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Running title: MPK6-ERF6-ROSE7 transcriptional pathway

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Title: The MPK6-ERF6-ROSE7/GCC-box complex modulates oxidative gene transcription and the oxidative response in *Arabidopsis thaliana*

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ABSTRACT

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 herein, we identified seven potential ROS-responsive cis-acting elements (ROSEs) from the promoters of genes upregulated by ROS. We also found that the APETALA2 (AP2/EREBP)-type transcription factor ERF6 could bind specifically to the ROSE7/GCC box. Co-expression 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 kinase 6 (MPK6), and also served as a substrate of MPK6. MPK6-mediated ERF6 phosphorylation at both Ser 266 and Ser 269 affected the dynamic alternation of 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.

Keywords: Oxidative stress; MAP Kinase; gene expression; ERF6; ROS-responsive cis-element
Introduction

Reactive oxygen species (ROS) have been characterized as both important signaling molecules and universal stressors that mediate many developmental and physiological processes in plants. These processes include responses to biotic and abiotic stresses, stomatal movement, programmed cell death, photoprotection, growth, and development (Neill et al., 2002; Laloi et al., 2004; Wang and Song 2008; Takahashi et al., 2008; Tsukagoshi et al., 2010). It is well documented that ROS play important roles in transcriptional regulation. For example, 175 non-redundant expressed sequence tags that are regulated by hydrogen peroxide (H₂O₂) have been identified by cDNA microarray analysis. Of these, 113 are induced and 62 are repressed by H₂O₂ (Desikan et al., 2001). Similarly, analysis of an Affymetrix whole genome GeneChip array has shown that 459 genes are induced two-fold or more in Arabidopsis seedlings upon exposure to H₂O₂ for 6 h, whereas 221 are downregulated (Wang et al., 2006). Moreover, deficiency of ROS scavengers, such as catalase (CAT), ascorbate peroxidase (APX), alternative oxidase (AOX) or superoxide dismutase (SOD), causes the accumulation of endogenous ROS and also triggers the expression of diverse genes (Rizhsky et al., 2002; Pnueli et al., 2003; Vandenabeele et al., 2004; Vanderauwera et al., 2005). These genes include those that encode transcription factors, channels, and transporters. The gene products play active roles in modulating the damage to cells, defense and stress responses, and plant development. In addition, ROS-responsive genes show different patterns of expression depending on the type of ROS, such as H₂O₂, superoxide, and singlet oxygen, to which the cell is exposed, or the subcellular site of production of the ROS (Gadjev et al., 2006). These data clearly indicate the complex way in which oxidative signaling is regulated.

Several transcription factors that are redox controlled have been identified in different organisms. For example, OxyR, PerR, and SoxR were first identified in Escherichia coli and Salmonella species as transcription factors that could sense different types of ROS signals and coordinate distinct redox-dependent genetic responses by binding directly to regulatory sequences in DNA (Kim et al., 2002; Stone, 2004; Lee and Helmann 2006; D'Autreaux and Toledano, 2007). OxyR, PerR, and SoxR are activated by H₂O₂ via a redox switch that consists of a thiol-disulfide bond or iron-sulfur cluster that induces significant structural changes in the protein (Hidalgo and Demple, 1994; Stone, 2004). Thus, these transcription factors also function as bacterial ROS sensors. In eukaryotes, the signaling networks that regulate transcription are highly complicated, and the transcriptional regulator is usually
separate from the sensor or transducer of ROS signaling. For example, the yeast 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 oxidation results in its accumulation in the nucleus. Therefore, in this case, the enzyme Gpx3 acts as a receptor or sensor of H₂O₂ and also functions as a redox-transducer to transduce the oxidative signal to the transcription factor (Delaunay et al., 2000; Delaunay et al., 2002).

In plants, several families of transcription factors, e.g., MYB, WRKY, zinc transporter (ZAT), heat shock transcription factor (HSF), 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; Vranova 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 H₂O₂ 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 non-expressor of pathogenesis-related genes 1 (NPR1) and the TGACG motif binding proteins (TGAs) (Despres 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 ERF1-4, Arabidopsis ERF1-5, and ERF7, and tomato Pto-interacting 4-6 and LeERF1-4 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 ocs- or asl-element, is the only well-characterized ROS-related cis-acting element in plants (Chen and Singh, 1999; Garreton 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 NtPR-1a, NtParA, Nt103, Gmhsp26-A, AtGST, and AtPR-1, contain TGA
motifs (Krawczyk et al., 2002). Another cis-acting element, CORE (coordinate regulatory element for antioxidant defense), has been identified in three antioxidant defense genes in rice that are 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 MPK1-3, 6-9, and 12, MKK4/5, MKK7/9 and MKK1/2, 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 kDa MAP kinases. 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 herein, we identified seven ROS-responsive elements, designated ROSEs, by using bioinformatical analysis to search for promoters that were upregulated 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-ERF6WT and 35S-ERF6DD transgenic plants. These data suggest that an H$_2$O$_2$-activated MAPK cascade
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 Camp et al., 2003; Rizhsky et al., 2003; Takahashi et al., 2004; Gechev et al., 2005; Umbach et al., 2005; Vanderauwera et al., 2005; Gadjev et al., 2006; Wang et al., 2006), ROS-responsive genes were clustered into three groups (Gadjev et al., 2006): Cluster I includes 111 genes that are highly induced by H$_2$O$_2$ treatment and named $hih$. Cluster II contains 65 fluorescent (flu) mutant, O$_3$, and methyl viologen (MV) induced genes, which are named $fom$. Cluster III only contains six genes that are induced by 3-aminotriazole (3-AT) treatment in catalase-deficient mutant plants (Supplementary Table S1). Genes from cluster III were not used for the promoter analysis in this study in order to avoid obtaining misleading results from similar random sequences.

Promoters from the $hih$ and $fom$ clusters were selected for analysis with the program MEME to identify potential transcription factor binding sites. Then the present frequencies of each motif in $hih$ and $fom$ cluster pools were calculated against a whole genome set by MAST program (http://meme.nbcr.net/meme4_6_1/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 (Supplementary Table S2). As a result, a total of seven ROSEs were identified in the $hih$ and $fom$ clusters (Figure 1A). Table I provides detailed information about the seven ROSEs, including homologous sequences, examples of ROS-responsive genes in whose promoters they were found, etc. Several ROSE motifs matched known cis-acting elements, for example, the ABA-responsive element (ABRE; ROSE1), brassinosteroid responsive element (BRRE; ROSE2), W box (ROSE4), and GCC box (ROSE7). However, ROSE3, 5, and 6 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 4×ROSE 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$_2$ (Figure 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 (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 bind the GCC box (Buttner and Singh, 1997). The expression of ERF6 was also increased by the application of singlet oxygen (Danon et al., 2005). The results of our quantitative real-time PCR (qRT-PCR) analysis also showed that ERF6 transcription was induced strongly by 3-AT, H$_2$O$_2$, and MV. After treatment of Arabidopsis seedlings for 6 h with 3-AT or H$_2$O$_2$, a 22- or 12-fold increase in ERF6 transcription was observed, respectively, as compared with untreated seedlings (Figure 2A). We also measured the expression of ERF6 after cold, drought, and HL (2000 μmol/m$^2$·s) treatments. Both cold and HL stresses enhanced the expression of ERF6 significantly (Figure 2B). On the basis of our results and previous reports (Danon et al., 2005; Wang et al., 2006; Jing et al., 2008), we hypothesized that ERF6 and the GCC box are involved in the regulation of ROS-responsive gene expression.

To test the hypothesis, we first analyzed the ability of ERF6 to bind to the GCC box by electrophoretic mobility shift assay (EMSA). We expressed recombinant His-tagged ERF6 protein in E. coli. Although the majority of the recombinant ERF6 was present in inclusion bodies, the small proportion of soluble ERF6 bound strongly to the ROSE7/GCC-box (Figure 2C). When the ROSE7/GCC-box fragment was used as the probe, binding of ERF6 to the ROSE7/GCC-box fragment was competed
efficiently by unlabeled ROSE7/GCC box. The mutant GCC box, which contained two point mutations, could neither bind to ERF6 nor compete with the binding of the wild-type GCC box (Figure 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 the 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 (2000 μmol/m²·s 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 (Figure 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, 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 (Figure 2E). The expressions of several genes (At5g44420, At1g49450, At4g33720, At1g67810, At2g06000 and At2g15480) induced by ROS were also examined by qRT-PCR. The results show that these gene expressions were indeed induced by HL (Supplementary Figure S1). Thus, primer combinations were chosen that amplify fragments of ~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 (Figure 2E). Representative qRT-PCRs of ROS-responsive genes are shown in Figure 2F. Promoters from PDF1.2a, PDF1.2b, At2g37130, and At5g18470 exhibit significant enrichments of PCR products after HL treatment, whereas the negative control from 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 4×GCC-LUC as the reporter and 35S-ERF6, as the effector (Figure 2G) were co-transformed 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, \( P < 0.05 \) under \( t \) test) (Figure 2H), which suggests that ERF6 is an activator of GCC box-mediated transcription. Furthermore, when \( \text{H}_2\text{O}_2 \) was applied, the luciferase activity was increased 8.1-fold in the presence of ERF6 (\( P < 0.05 \) under \( t \) test, Figure 2H) as compared with the control, which indicates that \( \text{H}_2\text{O}_2 \) 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 ERF6 gene in the transcription of ROS-responsive genes, we identified T-DNA insertion mutant erf6-1 and erf6-2 from the Arabidopsis Biological Resource Center. The positions of the T-DNA insertions for the alleles are nucleotides 840 and 47, respectively. The qRT-PCR results showed that \( \text{erf6-1} \) (Salk_087356) is a null mutant, and \( \text{erf6-2} \) (Salk_030723) is a knock-down allele (Supplementary Figure S2). Under our normal growth conditions in soil (see Methods), adult erf6 mutant plants were smaller than wild-type plants, displaying an approximately 12.3% reduction of fresh weight compared to wild-type (\( n = 60, P < 0.05 \) under \( t \) test, Figure 3A and B).

Since we found that ERF6 accumulation was stimulated by HL and ROS in cells, it was reasonable to assume that ERF6 gene functions in photo-oxidative damage. Thus, we measured the chlorophyll fluorescence parameter \( \text{Fv/Fm} \), the maximal quantum yield of photosystem II (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 3C (see Methods). Before exposure to HL treatment, wild-type and \( \text{erf6} \) mutants showed no difference in adapted \( \text{Fv/Fm} \) under light inside the growth chamber (see Methods) (Figure 3D). However, after treatment with HL within 24, 36 and 48 h, leaves of \( \text{erf6-1} \) had a lower \( \text{Fv/Fm} \) value than the corresponding wild-type leaves (\( n = 9, P < 0.05 \) under \( t \) test, Figure 3D). Consistent with this finding, ROSE7-type genes lost their activation under HL treatment in \( \text{erf6-1} \) mutants (Figure 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., 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 expression of the 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* (S. cerevisiae) Y190 cells that had been transformed with pACT2-ERF6 together with pAS2-MPK3, pAS2-MPK6, or pAS2-MPK4 were grown on SD medium. The combination of pACT2-ERF6 with pAS2-MPK3 or pAS2-MPK6 yielded strong β-galactosidase expression (Figure 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 (Figure 4A).

To confirm the interaction between MPK3/6 and ERF6, we monitored the association of transiently expressed MPK3/6 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 co-expressed in protoplasts of *Arabidopsis* leaves, fluorescence from reconstituted yellow fluorescent protein (YFP) was observed in the nucleus (Figure 4B-a). However, co-expression of MPK3-YCE and ERF6-YNE did not result in fluorescence (Figure 4B-b). In addition, no YFP signal was detected with the combinations of MPK3/6-YCE and pSPYNE, or pSPYCE and ERF6-YNE (Figure 4B-c, -d and -e). Consistent with the localization of the interaction signal, confocal microscopy of protoplasts transformed with MPK3, MPK6, or ERF6 fused with green fluorescent protein (GFP) showed that all three fusion proteins were localized in both the cytoplasm and the nucleus (Figure 4B-f, -g and -h). These results confirmed that ERF6 and MPK6 could interact in planta, but ERF6 and MPK3 could not.

The observation that the GFP fluorescence of ERF6 and MPK6 proteins were

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emitted from both the cytoplasm and the nucleus, and that ERF6-MPK6 association only occurred in nucleus, prompted us to carefully examine compartments of the protein interactions by coimmunoprecipitation. To this end, we isolated the nucleus from 35S-ERF6 plant, and investigated ERF6 and MPK6 localization and interaction in planta. Under untreated condition, both ERF6 and MPK6 protein could be detected in total cell extracts, but not the nucleus (Figure 4C, input). Interestingly, after exposure of Arabidopsis seedlings to HL for 2 h, they could be detected by immunobloting from nucleus extracts (Figure 4C, output). The coimmunoprecipitation assays also indicated that the interaction between ERF6 and MPK6 mainly occurred in 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 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 able to be detected in the cytoplasm and nucleus. However, FLAG-ERF6 was almost not present in the nucleus of 35S-ERF6 transgenic plants without H2O2 treatment (Supplementary Figure S3). These results suggest that H2O2 or HL treatment triggers the shuttling of ERF6 protein from the cytoplasm to nucleus, as well as increasing ERF6 accumulation in 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 non-transgenic plants and no phosphorylation band was observed (Figure 4D). These results indicate that ERF6 is a substrate of MPK6.

Both ser-266 and 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 (S) residues are MPK6 phosphorylation sites, the recombinant GST-tagged C-terminus peptides of ERF6 (aa 261-282) (pep$^{S266A}$, pep$^{S269A}$, and pep$^{S266AS269A}$) were generated by mutating the Ser residues to Ala (A) by site-directed mutagenesis (Figure 5A). MPK6 was able to phosphorylate the pep$^{S266A}$ (pep$^{AS}$) and pep$^{S269A}$ (pep$^{SA}$), but not the pep$^{S266AS269A}$ (pep$^{AA}$) mutant proteins (Figure 5B). The single mutation pep$^{AS}$ or pep$^{SA}$ showed reduced phosphorylation as compared with that of pep$^{WT}$. 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 (Bethke et al., 2009; Yoo et al., 2009). We first assayed the MPK6/3 kinase activity induced by H$_2$O$_2$ or HL by measuring the levels of phosphorylation in wild-type plants that were exposed to 2 mM H$_2$O$_2$ or HL. As shown in Figure 5C, exogenous application of H$_2$O$_2$ 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 H$_2$O$_2$ or HL under the same experimental conditions. Importantly, in the conditional gain-of-function (DEX-inducible) MKK5$^{DD}$ 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 (Figure 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 respective basal level within 9 h (Figure 5D, top panel). MKK5$^{DD}$ on the other hand remained quite high and continued to rise. Alternatively, we determined the dynamic changes of ERF6 in MKK5$^{DD}$/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 (Figure 5D, bottom panel).

To confirm whether the dynamic changes of ERF6 are related to the phosphorylation of ERF6, we expressed FLAG-tagged ERF6 (35S-ERF6$^{WT}$), and its mutants, 35S-ERF6$^{AA}$ and 35S-ERF6$^{DD}$ in Arabidopsis, and then analyzed the levels of ERF protein in the nonphosphorylated form (ERF$^{AA}$) and phosphor-mimicking mutation of ERF6 (ERF$^{DD}$). Under HL treatment, the ERF protein levels were increased in both ERF$^{WT}$ and ERF$^{AA}$ transgenic plants. Consistent with the above
observation, 35S-ERF6\textsuperscript{DD} plants accumulated a much lower amount of ERF6 protein than plants that expressed 35S-ERF6\textsuperscript{WT} or 35S-ERF6\textsuperscript{AA} (Supplementary Figure S4). These data imply that there might be a feedback control mechanism to ensure that the expressions of ROS-responsive genes or protein levels are 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-ERF6\textsuperscript{WT}, 35S-ERF6\textsuperscript{AA}, and 35S-ERF6\textsuperscript{DD} transgenic plants, and the resultant protein-agarose complexes were immunoblotted with an anti-MPK6 antibody. Consistent with above analysis (Figure 4C), MPK6 protein could be detected in the complex that contained ERF6\textsuperscript{WT} protein. Importantly, the binding affinity of MPK6 for ERF6\textsuperscript{WT} was much stronger when the complexes were isolated from cells that had been treated with H\textsubscript{2}O\textsubscript{2} as compared with untreated cells (Figure 5E, right panel, lane 1 vs. lane 2), which was probably due to the phosphorylation of ERF6 by H\textsubscript{2}O\textsubscript{2}-activated MPK6. In accordance with this observation, the binding of MPK6 to ERF6\textsuperscript{DD}, which mimicked the ERF6 phosphorylated at Ser 266 and Ser 269, was found to be stronger than the binding of MPK6 to ERF6\textsuperscript{WT} (Figure 5E, right panel, lane 4 vs. lane 1). However, no clear band was observed for ERF6\textsuperscript{AA}, which mimicked the unphosphorylated form of ERF6 (Figure 5E, right panel, lane 3).

Amino acid sequence analysis of ERF6 indicated that the nuclear localization signal (NLS) of ERF6 overlapped with the MAPK docking sequence, and the leucine-rich NES overlapped with two independent phosphorylation sites of ERF6 (Figure 5F and 5G) (la Cour \textit{et al.}, 2004; Kosugi \textit{et al.}, 2008). Activated ERF6 (ERF6\textsuperscript{DD}-GFP) was localized mainly in the nucleus, i.e., >78% of all observed protoplasts showing the fluorescence of ERF6\textsuperscript{DD}-GFP (Figure 5G, bottom panel). In contrast, mutation of the phosphorylation sites of ERF6 (ERF6\textsuperscript{AA}-GFP) resulted in the accumulation of fluorescence in both the cytoplasm and nucleus (Figure 5G, top panel). This result was not surprising because of the regulation of the ERF6 feed-back control mechanism which 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 H\textsubscript{2}O\textsubscript{2}-activated MPK6 enhances ROSE7/GCC box transcriptional activity and expression of downstream genes**

Next, we used a transient expression assay to examine whether phosphorylation of ERF6 by MPK6 affected the transcriptional activity of target genes. As shown in
Figure 6A and 6B, co-expression of ERF6\textsuperscript{WT}, ERF6\textsuperscript{AA}, and ERF6\textsuperscript{DD} with the 4xGCC-LUC reporter dramatically increased the luciferase activity produced. Expression of ERF6\textsuperscript{DD}, the constitutively active form of ERF6, increased LUC expression by 2.4- and 3.2-fold as compared with ERF6\textsuperscript{WT} and ERF6\textsuperscript{AA} (\(P < 0.05\) under \(t\) test), respectively. As expected, after the protoplasts were transformed with ERF\textsuperscript{WT}, \(H_2O_2\) induced the expression of 4xGCC-LUC significantly, which resulted in a 1.9-fold increase as compared with protoplasts that transiently expressed ERF6\textsuperscript{WT} but had not been treated with \(H_2O_2\) (\(P < 0.05\) under \(t\) test). Surprisingly, luciferase activity in the presence of \(H_2O_2\) was inhibited in protoplasts that transiently expressed either ERF6\textsuperscript{DD} or ERF6\textsuperscript{AA}. It is likely that this effect was due to feed-back control by the high level of ERF\textsuperscript{DD} protein present and the inability of ERF6\textsuperscript{AA} to be phosphorylated by \(H_2O_2\)-activated MPK6, respectively. Importantly, the transcription was not enhanced by co-expression of MPK6 and the 4xGCC-LUC reporter in the absence of ERF6. In addition, co-expression of the ERF6\textsuperscript{WT} and MPK6 effectors enhanced the expression of LUC by more than twofold in the presence of \(H_2O_2\) as compared with control conditions where no effectors were present (\(P < 0.05\) under \(t\) test). In the \textit{mpk6-3} mutant plants, ERF6\textsuperscript{WT} induced luciferase activity was dramatically inhibited by the deficiency of MPK6, even under \(H_2O_2\) 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 \(H_2O_2\)-induced genes that contain a GCC box in their promoter in wild-type, \textit{erf6-1}, 35S-ERF6\textsuperscript{WT}, 35S-ERF6\textsuperscript{AA}, and 35S-ERF6\textsuperscript{DD} transgenic plants. As shown in Figure 6C-a, expression of ERF6 was increased by 195-, 131-, and 40-fold in 35S-ERF6\textsuperscript{WT}, 35S-ERF6\textsuperscript{AA}, and 35S-ERF6\textsuperscript{DD} plants, respectively, as compared with wild-type plants. The expression of downstream genes was also enhanced dramatically by treatment with \(H_2O_2\) or overexpression of ERF6. For example, the levels of \textit{PDF1.2a} and \textit{PDF1.2b} transcription were greatly increased in ERF6\textsuperscript{DD} transgenic plants, with an increase of more than 600- and 400-fold, respectively, as compared with levels in wild-type plants. Application of \(H_2O_2\) also resulted in a further increase in the transcription of both \textit{PDF1.2a} and \textit{PDF1.2b} in ERF6\textsuperscript{DD} transgenic plants (Figure 6C-b and c). The transcription factor WRKY33 is a ROS-induced gene that contains a GCC box in its promoter (Davletova \textit{et al}, 2005; Pitzschke \textit{et al}, 2009). When \(H_2O_2\) was applied to ERF6\textsuperscript{WT} lines, the expression of WRKY33 was increased 2.3-fold as compared with untreated plants (\(P < 0.05\) under \(t\) test). In addition, the abundance of \textit{WRKY33} mRNA in ERF6\textsuperscript{DD} plants was increased significantly. However, treatment with \(H_2O_2\) had
almost no effect on the expression of WRKY33 in 35S-ERF6\textsuperscript{AA} and 35S-ERF6\textsuperscript{DD} plants (Figure 6C-e). Similar results were obtained for other genes, which included PR5 and MYB51 (Figure 6C-d and -f). However, the transcription levels of all the checked genes in erf6-1 mutants were not significantly affected, even under H\textsubscript{2}O\textsubscript{2} treatment.

To determine whether the increased expression of the ROS-responsive genes in 35S-ERF6\textsuperscript{DD} plants is dependent on MPK6, we conducted qRT-PCR experiments by using MKK5\textsuperscript{DD}/mpk6-3/35S-ERF6 or mpk6-3 mutant plants. Impressively, the expression of nearly all the ROS-responsive genes examined (PDF1.2\textsubscript{a}, PDF1.2\textsubscript{b}, WRKY33, PR5 and MYB51) was significantly reduced in both mpk6-3 mutant plants and MKK5\textsuperscript{DD}/mpk6-3/35S-ERF6 compared with the 35S-ERF6\textsuperscript{DD} transgenic plants, with or without H\textsubscript{2}O\textsubscript{2} treatments (Figure 6C). For example, PDF1.2\textsubscript{a} and PDF1.2\textsubscript{b} transcript abundance under 2 mM H\textsubscript{2}O\textsubscript{2} treatment in 35S-ERF6\textsuperscript{DD} transgenic plants were 28 or 14 times higher than that in MKK5\textsuperscript{DD}/mpk6-3/35S-ERF6, respectively. Meanwhile, the low basal level of these gene expressions in mpk6-3 did not differ much from that of erf6-1 mutant plants (Figure 6C). We noticed that PR5, WRKY33 and MYB51 were still induced by H\textsubscript{2}O\textsubscript{2} treatment in mpk6-3. The reason might be that other MPKs (e.g. MPK3) have been shown to be activated in the MKK5/MPK6/3 module in the presence of H\textsubscript{2}O\textsubscript{2} (Moon \textit{et al.}, 2003; Takahashi \textit{et al.}, 2011), which is involved in the regulation of these gene expressions. 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 upregulated by ROS, seven potential ROSEs were identified (Figure 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 H\textsubscript{2}O\textsubscript{2}, MV, and 3-AT (Figure 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 I). Indeed, the results of the transient assay indicate that ABA could strongly induce an increase in luciferase activity under the control of Gal4-ROSE1 (Figure 1B), which corresponded to ABRE. The ABRE-binding protein ABI3 mediates the expression of the peroxiredoxin antioxidant AtPER1 gene in response to oxidative stress (Camilla et al, 2003). The tomato Ep5C gene, which encodes an extracellular cationic peroxidase, is transcriptionally activated by the H$_2$O$_2$ that is generated during the course of plant interaction with pathogens (Coego et al, 2005). The promoter of Ep5C contains an ABRE and 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 ROSE 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 (Buttner 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 dehydration 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 (Figure 2), and that the transcription of the luciferase gene was enhanced by co-transfection of the effector ERF6 or the application of H$_2$O$_2$ 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 (EpRE) is found in the promoters of some animal glutathione S-transferase (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 EpRE and ocs elements 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
AtPRI (Krawczyk et al., 2002). Although previous data strongly suggest that ocs1/as1
elements are oxidative stress responsive elements (Garreton et al., 2000), 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 which
controls oxidative signaling through 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 6 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$_2$ (Figure 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.
Firstly, the expression of ERF6 was induced dramatically by treatment with H$_2$O$_2$,
MV or 3-AT (Figure 2A). Secondly, an ERF6 fusion protein was able to bind
specifically to the ROSE/GCC box in vitro and in vivo (Figure 2C and 2F). Co-
expression of the ERF6 effector enhanced the expression of a luciferase gene under
the control of a GAL4 promoter that harbored a GCC box (Figure 2H). Thirdly,
mutation of erf6-1 resulted in the changes in the transcriptions of ROS- or HL-
responsive genes (Figure 6C; Supplementary Figure S1). The profile of ERF6
expression during the HL and H$_2$O$_2$ exposure reported in this article (Figure 2A and
2B) 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 (Figure 2A, 2B 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 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, 2011; Moffat et al, 2012). These are quite complementary to the role ERF6 plays in 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 biotic and abiotic responses in plants (Buttner 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 accumulations in nucleus (Figure 2D and 4C), the increment of gene expressions related to oxidative stress, and stronger photooxidative damage in erf6-1 (Figure 3D and 3E). 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 (Figure 4C and 5E, 5G). ERF6 protein first appeared to be induced faster and reached a peak at 3 h after ROS and HL treatment, and then declined to its un-induced level after 6 h (Figure 5D), a finding...
that is coherent with ERF6 transcription pattern under HL (Figure 2B). Under MKK5DD background, the induction pattern of ERF6 protein after the application of DEX was similar to those of wild-type treated with ROS and HL (Figure 5C and D). ERF6 protein was nearly undetected in phosphor-mimicking of ERF6 transgenic plants (Supplementary Figure 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 over-accumulation, and repair of damaged proteins, lipids, and photosystems (Bennet, 1977; Kao and Forseth, 1991; Allen, 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 transcript accumulation of ROS- or HL-responsive genes. Therefore, H2O2- 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 modulating 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 (Figure 6). Co-expression of ERF6DD, the constitutively active form of ERF6, resulted in a significant increase in LUC expression as compared with ERF6WT and ERF6AA (Figure 6B). By contrast, application of H2O2 to plants that expressed ERF6WT stimulated LUC expression, but lowered LUC expression in plants that expressed ERF6DD (Figure 6B). Transgenic seedlings that harbored ERF6DD 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 (Figure 6C). Meanwhile, a deficiency of either ERF6 or MPK6 almost inhibited the enhanced transcription of these genes (Figure 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 coimmunoprecipitation indicate that a complex of MPK6/ERF6 exists in plant cells. Interestingly, the expression of all tested ROS-responsive genes showed high activation in MKK5<sup>DD</sup> transgenic plants treated with DEX (Figure 6C), which increased MPK3 and MPK6 activities in a time-dependent manner (Figure 5D). By contrast, the transactivation of these genes was almost completely blocked in erf6 and mpk6 mutants, even in MKK5<sup>DD</sup>/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 (Figure 5B, E and G). ERF6<sup>DD</sup> binds to MPK6 with high affinity, in contrast to ERF6<sup>AA</sup> (Figure 5E), and lower levels of ERF6<sup>DD</sup> proteins and transcription of ROS-responsive genes were accumulated in ERF6<sup>DD</sup> transgenic plants (Figure 6C; Supplementary Figure S4). These observations suggest that MPK6-mediated phosphorylation controls both the stability of the MPK6-ERF6 complex and the nucleo-cytoplasmic shuttling of ERF6, and leads to an increase in oxidative gene transcription.

In summary, H<sub>2</sub>O<sub>2</sub> 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, erf6-1, erf6-2, mpk6-3 (Wang et al, 2010), and transgenic Arabidopsis thaliana (ecotype Columbia-0) plants were grown in a 16 h light / 8 h dark photoperiod at 22°C. For treatment, three-week old seedlings grown on a Murashige and Skoog (MS) medium that contained 2 % sucrose and 0.8 % phytagel (Sigma-Aldrich, St. Louis, MO) were transferred to incubation buffer (50 mM KCl and 10 mM MES-KOH, pH 6.2) containing the indicated concentrations of H<sub>2</sub>O<sub>2</sub>, MV, 3-AT or ACC for the indicated times. For HL treatment, four-week-old seedlings grown on soil were exposed to light at the indicated intensity.

The primers used in all PCRs are listed in Supplementary Table S3.
Analysis of statistically significant promoter motifs

Promoter sequences were defined as 1000-bp sequences upstream of the translation start codon and were downloaded from the Arabidopsis Information Resource (TAIR) database (ftp://ftp.arabidopsis.org/home/tair/sequence/blast_datasets/OLD/At_upstream_1000_200630310.gz). The upstream regions were analyzed with the MEME_3.5.1 program (http://meme.sdsc.edu) as follows. All of the genes from the hih and fom cluster were pooled randomly into two groups. Each group was analyzed using MEME with a fixed motif length from 6 to 30, and thirty requested motifs. Conserved motifs present in all two pools were selected. The statistically significant 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 (Supplementary Table S3). To prepare effector plasmids, the coding regions of MPK6, and wild-type and mutant ERF6 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, Madison, WI) and a TD-20/20 luminometer (Promega). To normalize values after each transfection, 0.4 mg of the plasmid pPTRL, which contained the Renilla 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 electrophoretic mobility shift assay

For in vitro protein expression, the coding region of ERF6 was inserted in frame into the plasmid pET-28a. The resultant pET-ERF6 construct was introduced into E. coli BL21 (DE3) cells. The recombinant His-tagged proteins were purified using Ni-NTA agarose (Qiagen, Valencia, CA), in accordance with the manufacturer’s protocol. Individual synthetic DNA oligonucleotides that corresponded to the 16-bp GCC box
fragment 5'-AGCCGCCAGCCGCCAGCCGCC-3' and its mutant 5'-ATCCATCCTCCATCCTCC-3' were annealed with their complementary oligonucleotides. The resultant double-stranded oligonucleotides were end-labeled with [γ-32P] ATP and T4 polynucleotide kinase (Invitrogen, Carlsbad, CA) 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 ERF6 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 fmol of the wild-type or mutant forms 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 under non-denaturing conditions.

Yeast two-hybrid system

For yeast two-hybrid assays, the coding regions of MPK3, MPK6, and MPK4 were amplified by RT-PCR using primers that contained appropriate restriction enzyme sites. The amplified fragments were inserted into the plasmid pAS2 as the baits (Clontech, Palo Alto, CA) 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 S. cerevisiae strain Y190 were transformed simultaneously with pAS-MPK6/3/4 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. Protoplasts isolated from Arabidopsis leaves were transformed with the following combinations of plasmids: (1) pSPYCE-MPK6/3 and pSPYNE; (2) pSPYCE and pSPYNE-ERF6; or (3) pSPYCE-MPK6/3 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 pHBT-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 16-20 h, the fluorescence of
the protoplasts was measured with an FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan). All figures show representative images from the three independent experiments.

**Transgenic plants**

The coding region of ERF6 was amplified from cDNA isolated from Arabidopsis ecotype Columbia (Col-0) using primers that contained EcoRI and SalI sites, respectively. Mutations were introduced into the ERF6 coding sequence using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The wild-type and mutant ERF6 coding regions were cloned together with an N-terminal FLAG-epitope into the vector pCAMBIA1205 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 ERF6WT, ERF6AA, and ERF6DD seedlings were ground in IP buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 1 mM NaF, 10 mM β-glycerophosphate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.1% Triton X-100). Aliquots that contained 200 μg of total protein were added to 40 μl of anti-FLAG M2 affinity gel (Sigma-Aldrich) and incubated at 4 °C for 4 h on a rocking platform. After washing three times with TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), the resultant agarose bead-protein complexes were used for either the kinase assay or immunoblotting.

For immunoblotting, the agarose bead-protein complexes were separated by electrophoresis on 12.5% SDS-polyacrylamide gels, and the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland) by electrophoretic transfer. After blocking at room temperature, the membranes were incubated with monoclonal anti-FLAG M2 antibody, anti-AtMPK3, or anti-AtMPK6 antibody (Sigma-Aldrich). After washing three times, the membranes were incubated with horseradish-peroxidase-conjugated secondary antibody, and visualized using Lumi-Light Western Blotting Substrate (Roche) in accordance with the manufacturer’s instructions.

The kinase assay was performed as described previously (Liu and Zhang, 2004).
In brief, recombinant His-tagged MPK6 (10 μg) was activated by incubation with recombinant MKK5DD (1 μg) in the presence of 50 μM ATP in 50 μl of reaction buffer (20 mM HEPES, pH 7.5, 10 mM MgCl₂, and 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 c-terminus peptides terminus (aa 261-282, 1:10 enzyme: substrate ratio) in the same reaction buffer with 50 μM ATP and [γ-32P] ATP (0.1 μCi per reaction). The reactions were stopped by the addition of SDS loading buffer after 30 min. The phosphorylated native ERF6 proteins were visualized by autoradiography after separation on a 10% SDS-polyacrylamide gel. After electrophoresis, the gel was exposed to a Kodak X-Omat film (Kodak, Rochester, NY) for 1 h. A separate phosphorylation reaction that did not contain [γ-32P]ATP was used for immunoblotting with the monoclonal anti-FLAG M2 antibody (Sigma-Aldrich). Another phosphorylation without the addition of [γ-32P]ATP was used for the immunoblot assay with monoclonal anti-Flag M2 antibody (Sigma-Aldrich, St. Louis, MO).

**Quantitative real-time PCR**

Total RNA from *Arabidopsis* seedlings was extracted using TRIzol reagent (Invitrogen). Reverse transcription was performed using 5 μg 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 were used as described in Supplementary Table S3. The qRT-PCR was performed with the Stratagene Mx3005 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: forward 5'-CACACTCCACCTTGCTTGCCT-3' and reverse 5'-'TGGTCTTTCCGGTGAGAGAGTCTT-3'.

**Chromatin immunoprecipitation and PCR**

ChIP was performed as described previously (Chen et al., 2008). In brief, wild-type and 35S-FLAG-ERF6 seedlings were grown on MS plates for 14 d, and then treated with HL (2000 μmol/m²·s) 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 VCX130 ultrasonic processor (Sonics). Immunoprecipitation was performed by incubating chromatin with 200 μL 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. The primer pairs (forward and reverse) used for ChIP-qPCR were PDF1.2, PDF1.2b, ATPN, At5g18470 and UBQ10 (see Table S3).

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Whitbred JM, Schuler MA (2000) Molecular characterization of CYP73A9 and


Figure Legends

**Figure 1.** Summary of putative ROS-responsive cis-elements (ROSE). 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 transfection, the luciferase gene from *Renilla* was used as an internal control. Luciferase activity is expressed in arbitrary units relative to *Renilla* luciferase activity. Each value represents the mean (± SD) of six independent experiments. The asterisk means that there was a statistically significant difference in luciferase activity (p < 0.05 under Student’s *t* test) between the control and treatment conditions.

**Figure 2.** ROS enhanced ERF6 expression and the binding of ERF6 to the ROSE7/GCC box. A, An increase in the expression of ERF6 was induced by 3-AT, H$_2$O$_2$, and MV. B, HL and cold treatments, but not drought, increased expression of ERF6. In (A) and (B), total RNA was extracted from seedlings treated with ROS, HL, cold or drought, and the abundances of ERF6 mRNA were assayed by qRT-PCR. *UBQ10* primers were used in the PCR as internal controls. Three independent qRT-PCR assays were performed. Error bars indicate SD. C, ERF6 bound specifically to the ROSE7/GCC box. EMSA was performed with radiolabeled (hot probe) or unlabeled (cold probe) GCC and mGCC DNA fragments (top panel) and recombinant ERF6 protein; specific combinations are shown above the autoradiograph. Unlabeled fragments were added gradually in 100- or 400-fold excess, as indicated. D, ERF6 abundance was increased by HL, ACC and MV in 35S-FLAG-ERF6 transgenic lines. 35S-FLAG-ERF6 transgenic plants were kept under non-stress conditions (control) or subjected to HL (2,000 μmol m$^{-2}$s$^{-1}$), 50 μM ACC and 10 μM MV at the indicated time points. Proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-FLAG antibody. E, ERF6 binding target genes containing the typical ROSE7/GCC box in their promoters. F, Levels of H$_2$O$_2$- and HL-responsive genes containing the ROSE7/GCC element in the promoters in 35S-FLAG-ERF6 transgenic plants with or without HL treatment by ChIP-qRT-PCR amplification. The qRT-PCR values are expressed as the mean ± SD compared to that of the untreated plants with HL. An asterisk p<0.05 was considered statistically significant under Student’s *t* test. Quantitative data from FLAG-ERF6 seedlings were normalized to those from wild-
type seedlings. Assays were done in triplicate, and *ubiquitin 10* (*UBQ10*) served as a negative control. G, Schematic representations of the reporter and effector plasmids. The Gal4 binding site and ROSE7/GCC box sequence were fused to a minimal TATA box and the LUC gene. H, Relative luciferase activity in leaves transformed with 35S-ERF6 and the luciferase reporter after application of 2 mM H$_2$O$_2$. Luciferase activity was determined as described in the legend of Figure 1B. Each value represents the mean (± SD) of three independent experiments.

**Figure 3.** Phenotype of *ERF6* mutation in response to oxidative stress. A, Wild-type and *erf6* mutant plants in soil under normal growth conditions. The picture was taken for 15-d-old seedlings grown at 21°C. B, Comparison of fresh weight of wild type and *erf6* mutant plants. C, Morphology (left) and chlorophyll fluorescence images for Fv/Fm (right) of wild-type and *erf6-1* seedlings. 14-d-old seedlings were acclimated at 600 μmol m$^{-2}$s$^{-1}$ for 6 h and then were exposed to HL (2000 μmol m$^{-2}$s$^{-1}$). The chlorophyll fluorescence images were collected after 12 h of HL treatment. D, The Fv/Fm was measured for seedlings from wild-type plants (squares) and *erf6* mutant plants (circles) following light acclimation (600 μmol m$^{-2}$s$^{-1}$) and HL illumination (2,000 μmol m$^{-2}$s$^{-1}$) at indicated time points. Each value represents the mean (± SD) of six independent experiments. The asterisk means that there was a statistically significant differences (p<0.05 under t test) between the wild-type and *erf6-1* mutant.

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Putative NES in ERF6 overlapped with phosphorylation sites. The conserved residues of the NES are highlighted in green and the phosphorylation sites in red. G, ERF6^{AA}·GFP was localized in both cytoplasm and nucleus (top panel) and ERF6^{DD}·GFP in the nucleus (bottom panel) (bar = 5 μm).

**Figure 6.** Transcription activation of ROSE7/GCC box-mediated expression by phosphorylated ERF6. A, Constructs used in the co-transformation experiments. The wild-type and mutant ERF6 (ERF6^{WT}, ERF6^{AA}, and ERF6^{DD}) and MPK6 effector plasmids were under the control of the CaMV 35S promoter. B, Relative luciferase activities of reporter genes in the protoplasts from WT or mpk6-3 null mutant co-transfected with WT or mutant ERF6 and MPK6 effectors, with or without H₂O₂ application. Each value represents the mean (± SD) of six independent experiments. An asterisk and **p<0.05 were considered statistically significant under Student’s t test (control and H₂O₂ treatment, respectively). C, Expression of ROS-responsive genes that contained a GCC box in their promoters in wild-type, erf6-1, 35S-ERF6^{WT}, 35S-ERF6^{AA}, 35S-ERF6^{DD}, mpk6-3 mutants and mpk6-3/35S-ERF6^{WT} transgenic plants. Total RNA was extracted from the control and H₂O₂-treated wild-type and transgenic seedlings. Expression of ERF6, PDF1.2a, PDF1.2b, PR5, WRKY33, and MYB51 was assayed by qRT-PCR with gene-specific primers. UBQ10 primers were used in the PCR as internal controls. Three independent real-time RT-PCR assays were performed. Error bars indicate SD. An asterisk and **p<0.05 were considered statistically significant under Student’s t test (control and H₂O₂ treatment, respectively).

**Supplemental Figure Legend**

**Figure S1.** The expression of ROS-responsive genes containing typical ROSE7/GCC box in their promoters in wild-type and erf6-1 under HL treatment. Total RNA was extracted from seedlings treated with HL (2,000 μmol m⁻²s⁻¹) for 2 h, and the abundances of mRNA of these genes were assayed by qRT-PCR with the following primers: PDF1.2a: forward 5'-TTTGCTTCCATCATCACCCCTA-3' and reverse 5'-GCGTCAAAGCAGCAGAAAG-3'; At1g49450: forward 5'-ACCGAAGTCACAACGATTAT-3' and reverse 5'-GGGTAGATTGGTGATGACGA-3'; At4g33720: forward 5'-AAGCCCAAGACAGTCCTCAA-3' and reverse 5'-GCTGGTTAGCATAGTACGG-3'; At1g67810: forward 5'-GGTTTCGTTGTAGCTCC-3' and reverse 5'-ATCCCGTGACTCGGTTC-3';
At2g06000: forward 5'-ATCCGTTTATCTCCTTTG-3' and reverse 5'-TAAGAACCCTAATAACCGAT-3'; At2g15480: forward 5'-GCGAGAACGCTGACTTTA-3' and reverse 5'-TGGGAGACCAGGGATTAC-3'. 

UBQ10 primers were used in the PCR as internal controls. Three independent qRT-PCR assays were performed. Error bars indicate SD.

Figure S2. Expression of ERF6 in wild-type, erf6-1 and erf6-2 plants. A, The insertion positions of T-DNA in the ERF6 gene. B, ERF6 expression in wild-type, erf6-1 and erf6-2. Total RNA was extracted from wild-type (white column) and erf6-1 and erf6-2 mutant seedlings (black and gray column, respectively). The fragments were amplified using following primers: 200: forward 5'-TTGTCTCCGTTGCTACTA-3' and reverse 5'-CAATTCTTCCGTTGCTACTA-3'; 400: forward 5'-TCGCGTTACTGTTCAATCC-3' and reverse 5'-TGCTCTTCTCTCAGCTGC-3'; 700: forward 5'-GGTGGTTGATGGTCTACTA-3' and reverse 5'-ATGCTACTGTTGCTACTA-3'; 800: forward 5'-GATTATACTGTTGCTACTA-3' and reverse 5'-TGCCTCTTCTCTCAGCTGC-3'; 900: forward 5'-ATGCTAATCCGTTGCTACTA-3' and reverse 5'-TACAACAAACGCTGCTGCTAC-3'. Three independent qRT-PCR assays were performed. Error bars indicate SD. UBQ10 primers were used in the PCR as internal controls.

Figure S3. H2O2 enhances the accumulation of ERF6 protein in the nucleus. Total cell and nucleus extracts from FLAG-ERF6 transgenic plants with or without H2O2 treatment were used for immunoprecipitation. ERF6 and MPK6 protein were detected by immunoblotting using the anti-FLAG antibody.

Figure S4. HL enhances ERF6 accumulation in 35S-ERF6WT and 35S-ERF6AA transgenic plants, but not in 35S-ERF6DD. FLAG-ERF6WT, FLAG-ERFAA and FLAG-ERFDD transgenic plants were treated with HL (2,000 μmol m⁻²s⁻¹) for 2 h. Total cell extracts from the treated and untreated plants were used for immunoprecipitation. ERF6 protein was detected by immunoblotting using the anti-FLAG antibody.
Table S1. Gene lists used for ROSEs prediction.

Table S2. Enrichment of ROSEs in ROS-responsive gene clusters and whole genome set.

Table S3. A list of the primers used in all experiments.
### Table I. Similar cis-acting elements and putative functions of ROSEs

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<th>Core seq</th>
<th>Function</th>
<th>Reference</th>
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<td>+</td>
<td>W-box</td>
<td>(T)TGAC</td>
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