Induction of Isoforms of Tetrapyrrole Biosynthetic Enzymes, AtHEMA2 and AtFC1, under Stress Conditions and Their Physiological Functions in Arabidopsis1,2[W][OA]

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In the tetrapyrrole biosynthetic pathway, isoforms of glutamyl-tRNA reductase (HEMA2) and ferrochelatase1 (FC1) are mainly expressed in nonphotosynthetic tissues. Here, using promoter-β-glucuronidase constructs, we showed that the expressions of Arabidopsis (Arabidopsis thaliana) HEMA2 (AtHEMA2) and FC1 (AtFC1) were induced in photosynthetic tissues by oxidative stresses such as wounding. Transcript levels and β-glucuronidase activity were rapidly induced within 30 min, specifically in the wound area in a jasmonate-independent manner. Transcriptome analysis of wound-specific early inducible genes showed that AtHEMA2 and AtFC1 were coinduced with hemoproteins outside plastids, which are related to defense responses. Ozone fumigation or reagents generating reactive oxygen species induced the expression of both genes in photosynthetic tissues, suggesting that reactive oxygen species is involved in the induction. Since cycloheximide or puromycin induced the expression of both genes, inhibition of cytosolic protein synthesis is involved in the induction of these genes in photosynthetic tissues. The physiological functions of AtHEMA2 and AtFC1 were investigated using insertion knockout mutants of each gene. Heme contents of the roots of both mutants were about half of that of the respective wild types. In wild-type plants, heme contents were increased by ozone exposure. In both mutants, reduction of the ozone-induced increase in heme content was observed. These results suggest the existence of the tetrapyrrole biosynthetic pathway controlled by AtHEMA2 and AtFC1, which normally functions for heme biosynthesis in nonphotosynthetic tissues, but is induced in photosynthetic tissues under oxidative conditions to supply heme for defensive hemoproteins outside plastids.

Heme, an essential molecule with various biological functions, is widely distributed in all eukaryotic cells. In fact, heme is (1) a prosthetic group for photosynthetic and respiratory cytochromes involved in energy transduction, (2) oxidase, including catalase, peroxidase, and NADPH oxidase, involved in scavenging reactive oxygen species (ROS), (3) hemoglobin, including leghemoglobin, involved in oxygen homeostasis, and (4) cytochrome P450 involved in the biosynthesis of secondary metabolites.

In plants and many bacteria, all tetrapyrroles including heme and chlorophylls (Chls) originate from a common biosynthetic pathway (Beale, 1999). They are synthesized exclusively from glutamyl-tRNA5-glü via 5-aminolevulinic acid (ALA), a linear five-carbon molecule. ALA destined for all cellular tetrapyrroles is formed by two-step conversion from glutamyl-tRNA; glutamyl-tRNA is first reduced to Glu 1-semialdehyde (GSA) in a NADPH-dependent reaction by glutamyl-tRNA reductase, and then GSA is transaminated by GSA aminotransferase (Kannangara et al., 1988). Among these steps, reduction of glutamyl-tRNA is a regulatory point for the synthesis of heme and Chls, since it is the first step unique to ALA formation and glutamyl-tRNA reductase is subjected to feedback regulation by heme (Beale, 1999). Glutamyl-tRNA reductase is encoded by HEMA genes. In Arabidopsis (Arabidopsis thaliana) and cucumber (Cucumis sativus), two HEMA genes have been isolated (Ilag et al., 1994; Kumar et al., 1996; Tanaka et al., 1996; Ujwal et al., 2002). In both plants, HEMA1 gene is expressed in photosynthetic tissues and was induced by illumination, but no transcripts were detectable in roots. On the other hand, HEMA2 gene is preferentially expressed in nonphotosynthetic
tissues, and its expression did not alter by illumination. Recently, a third HEMA gene, HEMA3, has been identified in Arabidopsis, but its expression was very low under all experimental conditions tested (Matsumoto et al., 2004).

Meanwhile, the branch point of the biosynthesis pathway of heme and Chls is protoporphyrin IX, a closed macrocycle without chelated ions. Ferrochelatase (protoheme ferrolyase, EC 4.99.1.1) is the terminal enzyme of heme biosynthesis, and catalyzes the insertion of ferrous ion into protoporphyrin IX. This enzyme is important for regulation of the branch point, and may also play a role in the coordination of heme and apoprotein production. In Arabidopsis and cucumber, two ferrochelatase (FC) genes have been isolated (Miyamoto et al., 1994; Smith et al., 1994; Chow et al., 1998; Suzuki et al., 2002). Interestingly, the expression profiles of two FC isoforms were very similar to those of HEMA; FC2 showed a light-induced and predominant expression in photosynthetic tissues like HEMA1, whereas FC1 showed a light-independent and preferential expression in nonphotosynthetic tissues like HEMA2. These similar expression profiles of HEMA and FC genes suggested two separate pathways of tetrapyrrole biosynthesis operating in plant cells; one involving HEMA1 and FC2 for Chls and heme biosyntheses in photosynthetic tissues, and the other involving HEMA2 and FC1 for heme biosynthesis in nonphotosynthetic tissues. In fact, a model showing functional heterogeneity in the pool of ALA was proposed after inhibition studies in chloroplasts of developing cucumber seedlings (Huang and Castelfranco, 1990), although this distinction between ALA used for Chls versus other tetrapyrroles is now considered that plastids are the major site of heme synthesis, cycloheximide (CHX; Suzuki et al., 2002). Interestingly, these expression profiles were observed in

RESULTS

Induction of the Expression of AtHEMA2 and AtFC1 by CHX

It has been shown in cucumber that the expressions of CsFC1 and CsHEMA2 were induced by CHX (Suzuki et al., 2002). Since reminiscent tissue- and light-specific expression profiles were observed in AtHEMA2 (Ujwal et al., 2002) and AtFC1 (Singh et al., 2002), we analyzed whether the expression of these genes was affected by CHX. As shown in Figure 1A, 100 μM CHX increased both AtHEMA2 and AtFC1 mRNA. Although the levels of AtFC1 transcripts were much higher than AtHEMA2,
the induction profiles of both mRNA were similar, and peaked at 6 h after CHX treatment. After 6 h of CHX treatment, the expression of other isoforms, AtHEMA1 and AtFC2, was rather decreased, while that of AtHEMA3 was undetectable before and after treatment (Fig. 1B).

Effects of Wounding on the Expression of AtHEMA2 and AtFC1

In transgenic tobacco (Nicotiana tabacum) carrying AtFC1 promoter fused with reporter genes, Suc, wounding, oxidative stress, and tobacco mosaic virus infection induced the promoter activity of AtFC1 (Singh et al., 2002). In addition, it is reported that CHX acted as a signal to elicit the expression of wounding-responsive genes (Nishiiuchi et al., 2002). Thus, we examined whether mechanical wounding treatment induced the expression of AtHEMA2 and AtFC1.

To carry out a detailed study of the expression profiles of AtHEMA2 and AtFC1 genes, the promoter region was fused to a reporter gene uidA encoding GUS and introduced into transgenic plants. For AtFC1, a fragment ranging from −1,018 to +69 bp upstream of the translation start site, including 5’ untranslated region and the sequence encoding the first 23 amino acids, was translationally fused to uidA. The resultant construct was introduced into Arabidopsis via Agrobacterium- mediated transformation and designated AtFC1::GUS. For AtHEMA2, the transgenic line (AtHEMA2::GUS) was provided by Dr. M.J. Terry (Ujwal et al., 2002).

As shown in Figure 2A, the levels of endogenous AtHEMA2 and AtFC1 mRNA rapidly increased within 30 min of wounding treatment. The inductions of both genes were transient and returned to the initial levels within 3 h. We carried out histochemical analysis of transgenic lines, AtHEMA2::GUS and AtFC1::GUS. A

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**Figure 2.** Induction of AtHEMA2 and AtFC1 mRNA by wounding treatment. A, Changes in AtHEMA2 and AtFC1 mRNA by wounding were quantified by RNA gel-blot analysis. Bars indicate relative transcript levels of AtHEMA2 and AtFC1 mRNA to 0 time control, after normalizing the density of AtHEMA2 and AtFC1 mRNA to that of ACT. Circles indicate quantified data of cDNA macroarray. White circles indicate the fold expression value of each mRNA after wounding, and black circles indicate the value of 30 μM MeJA-treated samples. B, Transgenic lines of AtHEMA2::GUS and AtFC1::GUS were wounded by forceps or untreated and incubated for 6 h, and then total soluble proteins were extracted for the assay of GUS activity. The amount of 4-Mu produced in the reaction was determined by measuring 4-Mu-specific fluorescence. C and D, GUS activities in wound-treated AtHEMA2::GUS (C) and AtFC1::GUS (D) were stained histochemically. Wound-treated (b and d) and untreated (a and c) seedlings (a and b) and leaves (c and d) are presented. The regions wounded by forceps were the middle of the leaves (d).
leaf was wounded with forceps and then the entire plant was stained histochemically for GUS activity after 6 h. Consistent with the previous report (Ujwal et al., 2002), hardly any staining was found in the aerial parts of unwounded AtHEMA2::GUS plants (Fig. 2C). For AtFC1::GUS, GUS staining was observed in roots and petioles (Fig. 2D). By contrast, in wounded AtHEMA2::GUS and AtFC1::GUS plants, strong staining was evident in tissue around the wounding region (Fig. 2, C and D). In AtHEMA2::GUS, untreated leaves were not affected by wounding, while some systemic induction was observed in AtFC1::GUS. Quantitative analysis confirmed the increase of GUS activity in wounded leaves (Fig. 2B). These results suggested that wounding triggered the rapid and local induction of AtHEMA2 and AtFC1.

Global Analysis of Wounding for Responsive Arabidopsis Genes Related to Hemoproteins and Heme Biosynthesis

Wounding is known to induce the expression of hemoproteins, in particular cytochrome P450 and peroxidase for the repair of cell walls via suberin and lignin synthesis (Lagrimini and Rothstein, 1987; Narusaka et al., 2004). Furthermore, heme itself has been shown to increase after wounding of Jerusalem artichoke (Helianthus tuberosus; Werck-Reichhart et al., 1988). Thus, it is possible that the wounding-inducible expression of AtHEMA2 and AtFC1 functions for heme supply to such hemoproteins for defensive purposes. Therefore, we performed transcriptome analysis to identify coordinately expressed genes related to defensive functions of homoproteins and enzymes for heme biosynthesis that are possibly controlled by the same wounding-responsible signaling pathway with AtHEMA2 and AtFC1.

The wounding-inducible expression occurs not only in the wounded leaves (local response) but also in distal unwounded leaves (systemic response; Bowles, 1993). Studies on the wounding-inducible expression in tomato (Solanum lycopersicum) have demonstrated that an octadecanoid pathway including jasmonate (JA) is involved in signaling that leads to the transcriptional activation of wounding-responsive genes (Farmer and Ryan, 1992; Farmer et al., 1998). It is thought that JA is biosynthesized at the site of wounding and acts as a mobile wounding signal for the systemic response (Schilmiller and Howe, 2005). Previous study suggested that AtFC1 promoter activity in transgenic tobacco was not affected by JA methyl ester (MeJA) and arachidonic acid (Singh et al., 2002). Our transcriptome analysis of JA-responsive genes (Sasaki et al., 2001; Sasaki-Sekimoto et al., 2005) and a JA precursor, 12-oxo-phytodienoic acid-responsive genes (Taki et al., 2005), observed that neither AtHEMA2 and AtFC1 were responsive to JA treatment. In fact, MeJA treatment did not affect the expression of AtHEMA2 and AtFC1 (Fig. 2A), showing that the rapid and transient wounding-inducible expression of AtHEMA2 and AtFC1 is not mediated by the octadecanoid signaling pathway. Thus, we selected the Arabidopsis gene related to heme biosynthesis and hemoproteins, which showed the wounding-responsive expression, but was not affected by JA treatment. To detect rapidly and transiently induced wounding-specific genes, a time-course experiment was performed at 0, 0.25, 0.5, 1, 3, and 6 h after mechanical wounding using cDNA macroarray containing 13,516 ESTs covering 8,384 loci (Supplemental Tables S1–S3), and compared with already identified JA-responsive genes (Sasaki et al., 2001; Sasaki-Sekimoto et al., 2005). After global normalization, we calculated the logarithmic fold expression value of each gene (see “Materials and Methods”). Genes specifically responsive to wounding with fold expression of more than 0.4 or less than −0.4 and a peak maximal fold within 1 h were defined as wounding-specific early responsive genes. Among these genes, we identified two genes encoding hemoproteins and one related protein, the expression of which was specifically responsive to wounding (Table I). Two genes are involved in the cytochrome P450 family and one gene encoding NADPH-cytochrome P450 reductase. For the cytochrome P450 family, cinnamate-4-hydroxylase (C4H; CYP73A5 [At2g30490]) and CYP82G1 (At3g25180) were identified. The peak induction of the former genes was 1 h after wounding.

Table I. Wounding-specific early responsive genes related to defensive functions of hemoproteins and enzymes for heme biosynthesis

<table>
<thead>
<tr>
<th>Annotation</th>
<th>Locus</th>
<th>Peak</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P450 family</td>
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<td></td>
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<tr>
<td>C4H (CYP73A5)</td>
<td>AT2G30490</td>
<td>1</td>
<td>0.76***</td>
</tr>
<tr>
<td>CYP82G1</td>
<td>AT3G25180</td>
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<td>0.56*</td>
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<td>NADPH-cytochrome P450</td>
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<tr>
<td>reductase</td>
<td>AT4G30210</td>
<td>0.5</td>
<td>0.51***</td>
</tr>
<tr>
<td>NADPH-cytochrome P450</td>
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<td></td>
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<tr>
<td>reductase (ATR2, AR2)</td>
<td>AT4G30290</td>
<td>0.5</td>
<td>0.47**</td>
</tr>
<tr>
<td>Cell wall biosynthesis</td>
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<tr>
<td>Xyloglucan endotransglycosylase, putative</td>
<td>AT4G30280</td>
<td>0.5</td>
<td>0.49***</td>
</tr>
<tr>
<td>Xyloglucan endotransglycosylase, putative</td>
<td>AT4G30290</td>
<td>0.5</td>
<td>0.47**</td>
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<tr>
<td>Peroxidase</td>
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<tr>
<td>Cytosolic APX1</td>
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<td>0.48***</td>
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<td>Heme biosynthesis</td>
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<tr>
<td>Glutamyl-tRNA reductase</td>
<td>AT1G09940</td>
<td>0.5</td>
<td>0.47***</td>
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<tr>
<td>Ferrocobalaminase (ATFC1)</td>
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<td>0.40***</td>
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<td>Transcription factor</td>
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<tr>
<td>WRKY family transcription factor (WRKY48)</td>
<td>AT5G49520</td>
<td>0.5</td>
<td>0.53***</td>
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</table>

*Peak means the time (h) after wounding, at which the maximum fold expression was observed. **Fold means the logarithmic fold expression value, which indicates the maximal changes of each gene expression after wounding.
while the peak of the latter was at 0.5 h. C4H is localized on the cytosolic surface of the endoplasmic reticulum membrane, and is known to be involved in the general phenylpropanoid pathway, which provides a variety of secondary metabolites that are involved in differentiation and the protection of plant tissues against environmental stresses (Hahlbrock and Scheel, 1989). The expression of C4H is known to be coinduced with Phe ammonia lyase1 (PAL1; At2g37040) by wounding (Mizutani et al., 1997). In fact, in our array system, we detected the induction of PAL1 with a peak at 1 h after wounding with fold expression of 0.26. For NADPH-cytochrome P450 reductase, ATR2 (AR2; At4g30210) was induced with a peak at 0.5 h after wounding, consistent with a previous report (Mizutani and Ohta, 1998). The biochemical characterization of ATR2 (AR2) in yeast (Saccharomyces cerevisiae) coexpressed with C4H demonstrated that ATR2 is physiologically coupled with C4H, showing that this protein transports electrons to C4H (Urban et al., 1997). These results confirmed the hypothesis that the expression of the phenylpropanoid pathway is regulated in concert to meet the cellular requirement for phenylpropanoid-derived secondary metabolites (Dixon and Paiva, 1995; Mizutani et al., 1997). In addition, we identified two genes encoding putative xylologucan endotransglycosylase involved in cell wall biosynthesis, both of which were induced with a peak at 0.5 h after wounding. The gene encoding cytosolic ascorbate peroxidase1 (APX1; At1g07890) was induced with a peak at 1 h after wounding with fold expression of 0.48 (Sasaki-Sekimoto et al., 2005). APX1 is a key hydrogen peroxide (H2O2) removal enzyme (Prueli et al., 2003), the expression of which is known to be induced by wounding and other oxidative stresses (Rizhsky et al., 2004).

Among genes corresponding to heme and tetrapyrrole biosynthesis, AtHEMA2 and AtFC1 were the only genes whose expression was induced by wounding. The peak induction of AtHEMA2 and AtFC1 was 0.5 and 0.25 h after wounding with fold expressions of 0.47 and 0.40, respectively, confirming the rapid and transient induction of these genes (Fig. 1A). No other tetrapyrrole biosynthetic genes were induced but rather decreased by wounding. For instance, the transcripts of AtHEMA1 and CHLH, a subunit of Mg chelatase, both of which were recognized as the most important regulatory enzymes for Chl biosynthesis (Matsumoto et al., 2004), were decreased with fold expressions of −0.35 and −0.26, respectively. Interestingly, such wounding-dependent reduction was also observed in transgenic tobacco carrying AtFC2 promoter fused with reporter genes (Singh et al., 2002). Specific induction of AtHEMA2 and AtFC1 by wounding was also confirmed using a miniarray system covering all tetrapyrrole biosynthetic genes of Arabidopsis (Matsumoto et al., 2004; data not shown). Therefore, it is likely that in photosynthetic tissues Chl and heme biosynthesis driven by predominantly expressed light-regulated enzymes under normal conditions, such as AtHEMA1, Mg chelatase, and AtFC2, falls under wounding conditions, whereas heme biosynthesis distinctly controlled by AtHEMA2 and AtFC1, which are repressed in photosynthetic tissues under normal conditions, is induced under wounding conditions. It is probable that wounding-responsive AtHEMA2 and AtFC1 function to immediately supply heme to such defensive hemoproteins outside plastids.

Effects of Ozone and ROS Generating Reagents on the Expression of AtHEMA2 and AtFC1

It is well known that ROS such as H2O2 and superoxide are generated in plant tissues in response to wounding (Orozco-Cardenas and Ryan, 1999). Wound-induced H2O2 accumulation is observed locally and systemically and acts as a local signal for hypersensitive cell death and also as a diffusible signal for the induction of defensive genes in adjacent cells (Alvarez et al., 1998). To examine whether ROS are involved in the induction of AtHEMA2 and AtFC1, we performed an ozone exposure experiment on AtHEMA2::GUS and AtFC1::GUS plants, since ozone presumably degrades into H2O2, O2, and hydroxyl radicals in apoplasts after entering the plant through open stomata (Conklin and Last, 1995). It has been reported that ozone distinctly affected the synthesis of chlorophyll and isoprenoid in pine (Pinus halepensis; Shammay et al., 2001), but effects on the expression of tetrapyrrole biosynthetic genes have not been elucidated.

The exposure of 200 nL L−1 ozone for 6 h resulted in the increase of mRNA levels of both genes (Fig. 3A). Actually, quantitative analysis confirmed the increase of GUS activity in ozone-fumigated AtHEMA2::GUS and AtFC1::GUS (Fig. 3B). Histochemical staining showed that GUS activity was induced in the whole body of AtHEMA2::GUS and AtFC1::GUS plants, although the intensities of GUS staining varied among developmental leaves (Fig. 3, C and D).

Then, we assessed the effects of two different reagents that generate ROS on the expression of both genes. Paraquat (methyl viologen) promotes the formation of ROS by diverting electrons from photosynthetic electron transport to reduce O2 to superoxide (Suntrès, 2002). Rose bengal (4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodoflorescein) is a photosensitizer that generates singlet oxygen when exposed to light (Wieranni et al., 2002). As shown in Figure 4A, treatments of 10 μM paraquat or 10 μM Rose bengal resulted in the increase of endogenous mRNA levels of AtHEMA2 and AtFC1. Histochemical GUS staining also confirmed the induction of AtFC1 (Fig. 4B) and AtHEMA2 (data not shown).

These results demonstrated that the generation of ROS is involved in the induction of AtHEMA2 and AtFC1, and wounding-induced accumulation of ROS is likely to be involved in the local induction of these genes.

CHX-Induced Expression of AtHEMA2 and AtFC1 Involves Inhibition of Cytoplasmic Protein Synthesis

It has been suggested that CHX, an inhibitor of cytoplasmic protein synthesis, also functions as an
agonist to initiate the signaling pathway that regulates the expression of genes responsive to extracellular stimuli such as wounding (Nishiuchi et al., 2002). To clarify whether CHX acts as a protein synthesis inhibitor or a signal for the induction of \textit{AtHEMA2} and \textit{AtFC1}, we determined the effects of CHX on the transcription and translation of the reporter gene by measuring the levels of \textit{uidA} mRNA and GUS activity in each transgenic line treated with various concentrations of CHX for 6 h. As shown in Figure 5A, the increase in \textit{uidA} mRNA in both transgenic lines was clearly detectable at 1 \( \mu \)M CHX, and dependent on the concentration of CHX. The expression of \textit{uidA} showed a similar profile and level to those of endogenous \textit{AtHEMA2} and \textit{AtFC1} mRNA, showing that the reporter gene system reflects the expression of each endogenous gene (Fig. 5A). In both transgenic lines, however, GUS activity retained the basal levels under all CHX concentration tested (Fig. 5, B and C).

To clarify whether the induction of \textit{AtHEMA2} and \textit{AtFC1} expression is specific to inhibition of cytoplasmic protein synthesis, we further examined the effects of other protein synthesis inhibitors, puromycin and chloramphenicol, which inhibit cytoplasmic and plastidic protein synthesis, respectively. As shown in Figure 5D, as well as 100 \( \mu \)M CHX, treatment of 100 \( \mu \)M puromycin but not 100 \( \mu \)M chloramphenicol, increased the endogenous mRNA levels of \textit{AtHEMA2} and \textit{AtFC1}, showing that inhibition of cytoplasmic protein synthesis is involved in the induction of both genes.

Identification and Characterization of Insertional Mutants of \textit{AtHEMA2} and \textit{AtFC1}

To investigate the physiological function of \textit{AtHEMA2} and \textit{AtFC1}, we isolated insertional mutants of \textit{AtHEMA2} and \textit{AtFC1}. A transposon-tagged mutant

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3}
\caption{Induction of \textit{AtHEMA2} and \textit{AtFC1} mRNA by ozone exposure. A, Semiquantitative RT-PCR analysis of \textit{AtHEMA2} and \textit{AtFC1}. Total RNA was isolated from wild type (Col) exposed to 0.2 ppm ozone for 6 h of control. Transcript level of each gene was analyzed by RT-PCR. B, Transgenic lines of \textit{AtHEMA2::GUS} and \textit{AtFC1::GUS} were exposed to 200 nL L\textsuperscript{-1} ozone or fresh air for 6 h. GUS activities in these plants were measured, together with that in 0 time control. The amount of 4-Mu produced in the reaction was determined by measuring 4-Mu-specific fluorescence. C and D, Transgenic lines of \textit{AtHEMA2::GUS} and \textit{AtFC1::GUS} were exposed to 0.2 ppm ozone or fresh air for 6 h. GUS activities in ozone-treated \textit{AtHEMA2::GUS} (C) and \textit{AtFC1::GUS} (D) were stained histochemically. Ozone-treated (b) and fresh air-treated (a) seedlings are presented.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{Induction of \textit{AtHEMA2} and \textit{AtFC1} mRNA by reagents generating ROS. A, Semiquantitative RT-PCR analysis of \textit{AtHEMA2} and \textit{AtFC1}. Total RNA was isolated from wild type (Col) treated with 10 \( \mu \)M paraquat (MV) or Rose bengal (RB) for 6 h. Transcript level of each gene was analyzed by RT-PCR. B, Transgenic lines of \textit{AtFC1::GUS} were treated by placing a droplet of 10 \( \mu \)M paraquat or Rose bengal for 6 h. After treatment, GUS activities in \textit{AtFC1::GUS} were stained histochemically.}
\end{figure}
of AtHEMA2 (15-2026-1) was obtained from the transposon-tagging line collection of RIKEN, Japan (Ito et al., 2002). The ecotype of this line is Nossen (Nos). For AtFC1 (SALK_150001), a T-DNA insertional mutant of Columbia-1 (Col-1) background was obtained from the collection of the SALK Institute (Alonso et al., 2003). As shown in Figure 6A, in athema2 plants, a single copy of transposon was inserted into the first exon of AtHEMA2. Reverse transcription (RT)-PCR analysis failed to detect any AtHEMA2 transcript, while the transcript levels of other AtHEMA isoforms were unaffected in these mutants (Fig. 6B). In atfc1 plants, a single T-DNA was inserted --105 bp upstream from the transinitiation codon of AtFC1 (Fig. 6A). RT-PCR analysis detected a very faint band of AtFC1. Wounding treatment of the wild type resulted in an increased AtFC1 transcript; however, in atfc1 plants, the AtFC1 transcript was hardly detectable, even under wounding conditions (Fig. 6C). Thus, it is likely that atfc1 plants are knockout or knockdown mutants of AtFC1, resulting in severe reduction of its expression.

Visible phenotypes of these insertional mutants were almost comparable to each ecotype background (Supplemental Fig. S1). In fact, growth and various parameters concerning photosynthesis, such as Chl and carotenoid contents, and the efficiency of PSII, were almost equal in the respective wild types (Supplemental Table S4). As it has been suggested that AtHEMA2 and AtFC1 function in heme biosynthesis in nonphotosynthetic tissues, we determined the heme contents in rosette leaves and roots in these mutants using a recently developed sensitive heme assay (Masuda and Takahashi, 2006). In athema2 and atfc1 plants, we found decreased heme contents in roots when compared with each ecotype background (Fig. 6D). In both mutants, the reduction of heme was more apparent in roots than leaves, confirming the function of AtHEMA2 and AtFC1 in nonphotosynthetic tissues.

Effects of Ozone-Induced Increase in Heme Content

As shown in Figure 2, it appeared that the expression of AtHEMA2 and AtFC1 was induced by wounding, suggesting that AtHEMA2 and AtFC1 function in heme synthesis in wounding regions. The measurement of heme content, however, could not detect significant differences between wounded leaves and untreated leaves of athema2 and atfc1 (data not shown), probably because the wounding-dependent induction is limited to the surrounding local area of wounding regions. Since ozone exposure induced AtHEMA2 and AtFC1 in a whole body of seedlings (Fig. 3), we measured the heme content in ozone-exposed seedlings. As shown in Figure 7, in wild-type seedlings, the exposure of 200 nL L^-1 ozone for 6 h resulted in the 1.6- to approximately 2.3-fold induction of heme content. In both mutants, the ozone-induced increase in heme content was affected. The levels of heme in atfc1 mutant were rather decreased after ozone exposure (Fig. 7). In athema2, the level of heme remained unchanged after ozone exposure. These results suggest that both AtHEMA2 and AtFC1 are involved in ozone-induced increase in heme content in Arabidopsis.

**DISCUSSION**

**Expressions of AtHEMA2 and AtFC1 Are Regulated in the Same Manner**

It has been shown that AtHEMA2 and AtFC1 possess similar expression profiles (Singh et al., 2002; Ujwal
et al., 2002). Our results consistently showed that both genes were preferentially expressed in nonphotosynthetic tissues, although the basal levels and tissue specificity of expression were somewhat different (Figs. 1 and 2). Such similarity was also observed in the expression of HEMA2 and FC1 isoforms of cucumber (Tanaka et al., 1996; Suzuki et al., 2002). Furthermore, as well as the CHX-induced expression of cucumber HEMA2 and FC1 (Suzuki et al., 2002), here we showed that AtHEMA2 and AtFC1 were also induced in photosynthetic tissues by CHX treatment. Comeasurement of GUS mRNA and activity suggested that CHX acted as an inhibitor of cytosolic protein synthesis, rather than as an inductive signal for expression. It has been suggested that the increase in mRNA after CHX treatment is due to the inhibition of de novo biosynthesis of labile transcriptional repressors and/or mRNA-degrading enzymes (Berberich and Kusano, 1997). Thus, it is possible that such a labile transcription factor represses the expression of AtHEMA2 and AtFC1 under normal condition. Alternatively, inhibition of cytoplasmic protein synthesis may cause accumulation of metabolic intermediate that generates ROS, resulting in the induction of AtHEMA2 and AtFC1. We presumed that such induction would occur under stress conditions, such as wounding, since in transgenic tobacco carrying the AtFC1 promoter fused with reporter genes, Suc, wounding, oxidative stress, and tobacco mosaic virus infection induced the promoter activity of AtFC1 (Singh et al., 2002), and CHX acted as a signal to elicit the expression of wounding-responsive genes (Nishiuchi et al., 2002). Indeed, the local induction of AtHEMA2 and AtFC1 was observed after wounding (Fig. 2). Induction occurred rapidly and transiently within 30 min (Fig. 2; Table I). For the local induction of AtHEMA2 and AtFC1, it is likely that ROS species were generated locally in the area surrounding the wound, rather than octadecanoid signaling molecules such as JA triggering the induction of these genes, since the ozone exposure experiment induced both genes in whole bodies, while JA, MeJA, 12-oxo-phytodienoic acid, and arachidonic acid were unaffected by the expression (Sasaki et al., 2001; Singh et al., 2002; Sasaki-Sekimoto et al., 2005; Taki et al., 2005).

In fact, as AtHEMA2 and AtFC1 was induced by paraquat and Rose bengal, ROS may be possible candidates for the signal molecules that mediated

Figure 6. Isolation of insertional mutants of AtHEMA2 and AtFC1. A, Schematic representation of isolated insertional mutants of AtHEMA2 (athema2) and AtFC1 (atfc1) in Arabidopsis. A single transposon and T-DNA were inserted into the first exon of AtHEMA2 and 5' untranslated region of AtFC1, respectively. The positions of identified W boxes are indicated by black boxes. B and C, RT-PCR analysis of AtHEMA isoforms in athesa2 (B) and AtFC isoforms in AtFC1 (C). Using gene-specific primers of each isoform, RT-PCR analysis was performed with total RNA isolated from wild-type and insertional mutants. For atfc1, seedlings were wounded induced AtFC1 mRNA. D, Heme contents in wild types (Nos and Col), athesa2, and atfc1. Heme was extracted from rosette leaves and roots of Arabidopsis seedlings by the acid acetone method and determined by the chemiluminescent-based method. Heme contents in athesa2 and atfc1 were presented as relative to the value from tissues of wild type Nos and Col, respectively. Each bar represents the mean from five independent experiments ± SD. Asterisk indicate values, where the mutants are significantly different from the respective wild-type values (P < 0.05).
It has been shown that H$_2$O$_2$, a substrate providing energy for the induction of both genes, is likely that singlet oxygen is involved in the induction of these genes. As Rose bengal that photodynamically produces singlet oxygen was effective for the induction of both genes, it is probable that ROS species or other signaling molecules are required for the induction of both genes. Currently, we do not know whether ROS species directly affect the biosynthesis of the putative protein factor that represses the expression of AtHEMA2 and AtFC1 or act as a signaling molecule for regulation, and which ROS species are involved in this regulation. As Rose bengal that photodynamically produces singlet oxygen was effective for the induction of both genes, it is likely that singlet oxygen is involved in the induction. It has been shown that H$_2$O$_2$, a substrate produced in the oxidative burst, has less effect on AtFC1 promoter activity (Singh et al., 2002). Thus, it is probable that H$_2$O$_2$ itself is not sufficient for induction and the presence of and/or cross talk with other ROS species or other signaling molecules are required for the induction of AtHEMA2 and AtFC1.

It should be noted that the induction profiles of AtHEMA2 and AtFC1 after wounding are similar to those of wound-responsive genes, so-called immediate early responsive genes, which are activated rapidly after wounding (Suzuki et al., 1998; Cheong et al., 2002). It is reported that immediate early responsive genes often encode transcription factors, which are expected to play important roles in regulating the transcription of genes involved in wound healing and defense against the invasion of wounding tissue by pathogens (Nishiuchi et al., 2004). We analyzed cis-acting elements in the promoters of AtHEMA2 and AtFC1 using the PLACE database (Higo et al., 1999), and identified an inverted repeat of the DNA binding sites of WRKY transcription factors (W boxes) in the proximal regions of the promoters of AtHEMA2 and AtFC1 (Fig. 6A). The presence of such W boxes was only observed in the promoters of AtHEMA2 and AtFC1, and no other HEMA and FC isoforms and tetrapyrrole biosynthetic genes contained such a cis-acting element. Thus, it is possible that W boxes in the promoter of AtHEMA2 and AtFC1 function as cis-acting elements for wound-inducible immediate expression. In our array system, we detected the induction of WRKY48 (At5g49520) with a peak at 0.25 h after wounding with fold expression of 0.53 (Table I).

**Physiological Function of AtHEMA2 and AtFC1**

The preferential expression in nonphotosynthetic tissues under normal conditions suggested that AtHEMA2 and AtFC1 function in heme biosynthesis in nonphotosynthetic tissues. Here, using insertion knockout mutants of these genes, we demonstrated that these genes are actually responsible for heme synthesis in these organs. In both mutants, although the heme contents were reduced by nearly half in the roots, the growth rate and photosynthetic parameters were almost equal to the respective wild types. These results suggest that neither gene is essential for photosynthetic growth under normal conditions, probably because the predominantly expressed other isoforms, AtHEMA1 and AtFC2, redundantly complement the loss of function of AtHEMA2 or AtFC1.

In this study, we demonstrated that the expression of AtHEMA2 and AtFC1 was induced under oxidative stress conditions, such as wounding and ozone exposure. Actually, ozone exposure induced heme in wild types and this induction was significantly reduced in both mutants. The reduction was less pronounced in athema2 than atfc1, probably because of their basal expression levels. These results suggest that AtFC1, which showed a higher expression than AtHEMA2, is primarily responsible for the ozone-induced increase in heme content in this condition.

The selection of wounding-specific early responsive genes by cDNA macroarray identified defensive hemoproteins outside plastids, such as cytochromes P450, C4H, and APX1. Since these genes are related to defensive responses such as cell wall biosynthesis or antioxidants, it is reasonable to consider that AtHEMA2 and AtFC1 function for rapid heme supply to these hemoproteins.

C4H is one of the most abundant P450s in plants and is involved in the general phenylpropanoid pathway, which provides a variety of secondary metabolites that are involved in differentiation and the protection of plant tissues against environmental stresses (Hahlbrock and Scheel, 1989). The wounding- and light-induced expression of C4H is regulated in concert with other genes of the phenylpropanoid pathway, such as PAL1 and 4-coumarate:CoA ligase, but not with chalcone synthase for flavonoid biosynthesis (Mizutani et al., 1997). In addition, we also identified the cytochrome P450 reductase ATR2 (AR2) as a wounding-specific early responsive gene. It has been shown that ATR2 (AR2) is physiologically coupled with C4H (Urban et al., 1997) and is coinduced with the above three genes involved in the phenylpropanoid pathway (Mizutani and Ohta, 1998). Considering that these

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**Figure 7.** Effects of ozone-induced increase of heme in athema2 and atfc1. Wild types and insertion mutants were exposed to 200 nL L$^{-1}$ ozone for 0 and 6 h. Heme was extracted from Arabidopsis seedlings by the acid acetone method and determined by the chemiluminescent-based method. Changes in heme contents in each seedling are presented as the relative value of tissues from 6 to 0 h. Each bar represents the mean from five independent experiments ± SD. Asterisks indicate significant difference ($P < 0.05$) between 0 h control and ozone-treated plants.
genes are involved in the biosynthesis of lignin, one of the major components of the cell wall, and putative xyloglucan endotransglycosylase genes, which may be involved in cell wall biosynthesis, were coinduced with AtHEMA2 and AtFC1, it is likely that AtHEMA2 and AtFC1 function in the rapid heme supply for cell wall biosynthesis. Further investigation of cell wall biosynthesis in the wounding region of athema2 and atfc1 is necessary to clarify their precise defensive functions.

Another possible function of AtHEMA2 and AtFC1 is production of the heme molecule that acts as a signal for stress-inducible genes. Heme and its derivatives have been shown to regulate a variety of cellular processes in animal and bacterial systems, such as transcription (Guarente and Mason, 1983), translation (Chen et al., 1989; Joshi et al., 1995), and posttranslational protein translocation (Lathrop and Timko, 1993). Since the immediate early responsive genes are expected to play important roles in regulating the transcription of genes involved in wound healing and defense against invasion of wound tissue by pathogens (Nishiuchi et al., 2004), the possibility cannot be excluded that heme acts as a signal for transcriptional regulation. Although signaling functions of tetrapyrrole biosynthetic enzymes tested, genes encoding these two enzymes are specifically coregulated in Arabidopsis. Among tetrapyrrole biosynthetic enzymes tested, genes encoding these two enzymes are specifically coregulated in Arabidopsis. Since CHX-sensitive induction of cucumber HEMA and FC isoforms was observed, it is possible that such distinctly controlled tetrapyrrole biosynthetic pathways are generally conserved in dicot plants or in higher plants. Analysis of insertion knockout mutants showed that these enzymes function for heme biosynthesis in nonphotosynthetic tissues under normal conditions, but are induced in photosynthetic tissues under oxidative conditions.

It should be noted that the expression of genes encoding key enzymes for Chl biosynthesis, such as AtHEMA1 and CHLH, was repressed by wounding. Thus, it is likely that switching of Chl to heme biosynthesis may occur in photosynthetic tissues under oxidative conditions, which is attributed by repression of AtHEMA1 and CHLH, and induction of AtHEMA2 and AtFC1. The produced heme would be transported outside plastids, and bind to defensive hemoproteins, such as APX1 and C4H, forming an active holoenzyme. It is not known how heme is transported outside plastids. Complete understanding of the regulation of these networks will require further physiological and molecular genetic studies, probably with multiple mutants of corresponding genes.

MATERIALS AND METHODS

Plant Material

Seeds of Arabidopsis (Arabidopsis thaliana) were germinated and grown on solidified Murashige and Skoog medium (Murashige and Skoog, 1962) containing 1% Suc and 0.8% agar at 23°C under continuous white light (30 μmol photons m⁻² s⁻¹). For reagent treatment, seedlings were incubated on an orbital shaker at 23°C under continuous light (30 μmol photons m⁻² s⁻¹). After 10 d, the plants were treated with various concentrations of reagents. A transposon-tagged mutant of AtHEMA2 (15-2026-1) was obtained from the transposon-tagging line collection of RIKEN, Japan (Ito et al., 2002). For AtFC1 (SALK_150001), a T-DNA insertion mutant of Col-1 background was obtained from the collection of the SALK Institute (Alonso et al., 2003). Homozygous mutants of athema2 and atfc1 were screened by PCR amplification with specific primers for each gene and border sequences of transposon and T-DNA, respectively.

Molecular Analyses

Isolation of genomic DNA and genomic Southern hybridization were performed as described previously (Masuda et al., 2003). Total RNA was prepared using RNeasy Mini kit (Qiagen). Total RNA was electrophoresed on 1.2% agarose/formaldehyde gel and blotted onto a nylon membrane. Specific probes for AtHEMA and AtFC isoforms (Matsumoto et al., 2004) were labeled with [α-32P]dCTP and hybridization was performed as described previously (Suzuki et al., 2002). For RT-PCR analysis of insertional mutants, primers specific to AtHEMA and AtFC isoforms were designed to amplify particular genes. RT-PCR was carried out with RNA PCR kit (AMV) Ver.2.1 (Takara) with 1 μg of total RNA isolated from 2-week-old mature leaves according to manufacturer’s instructions. PCR amplification was carried out as follows: 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. For RT-PCR analysis of athema2, 40 cycles of PCR reaction were carried out to amplify low-expressed AtHEMA3. For atfc1, 25 cycles of PCR reaction were carried out. For semiquantitative RT-PCR analysis of AtHEMA2, AtFC1, and ACT8, PCR reaction was performed 30, 25, and 20 cycles, respectively.

Construction of AtFC1::GUS Fusion Vector and GUS Assay

The 5′-upstream region of AtFC1, which was amplified by PCR between −1,018 to +69 bp from the translation initiation site, was translationally ligated into a uidA gene in BarHI and SalI sites of a pBI101 vector. The fragment included the region coding for the first 23 amino acids of the AtFC1 gene. The PCR fragment was sequenced and checked for PCR errors. The obtained construct was introduced into Arabidopsis via Agrobacterium-mediated transformation.

Histochimical analyses for GUS expression were carried out in independent transgenic lines for each construct. Plant samples were soaked at 37°C for 1 d in GUS assay solution, which included 1 mm 5-bromo-4-chloro-3-indolyl-galacturonide, 0.5 mm K₂Fe(CN)₆, 0.5 mm K₃Fe(CN)₆, 0.3% (v/v) Triton X-100, 20% (v/v) methanol, and 50 mm inorganic phosphate-buffered saline. Samples were then soaked in 70% (v/v) ethanol for 1 d to stop the reaction and remove Chl. For quantitative GUS assay, three independent samples of frozen plant material were homogenized with GUS extraction buffer (50 mm phosphate buffer, pH 7.0, 10 mm β-mercaptoethanol, 10 mm EDTA, 0.1% (v/v) Triton X-100, and 0.1% (w/v) SDS), and centrifuged at 12,000g for 5 min at 4°C to prepare the supernatant as the enzyme mixture. Total proteins of the enzyme mixture were quantified by the method of Bensadoun and Weinstein (1976) with bovine serum albumin as a standard. The enzyme mixture was brought to 190 μL with GUS extraction buffer and then the reaction was initiated by mixing the enzyme solution with 10 μL of 20 mm 4-methylumbelliferyl (4-Mu)-β-D-glucuronide. After incubation at 37°C for 10 min, the reaction was terminated by mixing 800 μL of 200 mM Na₂CO₃. The amount of 4-Mu produced
in the reaction was determined by measuring 4-Mu-specific fluorescence (365 nm excitation, 455 nm emission).

**Radiolabeling, Hybridization, and Image Analysis of cDNA Microarrays**

The 13,516 EST clones covering 8,384 loci (Asamizu et al., 2000) were spotted onto nylon filters as described previously (Sasaki et al., 2001). Samples were collected from 2-week-old Arabidopsis rosette leaves at 0, 0.25, 0.5, 1, 3, and 6 h after wounding treatment. Total RNA was prepared using RNeasy Mini Kit (Qiagen) and 10 μg of total RNA was used as a template for radiolabeling by RT (Sasaki et al., 2001). The reaction was performed in the presence of [α-33P]dATP and 0.5 μg of oligo(dT)16 to 18 primer using the SuperScript First-Strand synthesis system (Invitrogen) according to the manufacturer’s instructions. The labeled cDNA was denatured and used as target DNA for hybridization. Hybridization with the labeled target was performed in the presence of 0.5 μg Na2HPO4 (pH 7.2), 1 μl EDTA, and 7% SDS (Church and Gilbert, 1984) at 65°C for 16 h. The membranes were then washed twice with 0.2 x SSC containing 0.1% SDS at 65°C and exposed to an imaging plate (Fuji Film) for 1 to 3 d under a shield box made of lead to reduce the effect of naturally occurring background radiation. This shield box improved the quality of the raw images, which were obtained using a high-resolution scanner (Strom, Amersham). Signal intensity was quantified using Array Vision software (version 6.0, Amersham).

**Data Normalization and Selection of Wound-Specific Early Responsive Genes**

Quality evaluation and global normalization of cDNA microarray data were performed as described previously (Sasaki-Sekimoto et al., 2005). The normalized data for ESTs were then transformed to normalized expression values for each locus (Obayashi et al., 2003) and statistically analyzed by one-way ANOVA variance analysis (Kerr et al., 2000). To select genes responsive to wounding, we calculated the difference between normalized expression at each time point and that at 0 time, and defined this as the relative expression. From the maximal or minimal value of relative expression, we calculated the difference between normalized expression at 0 time and that at 0 time, and defined this as the relative expression.

**Determination of Photosynthetic Parameters and Heme**

Seedlings were harvested and homogenized in 80% acetone, followed by centrifugation to remove debris. In mature leaves, Chl (and β and γ) and carotenoids contents of the samples were determined according to Arnon (1949), with corrections as outlined in Melis et al. (1987) and according to Lichtenthaler (1987), respectively. To determine the efficiency of PSI, a Mini-PAM (Heinz Walz Gmbh) was used to measure the maximum photochemical efficiency of PSI in the dark-adapted state value.

Heme was extracted from Arabidopsis according to the acid acetone extraction method (Stillman and Gassman, 1978). Seedlings (0.1 g) were homogenized with a mortar and pestle in five volumes of 0.1 M sodium acetate (9.1, v/v). After centrifugation at 15,000 g for 10 min at 4°C, the pellet was washed with 80% acetone and centrifuged again. Heme was extracted twice from the pellet by suspending in 100 μL of 2% HCl in acetone. The resultant supernatants were combined and diluted 100-fold with 100 μL Tris-HCl, pH 8.4. Heme was determined enzymatically using a chemiluminescent-based method (Masuda and Takahashi, 2006). The GraphPad Prism software (GraphPad Software Inc.) was used for statistical evaluation of the data.

**Ozone Treatment**

Arabidopsis seeds were germinated on blocks of glass wool and grown in a chamber at 22°C at a relative humidity of 50% to 60% under a photosynthetic photon flux density of 80 μmol photons m−2 s−1 in 14 h light/10 h dark cycles. Plants were watered with a liquid fertilizer (Hyponex 5-10-5) and diluted 2,000-fold. Sixteen-day-old plants were exposed to a single dose of 200 μL L−1 ozone in a chamber as described by Matsuyma et al. (2002). The ozone chamber was maintained at 25°C at a relative humidity of 70% under a photosynthetic photon flux density of 350 μmol m−2 s−1 of continuous light. Ozone was generated by an ozone generator (Sumitomo Seika Chemicals). After 6 h of exposure, plants were sampled for further assay.

**Supplemental Data**

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

- **Supplemental Figure S1.** Visible phenotypes of insertionional mutants.
- **Supplemental Table S1.** Expression analysis of wounding-responsive genes by cDNA macroarray.
- **Supplemental Table S2.** Correspondence of spot to gene locus on cDNA macroarray.
- **Supplemental Table S3.** MIAME checklist.
- **Supplemental Table S4.** Photosynthetic parameters of insertionional mutants.

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**LITERATURE CITED**


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