AtERF14, a Member of the ERF Family of Transcription Factors, Plays a Nonredundant Role in Plant Defense[1][C][W][OA]

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We had previously shown that several transcription factors of the ethylene (ET) response factor (ERF) family were induced with different but overlapping kinetics following challenge of Arabidopsis (*Arabidopsis thaliana*) with *Pseudomonas syringae* pv *tomato* DC3000 (*avrRpt2*). One of these genes, a transcriptional activator, *AtERF14*, was induced at the same time as *ERF*-target genes (*ChiB*, basic chitinase). To unravel the potential function of AtERF14 in regulating the plant defense response, we have analyzed gain- and loss-of-function mutants. We show here that AtERF14 has a prominent role in the plant defense response, since overexpression of *AtERF14* had dramatic effects on both plant phenotype and defense gene expression and *AtERF14* loss-of-function mutants showed impaired induction of defense genes following exogenous ET treatment and increased susceptibility to *Fusarium oxysporum*. Moreover, the expression of other *ERF* genes involved in defense and ET/jasmonic acid responses, such as *ERF1* and *AtERF2*, depends on *AtERF14* expression. A number of ERFs have been shown to function in the defense response through overexpression. However, the effect of loss of *AtERF14* function on defense gene expression, pathogen resistance, and regulation of the expression of other *ERF* genes is unique thus far. These results suggest a unique role for AtERF14 in regulating the plant defense response.

Plants defend themselves from pathogen attack by an array of mechanisms, including preformed and induced responses. The defenses may be induced throughout the plant and depend on the perception of the pathogen. Localized and systemic defenses rely on activation of one or more signaling pathways that lead to the induction of defense gene expression. The most studied of these pathways are regulated by salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) or their derivatives (for review, see Thatcher et al., 2005). These pathways have been associated with resistance to different types of pathogens, with the SA-dependent pathway mainly providing resistance to biotrophic pathogens while the JA and ET pathways provide resistance predominantly to necrotrophic pathogens (Thomma et al., 1998; Glazebrook, 2005). In many instances, the JA and ET pathways have been shown to regulate similar types of defense genes (Schenk et al., 2000; Lorenzo and Solano, 2004).

The regulation of plant defense responses is complex, with a number of transcription factor families playing important roles (Rushton and Somssich, 1998; Singh et al., 2002). There is considerable interest in identifying and utilizing key transcription factors in plant defense for engineering increased resistance to plant pathogens in agriculture (Gurr and Rushton, 2005). One transcription factor family that is being explored is the ET response factor (ERF) family, members of which are a point of integration of the JA and ET pathways (Lorenzo et al., 2003). In Arabidopsis (*Arabidopsis thaliana*), there are thought to be 147 members of the AP2/EREBP family of plant transcription factors (Feng et al., 2005; Nakano et al., 2006). The proteins encoded by the *AP2/EREBP* gene family have diverse functions throughout the plant life cycle, including regulation of development, responses to abiotic stresses such as drought and cold, as well as to biotic stresses such as fungal pathogen infections (Feng et al., 2005). The AP2/EREBP family is divided into the RAV, AP2, and EREBP subfamilies, with the EREBP subfamily being divided into DREB or A subgroup and the ERF or B subgroup. The ERF or B subgroup contains 65 *ERF* genes and contains all of the *AP2/EREBP* genes that have been linked to disease resistance responses (Gutterson and Reuber, 2004). *ERF* genes have been shown to be responsive to both JA and ET (Oñate-Sánchez and Singh, 2002; Lorenzo et al., 2003; Gutterson and Reuber, 2004; McGrath et al., 2005), while work in tomato (*Lycopersicon esculentum*) has revealed direct regulation of the ERFs *Pti4* and *Pti5* by the PTO R protein following recognition of *Pseudomonas syringae* pv *tomato* (Zhou

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Figure 1. Phenotype of two representative lines overexpressing *AtERF14* in the Col background (left) compared to wild-type Col-0 (right). Top, Plants grown for 3 weeks on MS containing kanamycin for ox-*AtERF14* or MS for wild type; bottom, plants at 7 weeks after germination. [See online article for color version of this figure.]

et al., 1997). ERFs are known to bind to the GCC box and related elements in the promoters of JA/ET-inducible, pathogenesis-related (*PR*) genes, such as the defensin *PDF1.2*, basic chitinase (*ChiB*), and thionin (*Thi2.1*), and either induce or repress the expression of these genes (Menke et al., 1999; Fujimoto et al., 2000; Ohta et al., 2001; Tournier et al., 2003). Several members of the *ERF* gene family have been shown to be functionally involved in plant defense against pathogens, as overexpression leads to increased expression of *PDF1.2*, *ChiB*, and *Thi2.1* and increased resistance to a range of pathogens, both necrotrophic and biotrophic (Berrocal-Lobo et al., 2002; Gu et al., 2002; McGrath et al., 2005). Although most ERFs described so far are activators, 14 Arabidopsis ERF proteins contain an ERF-associated amphiphilic repression (EAR) motif (Nakano et al., 2006), which has been shown to function as a repression domain (Fujimoto et al., 2000; Ohta et al., 2001). Overexpression of *AtERF4*, an EAR-containing ERF, reduces *PDF1.2* induction by methyl jasmonate (MeJA) and plant resistance to *Fusarium oxysporum* (McGrath et al., 2005).

Although overexpression of several *ERF*s has been shown to modify defense gene expression and resistance to pathogens, little has been reported on defense phenotypes caused by silencing, mutation, or knockout of *ERF*s (McGrath et al., 2005). Since the ERF family in Arabidopsis contains 65 members (Feng et al., 2005; Nakano et al., 2006), many of which are regulated by the same stimuli and potentially bind the same promoter element, it may be expected that a high level of functional redundancy exists and, thus, isolation of mutant phenotypes with knockout of a single *ERF* is uncommon. This notion is supported by the observation that few *AP2/EREBP* genes have been isolated through loss-of-function mutant screens. Exceptions are *BD1* (Chuck et al., 2002) and its ortholog *FZP* in maize (*Zea mays*; Komatsu et al., 2003), and the *DREB* or A subfamily genes *ABI4* (Finkelstein et al., 1998) and *CBF2* (Novillo et al., 2004) that control development or response to cold and drought conditions. To date, to our knowledge, no gene of the 65 member *ERF* or A subfamily that is associated with pathogen defense has been isolated through a mutant screen.

Previously, we identified Arabidopsis *ERF* genes whose expression was specifically induced by *P. syringae* pv *tomato* DC3000 (*avrRpt2*) infection with overlapping but distinct induction kinetics (Oñate-Sánchez and Singh, 2002). We chose *AtERF14* for further characterization since it was the only *ERF* whose induction started later than 6 h following *P. syringae* pv *tomato* DC3000 (*avrRpt2*) infection when potential downstream genes were also being induced. This unique expression pattern suggested that AtERF14 may play a different role than the other studied *ERF* genes that were induced prior to defense gene induction. We show that overexpression of *AtERF14* leads to increased *ERF* and defense gene expression and pleiotropic effects, including severe growth retardation and loss of seed set. Interestingly, loss-of-function mutations of *AtERF14* lead to loss of ET-mediated induction of defense genes and other *ERF*s. These results suggest a nonredundant role for AtERF14 in the coordination of *ERF* and defense gene expression. Moreover, loss-of-function mutants showed increased susceptibility to *F. oxysporum*, confirming that AtERF14 plays a key role in defense against some pathogens. These results are the first report of a loss-of-function mutant phenotype for an ERF activator and show that the *AtERF14* gene is important for ET responses and pathogen resistance.

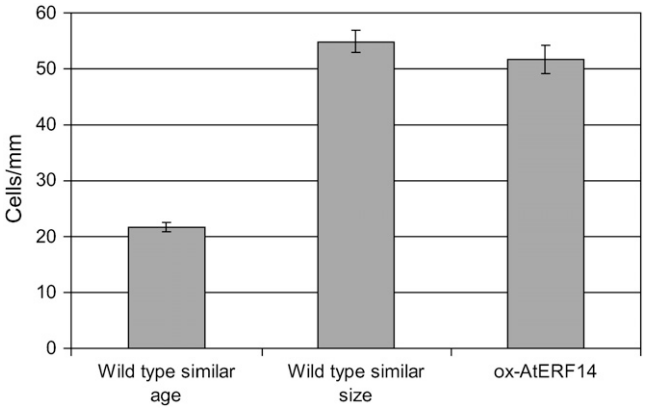

Figure 2. Examination of cell number and cell size in *AtERF14*-overexpressing plants and wild type. The number of palisade mesophyll cells per 1-mm region along transverse sections of leaves is shown for wild type and ox-*AtERF14* lines. "Wild type similar age" are leaves of a similar age to those selected for ox-AtERF14 but are larger in size. "Wild type similar size" are younger leaves than those selected for ox-*AtERF14* but are of a similar size. The average and SE of 10 replicate regions from two leaves are presented.

RESULTS

Overexpression of *AtERF14* Causes Severe Growth Retardation and Enhanced Defense Gene Expression

To obtain *AtERF14*-overexpressing plants (ox-*AtERF14*), the coding region of *AtERF14* was fused to a double 35S promoter and the construct introduced into Arabidopsis Columbia (Col)-0 plants. Transgenic plants overexpressing *AtERF14* showed a stunted phenotype from early stages of development, and these plants kept on producing rosette leaves, never bolted, and never produced seed (Fig. 1). To further investigate the phenotype caused by overexpression of *AtERF14*, the number of palisade mesophyll cells was counted per millimeter of transverse sections taken through the midpoint of the leaves of wild type and ox-*AtERF14* lines. A comparable number of cells per millimeter was found in ox-*AtERF14* lines when compared to wild-type leaves of a similar size (Fig. 2). However, comparison of leaves from ox-*AtERF14* lines to the much larger wild-type leaves of the same age revealed fewer individual cells per millimeter due to cell expansion (Fig. 2). These results therefore demonstrate that the stunted phenotype resulting from *AtERF14* overexpression is due to a reduction in cell size.

To confirm the increased level of expression of the transgenes and to assess the effects on the level of expression of several defense genes, we conducted quantitative real-time PCR (qRTPCR) on RNAs extracted from leaves of the transgenic plants. We selected two independent transgenic lines containing the *35S::AtERF14* construct for gene expression analysis. The lines *ox-AtERF14-2* and *ox-AtERF14-5* possessed *AtERF14* expression levels approximately 2,500- and 30,000-fold higher than in wild-type Col-0, respectively (Fig. 3A), although calculation of exact levels of *AtERF14* overexpression were hindered due to variation caused by very low basal levels in the controls. Overexpression of *AtERF14* caused a dramatic effect on defense gene expression. The levels of *ChiB* were increased by 270- and

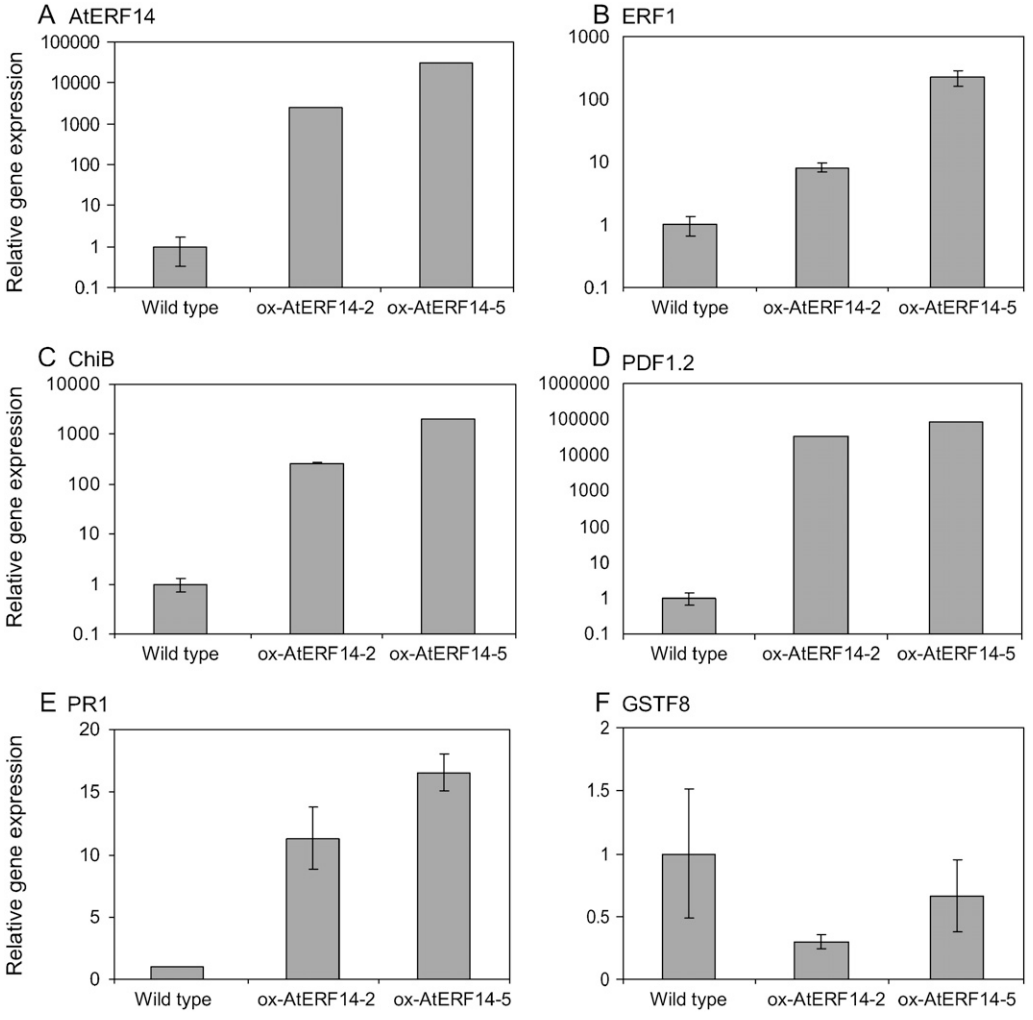

Figure 3. Expression of defense-associated genes in *AtERF14*-overexpressing lines. Gene expression is presented relative to average wild-type levels. The average and SE of two technical replicates are presented.

2,030-fold and *PDF1.2* levels were increased 30,000- and 87,000-fold higher than wild-type Col-0, respectively (Fig. 3, C and D). Interestingly, in addition to higher levels of *AtERF14* mRNA, the expression of *ERF1* was also increased 8- and 220-fold in lines *ox-AtERF14-2* and *ox-AtERF14-5*, respectively (Fig. 3B). The SA- and stress-induced *GSTF8* gene, formerly called GST6, showed no increase in expression level in *ox-AtERF14-2* and *ox-AtERF14-5*, while *PR1* did show an up-regulation in the *AtERF14* overexpression lines (Fig. 3, E and F), albeit small in comparison to those of *PDF1.2* and *ChiB*.

Defense Genes Are No Longer ET Inducible in *AtERF14* Loss-of-Function Mutants

Two SALK T-DNA insertion lines (Δ*aterf14-1*, SALK_140578; and Δ*aterf14-2*, SALK_118494) were obtained from the Arabidopsis Biological Resource Center (ABRC). These lines contained insertions in the *AtERF14* coding sequence as shown in Figure 4A. The position of the T-DNA insertions was confirmed using PCR, and homozygous lines were chosen based on segregation analysis. We used qRTPCR to examine *AtERF14* expression levels in wild-type Col and the two T-DNA insertion lines. The basal *AtERF14* expression level in wild type was very low, while the T-DNA insertion lines had undetectable expression levels. Following treatment with ET, *AtERF14* wild-type expression levels increased an average of 14-fold, while the T-DNA lines still had no detectable *AtERF14* transcript (Fig. 4B). Likewise, wild-type plants showed an induction of *PDF1.2* of approximately 30-fold following ET treatment, while the *AtERF14* T-DNA insertion lines showed no induction of the *PDF1.2* gene (Fig. 4C). A similar pattern was seen for *ChiB*, although a low level of induction was still

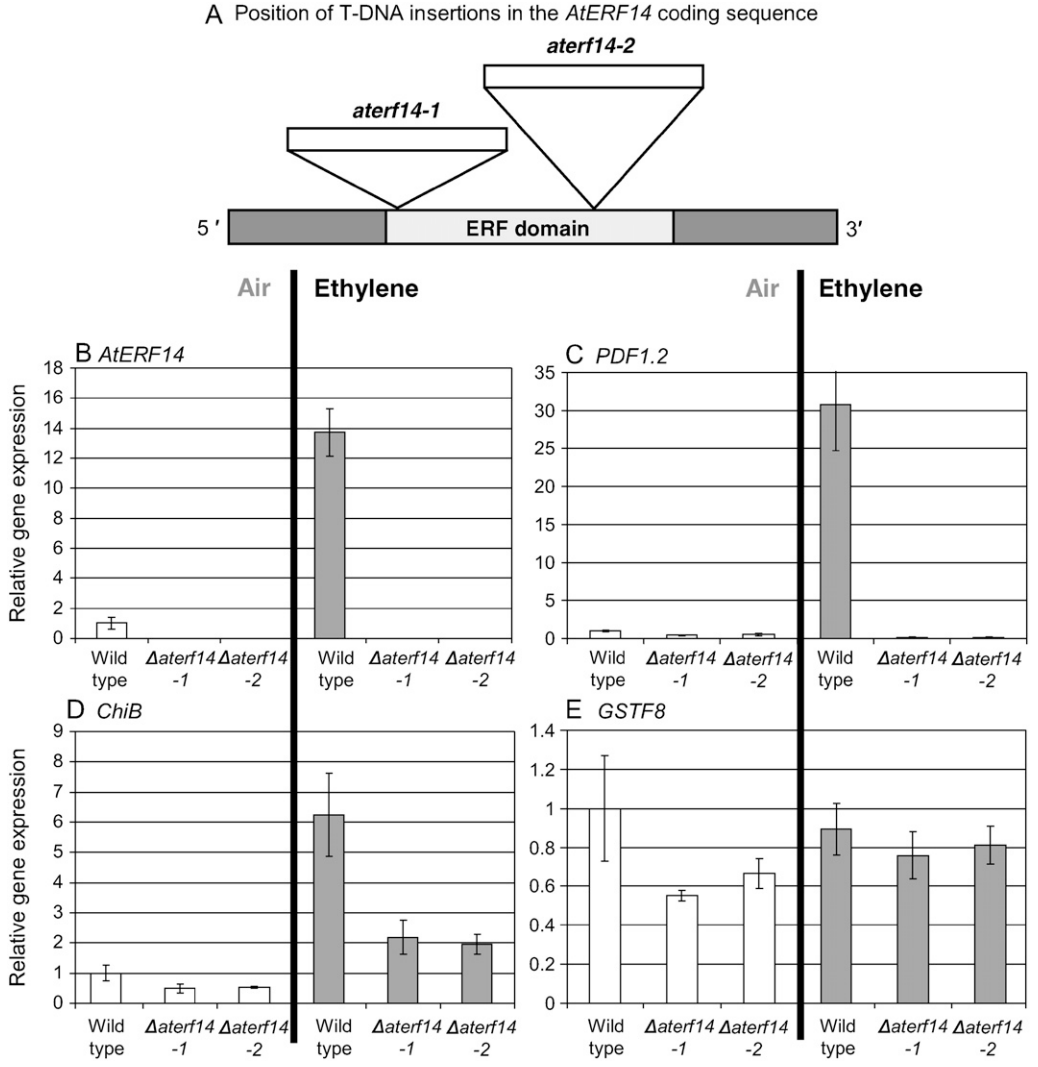

Figure 4. Mutant lines containing T-DNA insertions in the *AtERF14* gene. A, Schematic diagram showing the position of T-DNA insertions in the *AtERF14* gene. The *AtERF14* open reading frame does not contain any introns. B to E, Defense gene expression in wild type, Δ*aterf14-1*, and Δ*aterf14-2* lines with and without 24 h of ET treatment. Gene expression is presented relative to untreated wild-type levels. Average and SE of two biological replicates are presented.

visible in the *AtERF14* T-DNA insertion lines (Fig. 4D). The SA pathway defense gene *GSTF8* did not show substantial regulation following ET treatment in either the Col-0 or *AtERF14* T-DNA insertion lines (Fig. 4E).

The expression of *ERF1* was induced 18-fold in Col-0 following treatment with ET but, interestingly, the level of induction in the T-DNA insertion lines was much lower, suggesting that AtERF14 is required for full ET-mediated induction of *ERF1* (Fig. 5A). These results prompted us to analyze the expression levels of other *ERF* genes. *AtERF2* and *AtERF15* both showed a lower level of induction in the T-DNA lines in comparison to the clear induction seen in Col-0 following ET treatment (Fig. 5, B and C).

Regulation of Defense Genes by JA and SA Is Not Altered in *AtERF14* T-DNA Insertion Lines

To determine if the regulation of defense genes by other defense signals was also altered in the *AtERF14* T-DNA insertion lines, we treated these lines and wild type with either MeJA or SA for 24 h. The expression of the JA marker gene *Thi2.1* showed similar levels of induction in both the wild type and Δ*aterf14* lines (Fig. 6A). Similarly, the JA and ET marker gene *PDF1.2* showed comparable induction in the wild type and Δ*aterf14* lines following MeJA treatment (Fig. 6B). The SA-responsive genes *PR1* and *PR2* showed similar levels of induction in both the wild type and Δ*aterf14* lines following SA treatment (Fig. 6, C and D). These results suggest that AtERF14 does not play a significant role in the regulation of defense genes by other defense signals.

AtERF14 Loss-of-Function Mutants Are More Susceptible to Infection by *F. oxysporum* But Not by *Rhizoctonia solani*

Since *AtERF14* loss-of-function mutants had reduced expression of a number of defense genes, we were interested in seeing whether these lines were also more susceptible to pathogen attack. We inoculated wild type, Δ*aterf14-1*, and Δ*aterf14-2* with *Rhizoctonia solani*. Lupin (*Lupinus albus*) and canola (*Brassica napus*), natural hosts for strains ZG3 and ZG5, respectively, were included as positive controls for infection. These two strains of *R. solani* were previously shown to be pathogenic on Arabidopsis (Perl-Treves et al., 2004). Successful infection was shown by the susceptibility of the natural host species to their respective pathogenic strain, with complete susceptibility to *R. solani* demonstrated by the uniform death of the canola (data not shown). In contrast, the Arabidopsis lines predominantly showed reduced plant size, suggesting that wild-type Arabidopsis possesses an ability to partially resist the pathogen. Although substantial reduction in dry root weight was observed in the Arabidopsis lines following infection with ZG3 or ZG5, no significant difference ($P < 0.05$ according to Tukey-Kramer honestly significant difference test) was observed between the performance of wild type and the Δ*aterf14* mutants (Fig. 7). These results demonstrate that the ability of wild-type Arabidopsis to partially resist these *R. solani* strains is not compromised in the Δ*aterf14* mutants.

We also studied the response of wild type, Δ*aterf14-1*, and Δ*aterf14-2* plants to infection with *F. oxysporum*, previously shown to cause more damage on lines with altered JA signaling (Anderson et al., 2004). Susceptibility to *F. oxysporum* was determined by the rate of survival and dry root weight 10 d after inoculation. In comparison to the wild type, Δ*aterf14-1* and Δ*aterf14-2* showed substantially lower survival rates and dry root

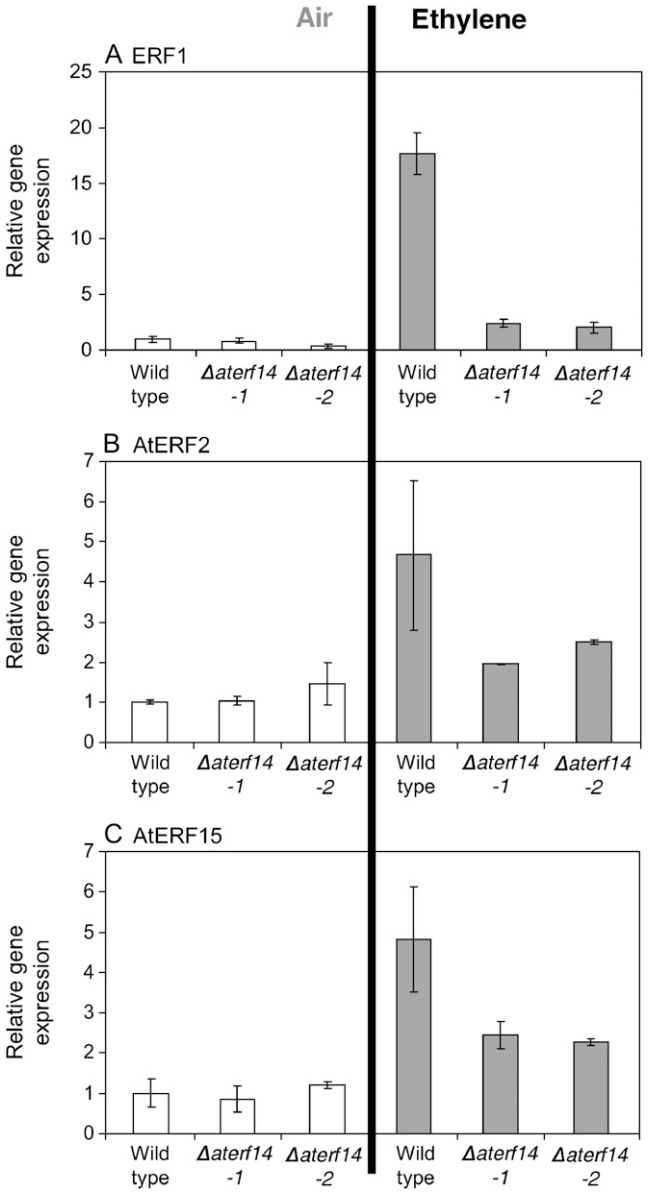

Figure 5. *ERF* gene expression in wild type, Δ*aterf14-1*, and Δ*aterf14-2* with and without 24 h of ET treatment. Gene expression is presented relative to untreated wild-type levels. Average and SE of two biological replicates are presented.

weight (Fig. 8), suggesting AtERF14 is important for resistance to *F. oxysporum* and the loss of AtERF14 function cannot be compensated by other proteins. This finding is unique for an ERF activator involved with plant defense as no studies have previously reported an alteration of defense gene expression or pathogen susceptibility by knocking out a single *ERF* activator gene, possibly due to redundancy of function in the large *ERF* gene family. Since the lines overexpressing *AtERF14* were not viable, pathogen inoculation experiments could not be conducted using these lines.

DISCUSSION

The majority of studies on the function of *ERF* genes in defense responses have focused on genes that are induced early during pathogen infection, prior to the induction of potential downstream genes such as *ChiB*. Previously, we identified pathogen-responsive *ERF* genes with distinct but overlapping induction kinetics following inoculation of Arabidopsis with *P. syringae* pv *tomato* DC3000 (*avrRpt2*; Oñate-Sánchez and Singh, 2002). *AtERF14* induction was the latest among the ERF genes tested, occurring between 6 and 12 h after pathogen infection, and the expression declined again by 36 h. Potential downstream defense genes such as *ChiB* were also induced by 12 h but remained high throughout the experiment time course. This unique expression pattern suggested that AtERF14 may play a different role than the other ERFs that were induced prior to defense gene induction. To study the function of AtERF14 in the regulation of the plant defense responses, we generated lines with increased expression of *AtERF14* and obtained T-DNA insertion lines for *AtERF14*.

Overexpression of *AtERF14* had dramatic effects on plant development and defense gene expression. The reduced cell expansion, overall plant size, and loss of seed set suggest the overexpression of AtERF14 is sufficient to induce widespread developmental defects. A similar phenotype was observed in plants having ectopic overexpression of the TINY AP2 transcription factor. In this gain-of-function mutant, reduced cell expansion was seen in the hypocotyls; however, unlike the ox-*AtERF14* lines, *tiny* plants continued to set viable seed (Wilson et al., 1996).

The increase in expression of *PDF1.2* and *ChiB* observed in the overexpression lines suggests AtERF14 is able to activate their expression either directly or

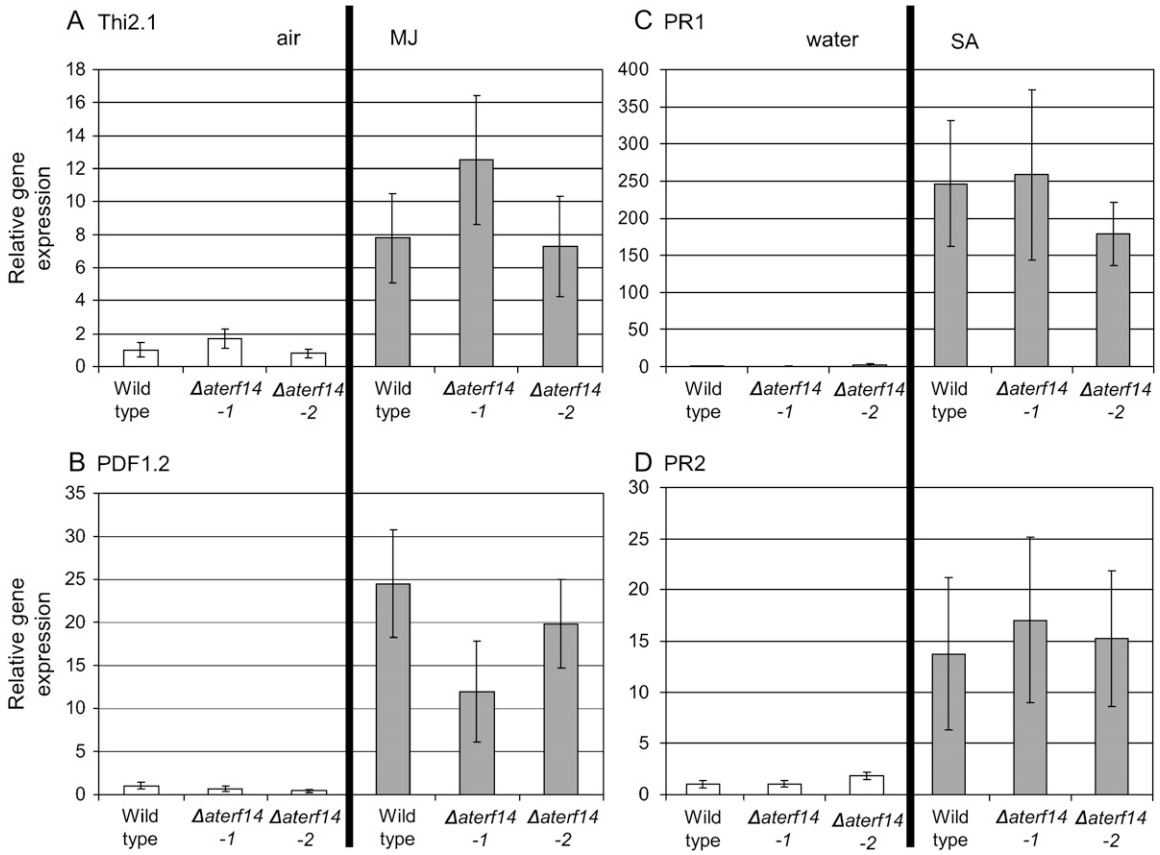

Figure 6. Defense gene expression in wild type, Δ*aterf14-1*, and Δ*aterf14-2* lines with and without 24 h of MeJA (MJ) treatment (A and B) or SA treatment (C and D). Gene expression is presented relative to untreated wild-type levels. The average and SE of five biological replicates are presented.

Figure 7. Response of wild type, Δ*aterf14-1*, and Δ*aterf14-2* to inoculation with *R. solani*. Dry root weight of plants inoculated with *R. solani* is expressed as the percentage of dry root weight of control, noninoculated plants.

indirectly, possibly through the GCC box as has been shown for several other *AtERF* genes (Fujimoto et al., 2000). In particular, *ERF1* and *AtERF2* are regulated through the ET/JA pathway and are induced early during pathogen infection (Oñate-Sánchez and Singh, 2002; McGrath et al., 2005). Although the overexpression of these genes produces up-regulation of defense genes and increased resistance to necrotrophic pathogens (Berrocal-Lobo et al., 2002; McGrath et al., 2005), the set of genes regulated by these ERFs must be different than that of AtERF14 since the phenotypes associated with their overexpression differ: *AtERF14*-overexpressing plants have severe growth retardation and loss of seed set, *ERF1*-overexpressing plants have a stunted phenotype but produce seeds (Solano et al., 1998), and AtERF2-overexpressing plants do not show a visible phenotype (McGrath et al., 2005).

Interestingly, the activation of defense genes in *AtERF14*-overexpressing plants also included elevation of *ERF1* expression, suggesting that activation of *PDF1.2* and *ChiB* could be occurring indirectly through the activity of ERF1 or other AtERFs. The absence of the GCC box in the promoter of *ERF1* suggests that AtERF14-mediated elevation of *ERF1* expression may be through an alternate mechanism or activation is indirect, for example, via positive feedback through increased ET/JA levels. If the activation of *ERF1* and other genes is through the stressed state of the plants, as manifested by the stunted growth, delayed flowering, and disease symptoms on the leaves, then other defense/stress genes may also be expected to be induced. However, the expression of *GSTF8* was not significantly changed in the *AtERF14*-overexpressing plants, indicating that the elevation of other defense genes is not a general stress response.

The influence of AtERF14 on defense gene and *ERF* expression was further studied using T-DNA insertion lines. Analysis of two lines containing a T-DNA insertion into the coding sequence of *AtERF14* revealed little change in defense gene expression or phenotype in untreated plants. However, following treatment of plants with ET, the T-DNA insertion lines failed to induce the expression of both *PDF1.2* and *ChiB*, suggesting that AtERF14 is not only sufficient but also essential for the activation of these genes. Moreover, *ERF1*, *AtERF2*, and *AtERF15* showed reduced induction by ET in Δ*aterf14-1* and Δ*aterf14-2*, while the wild type showed clear ET responsiveness of these genes. *ERF1* and *AtERF2* have been linked to the activation of defense genes in a number of studies (Solano et al., 1998; Berrocal-Lobo et al., 2002; McGrath et al., 2005).

This reduction in the induction of *AtERF*s following exogenous ET suggests that AtERF14 is required not only for regulation of defense genes through the GCC box but also for the regulation of *AtERF* genes that do not contain the GCC box in their promoters. One possibility is that AtERF14 may be able to bind to an unidentified promoter element or interact with another protein(s) to achieve this. Alternatively, AtERF14 may regulate *AtERF* genes lacking a GCC box by binding through the GCC box to the promoter of an intermediate transcription factor that in turn activates these *AtERF*s. The tomato Pti4 protein, when overexpressed in Arabidopsis, was shown to bind to promoters lacking a GCC box, suggesting it may bind to an alternate element or form interactions with other transcription factors that bind to those promoters (Chakravarthy et al., 2003). Although Büttner and Singh (1997) showed that AtEBP (an ERF) was able to interact with OBF4 (a bZIP transcription factor), very little is known about ERF-interacting proteins. In addition, the reduction of the ET-mediated expression of *AtERF*s, rather than a complete loss of response, suggests that another *AtERF14*-independent regulation of

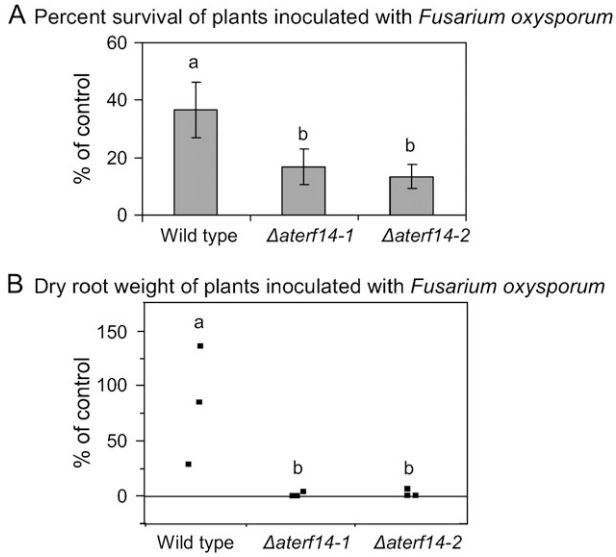

Figure 8. Response of wild type, Δ*aterf14-1*, and Δ*aterf14-2* to inoculation with *F. oxysporum*. A, The percentage of survival of plants inoculated with *F. oxysporum*. B, The dry root weight of *F. oxysporum*-inoculated plants expressed as the percentage of control, noninoculated plants. Points represent the average of five plants from each pot. Levels not connected by the same letter are significantly different ($P < 0.05$) according to Tukey-Kramer honestly significant difference test.

*AtERF*s also exists. The quantitative differences in the expression of some *ERF*s in the Δ*aterf14* lines may explain why ET-mediated *PDF1.2* induction is abolished while *ChiB* still shows a low level of response.

Interestingly, AtERF14 appears not to play a significant role in the regulation of *PDF1.2* following MeJA treatment, suggesting an alternate set of transcription factors functions in this response or the JA pathway can activate the same transcription factors independently of AtERF14. The independence of JA-mediated gene expression is supported by the similar levels of induction of *Thi2.1* in wild type and Δ*aterf14* lines following MeJA treatment. Similarly, the comparable responses of *PR1* and *PR2* to SA treatment in the Δ*aterf14* lines and wild type suggest that AtERF14 is not required for SA signaling.

Since the response of defense genes to ET treatment was reduced in the Δ*aterf14* lines, we wanted to test if these lines also showed greater susceptibility to pathogens. Inoculation of Δ*aterf14* lines and wild type with two isolates of *R. solani* revealed no increase in susceptibility to this root rot pathogen. These results suggest that AtERF14 and the downstream defense gene expression are not recruited for the response to this pathogen under the conditions tested or that other mechanisms regulating the AtERFs, possibly involving the JA pathway, are involved in the response. However, inoculation of Δ*aterf14* lines with *F. oxysporum* revealed an increase in susceptibility to the vascular wilt pathogen, suggesting that AtERF14 is an essential component of the defense response activated in wild-type ecotype Col. These results demonstrate that AtERF14 plays an integral role in the regulation of the defense not only to exogenous ET but also in response to a necrotrophic pathogen.

The large ERF subfamily in Arabidopsis includes positive and negative regulators of defense gene expression that are often induced by the same or similar conditions, and this is thought to provide tight regulation of defense gene expression (Zhou et al., 1997; Fujimoto et al., 2000; Gu et al., 2002; Feng et al., 2005; McGrath et al., 2005). The presence of many transcription factors with similar regulation and binding preference suggests there may be a large amount of functional redundancy and the disruption of *AtERF*s is unlikely to have an effect on defense gene expression or pathogen resistance. This notion is supported by the absence of ERF mutants showing a phenotype. The effect of loss of *AtERF14* function on *ERF* and defense gene expression and pathogen resistance suggests this gene may be one of the few members of the large ERF family to have a nonredundant function. Two other loss-of-function mutants of the ERF or B subfamily have been described and both are repressors of transcription (McGrath et al., 2005; Nasir et al., 2005). *AtERF4* is a negative regulator of JA-responsive defense gene expression and null mutants are more resistant to *Fusarium oxysporum* (McGrath et al., 2005). Silencing of the tobacco (*Nicotiana tabacum*) gene *nbCD1* lead to significantly increased growth of *Pseudomonas cichorii* in the silenced leaves, and results from *nbCD1* overexpression suggest a role in the negative regulation of a repressor of hypersensitive-like cell death (Nasir et al., 2005). This low number of *ERF* genes shown to cause a phenotype when mutated supports the notion of a high level of redundancy within the ERF family.

While a number of ERFs have been shown to function in the defense response through overexpression, this is the first ERF transcriptional activator shown to have a nonredundant effect on defense gene expression and pathogen resistance in loss-of-function mutants and to regulate the expression of other *ERF* genes. Altogether, the results presented in this article suggest that AtERF14 plays a unique and pivotal role in responses to ET and challenge with a fungal pathogen.

MATERIALS AND METHODS

Plant Material and Treatments

Plants were grown in 16 h light per day at a constant temperature of 22.5°C either on Murashige and Skoog (MS) plates or in soil. To generate the *AtERF14* overexpression lines, the coding sequence of the gene was introduced into the pGreenII0029 plasmid (Hellens et al., 2000) and transformed into Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 using the floral-dip method (Clough and Bent, 1998). Following selection on MS plates containing kanamycin, plants were transferred to soil and kept under clear plastic mini-glasshouses to maintain humidity. Because the *AtERF14*-overexpressing lines did not produce seed, leaf tissue from T1 lines was collected for gene expression analysis at 4 weeks after germination and immediately frozen in liquid nitrogen and stored at −80°C until RNA was isolated. Transverse sections were taken though the midpoint of fresh leaves from wild-type and ox-*AtERF14* plants. The number of palisade mesophyll cells per millimeter of section was averaged from 10 replicate regions across two leaves.

Arabidopsis lines SALK_140578 and SALK_118494 (Alonso et al., 2003), named Δ*aterf14-1* and Δ*aterf14-2*, respectively, containing T-DNA insertion in the *AtERF14* gene, were obtained from the ABRC and a nonsegregating homozygous T4 line selected for further analysis. PCR on genomic DNA with left border and specific primers (primer sequences are presented in Supplemental Table S1) and sequencing confirmed the position of the T-DNA insertions within the *AtERF14* gene.

The experiments to study the effect of ET, MeJA, and SA on gene expression in the Δ*aterf14* lines and wild-type Col were conducted in a randomized split plot design. Seeds were transferred to soil for 2 weeks prior to ET treatment. Plants were sealed in an airtight clear plastic container and treated with 200 ppm ET in air or 0.025 μL MeJA per L air. Control plants were sealed in separate containers without ET or MeJA. Plants for SA treatment were sprayed until run off with 1 mM SA sodium salt; control plants were sprayed with water only. After 24 h of treatment, plants were removed from the containers and leaves harvested and frozen in liquid nitrogen for RNA extraction. RNA was extracted from two to five biological replicates of nine to 20 plants each.

Gene Expression Analysis

RNA isolation and cDNA synthesis were performed using the Purescript RNA isolation kit (Gentra Systems) according to Oñate-Sánchez and Singh (2002). RNA was reverse transcribed using MLV (Promega) and the equivalent of 16 ng was used for qRTPCR. qRTPCR was performed using a MyCycler (Bio-Rad). Reactions were setup according to Klok et al. (2002) with the following thermal profile: 95°C for 2.5 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, followed by a melt curve program of 70°C to 95°C with 0.5°C increase per cycle. To compare data from different PCR runs or cDNA samples, C_T values for all selected genes were normalized to the C_T value of a tubulin gene (At5G23860) whose expression remained constant among various treatments and tissues (data not shown). Relative gene expression was derived

from using $2^{-\Delta CT}$, where ΔC_T represents C_T of the gene of interest minus C_T of tubulin. Primer sequences are presented in Supplemental Table S1.

Assessment of Susceptibility of Arabidopsis Lines to Pathogens

Rhizoctonia solani strains ZG3 and ZG5 were grown in potato dextrose broth (Booth, 1977). Inoculation of plants was done by transplanting 1-week-old soil-grown seedlings into 5- × 5- × 6-cm pots containing vermiculite inoculated with 5 mL of inoculum containing the equivalent of 2.2 mg of dry homogenized mycelium per mL or 5 mL of sterile water for controls. Five plants were sown per pot with four replicate pots in a randomized split plot design, making a total of 20 plants for each treatment-genotype combination. Plants were grown at 24°C with constant 100% soil moisture and 16 h light per day for 3 weeks. After 3 weeks, root dry weight was measured to give an indication of the extent of root rot.

Fusarium oxysporum was obtained from Dr. Kemal Kazan (CSIRO Plant Industry), and inoculations were performed according to Anderson et al. (2004) with the following exceptions. Plants were grown under 16 h light per day with five plants per 5- × 5- × 6-cm pot per genotype with three replicate pots per treatment. Following inoculation with *F. oxysporum*, plants were incubated at 28°C under clear plastic mini-glasshouses to maintain high humidity. Plants were harvested 10 d after inoculation, and percentage of plants surviving per pot and dry weights were measured.

Supplemental Data

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

Supplemental Table S1. Primers used for qRTPCR and sequence analysis.

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