as compared with ERF6WT and ERF6AA (P < 0.05 by Student’s t test), respectively. As expected, after the leaves
were transformed with ERF6WT, H2O2 induced the expression of 4×GCC-LUC significantly, which resulted in a 1.9-fold increase as compared with leaves that transiently expressed ERF6WT but had not been treated with H2O2 (P < 0.05 by Student’s t test). Surprisingly, luciferase activity in the presence of H2O2 was inhibited in leaves that transiently expressed either ERF6DD or ERF6AA. It is likely that this effect was due to feedback control by the high level of ERF6DD protein present and the inability of ERF6AA to be phosphorylated by H2O2-activated MPK6, respectively. Importantly, the transcription was not enhanced by coexpression of MPK6 and the 4×GCC-LUC reporter in the absence of ERF6. In addition, coexpression of the ERF6WT and MPK6 effectors enhanced the expression of LUC by more than 2-fold in the presence of H2O2 as compared with control conditions where no effectors were present (P < 0.05 by Student’s t test). In the mpk6-3 mutant plants, ERF6WT-induced luciferase activity was dramatically inhibited by the deficiency of MPK6, even under H2O2 treatment. This suggests that the effect of ERF6 on transcription activation is dependent on MPK6.

We also used qRT-PCR to analyze the expression of some H2O2-induced genes that contain a GCC box in the presence of H2O2. The expression of these genes was significantly induced in the presence of H2O2 as compared with control conditions where no H2O2 was present (P < 0.05 by Student’s t test). Importantly, the expression of these genes was not enhanced by coexpression of MPK6 and ERF6.
their promoter in wild-type, erf6-1, 35S-ERF6WT, 35S-ERF6AA, and 35S-ERF6DD transgenic plants. As shown in Figure 6C, a, expression of ERF6 was increased by 195-, 131-, and 40-fold in 35S-ERF6WT, 35S-ERF6AA, and 35S-ERF6DD plants, respectively, as compared with wild-type plants. The expression of downstream genes was also enhanced dramatically by treatment with H2O2 or overexpression of ERF6. For example, the levels of PDF1.2a and PDF1.2b transcription were greatly increased in ERF6DD transgenic plants, with increases of more than 600- and 400-fold, respectively, as compared with levels in wild-type plants. Application of H2O2 also resulted in a further increase in the transcription of both PDF1.2a and PDF1.2b in ERF6DD transgenic plants (Fig. 6C, b and c). The transcription factor WRKY33 is a ROS-induced gene that contains a GCC box in its promoter (Davletova et al., 2005; Pitzschke et al., 2009). When H2O2 was applied to ERF6WT lines, the expression of WRKY33 was increased 2.3-fold as compared with untreated plants (P < 0.05 by Student’s t test). In addition, the abundance of WRKY33 mRNA in ERF6DD plants was increased significantly. However, treatment with H2O2 had almost no effect on the expression of WRKY33 in 35S-ERF6AA and 35S-ERF6DD plants (Fig. 6C, e). Similar results were obtained.
for other genes, which included PR5 and MYB51 (Fig. 6C, d and f). However, the transcription levels of all the checked genes in erf6-1 mutants were not significantly affected, even under H2O2 treatment.

To determine whether the increased expression of the ROS-responsive genes in 35S-ERF6DD plants is dependent on MPK6, we conducted qRT-PCR experiments by using MKK5DD/mpk6-3/35S-ERF6 or mpk6-3 mutant plants. Impressively, the expression of nearly all the ROS-responsive genes examined (PDF1.2a, PDF1.2b, WRKY33, PR5, and MYB51) was significantly reduced in both mpk6-3 mutant plants and MKK5DD/mpk6-3/35S-ERF6 compared with the 35S-ERF6DD transgenic plants, with or without H2O2 treatments (Fig. 6C). For example, PDF1.2a and PDF1.2b transcript abundance under 2 mM H2O2 treatment in 35S-ERF6DD transgenic plants was 28 or 14 times higher than that in MKK5DD/mpk6-3/35S-ERF6, respectively. Meanwhile, the low basal level of this gene expression in mpk6-3 did not differ much from that of erf6-1 mutant plants (Fig. 6C). We noticed that PR5, WRKY33, and MYB51 were still induced by H2O2 treatment in mpk6-3. The reason might be that other MPKs (e.g. MPK3) have been shown to be activated in the MKK5/MPK6/MPK3 module in the presence of H2O2 (Moon et al., 2003; Takahashi et al., 2011), which is involved in the regulation of the expression of these genes. These data clearly indicate that MPK6-mediated phosphorylation of ERF6 is involved in the regulation of ROS-responsive gene expression.

**DISCUSSION**

The ROSE7/GCC Box as a ROS-Responsive cis-Element

Through a bioinformatics analysis of the promoters of genes up-regulated by ROS, seven potential ROSEs were identified (Fig. 1A). Four of them, which include the GCC and W boxes, were sufficient to increase luciferase activity in a transient assay in response to different ROS signals, such as H2O2, MV, and 3-AT (Fig. 1B), which indicates that ROSEs play an important role in the transcription of ROS-responsive genes.

Whether these putative elements function in oxidative signaling, and to what extent they contribute to the regulation of oxidative stress responses, are issues yet to be resolved. Among the seven putative elements, ROSE1 and ROSE2 are similar to the ABA-responsive cis-acting element ABRE and the core sequence of CE3, respectively (Table 1). Indeed, the results of the transient assay indicate that ABA could strongly induce an increase in luciferase activity under the control of GAL4-ROSE1 (Fig. 1B), which corresponded to ABRE. The ABRE-binding protein ABI3 mediates the expression of the peroxiredoxin antioxidant AtPER1 gene in response to oxidative stress (Haslekås et al., 2003). The tomato Ep5C gene, which encodes an extracellular cationic peroxidase, is transcriptionally activated by the H2O2 that is generated during the course of plant interaction with pathogens (Coego et al., 2005). The promoter of Ep5C contains an ABRE and an H box. MV was able to slightly activate the expression of a reporter that harbored ROSE1. Therefore, the ROSE1 motif might represent a point of convergence of the ABA and ROS signaling pathways. Further experiments are in progress to clarify the roles of the identified ROSE motifs in oxidative signaling, including the characterization of ROSE-binding factors by yeast one-hybrid assays and analysis of the phenotypes of homozygous Arabidopsis plants in which ROSE-binding factors have been knocked out.

Our study mainly focused on the ROSE7/GCC box motif because it appeared to have the most universal role in gene transcription among the different ROS motifs identified (Brown et al., 2003; Song et al., 2005; Wang et al., 2006). Indeed, the GCC box has been defined as a cis-acting element that binds AP2/EREBP-type transcription factors (Büttner and Singh, 1997; Hao et al., 1998). Surveys of databases have estimated that 125 genes in the Arabidopsis genome encode AP2/ERF proteins (Nakano et al., 2006). In addition, it has been found that many stress-inducible genes, including ARSK1 and dehydrogenation genes, both of which are induced by ABA, NaCl, cold, and/or wounding (Hwang and Goodman, 1995; Rouse et al., 1996), possess a GCC box in their 5’ upstream promoter regions. The findings that the ROSE7/GCC box motif could bind to ERF6 in vitro and in vivo (Fig. 2), and that the transcription of the luciferase gene was enhanced by cotransfection of the effector ERF6 or the application of H2O2, raise the possibility that the GCC box might represent a cis-regulatory element for signal transduction in response to biotic and abiotic stress.

The electrophile-responsive element is found in the promoters of some animal GST genes (Friling et al., 1990). Meanwhile, a short cis-acting element known as the ocs element is present in the promoters of GST genes of plants (Chen et al., 1996). The ocs element in the CaMV promoter is also referred to as the as-1 site (Lam et al., 1989). The electrophile-responsive element and the ocs element share a number of features, which raises the possibility that the ocs element might function as an oxidative stress-responsive element in plants. The ocs/as1 elements have only been found in a few genes, such as NtPR1, NtParA, Nt103, GmHsp26-A, AtGSTs, and AtPR1 (Krawczyk et al., 2002). Although previous data strongly suggest that ocs1/as1 elements are oxidative stress-responsive elements, it is unlikely that they are universal elements in oxidative signaling. Our data suggest that the ROSE7/GCC box motif functions as a ROS-responsive cis-element to regulate oxidative gene expression. The frequency of appearance of the ROSE7/GCC box might hint at its important role in the regulation of oxidative stress-responsive gene transcription. These observations suggest that a dynamic system exists in plants that controls oxidative signaling through the modulation of GCC box-dependent transcription.

There are hints that different ROSEs might be able to activate distinct genetic programs in response to different ROS signals, although decisive evidence is lacking. For
example, ROSE4 and ROSE6 were activated strongly by MV, whereas expression under the control of ROSE5 was increased by the application of H$_2$O$_2$. However, the promoter that contained ROSE5 was only activated effectively by 3-AT, and the same promoter was not induced by H$_2$O$_2$ (Fig. 1B). Thus, the oxidative responsiveness of individual target genes might be determined by distinct DNA-binding selectivity of the ROSE-binding factors in the presence of different ROS.

ERF6 Is an H$_2$O$_2$-Responsive Element-Binding Factor

We investigated the function of the transcription factor ERF6 in H$_2$O$_2$ signaling. First, the expression of ERF6 was induced dramatically by treatment with H$_2$O$_2$, MV, or 3-AT (Fig. 2A). Second, an ERF6 fusion protein was able to bind specifically to the ROSE/GCC box in vitro and in vivo (Fig. 2, C and F). Coexpression of the ERF6 effector enhanced the expression of a luciferase gene under the control of a GAL4 promoter that harbored a GCC box (Fig. 2H). Third, mutation of erf6-1 resulted in changes in the transcriptions of ROS- or HL-responsive genes (Fig. 6C; Supplemental Fig. S1). The profile of ERF6’s expression during the HL and H$_2$O$_2$ exposure reported in this article (Fig. 2, A and B) was consistent with the time course of transcription in the typical target gene (e.g. PDF1.2a and PDF1.2b) induced gradually by oxidative stress under UV-B treatment (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi). We also found that PDF1.2a and PDF1.2b were elevated at the time point of ERF6 induction (Figs. 2, A and B, and 6C). These results reveal that ERF6 could act as an activator of the ROSE7/GCC box and play a central role in ROS-mediated gene transcription.

ERF proteins were first identified as GCC box-binding proteins in tobacco and have been shown to take part in ethylene-related pathogen resistance, plant development, and responses to various types of environmental stress by regulating the expression of stress-responsive genes (Ohme-Takagi and Shinshi, 1995; Shinozaki and Yamaguchi-Shinozaki, 1997; Kizis et al., 2001; Chakravarthy et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2005). Application of exogenous H$_2$O$_2$ singlet oxygen or changes in cellular redox balance induced the expression of ERF6 in Arabidopsis seedlings (Danon et al., 2005; Wang et al., 2006; Jing et al., 2008). ERF6 has also been identified as a positive regulator in Botrytis cinerea and chitin-induced innate immunity responses, which show reduced expression of PDF1.1 and PDF1.2a in a double erf5 erf6 mutant (Son et al., 2012; Moffat et al., 2012). These are quite complementary to the role that ERF6 plays in the transcription regulation of ROS-responsive genes. The above findings, combined with previous discoveries that ERF proteins interact with other transcription factors, such as bZIP, strongly suggest that the complex regulatory network involving ERF proteins is important in both biotic and abiotic responses in plants (Büttner and Singh, 1997; Xue and Loveridge, 2004; Moffat et al., 2012). Therefore, elucidation of these regulatory pathways in detail will be crucial to revealing the mechanisms by which ERF proteins act in diverse plant responses.

ERF6 Was Possibly Involved in the Regulation of Photooxidative Damage

In photosynthetic cells, excess light affects the intracellular redox homeostasis and can evoke oxidative stress and eventually cause photoinhibition, which results from an overexcitation of the photosystems and a potential to generate ROS (Niyogi, 1999; Noctor et al., 2002). It was found that HL could induce ERF6 accumulation in the nucleus (Figs. 2D and 4C), the increment of gene expression related to oxidative stress, and stronger photooxidative damage in erf6-1 (Fig. 3, D and E). These results suggest that ERF6 plays an important role in the regulation of photooxidative damage under HL stress.

This work provides several lines of evidence suggesting that there exists a feedback-control mechanism for modulating dynamic changes of ERF6 activity, which operates via MAPK cascade-mediated phosphorylation and leads to an increase in the transcription of ROS-responsive genes through both the sorting of the nucleus and the degradation of transcription factors (Figs. 4C and 5, E and G). ERF6 protein first appeared to be induced faster and reached a peak at 3 h after ROS and HL treatment, then declined to its uninduced level after 6 h (Fig. 5D), a finding that is coherent with the ERF6 transcription pattern under HL (Fig. 2B). Under the MKK5$^{D103}$ background, the induction pattern of ERF6 protein after the application of DEX was similar to those of the wild type treated with ROS and HL (Fig. 5, C and D). ERF6 protein was nearly undetected in phosphor mimicking of ERF6 transgenic plants (Supplemental Fig. S4). Since plants are unable to avoid oxidative damage caused by HL or other stresses, they employ a broad repertoire of protective measures, including minimization of light absorption, avoidance of ROS overaccumulation, and repair of damaged proteins, lipids, and photosystems (Bennet, 1977; Kao and Forseth, 1991; Allen and Staehelein, 1992; Demmig-Adams and Adams, 1996; Asada, 1999; Niyogi, 1999; Pfannschmidt et al., 1999; Kasahara et al., 2002). Phosphorylation of the transcription factors at particular stages during photoinhibition may represent an efficient way to regulate the transcript accumulation of ROS- or HL-responsive genes. Therefore, H$_2$O$_2$ and HL-mediated activation of the MAPK cascade modulates oxidative stress responses by relocation and stability alternation of ERF6 for the regulation of gene transcription activity. These dynamic changes of ERF6 activity are probably important for photoprotection, because timely and efficient modulation of ROS homeostasis benefits plants faced with fluctuations in their light environment, such as hourly variations of light emission in a day or sudden exposure to different stresses.
MPK6 Mediated the Transcription of H2O2-Responsive Genes

Here, we found that MPK6 interacted physically with ERF6. Phosphorylation of ERF6 activated by H2O2 enhanced its ability to bind to the ROSE7/GCC box and activate oxidative gene transcription (Fig. 6). Coexpression of ERFF6DD, the constitutively active form of ERF6, resulted in a significant increase in LUC expression as compared with ERFF6WT and ERFF6AA (Fig. 6B). By contrast, application of H2O2 to plants that expressed ERFF6DD stimulated LUC expression but lowered LUC expression in plants that expressed ERFF6DD (Fig. 6B). Transgenic seedlings that harbored ERFF6DD were more sensitive to H2O2 than wild-type plants, and the transcription of oxidative-responsive genes, such as PDF1.2 and WRKY33, was strongly enhanced (Fig. 6C). Meanwhile, a deficiency of either ERF6 or MPK6 almost inhibited the enhanced transcription of these genes (Fig. 6C). These data suggest that the H2O2-activated MAPK cascade modulates ERF6-mediated gene transcription in seedlings in response to oxidative stress. We have designated this cascade the MPK6-ERF6-ROSE7 pathway.

How does the MPK6-ERF6-ROSE7 pathway affect the transcription of target genes? The results of commounprecipitation indicate that a complex of MPK6/ERF6 exists in plant cells. Interestingly, the expression of all tested ROS-responsive genes showed high activation in MKK5DD transgenic plants treated with DEX (Fig. 6C), which increased MPK3 and MPK6 activities in a time-dependent manner (Fig. 5D). By contrast, the transactivation of these genes was almost completely blocked in erk and mpk6 mutants and even in MKK5DD/mpk6-3/35S-ERF6 transgenic plants. As mentioned above, Ser-266 and Ser-269 of ERF6, which have been identified as MPK6 phosphorylation sites, are indeed crucial for both the dynamic changes of the MPK6-ERF6 complex and the subcellular localization of ERF6 (Fig. 5, B, E, and G). ERFF6DD binds to MPK6 with high affinity, in contrast to ERFF6AA (Fig. 5E), and lower levels of ERFF6DD proteins and transcription of ROS-responsive genes were accumulated in ERFF6DD transgenic plants (Fig. 6C; Supplemental Fig. S4). These observations suggest that MPK6-mediated phosphorylation controls both the stability of the MPK6-ERF6 complex and the nucleocyttoplasmic shuttling of ERF6 and leads to an increase in oxidative gene transcription.

In summary, H2O2 is unquestionably ubiquitous in tissues, and redox reactions are fundamental processes that play very important roles in many aspects of living cells, such as physiological and biochemical events and gene expression. Meanwhile, there are many ROS-responsive genes containing the ROSE7/GCC box in their promoters in the plant genome. Therefore, phosphorylation of ERF6 by MPK6 could initiate downstream signaling in both the cytoplasm and nucleus and thus affect gene expression.

MATERIALS AND METHODS

Plant Growth Conditions and the Primers Used in All PCRs

Wild-type, erk-1, erk-2, mpk6-1 (Wang et al., 2010), and transgenic Arabidopsis (Arabidopsis thaliana ecotype Columbia-0) plants were grown in a 16-h-light/8-h-dark photoperiod at 22°C. For treatment, 3-week-old seedlings grown on Murashige and Skoog medium that contained 2% Suc and 0.8% phytagel (Sigma-Aldrich) were transferred to incubation buffer (50 mM KCl and 10 mM MES-KOH, pH 6.2) containing 2 mM H2O2, 10 µM MV, 1 mM 3-AT, or 50 µM ACC for the indicated times. For HL treatment, 4-week-old seedlings grown on soil were exposed to light at the indicated intensity.

The primers used in all PCRs are listed in Supplemental Table S3.

Analysis of Statistically Significant Promoter Motifs

Promoter sequences were defined as 1,000-bp sequences upstream of the translation start codon and were downloaded from The Arabidopsis Information Resource database (http://ftp.arabidopsis.org/home/tair/sequence/blast_datasets/OLD/At_upstream_1000_20063010.gi). The upstream regions were analyzed with the MEME 3.5.1 program (http://meme.sdsc.edu) as follows. All of the genes from the Z惠民 and 50 clusters were pooled randomly into two groups. Each group was analyzed using MEME with a fixed motif length from 6 to 30 and 30 requested motifs. Conserved motifs present in both pools were selected. Statistically significantly conserved motifs were used to create sequence logos using the Web-based application WebLogo (http://weblogo.berkeley.edu/logo.cgi).

Transient Expression Assay

Four tandem copies of putative ROSEs were introduced into a LUC gene expression vector. The primers from the promoters of genes containing the ROSes were used (Supplemental Table S3). To prepare effector plasmids, the coding regions of MPK6 and wild-type and mutant ERFF6 were amplified and inserted into the plasmid 35S-LUC.

Transient expression was analyzed in Arabidopsis leaves by particle bombardment as described previously (Fujimoto et al., 2000). In brief, the reporter plasmid (1.6 µg) and the effector plasmid (1.2 µg) were bombarded into Arabidopsis leaves in each experiment. The LUC assay was performed with the Dual-Luciferase Reporter Assay System (Promega) and a TD-20/20 luminometer (Promega). To normalize values after each transfection, 0.4 mg of the plasmid pCTRL, which contained the Renilla spp. luciferase coding sequence under the control of the CaMV 35S promoter, was used as an internal control. Normalized LUC activity measured after transfection with the reporter plasmid alone was set arbitrarily at 1.

Recombinant Protein Expression and EMSA

For in vitro protein expression, the coding region of ERFF6 was inserted in frame into the plasmid pET-28a. The resultant pET-ERF6 construct was introduced into Escherichia coli BL21 (DE3) cells. The recombinant His-tagged proteins were purified using nickel-nitrilotriacetic acid agarose (Qiagen), in accordance with the manufacturer’s protocol.

Individual synthetic DNA oligonucleotides that corresponded to the 16-bp GCC box fragment, 5'-AGCAGCGCGCAGGACC-3' and its mutant 5'-ATCCCTCAGCCAGCAGGACC-3', were annealed with their complementary oligonucleotides. The resultant double-stranded oligonucleotides were end labeled with [γ-32P]ATP and T4 polynucleotide kinase (Invitrogen) in accordance with the manufacturer’s instructions. DNA-binding reactions were performed as described previously (Fujimoto et al., 2000). Briefly, 0.1 µg of recombinant ERFF6 protein was added to a total volume of 20 µL in a binding buffer that contained 10 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, 2 mM dithiothreitol, 10% glycerol, and 10 mM of the wild-type or mutant form of the 16-bp double-stranded oligonucleotide. After incubation for 15 min, the reaction mixture was analyzed by electrophoresis through 5% polyacrylamide gels prepared in 0.5× Tris-borate-EDTA buffer under nondenaturing conditions.

Yeast Two-Hybrid System

For yeast (Saccharomyces cerevisiae) two-hybrid assays, the coding regions of MPK3, MPK6, and MPK4 were amplified by qRT-PCR using primers that


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contained appropriate restriction enzyme sites. The amplified fragments were inserted into the plasmid pAS2 as the bait (Clontech) for the constructs pAS-MPK3, pAS-MPK6, and pAS-MPK4. The full-length coding region of ERF6 was cloned in frame in the pACT2 vector to create the plasmid pACT2-ERF6. Yeast two-hybrid assays were performed as described previously (Song et al., 2005). Competent cells of yeast strain Y190 were transformed simultaneously with pAS-MPK6/MPK3/MPK4 and pACT2-ERF6. Empty pAS2 and pACT2 vectors were used as negative controls.

**BiFC Assay**

To analyze in vivo interactions, the coding regions of MPK6, MPK3, and ERF6 were inserted into the plasmids pSPYNE and pSPYCE to form pSPYCE-MPK6, pSPYCE-MPK3, and pSPYNE-ERF6, respectively. Proteolipids isolated from Arabidopsis leaves were transformed with the following combinations of plasmids: (1) pSPYCE-MPK6/MPK3 and pSPYNE; (2) pSPYCE and pSPYNE-ERF6; or (3) pSPYCE-MPK6/MPK3 and pSPYNE-ERF6, as described previously (Walter et al., 2004). For the GFP constructs, the coding regions of MPK6, MPK3, and ERF6 were inserted into the modified plasmid pHB7-GFP-NOS to form pMPK6-GFP, pMPK3-GFP, and pERF6-GFP, respectively. The protoplast transient expression assay was performed as described previously (Sheen, 2001). After incubation for 16 to 20 h, the fluorescence of the protoplasts was measured with an FV1000 confocal laser scanning microscope (Olympus). All figures show representative images from three independent experiments.

**Transgenic Plants**

The coding region of ERF6 was amplified from cDNA isolated from Arabidopsis ecotype Columbia-0 using primers that contained EcoRI and SalI sites. Mutations were introduced into the ERF6 coding sequence using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The wild-type and mutant ERF6 coding regions were cloned together with an N-terminal FLAG epitope into the pCAMBIA1305 under the control of the CaMV 35S promoter (Zhao et al., 2007). The constructs were introduced into Agrobacterium tumefaciens strain LBA4404 and transformed by floral infiltration into wild-type Arabidopsis. ERF6 proteins in the transgenic plants were detected by immunoblotting with the monoclonal anti-FLAG M2 antibody (Sigma-Aldrich).

**Immunoprecipitation, Immunoblotting, and Kinase Assays**

To obtain the native ERF6 proteins, 0.2 g of 2-week-old ERF6$_{WT}$, ERF6$_{AA}$, and ERF6$_{DD}$ seedlings was ground in immunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na$_3$VO$_4$, 1 mM dithiothreitol) at 25°C for 30 min. Activated MPK6 was used to phosphorylate the agarose bead-protein complexes or the recombinant GST-tagged ERF6 by electrophoresis on 12.5% SDS-polyacrylamide gels, and the proteins were detected by immunoblotting with the monoclonal anti-FLAG M2 antibody (Sigma-Aldrich). Another phosphorylation reaction that did not contain [$^{32}$P]ATP was used for immunoblotting with the monoclonal anti-FLAG M2 antibody (Sigma-Aldrich). Another phosphorylation without the addition of [$^{32}$P]ATP was used for the immunoblot assay with monoclonal anti-FLAG M2 antibody (Sigma-Aldrich).

**qRT-PCR**

Total RNA from Arabidopsis seedlings was extracted using TRizol reagent (Invitrogen). Reverse transcription was performed using 5 µg of total RNA and SuperScript II Reverse Transcriptase (Invitrogen). The cDNA diluted 10-fold was then used as a template for qRT-PCR amplification. The primer pairs used are listed in Supplemental Table S3. qRT-PCR was performed with the Stratagene Mx3000 QPCR system using SYBR Green to monitor double-stranded DNA products. UBQ10 was used as an internal control and was amplified with the primer pair 5′-CACACCTCCTTGGCCTTGGC-3′ (forward) and 5′-TGGCTTTCCGTAAGAGACTT-3′ (reverse).

**ChiP and PCR**

ChiP was performed as described previously (Chen et al., 2009). In brief, wild-type and 35S-FLAG-ERF6 seedlings were grown on Murashige and Skoog plates for 14 d and then treated with H$_2$O$_2$ (2,000 µmol m$^{-2}$ s$^{-1}$) for 2 h. Chromatin was isolated from 2.0 g of frozen tissue and sonicated for 8 min (20-s on and 40-s off cycles, 25% power settings) with a VCP130 ultrasonic processor (Sonics). Immunoprecipitation was performed by incubating chromatin with 200 µl of anti-FLAG M2 agarose (Sigma) for 4 h at 4°C. After proteinase K digestion, the immunoprecipitated DNA was extracted. The enrichment of DNA fragments was determined by qRT-PCR using the primers listed in Supplemental Table S3.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers ERF6 (A4174904), MPK3 (A0345640), MPK4 (A0401270), MKK5 (A3922120), T-DNA insertion lines used here are erf6-1 (SALK_087356, germplasm 4688944) and erf6-2 (SALK_030723, germplasm 463311).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Expression of ROS-responsive genes under HL treatment.

**Supplemental Figure S2.** Expression of ERF6 in wild-type and erf6 mutants.

**Supplemental Figure S3.** H$_2$O$_2$ enhances ERF6 accumulation in the nucleus.

**Supplemental Figure S4.** HL enhances ERF6 accumulation.

**Supplemental Table S1.** Gene lists used for ROSE prediction.

**Supplemental Table S2.** Enrichment of ROSEs in gene clusters.

**Supplemental Table S3.** Primers used in all experiments.

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**LITERATURE CITED**


Hobo T, Asada M, Kowzyama Y, Hattori T (1999) ACGT-containing abscisic acid response element (ABRE) and coupling element 3 (CE3) are functionally equivalent. Plant J 19: 677–689
Krawczyk S, Thurow C, Niggeweg R, Gatz C (2002) Analysis of the spacing between the two palindromes of activation sequence-1 with respect to binding to different TGA factors and transcriptional activation potential. Nucleic Acids Res 30: 775–781
Lam E, Benfey PN, Gilmartin PM, Fang RX, Chua NH (1989) Site-specific mutations alter in vitro factor binding and change promoter
expression pattern in transgenic plants. Proc Natl Acad Sci USA 86: 7890–7894
Song CP, Galbraith DW (2006) AtSAP18, an orthologue of human SAP18, is involved in the regulation of salt stress and mediates transcriptional repression in Arabidopsis. Plant Mol Biol 60: 241–257
Stockinger EJ, Gilmour SJ, Thomasow MF (1997) Arabidopsis thaliana CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc Natl Acad Sci USA 94: 1035–1040


