Identification of Arabidopsis Ethylene-Responsive Element Binding Factors with Distinct Induction Kinetics after Pathogen Infection[1][w]

Luis Oñate-Sánchez and Karam B. Singh*

Commonwealth Scientific and Industrial Research Organization, Plant Industry, Private Bag #5, Wembley, Western Australia 6913, Australia

Ethylene-responsive element binding factors (ERF) proteins are plant-specific transcription factors, many of which have been linked to stress responses. We have identified four Arabidopsis ERF genes whose expression was specifically induced by avirulent and virulent strains of the bacterial pathogen *Pseudomonas syringae* pv *tomato*, with overlapping but distinct induction kinetics. However, a delay in *ERF* mRNA accumulation after infection with the virulent strain was observed when compared with the avirulent strain. The induction of *ERF* gene expression in most cases preceded the mRNA accumulation of a basic chitinase gene, a potential downstream target for one or more of these ERFs. The expression of the *ERF* genes was examined among different Arabidopsis tissues, in response to the signaling molecules ethylene, methyl jasmonate, and salicylic acid (SA), and in Arabidopsis mutants with decreased or enhanced susceptibility to pathogens, and significant differences were observed. For example, in seedlings, some of the *ERF* genes were not induced by SA in the wild-type but were SA responsive in the *pad4-1* mutant, suggesting that PAD4-1, which acts upstream of SA accumulation, is also involved in repressing the SA-induced expression of specific *ERF* genes. The four ERF proteins were shown to contain transcriptional activation domains. These results suggest that transcriptional activation cascades involving ERF proteins may be important for plant defense to pathogen attack and that some ERF family members could be involved in the cross-talk between SA- and jasmonic acid-signaling pathways.

Many plant genes are transcriptionally regulated in response to pathogen attack or environmental stresses. Plant signals, like salicylic acid (SA), ethylene, and jasmonic acid (JA), which accumulate in plants during pathogen infection, are involved in the regulatory pathways mediating these responses (Glazebrook, 2001). These regulatory pathways require the coordination of highly specific DNA-protein and protein-protein interactions, most of which are not fully understood. A number of plant promoter elements that can respond to diverse environmental stimuli have been identified including the GCC box, an ethylene-responsive element initially found in several pathogenesis-related (*PR*) gene promoters (Hart et al., 1993; Ohme-Takagi and Shinshi, 1995; Sessa et al., 1995; Sato et al., 1996). Proteins that specifically bind to the GCC box were initially discovered in tobacco (*Nicotiana tabacum*) and are called ERFs (ethylene-responsive element binding factors; Ohme-Takagi and Shinshi, 1995; Suzuki et al., 1998). The tobacco ERFs share a well-conserved 58- to 59-amino acid domain called the ERF domain (Hao et al., 1998), which has only been found in plants. The ERF domain has a novel structure consisting of a β-sheet and an α-helix (Allen et al., 1998), which binds to DNA as a monomer (Hao et al., 1998).

There are numerous ERF proteins in plants (Riechmann et al., 2000), and the similarity is primarily confined to the ERF domain. ERF proteins play important roles in plant responses to various hormones or environmental cues. In Arabidopsis, ERF proteins are involved in mediating responses to dehydration, salt, and cold stress (Stockinger et al., 1997; Liu et al., 1998; Fujimoto et al., 2000; Park et al., 2001), abscisic acid (Finkelstein et al., 1998), ethylene (Büttner and Singh, 1997; Solano et al., 1998; Fujimoto et al., 2000), and pathogen infection (Solano et al., 1998; Maleck et al., 2000; Schenk et al., 2000; Park et al., 2001). ERF proteins have also been found to be involved in defense responses in other plants. In periwinkle, elicitor and/or jasmonate-inducible *ERF* genes have been identified (Menke et al., 1999; van der Fits and Memelink, 2000), whereas tobacco and tomato *ERF* genes are induced after infection by *Pseudomonas syringae* (Zhou et al., 1997; Thara et al., 1999), tobacco mosaic virus (Horvath et al., 1998), or *Cladosporium fulvum* (Durrant et al., 2000). Some of the tomato ERFs can interact specifically with the PTO protein, which confers resistance to *P. syringae* (Zhou et al., 1997). Overexpression of a tobacco ERF enhances resistance against pathogen attack and osmotic stress (Park et al., 2001).

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* Corresponding author; e-mail karam.singh@csiro.au; fax 61–8–93878991.

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To further analyze the role that ERFs play in plant defense responses, we tried to identify *ERF* genes in Arabidopsis whose expression was specifically induced after pathogen attack. We identified four Arabidopsis *ERF* genes that are specifically induced by infection with either an avirulent or virulent *P. syringae* strain, with the induction in most cases occurring earlier with the avirulent pathogen. The four *ERF* genes studied here displayed overlapping but distinct induction kinetics after pathogen attack and all contained transcriptional activation domains. Further characterization of the Arabidopsis *ERF* genes revealed that there were interesting differences in their expression in response to defense signaling molecules and in Arabidopsis mutants altered in their defense responses. These results suggest that the ERF proteins may form part of a transcriptional cascade that regulates the temporal response of plant gene expression in response to pathogen attack.

RESULTS

Identification of Arabidopsis *ERF* Genes Induced after Pathogen Attack

Previously we had isolated an Arabidopsis ERF-like protein called AtEBP by virtue of its interaction with an *ocs*-element binding protein (Büttner and Singh, 1997). Because *AtEBP* (At3g16770) was induced by ethylene and the encoded protein was able to bind to the GCC box, we tested whether *AtEBP* expression could also be induced by pathogen attack. We infiltrated leaves of Arabidopsis plants containing the *RPS2* resistance gene with either a mock solution or a suspension containing the bacterial pathogen *Pseudomonas syringae* pv *tomato* (*Pst*) strain DC3000 carrying the avirulence gene *avrRpt2* (Kunkel et al., 1993) and isolated RNA from the infiltrated leaves at different time points. The reverse transcriptase (RT)-PCR analysis shown in Figure 1A, demonstrated that *AtEBP* mRNA was not significantly induced at any of the time points analyzed although a small induction appeared 24 to 48 h after the inoculation. In contrast, mRNA levels of a basic chitinase (*CHIT-B*; Samac et al., 1990) started to accumulate between 6 and 12 h after inoculation, and by 24 h, a large induction had occurred that continued to increase at the 48-h time point. *CHIT-B* (also called PR3) had been shown previously to be inducible by pathogen infection (Thomma et al., 1998).

Because overexpression of ERF1 (At3g23240), another Arabidopsis ERF protein, resulted in enhanced expression of *CHIT-B* (Solano et al., 1998), we tested whether *ERF1* expression was induced by *Pst* DC3000(*avrRpt2*) infection. As shown in Figure 1A, a substantial induction in *ERF1* expression was observed, which first appeared between 3 and 6 h after inoculation and peaked around 24 h. We then searched the Arabidopsis database to identify other ERFs that were closely related to ERF1. We focused on the five most closely related proteins available at the time these studies were initiated, and these are shown in bold in Figure 1B. An alignment of the amino acid sequences for all of the ERF proteins shown in Figure 1B is available as supplemental data at www.plantphysiol.org. The extensive amino acid similarity among these ERF proteins is primarily confined to the ERF domain. In addition, there are stretches of amino acid similarity outside the ERF domain encoded by *AtERF1* (At4g17500; Fujimoto et al., 2000) and *AtERF2* (At5g47220; Fujimoto et al., 2000), *AtERF14* (At1g04370) and *At5g43410*, and *AtERF15* (At2g31230) and *At1g06160*, respectively (see supplemental data available at www.plantphysiol.org). Three of the *ERF* genes shown in bold in Figure 1B have not previously been characterized, and we named them *AtERF13* (At2g44840), *AtERF14*, and *AtERF15* after finding in the database that *AtERF12* (At1g28360; Ohta et al., 2001) was the last published member of the *AtERF* series.

As shown in Figure 1A, three of the *ERF* genes were specifically induced by *Pst* DC3000(*avrRpt2*) infection with induction patterns distinct from *ERF1*. *AtERF13* and *AtERF1* had quite similar induction patterns, with both showing a small increase in mRNA levels within 3 h that peaked at 12 h, although *AtERF1* also showed a small induction in expression after the mock treatment. In contrast, *AtERF14* first showed a response at 12 h that peaked at 24 h. The other two clones tested, *AtERF2* and *AtERF15*, were induced by *Pst* DC3000(*avrRpt2*) infection but were also induced by the mock treatment, although in the case of *AtERF2*, the response to *Pst* DC3000(*avrRpt2*) started earlier than *AtERF15*. Other *ERF* genes have also been shown to be induced by a mock treatment (Thara et al., 1999). For our subsequent studies we focused on the four *ERF* genes that showed a significant and specific increase in expression after infection by *Pst* DC3000(*avrRpt2*).

We tested whether the expression of the *ERF* genes changes after infection with the virulent pathogen *Pseudomonas syringae* pv *tomato* strain DC3000, which does not contain the *avrRpt2* gene. As shown in Figure 2, all four *ERF* genes tested were induced with distinct induction kinetics after infiltration with the virulent strain, similar to what was seen with the avirulent strain (Fig. 1A). Thus, *AtERF13* and *AtERF1* were the first to be induced followed by *ERF1* and *AtERF14*. *AtERF1*, *ERF1*, and, to a lesser extent, *CHIT-B* showed a delay in mRNA accumulation patterns when compared with the induction elicited by the avirulent pathogen (Fig. 1A), whereas *AtERF14* displayed lower levels of induction in the compatible interaction (Fig. 2).

mRNA Expression Patterns of the *ERF* Genes

The mRNA expression patterns of the *ERF* genes were examined by RT-PCR in different Arabidopsis

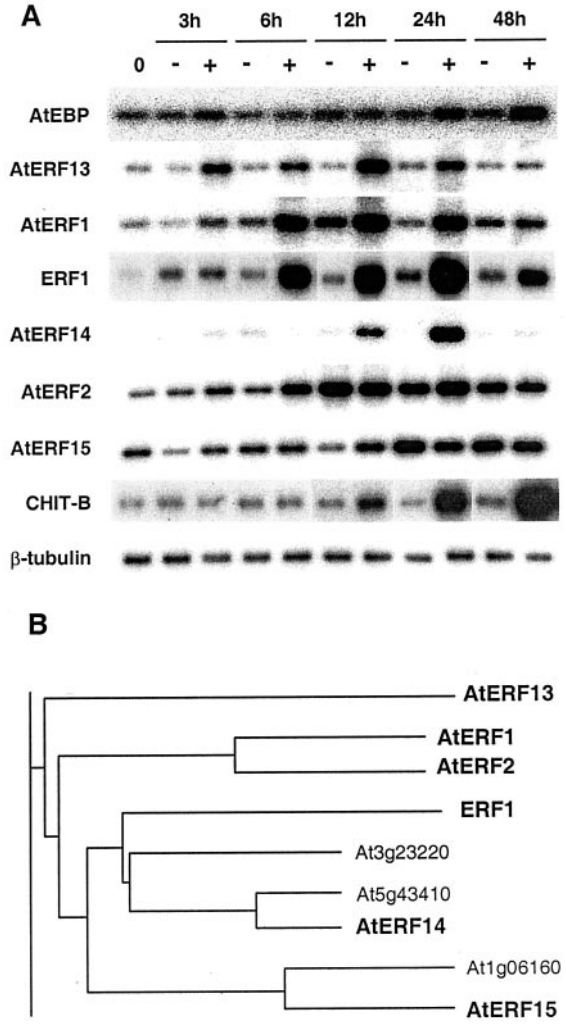

Figure 1. RNA expression after pathogen infection and dendrogram of Arabidopsis ERF1-related proteins. A, Induction of Arabidopsis *ERF* genes after pathogen attack. Arabidopsis leaves were infiltrated with a mock solution as a control (−) or with the same solution containing approximately 10^7 colony forming units (cfu)/mL of the avirulent pathogen *Pst* DC3000(*avrRpt2*) (+). Infiltrated leaves were harvested at the indicated time points, and the RNAs were isolated and subjected to RT-PCR using primers specific for each gene. An Arabidopsis basic chitinase (*CHIT-B*) and a constitutively expressed β-tubulin (Snustad et al., 1992) were used as controls in RT-PCR analysis. B, Fragment of the phylogenetic tree produced after 107 Arabidopsis ERF protein sequences were aligned. The Multiple Sequence Alignment application (AlignX) of the Vector NTI Suite program (InforMax, Inc., North Bethesda, MD), based on the Clustal W algorithm, was used. The genes in bold were available in the Arabidopsis database at the time this work was initiated and were chosen for further analysis. Protein identification numbers are AtERF13, AAK48967; AtERF1, BAA32418; AtERF2, BAA32419; ERF1, AAD03545; At3g23220, BAA95733; At5g43410, BAA97420; AtERF14, AAB70439; At1g06160, AAF80213; and AtERF15, AAD20668.

tissues. As shown in Figure 3A, the four *ERF* mRNAs were detected in all the tissues analyzed, although in some cases there were differences in the level of expression in specific tissues. Although *AtERF14* was equally expressed in all the tissues tested, *AtERF13*, *AtERF1*, and *ERF1* had the highest level of mRNA expression in flowers and rosette leaves compared with other tissues.

Ethylene has been shown to play important roles in a number of plant stress responses including responses to pathogens and in the expression of some *ERF* genes, including *ERF1* and *AtERF1* (Solano et al., 1998; Fujimoto et al., 2000). To determine whether *AtERF13* or *AtERF14* expression is regulated by ethylene, we treated 3-week-old Arabidopsis plants with ethylene. For this experiment we used the *ERF1* gene as a positive control for the ethylene treatment. Figure 3B shows that *AtERF14* and *ERF1* are upregulated by ethylene, whereas *AtERF13* is not induced after 24 h of treatment. We also did not observe any change in *AtERF13* expression after ethylene treatment for 12 h (data not shown). *AtERF14* expression in ein2-1, an ethylene insensitive mutant, was also examined. As shown in Figure 3C, the ethylene induction of *AtERF14* is reduced in *ein2-1* as was also the case for *ERF1*.

Like ethylene, SA and JA are important phytohormones involved in signaling in response to pathogen infection. To test the possible involvement of the *ERF* genes in SA or JA signaling pathways, we examined their mRNA expression after treatment of 2-week-old Arabidopsis seedlings with these hormones. As shown in Figure 4, *AtERF13* and *ERF1* were induced by methyl jasmonate (MeJA) but not by SA whereas *AtERF1* was induced by both hormones. *AtERF14* expression was not affected by either SA or MeJA. For these experiments, the SA-regulated *PR5* gene (Ward et al., 1991) and the MeJA-regulated *PDF1.2* gene (Penninckx et al., 1996) were used as controls and were induced by SA and MeJA, respectively.

To further characterize the role of SA and MeJA in the expression of the *ERF* genes, we investigated

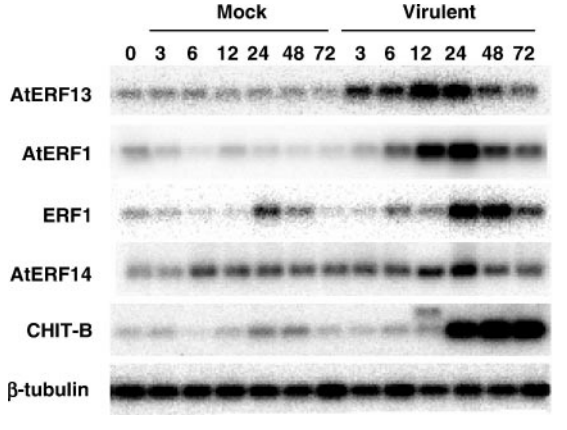

Figure 2. RNA expression of the Arabidopsis *ERF* genes in response to a virulent pathogen. Arabidopsis leaves were infiltrated with a mock solution (Mock) or with the same solution containing approximately 10^7cfu mL^{-1} of the virulent pathogen *Pst* DC3000 (Virulent). RNAs isolated from leaves at the indicated time points were subjected to RT-PCR.

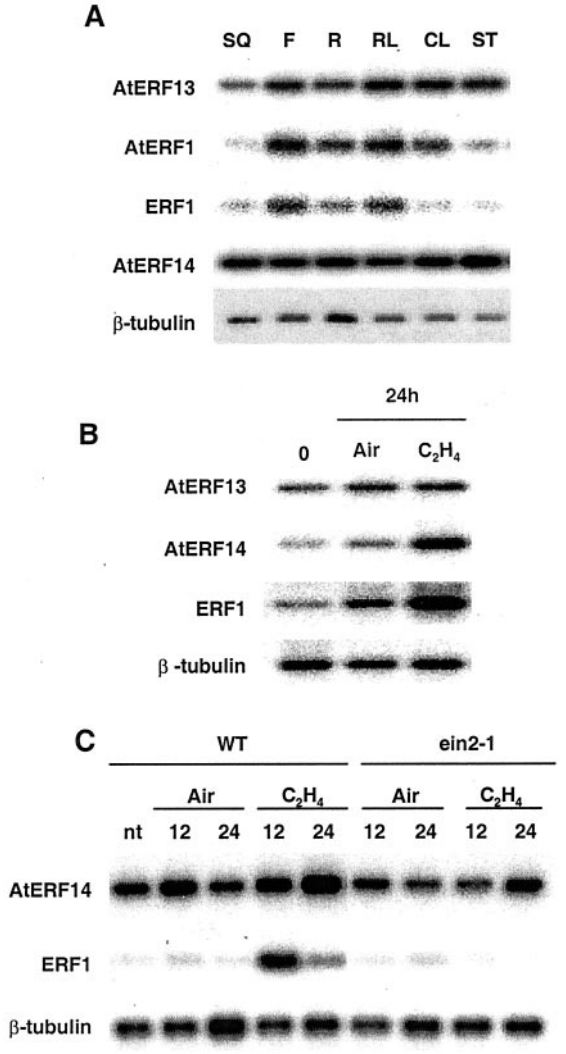

Figure 3. Expression of the *ERF* genes in Arabidopsis tissues and in response to ethylene and in an ethylene insensitive mutant. A, RNAs isolated from Arabidopsis siliques (SQ), flowers (F), roots (R), rosette leaves (RL), cauline leaves (CL), and stems (ST) were analyzed by RT-PCR. B, Arabidopsis plants were placed in a glass chamber and flushed with ethylene (C_2H_4) or air. Plants were harvested after 24 h for RNA extraction and RT-PCR analysis. *ERF1*, which is up-regulated by C_2H_4 (Solano et al., 1998), was used as a control for the ethylene treatment. C, As in B but including the Arabidopsis *ein2-1* mutant. Plant samples were collected after 12 and 24 h of treatment.

their mRNA levels in different Arabidopsis mutants with enhanced (*cpr5-2*, Bowling et al., 1997; *cep1*, Silva et al., 1999) or reduced (*npr1-5*, Cao et al., 1994; *pad4-1*, Glazebrook et al., 1996; *jar1-1*, Staswick et al., 1998) disease resistance to pathogens and/or altered responses to SA (*npr1-5*, *pad4-1*, *cpr5-2*, and *cep1*) or MeJA (*jar1-1* and *cpr5-2*). *AtERF13*, *AtERF1*, *ERF1*, and *PDF1.2* were induced by MeJA in *jar1-1* to levels similar to those observed in the wild-type (WT; Fig. 4). *AtERF1* was induced by SA in the WT, in the *pad4-1* mutant, and, to a lesser extent, in the *npr1-5* mutant. Although *AtERF13* and *ERF1* were not induced by SA in the WT, they showed an increase in mRNA accumulation after SA treatment in the *pad4-1* mutant. In contrast, *PDF1.2* exhibited reduced expression in all samples treated with SA. In the two mutants *cpr5-2* and *cep1*, in which the plant defense response is enhanced, the levels of *PDF1.2* were high. However, none of the *ERF* genes were significantly induced in *cpr5-2* or *cep1*, suggesting that they were not involved in the enhanced expression of *PDF1.2* in these mutant backgrounds. The small increase in *AtERF13* expression in *cep1* was not reproducibly seen in other experiments. The mRNA levels of *AtERF14* did not show significant changes after SA or MeJA treatments or in any of the mutant backgrounds.

We also looked to see whether the expression patterns of the *ERF* genes after pathogen infection were altered in the *pad4-1* mutant. As shown in Figure 5A, *AtERF13*, *AtERF1*, and *ERF1* were induced in a similar fashion after infection with either the avirulent or virulent *P. syringae* strains in the *pad4-1* mutant compared with WT. These experiments used 4-week-old plants grown in soil in contrast with the experiments presented in Figure 4, which used 2-week-old seedlings grown in Murashige and Skoog agar. Therefore, we repeated the SA treatment with 4-week-old plants grown in soil. As shown in Figure 5B, *AtERF13* and *ERF1* were induced by SA in the WT and *pad4-1*, in contrast to the results observed with the 2-week-old seedlings. These results suggest that the regulation of *AtERF13* and *ERF1* by PAD4 is complex and may be

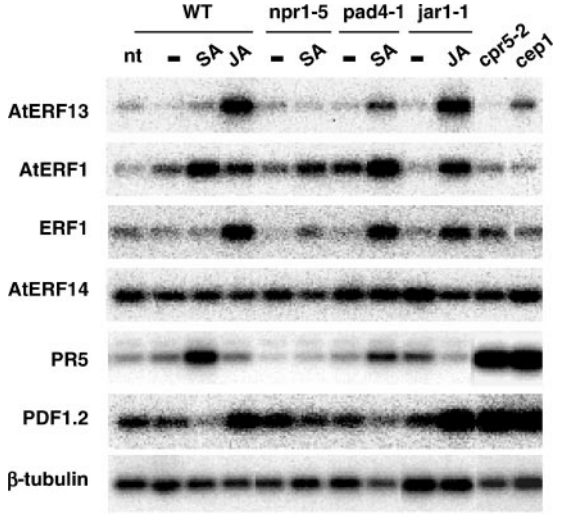

Figure 4. Analysis of ERF RNA levels in mutants with altered responses to defense signaling molecules and/or pathogens. RT-PCR analysis of RNA samples from Arabidopsis (WT) and the *npr1-5*, *pad4-1*, *jar1-1*, *cpr5-2*, and *cep1* mutants. Seedlings were treated for 6 h with 0.1% (v/v) ethanol (−), 1 mM SA (SA), or 100 μM MeJA (JA) or were not treated (nt). *PR5* and *PDF1.2*, which are up-regulated by SA (Ward et al., 1991) and MeJA (Penninckx et al., 1996), respectively, were used as controls. For the WT, results for the Columbia ecotype are shown, but similar results were obtained with the Nossen ecotype.

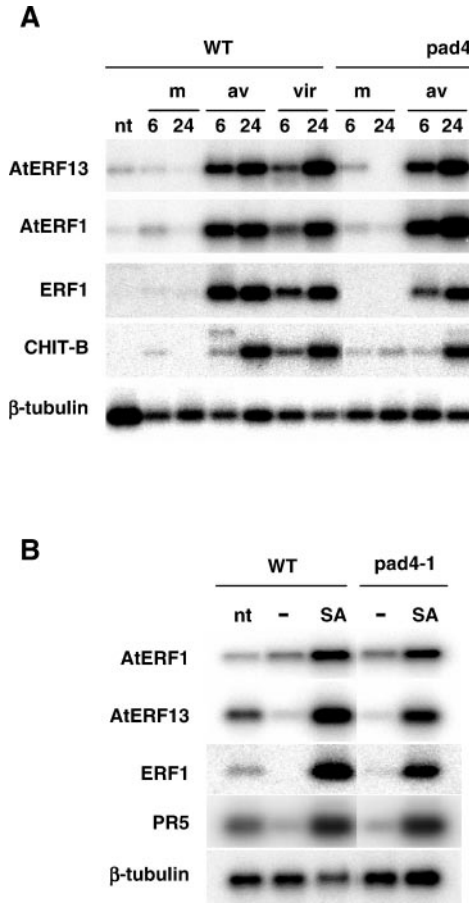

Figure 5. RNA expression of the Arabidopsis *ERF* genes in response to pathogen infection and SA treatment in the *pad4-1* mutant. A, Infection of WT and *pad4-1* with *P. syringae*. Arabidopsis leaves from 4-week-old plants were infiltrated with a mock solution (m) or with the same solution containing approximately 10^7cfu mL^{-1} of the avirulent pathogen *Pst* DC3000(*avrRpt2*) (av) or approximately 10^7cfu mL^{-1} of the virulent pathogen *Pst* DC3000 (vir). RNAs isolated from leaves at the indicated time points were subjected to RT-PCR. B, Treatment of WT and *pad4-1* with SA. Four-week-old Arabidopsis plants were sprayed with 0.1% (v/v) ethanol (−) or 1 mM SA or were not treated (nt). RNAs isolated from leaves after 6 h of treatment were analyzed by RT-PCR.

controlled by developmental and/or growth conditions.

Transcriptional Properties of the ERFs

ERF proteins have been shown to function as either transcriptional activators or repressors (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Solano et al., 1998; Fujimoto et al., 2000; van der Fits and Memelink, 2000; Ohta et al., 2001; Park et al., 2001). Of the four ERF proteins examined in this study, AtERF1 has been analyzed with regard to its transcriptional properties and shown to be a transcriptional activator (Fujimoto et al., 2000), whereas overexpression of ERF1 resulted in enhanced expression of *PDF1.2* and *CHIT-B* (Solano et al., 1998). We used a yeast one-hybrid system to examine the transcriptional properties of the Arabidopsis ERF proteins. As shown in Figure 6A, we generated effector plasmids containing translational fusions between the *ERF* and the *GAL4*-binding domain coding regions. Two reporter constructs integrated in the genome of two different strains of the yeast *Saccharomyces cerevisiae* were used. In both cases, the Gal4-binding site ($Gal4_{BS}$) was fused to a minimal promoter ($Gal1_{TATA}$) to control the expression of either the *LacZ* or the *His3* reporter genes. The effector constructs expressing the ERF proteins were introduced into the two yeast strains specified above. As shown in Figure 6B, all the ERFs were able to activate transcription, with AtERF13, ERF1, and AtERF14 showing significantly stronger activation compared with AtERF1, which was used as a positive control. ERF1, AtERF13, and AtERF14 contain acidic rich regions that may function as transcriptional activation domains, as has been reported for AtERF1 (Fujimoto et al., 2000).

DISCUSSION

ERF proteins comprise one of the largest families of transcription factors in plants with 124 family members present in Arabidopsis (Riechmann et al., 2000). We have identified four Arabidopsis *ERF* genes that are specifically induced after inoculation with avirulent or virulent *Pst* DC3000 strains. One possibility for why a number of ERF factors are enhanced in response to a specific pathogen may be to help orchestrate the correct temporal response in defense gene expression. Support for this possibility comes from our results showing distinct induction patterns among the four Arabidopsis *ERF* genes in response to *P. syringae* infection. The same pattern of induction kinetics was seen with both the avirulent and virulent strains of *P. syringae*, although, in most cases, the induction in *ERF* expression was delayed and/or reduced after inoculation with the virulent strain. These results suggest that the ERF proteins analyzed here play roles in both compatible and incompatible interactions.

Our results and, in the case of *AtERF1*, the results of Fujimoto et al. (2000), show that all four ERF proteins contain transcriptional activation domains. One possibility is that these ERF proteins form part of a transcriptional activation cascade whereby ERF proteins induced early in response to *P. syringae* infection, such as AtERF1 and AtERF13, are directly involved in regulating the expression of ERF members induced later in the infection, such as AtERF14. Although detailed analysis of the promoters of the ERF genes will be required to fully test this possibility, their promoters do not contain any obvious GCC box sequences. An alternative possibility is that the different ERF proteins induced in response to *P. syringae* infection regulate the expression of specific sets of plant defense genes. Support for this possibil-

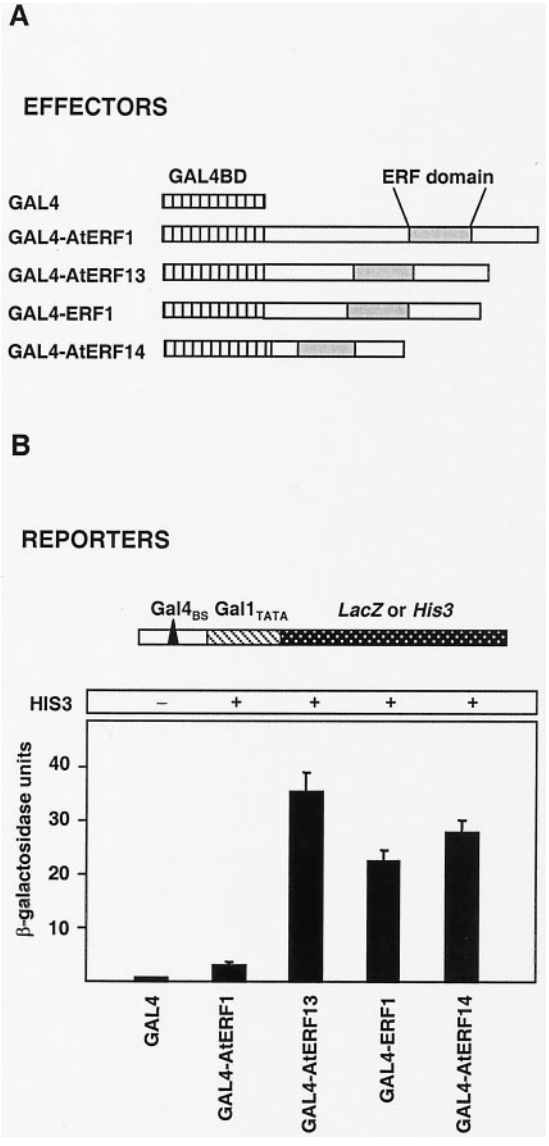

Figure 6. Transcriptional properties of the ERF proteins. A, Schematic diagram of the effector and reporter constructs used in the yeast assays. The effectors contained the GAL4 DNA-binding domain coding region (GAL4BD) fused to each of the ERFs (GAL4-AtERF1, GAL4-AtERF13, GAL4-ERF1, and GAL4-AtERF14). The reporter genes were the *LacZ* or *His3* gene under the control of a minimal promoter ($Gal1_{TATA}$) plus a GAL4 binding site ($Gal4_{BS}$). B, β-Galactosidase activity and growth in the absence of His induced by the effectors shown in A. β-Galactosidase values are from at least three independent replicates. Error bars represent SEs.

ity comes from studies with *AtERF1* and *ERF1*. *AtERF1*, one of the *ERF* genes that shows the earliest response to *P. syringae* inoculation, is a positive regulator of *Hookless-1*, an Arabidopsis gene containing a GCC box motif in its promoter (Fujimoto et al., 2000). In contrast, overexpression of *ERF1*, which is induced later than *AtERF1* after *P. syringae* inoculation, did not cause any change in *Hookless-1* expression. However, the expression of two other genes that contain potential GCC boxes in their promoters, *PDF1.2* and *CHIT-B*, was induced (Solano et al., 1998).

Our analysis of Arabidopsis ERF proteins induced after *P. syringae* inoculation has not been exhaustive. First, the cut-off for the genes initially chosen on the basis of similarity to ERF1 was arbitrary. Moreover, as shown in Figure 1B, there are other Arabidopsis ERF proteins that are as similar to ERF1 as the ones used in this study, but these were not present in the databases at the time that we initiated these studies. Some of these genes may also be induced by *P. syringae*. Because some ERF proteins have been shown to act as transcriptional repressors (Fujimoto et al., 2000; Ohta et al., 2001), it would be interesting to know whether any Arabidopsis ERF members that belong to this category are inducible by *P. syringae*, and, if so, what are their temporal accumulation patterns.

Although the mRNA levels of three of the *ERF* genes, *AtEBP*, *AtERF2*, and *AtERF15*, did not show specific and/or significant changes in response to *P. syringae* (avirulent) inoculation, these proteins may still play roles in plant defense gene expression, possibly in response to other pathogens and/or through post-transcriptional regulation. For instance, Hermsmeier et al. (2000) have found that *AtEBP* RNA levels are locally down-regulated at the syncytium in a compatible cyst nematode infection and have proposed that the nematode may actively suppress the plant defense response. Moreover, Schenk et al. (2000) have shown using a genomic approach that *AtEBP* expression, also called RAP2.3 (Okamuro et al., 1997), was increased 4.3 times in response to infection by the incompatible fungal pathogen *Alternaria brassicicola*. Post-translational control has been observed for some ERF proteins, for example the tomato Pti4 protein is specifically phosphorylated by the product of the disease resistance gene *Pto*, and this phosphorylation enhances the binding of Pti4 to the GCC box (Gu et al., 2000). In periwinkle, *ORCA3* regulates the JA-mediated expression of several terpenoid indole alkaloids biosynthetic genes (van der Fits and Memelink, 2001). The regulation by *ORCA3* does not depend on de novo protein synthesis, and the JA-inducible expression of at least two of these genes is sensitive to protein kinase inhibitors (Menke et al., 1999).

The large number of ERF proteins involved in defense responses may also be to help orchestrate the spatial response in defense gene expression to specific pathogens. Although we found that the four ERF genes examined here were expressed in all of the plant tissues analyzed, there were differences in the levels of expression in specific tissues for some of the genes. We also found interesting differences in the expression of the four ERF genes in response to defense signaling molecules. Although *ERF1* (Solano et al., 1998), *AtERF1* (Fujimoto et al., 2000), and *AtERF14* were responsive to ethylene, only *AtERF1* was responsive

to SA in 2-week-old seedlings. Treatment with MeJA resulted in enhanced expression for *ERF1*, *AtERF1*, and *AtERF13*, although the induction of *AtERF1* was less pronounced. Interestingly, whereas *AtERF1* expression is enhanced within 3 h after *P. syringae* inoculation, the response to ethylene treatment is significantly slower and starts between 6 and 12 h (Fujimoto et al., 2000). In contrast, *ERF1* expression is enhanced between 3 and 6 h after *P. syringae* infection but as soon as 15 min after ethylene treatment (Solano et al., 1998). These results demonstrate differences between the responses to a pathogen and a defense signal and are consistent with those of Thara et al. (1999), who showed that the induction of *Pti4* and *Pti5* by *P. syringae* was independent of ethylene, SA, and JA. Fujimoto et al. (2000) also found that the induction of *AtERF1* by wounding and cycloheximide treatment was both faster than and independent of the induction caused by ethylene.

There were also interesting differences in the expression of the *ERF* genes in Arabidopsis mutants altered in their responses to defense signaling molecules and/or pathogens. The *jar1-1* mutant has decreased sensitivity to JA inhibition of root elongation (Staswick et al., 1992). JAR1 has been linked to plant defense responses, because *jar1-1* mutants suppress resistance responses of *cpr5-2* and *cpr6* (Clarke et al., 2000) and are more susceptible to the soil fungus *Pythium irregulare* (Staswick et al., 1998). However, the expression of the JA-responsive *ERF* genes and the *PDF1.2* gene, commonly used as a marker for JA-mediated defense responses, were still inducible by MeJA in the *jar1-1* mutant. Because the MeJA induction of *PDF1.2* expression has been shown to be blocked in another JA signaling mutant called *coi1* (Penninckx et al., 1998), our results are consistent with multiple JA signaling pathways operating in the plant defense response.

Plant defense responses are constitutively expressed in the *cpr5-2* and *cep1* mutants. Thus, *cpr5-2* has elevated levels of SA, enhanced levels of *PR*, increased *PDF1.2* gene expression thought to reflect an activated JA and/or ethylene signaling pathways, and enhanced resistance to virulent strains of *P. syringae* and *Peronospora parasitica* (Bowling et al., 1997). The *cep1* mutant has higher levels of SA and *PR* gene expression (Silva et al., 1999), and our results show that *PDF1.2* expression is increased (Fig. 4). However, the SA- and/or JA-responsive *ERF* genes were not induced in either of these mutant backgrounds. Although the *PDF1.2* promoter contains a GCC box (Manners et al., 1998), our results make it unlikely that the *ERF* genes analyzed here are regulating *PDF1.2* expression in the *cep1* and *cpr5-2* mutants, although a post-transcriptional role cannot be ruled out. Whether *PDF1.2* is being induced in *cep1* or *cpr5-2* by other ERF proteins or through some other transcription factor/promoter system remains to be investigated.

Two mutants, *npr1-5* and *pad4-1*, that are altered in SA responses were also tested. NPR1 acts downstream of SA to promote the expression of genes like *PR-1*, although some SA responses are NPR1-independent (for review, see Glazebrook, 2001). The induction of *AtERF1* expression by SA seemed to be, in part, NPR1-independent because it was reduced but not eliminated in the *npr1-5* mutant, whereas *PR5* induction was abolished in this mutant background. The *PAD4-1* gene, encoding a lipase-like protein, is inducible by SA and virulent *P. syringae* infection (Jirage et al., 1999), and *pad4-1* Arabidopsis plants are defective in camalexin production, SA synthesis, and PR gene expression after infection with a virulent strain of *P. syringae* (Zhou et al., 1998). Our results demonstrating that in 2-week-old seedlings *AtERF13* and *ERF1* are induced by SA in *pad4-1* but not in the WT, suggest a new role for PAD4, whereby it regulates the expression of specific *ERF* genes by preventing their induction by SA. These results further support the evidence for cross-talk between the SA and JA signaling pathways.

ERF members clearly have significant differences in their RNA expression patterns and transcriptional properties. Superimposed upon these differences may be differences in DNA binding site preference (Park et al., 2001), post-translational control (Gu et al., 2000), and/or specific interactions with other proteins (Büttner and Singh, 1997; Xu et al., 1998) that may help to modulate the specificity of plant defense/stress gene expression in response to different signal transduction pathways. Given the prominent role ERF proteins play in plant stress responses and the large size of the ERF family, it will be an important task to determine the function of each member of this large family of transcription factors.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The Arabidopsis mutants *cep1*, *cpr5-2*, *jar1-1*, and *pad4-1* were obtained from the Nottingham Arabidopsis Stock Centre (University of Nottingham, Nottingham, UK), and the Arabidopsis mutants *ein2-1* and *npr1-5* were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus) and from Dr. Dan Klessig (Bruce Thompson Institute for Plant Research, Ithaca, NY), respectively. All the mutants were in the Columbia ecotype except for *npr1-5* (Nossen) and *cep1* (Wassilewskija). Seeds from these mutants and from Columbia ecotype (WT) were stratified for 2 d at 4°C and grown on Murashige and Skoog agar plates under 16-h-light/8-h-dark cycle at 22°C unless otherwise noted. Plant samples were harvested by freezing whole seedlings or plant tissues in liquid nitrogen, which were then stored at −80°C until RNA was isolated.

Pathogen Infection and Chemical Treatments

Pseudomonas syringae pv *tomato* (*Pst*) strain DC3000 and *Pst* DC3000 expressing the avirulence gene *avrRpt2* were a

gift from B. Staskawicz (University of California, Berkeley). Leaves from 4- to 5-week-old plants containing the *RPS2* resistance gene that were grown on soil under a 12-h-light/12-h-dark regime, were infiltrated with a mock (10 mM $MgSO_4$) solution as a control or with the same solution containing approximately 10^7cfu mL^{-1} of the plant pathogens *Pst* DC3000(*avrRpt2*) or *Pst* DC3000. Infiltrated leaves were harvested at the indicated time points and RNA was isolated. For the ethylene treatment, 3- to 4-week-old Arabidopsis plants that had been grown in pots were placed in a glass chamber, and ethylene was injected to a final concentration of 100 μL L^{-1}. Control plants were handled in the same way and flushed with air. For the SA and MeJA treatments, 2-week-old seedlings that had been grown on Murashige and Skoog plates in vertical position were used, unless otherwise stated. Murashige and Skoog solutions containing 0.1% (v/v) ethanol plus 1 mM SA or 100 μM MeJA were poured onto Murashige and Skoog plates so the liquid covered the roots but not the aerial tissues. No treatment or 0.1% (v/v) ethanol were used as controls. Whole seedlings were harvested after 6 h of treatment, and RNA was isolated.

RNA Isolation and RT-PCR Analysis

Total RNA was isolated from seedlings or leaves using the Purescript reagent (Gentra Systems, Minneapolis), treated with 1 unit of RNase-free DNase (Promega, Madison, WI) at 37°C for 1 h and repurified with Purescript. One microgram of total RNA was used for the first-strand cDNA synthesis after incubation at 65°C for 10 min. cDNA was synthesized in a volume of 25 μL that contained 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol, 4 μM poly(dT) primer, 0.5 mM dNTPs, 2 units of avian myeloblastosis virus RT (Promega), and 12.5 units of RNasin (Promega) at 37°C for 1 h. All PCR reactions were performed with 0.5 unit of *Taq* polymerase (Invitrogen, Carlsbad, CA), the buffer provided by the supplier, 0.2 mM dNTPs, and a pair of primers (0.1 μM each) in a final volume of 20 μL. The gene-specific primer pairs used for the RT-PCR are listed: *CHIT-B*, 5′-CGGTGGTACTCCTCCTGGACCCACCGGC-3′ and 5′-CGGCGGCACGGTCGGCGTCTGAAGGCTG-3′; *AtEBP*, 5′-GCCATGGATCCGAATTCAGCGGCG AAGCAG-3′ and 5′-TACACTCTAGACTCGAGACATCATCAGCAG-3′; *β-tubulin*, 5′-CGTGGATCACAGCAATACAGAGCC-3′ and 5′-CCTCCTGCACTTCCACTT CGTCTTC-3′; *AtERF1*, 5′-CAATCTTGTGTAACCGGTCAGAGC-3′ and 5′-CACCGTCAATCCCTTATCCATTCC-3′; *AtERF2*, 5′-TGTACGGACAGTGCAATA TAGAATCCG-3′ and 5′-CACCTCCGACGTCAGATTCTCTGC-3′; *ERF1*, 5′-ATTCTATCGGATCTTCTCCAGATTC-3′ and 5′-CCTAATCTTTCACCAAGT CCCACT-3′; *AtERF13*, 5′-CCATTATGAGCTCATCTGATTCCG-3′ and 5′-CAGAATAAGAACATTCTGATTGGTCC-3′; *AtERF14*, 5′-GGATCAAGGAGGT CGTAGCAGTGG-3′ and 5′-TTATTGCCTCTTGCCCATGTTG-3′; *AtERF15*, 5′-TCATTTGAGTCACCGGAGATGATG-3′ and 5′-CCACAAGTGTACCACTTTCT TGC-3′; *PDF1.2*, 5′-AATGGATCCATGGCTAAGTTTGCTTCCATC-3′ and 5′-AATGAATTCAATACACACGATTTAGCACC-3′; *PR5*, 5′-ATGGCAAATATCTC CAGTATTCACA-3′ and 5′-ATGTCGGGGCAAGCCGCGTTGAGG-3′.

When the PCR reactions were in the exponential phase of amplification, typically after 20 to 25 cycles, the products were run on a 1.5% (w/v) agarose gel, transferred onto Zeta-Probe GT Genomic blotting membranes (Bio-Rad, Hercules, CA), and hybridized with the appropriate randomly primed ^{32}P-labeled probes following standard procedures (Sambrook et al., 1989). Chronex #4 x-ray films (AGFA-Gevaert, Nunawading, Australia) or a Cyclone phosphor imager (Packard, Meriden, CT) were used to image the hybridized membranes.

Yeast Strains and LacZ Assays

The effector plasmid pGBT9 (CLONTECH, Palo Alto, CA) was used to generate in-frame C-terminal fusions of the complete coding sequences of *AtERF1*, *AtERF13*, *ERF1*, and *AtERF14* with the *GAL4*-DNA binding domain (*GAL4*BD). The constructs were generated after PCR amplification of the *ERFs* using the Pfu turbo DNA polymerase (Stratagene, La Jolla, CA) and appropriate oligonucleotides with engineered restriction sites for cloning. These constructs were introduced into two haploid strains of *Saccharomyces cerevisiae*; HF7c and SFY526 contained the *His3* and *LacZ* reporter genes, respectively, under the control of a minimal *Gal1* promoter ($Gal1_{TATA}$) containing an upstream *Gal4*-binding site ($Gal4_{BS}$). The bacterial *LacZ* gene encodes a β-galactosidase, and the *His3* gene encodes a *S. cerevisiae* imidazole glycerol-phosphate dehydratase, which catalyzes one of the enzymatic steps in His biosynthesis. Growth of HF7c transformants on minimal media without His indicated activation of transcription (+), which was quantified by measuring β-galactosidase activity in SFY526 cells as described by Ausubel et al. (1990). His media was supplemented with 2.5 mM of 3-amino-1,2,4-triazole (Sigma, St. Louis) to inhibit the HIS3 protein derived from the leaky expression of the *His3* gene.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this paper that would limit their use in noncommercial research purposes.

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