The B-3 Ethylene Response Factor MtERF1-1 Mediates Resistance to a Subset of Root Pathogens in *Medicago truncatula* without Adversely Affecting Symbiosis with Rhizobia[1][W][OA]

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The fungal necrotrophic pathogen *Rhizoctonia solani* is a significant constraint to a range of crops as diverse as cereals, canola, and legumes. Despite wide-ranging germplasm screens in many of these crops, no strong genetic resistance has been identified, suggesting that alternative strategies to improve resistance are required. In this study, we characterize moderate resistance to *R. solani* anastomosis group 8 identified in *Medicago truncatula*. The activity of the ethylene- and jasmonate-responsive GCC box promoter element was associated with moderate resistance, as was the induction of the B-3 subgroup of ethylene response transcription factors (ERFs). Genes of the B-1 subgroup showed no significant response to *R. solani* infection. Overexpression of a B-3 ERF, *MtERF1-1*, in *Medicago* roots increased resistance to *R. solani* as well as an oomycete root pathogen, *Phytophthora medicaginis*, but not root knot nematode. These results indicate that targeting specific regulators of ethylene defense may enhance resistance to an important subset of root pathogens. We also demonstrate that overexpression of *MtERF1-1* enhances disease resistance without apparent impact on nodulation in the A17 background, while overexpression in *sickle* reduced the hypernodulation phenotype. This suggests that under normal regulation of nodulation, enhanced resistance to root diseases can be uncoupled from symbiotic plant-microbe interactions in the same tissue and that ethylene/ERF regulation of nodule number is distinct from the defenses regulated by B-3 ERFs. Furthermore, unlike the stunted phenotype previously described for Arabidopsis (*Arabidopsis thaliana*) ubiquitously overexpressing B-3 ERFs, overexpression of *MtERF1-1* in *M. truncatula* roots did not show adverse effects on plant development.

Plant diseases cause major losses to agriculture worldwide (Anderson et al., 2004b). For the most part, strategies to reduce the impacts of disease involve the introduction of single resistance genes into cultivars to provide a strong resistance. For some intractable diseases, genes conferring strong resistance have not been identified, despite in-depth searching of germplasm. Furthermore, some pathogens, especially those that are soil borne, are not well controlled by fungicides. For these pathogens, alternative approaches to increase plant resistance, based on a thorough knowledge of plant defense responses, are necessary.

The fungal necrotroph *Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris*) is one such pathogen for which genes conferring a strong resistance have not been found. The fungus has a worldwide distribution and is considered a very destructive plant pathogen, with individual isolates infecting a broad range of hosts (Sweetingham and MacNish, 1994; González García et al., 2006). In particular, *R. solani* causes serious losses to cereals such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*; cereal bare patch), canola (*Brassica napus*), beet (*Beta vulgaris*), cotton (*Gossypium hirsutum*), lettuce (*Lactuca sativa*), melon (*Cucumis melo*), and grain and pasture legumes (seedling damping off, root and hypocotyl rots) as well as causing diseases on ornamental and forestry species (Anderson, 1982; Sneh et al., 1991; Sweetingham and MacNish, 1994; González García et al., 2006). *R. solani* typically causes seedling damping off of young plants and root and hypocotyl rot of mature plants, with the largest impact observed on young plants (Sweetingham and MacNish, 1994).

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The species complex that is referred to as *R. solani* has a large variability in morphology and pathology and is subdivided based on reproductively isolated anastomosis groups (AGs; Anderson, 1982). Typically, *R. solani* infects roots and stems by direct penetration of the host through the formation of multilayered infection cushions or simple appressoria. Colonization of the host is commonly characterized by cell wall degradation, plasmolysis, and cell death in advance of the hyphae, with the eventual collapse of roots and stems (for review, see Keijer, 1996). Keijer (1996) also proposes that this nonspecific cell damage preceding hyphal colonization may be one of the reasons why host resistance is typically only observed as an incremental reduction in disease severity. Increased host resistance has previously been associated with increased production of inhibitors of *R. solani* pectin lyase, increased cuticle thickness, and the accumulation of phytoalexins (for review, see Keijer, 1996), although the molecular responses of host cells have been largely unexplored.

Most pathogens are host specific and only infect one host or at worst a range of closely related species. As such, the focus of studies on host responses has overwhelmingly considered monospecific interactions. However, pathogen-host interactions including a broad host range pathogen such as *R. solani* may involve different mechanisms of pathogenesis and host responses. One advantage of studying *R. solani* over narrow host range pathogens is that pathosystems can be established between one isolate and numerous different hosts to identify common or unique defense strategies. The *R. solani* AG8 strain ZG1-1 causes bare patch in wheat and barley, crown and root rot of various legumes, and root rot on canola (Sweetingham and MacNish, 1994).

The model legume and important pasture crop in Australia, *Medicago truncatula* (barrel medic), has proven to be a valuable host for investigations of plant defense responses (Klingler et al., 2005; Colditz et al., 2007; Foster-Hartnett et al., 2007; Gao et al., 2007; Naoumkina et al., 2008; Pilet-Nayel et al., 2009). In this study, we investigate the molecular responses of *M. truncatula* to infection by *R. solani* AG8. As is the case for many other crops, useful resistance to *R. solani* in *M. truncatula* breeding populations has not yet been found, despite substantial screening efforts (Barbetti et al., 2006). However, we previously identified moderate resistance in A17 (the reference genotype for genome sequencing and the development of genomics tools; Kakar et al., 2008) and strong susceptibility in an ethylene-insensitive mutant of A17, *sickle* (*skl*; Penmetsa and Cook, 1997; Penmetsa et al., 2008). Here, we used promoter studies and gene expression profiling to further characterize the moderate resistance in A17. We show an early induction of the ethylene/jasmonate signaling pathway and a predominant regulation of genes encoding ethylene response factors (ERFs) of the B-3 subgroup. Furthermore, overexpression of one of the B-3 ERFs increased resistance to *R. solani* and an oomycete pathogen, *Phytophthora medicaginis*. While overexpression of *ERF* genes in other systems has led to adverse developmental effects (Solano et al., 1998; Oñate-Sánchez et al., 2007), overexpression of the *MtERF* in roots of composite plants (plants with wild-type shoots and transgenic roots) had no such effects on plant development. Root nodulation with *Sinorhizobium meliloti* was not affected in A17 roots overexpressing *MtERF1-1*. Taken together, these results suggest that appropriate overactivation of components of a moderate resistance response may be a useful strategy to increase resistance to pathogens for which no strong natural resistance has been found.

RESULTS

Involvement of Ethylene in Moderate Resistance to *R. solani*

Previously, it has been reported that the ethylene-insensitive mutant *skl* displays strong susceptibility to *R. solani* AG8 compared with the isogenic wild-type A17 (Penmetsa et al., 2008). To further explore the role of ethylene in resistance to *R. solani*, we treated A17 seedlings with ethylene 24 h prior to inoculation and scored plant survival through the seedling damping off stage of the disease at 1 week and longer term plant performance at 3 weeks after inoculation. Exogenous ethylene treatment reduced the impacts of seedling damping off compared with mock-treated plants at 1 week after inoculation (Fig. 1A). The effects of ethylene treatment at 3 weeks after treatment/inoculation were reduced. The number of healthy leaves at 3 weeks after inoculation did not differ significantly between mock- and ethylene-treated plants following *R. solani* inoculation (Fig. 1B). However, a trend toward a higher number of healthy leaves may be observed due to the absence of a significant difference between ethylene-treated *R. solani*-infected plants and their respective ethylene-treated noninoculated controls. In comparison, non-ethylene-treated plants showed a significant impact of *R. solani* inoculation.

R. solani inoculation showed no significant impact on shoot dry weight regardless of exogenous ethylene treatment (Fig. 1C). Although the root dry weight of *R. solani*-inoculated plants did not differ between mock- and ethylene-treated plants, *R. solani*-inoculated ethylene-treated plants did differ from their respective ethylene-treated noninoculated control (Fig. 1D). These findings suggest that overactivation of the ethylene pathway can quantitatively increase resistance to *R. solani* in terms of survival through the seedling damping off stage of the disease, which occurs immediately after the 24-h ethylene treatment; however, extended effects of ethylene treatment on healthy leaves and plant weight at time points more distant from the ethylene treatment are diminished. These findings suggest that manipulation of ethylene-mediated defenses may be a useful strategy to reduce the impacts of this recalcitrant disease.

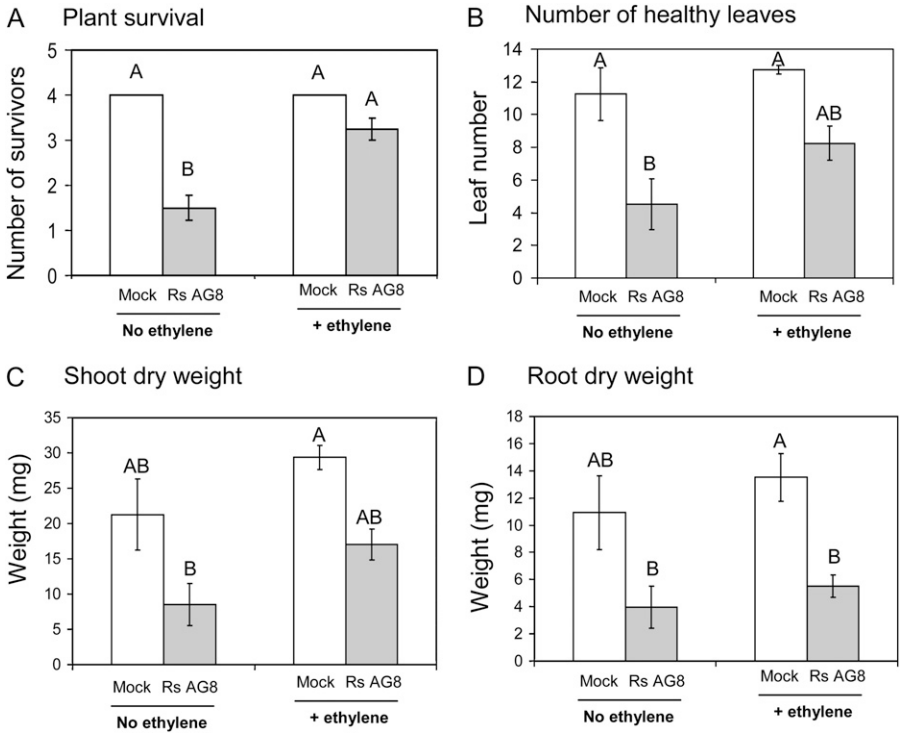

Figure 1. Effect of exogenous ethylene treatment of A17 on susceptibility to *R. solani*. A, The average number of survivors (out of four seedlings planted per pot) 1 week after inoculation. B, The number of healthy leaves at 3 weeks after inoculation. C and D, Shoot dry weight (C) and root dry weight (D) 3 weeks after inoculation. Columns present group means, and the vertical lines represent their respective SE values. For each graph, columns joined by the same letter are not significantly different according to the Tukey-Kramer HSD ($P < 0.05$). The experiment consisted of four complete blocks with four plants per pot, making a total of 16 plants per treatment.

Activity of a Defense-Responsive Promoter following *R. solani* Inoculation

To further explore the involvement of defense signaling pathways in response to *R. solani*, a promoter reporter construct was made by fusing four copies of the ethylene- and jasmonate-responsive GCC box promoter element (Shinshi et al., 1995) to a minimal promoter and the luciferase reporter gene. The construct was introduced into roots of *Medicago* genotypes A17 and *skl* via "hairy-root" transformation, and the composite plants were challenged with the fungus. The GCC promoter element was induced in a biphasic pattern following inoculation in A17, while in *skl* luciferase expression driven by the GCC promoter remained at basal levels (Fig. 2). Independent follow-up experiments confirmed these results.

Expression of Defense-Related and Transcription Factor Genes in Response to *R. solani*

The expression of several defense-related genes was analyzed in root tissue samples from a time course inoculation experiment involving A17 and *skl*. The typically salicylate-responsive *Pathogenesis related10* (*PR10*; Gao et al., 2007) showed a significant induction in *skl* but not A17 following *R. solani* AG8 infection, whereas *PR1* showed no significant changes in either genotype (Fig. 3, A and B). The expression of *PR10* was found to be responsive to exogenous ethylene, whereas *PR1* was not (Supplemental Table S2). The salicylate- and ethylene-responsive β-1,3-glucanase *BGL2* (Supplemental Table S2; Gao et al., 2007) was significantly induced in A17 but not in *skl*, suggesting a reliance on ethylene signaling for pathogen induction of this gene (Fig. 3C). The gene encoding 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase, an enzyme involved in ethylene synthesis, showed significant induction only in *skl* at 24 h after inoculation with *R. solani* AG8 (Fig. 3D). The ethylene-associated hevein-like gene, *HEL1*, showed a similar induction to *BGL2* in that it was only induced in A17 at 12 h after inoculation and was induced following application of

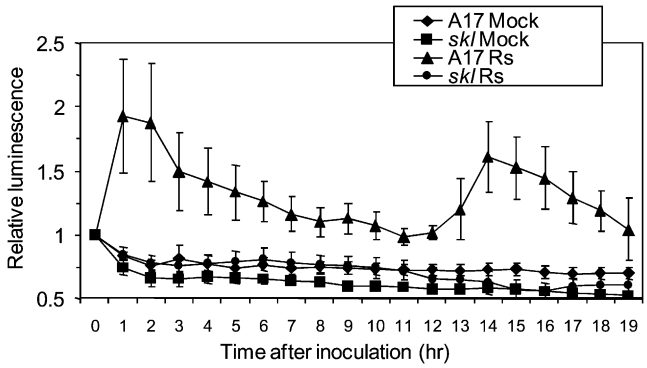

Figure 2. The response of a GCC box promoter element tetramer (ethylene and jasmonate responsive) to inoculation with *R. solani* AG8. Transgenic hairy roots containing a tetramer of the GCC box fused to the luciferase reporter gene were generated for A17 and *skl* and challenged with *R. solani*. Relative luminescence is the light emitted from the roots relative to that at time zero before inoculation. Averages and SE of four replicate plants are shown.

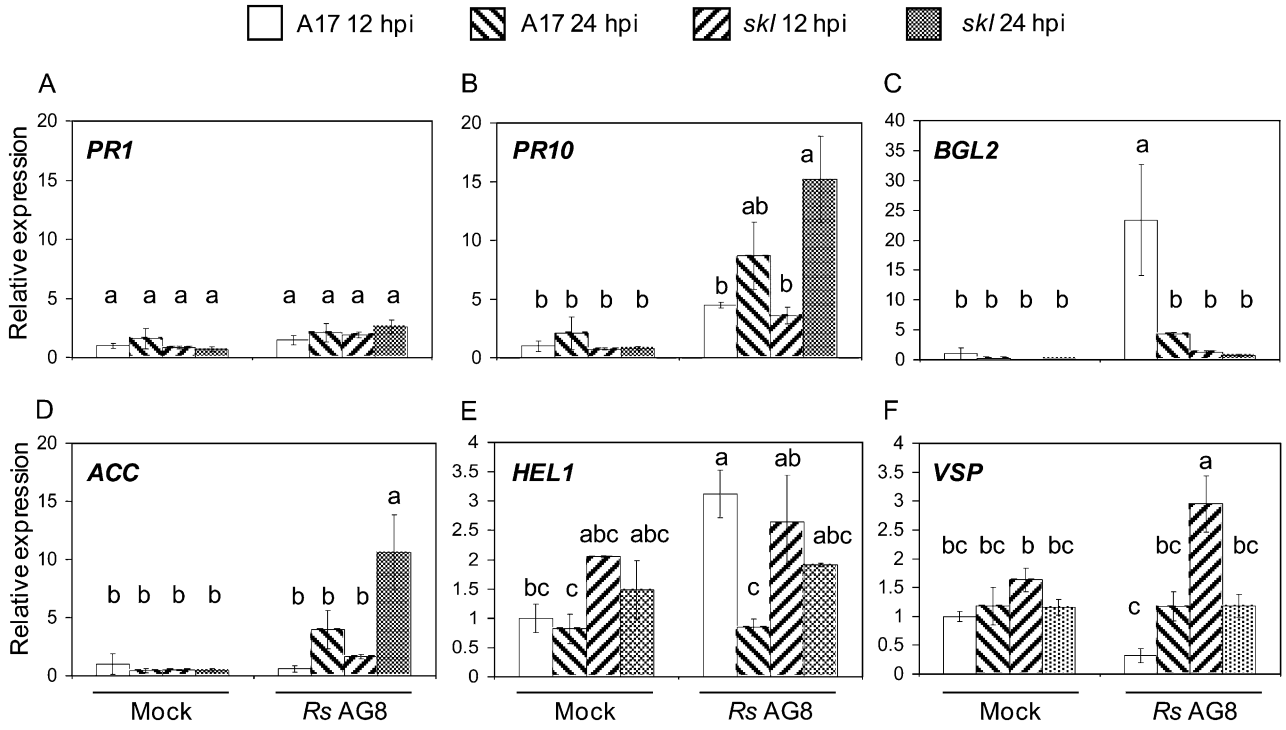

Figure 3. Expression of defense-related genes in response to inoculation with *R. solani* AG8 at 12 and 24 h post inoculation. A, Salicylate-associated gene *PR-1*. B and C, Salicylate- and ethylene-responsive genes *PR10* (B) and *BGL* (C). D and E, Ethylene-responsive genes *ACC oxidase* (D) and *Hel1* (E). F, Jasmonate-responsive gene *VSP*. The expression data are shown relative to Mock-A17-12 h. Columns represent group means, and the vertical lines represent their respective SE values. Comparison of means was done using the Tukey-Kramer HSD test; columns connected with the same letter are not significantly different ($P < 0.05$). Results of two-way ANOVA are presented in Supplemental Table S1.

exogenous ethylene (Fig. 3E; Supplemental Table S2), suggesting that its pathogen-induced expression may also be dependent on ethylene signaling. However, *VSP*, which is only induced by jasmonate (Gao et al., 2007) but not ethylene (Supplemental Table S1), was not significantly regulated by *R. solani* inoculation in A17. *VSP* was significantly regulated in *skl* following *R. solani* inoculation, indicating that the jasmonate pathway may be playing a more substantial role in regulating defense gene expression in the absence of ethylene signaling.

Given the importance of the ethylene pathway in moderate resistance to *R. solani* AG8 (Fig. 1), the up-regulation of the GCC box in moderately resistant A17 (Fig. 2), and a potential role for ethylene in defense gene regulation following *R. solani* infection, we tested the expression of members of the ERF family. *Medicago ERF* genes were identified through tBLASTX searching of the *M. truncatula* EST database (http://compbio.dfci.harvard.edu/tgi/plant.html) using the amino acid sequences of selected pathogen-responsive Arabidopsis (*Arabidopsis thaliana*) *ERF* genes. ClustalW analysis of the DNA-binding domain sequences of identified ERFs from *Medicago* and Arabidopsis was performed to confirm the classification of the *Medicago* ERFs into the subgroups proposed by Feng et al. (2005), also based on the protein sequence of the DNA-binding domains (Fig. 4). Members of subgroups B-3 and B-1 have previously been associated with the response to pathogens (Zhou et al., 1997; Oñate-Sánchez and Singh, 2002; Berrocal-Lobo and Molina, 2004; McGrath et al., 2005; Oñate-Sánchez et al., 2007); thus, members of these groups were chosen as targets for gene expression analysis following *R. solani* AG8 inoculation in *Medicago* (Fig. 5).

Significant changes were observed in the expression of all B-3 genes analyzed following *R. solani* inoculation of A17 (Fig. 5, A–F; Supplemental Table S1). *MtERF1-1*, *MtERF1-2*, *MtERF5-2*, *MtERF1-3*, and *MtERF1-4* showed significant up-regulation in response to inoculation, with the highest expression observed at the 12-h post inoculation time point (Fig. 5, A–E). Inoculation of *skl* also led to significant up-regulation of *MtERF1-3* and *MtERF1-4* compared with their respective mock-treated controls. Interestingly, the B-3 gene *MtERF5-1* was down-regulated in A17 and *skl* at 12 and 24 h post inoculation, respectively (Fig. 5F). In contrast, the B-1 genes *MtERF4-1*, *MtERF4-2*, and *MtERF7* did not show significant changes following *R. solani* challenge (Fig. 5, G–I; Supplemental Table S1). The B-3 genes *MtERF1-1*, *MtERF1-3*, *MtERF5-1*, and *MtERF5-2* were significantly induced

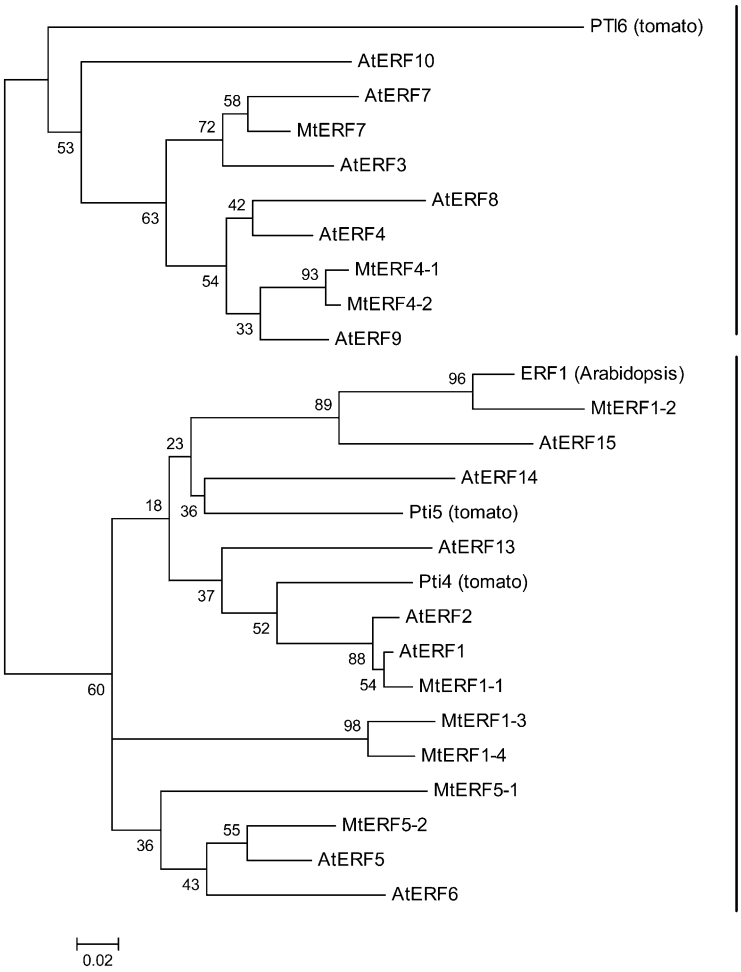

Figure 4. Phylogenetic tree of selected Arabidopsis and *Medicago ERF* genes of subgroups B-1 and B-3.

following exogenous ethylene application, as were the B-1 genes *MtERF4-1* and *MtERF7* (Supplemental Table S2).

Overexpression of an ERF of Subgroup B-3 Provides a Quantitative Increase in Resistance to *R. solani* AG8

The coding regions of the subgroup B-3 gene *MtERF1-1* (TC144328) and the subgroup B-1 gene *MtERF4-1* (TC161156) were fused to the cauliflower mosaic virus 35S promoter in overexpression constructs and introduced to A17 and *skl* roots (Fig. 6; Supplemental Fig. S1). Genomic DNA was extracted from root systems of composite plants and the presence of the constructs confirmed by PCR (data not shown). Composite plants with transgenic roots were challenged with *R. solani* AG8 in plate assays. The use of mature plants in this experiment, due to the requirement to produce transgenic hairy roots and hence the absence of the seedling damping off aspect of the disease, resulted in reduced severity of disease symptoms following *R. solani* inoculation compared with the seedlings used in the ethylene treatment experiments in Figure 1. A17 plants with roots overexpressing *MtERF1-1* showed an increased resistance to *R. solani*, as expressed by a higher number of healthy leaves (not significantly different from mock-inoculated controls according to a Tukey-Kramer honestly significant difference [HSD] test) and a reduced lesion length when compared with roots overexpressing *GFP* (Fig. 6, A and B) or the wild type (Supplemental Fig. S1). *R. solani* infection had no significant effect on the root and shoot weights of these more mature plants (Fig. 6, C and D). Quantification of relative *R. solani* biomass (as the relative abundance of fungal DNA to plant DNA measured by quantitative PCR) in the roots of these plants confirmed a reduction in fungal colonization in roots overexpressing *MtERF1-1* (Fig. 6E), suggesting that the plants were able to suppress pathogen growth. Assessment of *MtERF1-1* expression levels in *Ox-MtERF1-1* and *Ox-GFP* (control) roots confirmed overexpression of the B-3 ERF. Similar experiments testing the overexpression of *MtERF1-1* and the B-1 subgroup ERF, *MtERF4-1*, in A17 and *skl* (Supplemental Fig. S1) demonstrated that overexpression of *MtERF1-1* in *skl* was able to reduce the impact of *R. solani* infection on the number of healthy leaves (no significant difference [$P < 0.05$] from the respective mock-inoculated controls according to a *t* test) but did not significantly reduce lesion length. *R. solani* infec-

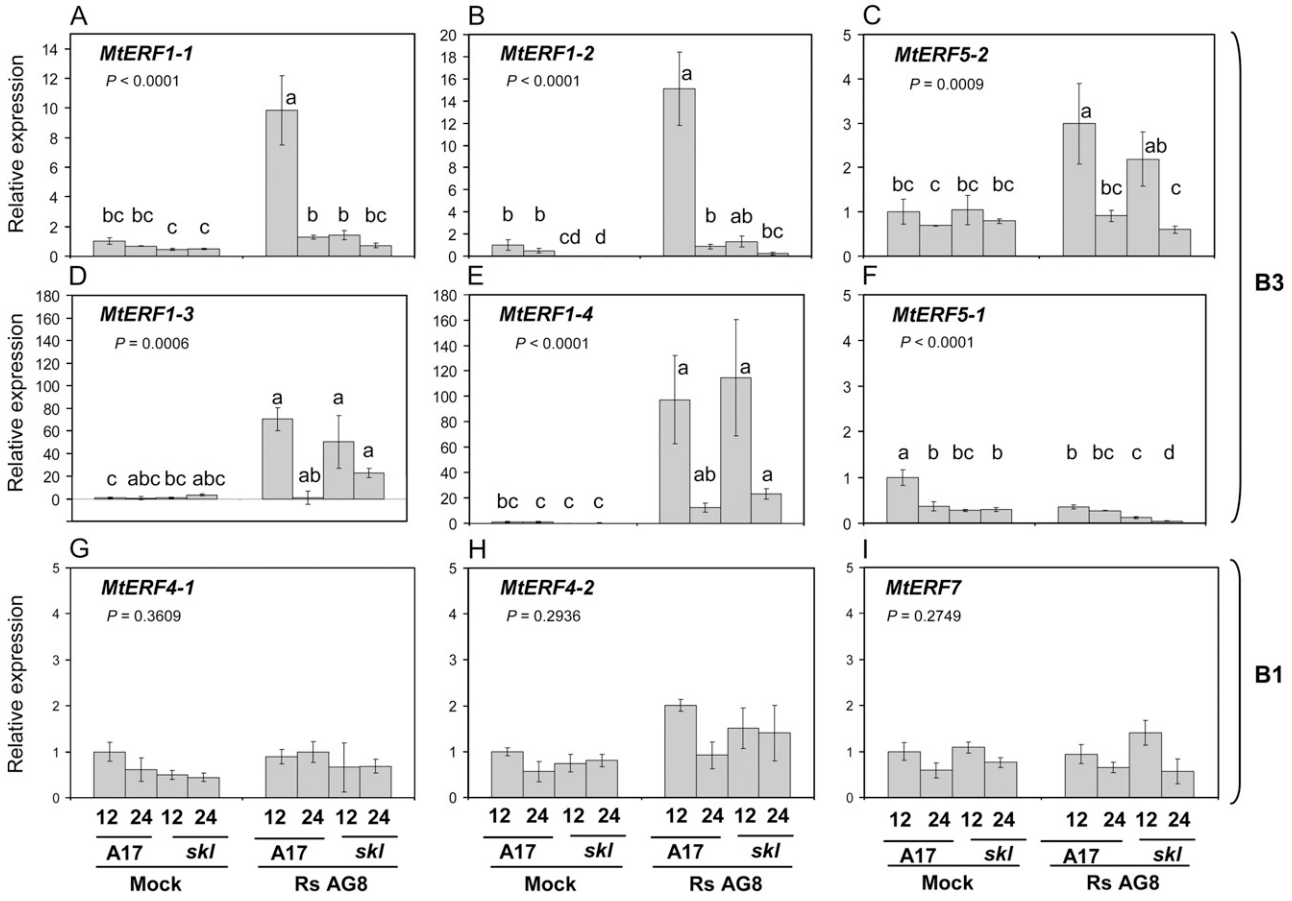

Figure 5. Expression of selected *Medicago ERF* genes in response to *R. solani* inoculation. B-3 subgroup *ERF* genes (A–F) and subgroup B-1 *ERF* genes (G–I) are represented at 12 and 24 h after inoculation with *R. solani* AG8. The expression data are shown relative to Mock-A17-12 h. All statistical analyses were done using the log-transformed values of the relative expression data. *P* values indicate significance levels of the linear statistical model used to evaluate the effect of the treatments (inoculation, genotype, and time; Supplemental Table S1). Columns represent group means, and the vertical lines represent their respective SE values. Comparison of means was done exclusively in cases where the linear model significantly fitted the data ($P < 0.05$; A–F) using the Tukey-Kramer HSD test; means connected with the same letter are not significantly different ($P < 0.05$).

tion of A17 with roots overexpressing *MtERF4-1* resulted in similar susceptibility to the wild type, with fewer healthy leaves compared with mock-inoculated controls and an average lesion length not significantly different from the wild type (Supplemental Fig. S1). Infection of *skl* overexpressing *MtERF4-1*, however, resulted in a reduced impact on healthy leaves and shorter lesion length, indicating that *MtERF4-1* had a greater effect when overexpressed in the *skl* background compared with overexpression in A17.

Overexpression of *MtERF1-1* Quantitatively Increases Resistance to *P. medicaginis* But Not Root Knot Nematode

It has previously been reported that *skl* displays higher susceptibility to *P. medicaginis* than A17 (Penmetsa et al., 2008). To test the ability of *MtERF1-1* to increase resistance to *P. medicaginis*, composite plants with roots overexpressing *MtERF1-1* or *GFP* (as a negative control) were inoculated with motile zoospores. Plants with roots overexpressing *GFP* showed significant reduction of healthy leaves and eventually death in response to inoculation. Similar to *R. solani* AG8 inoculation, overexpression of *MtERF1-1* resulted in increased resistance, with the number of healthy leaves in inoculated and mock-inoculated treatments showing no significant difference (Fig. 7A), while a significant reduction in pathogen biomass (as the relative abundance of *P. medicaginis* DNA to plant DNA measured by quantitative PCR) was observed in *Ox-MtERF1-1* roots (Fig. 7B). Follow-up experiments showed similar results (data not shown). To determine if the broad spectrum of enhanced resistance conferred by overexpression of *MtERF1-1* extended to biotrophic invertebrate root pathogens, transgenic hairy roots were challenged with the root knot nematode, *Meloidogyne javonica*. No significant differences were observed between roots overexpressing *MtERF1-1* or

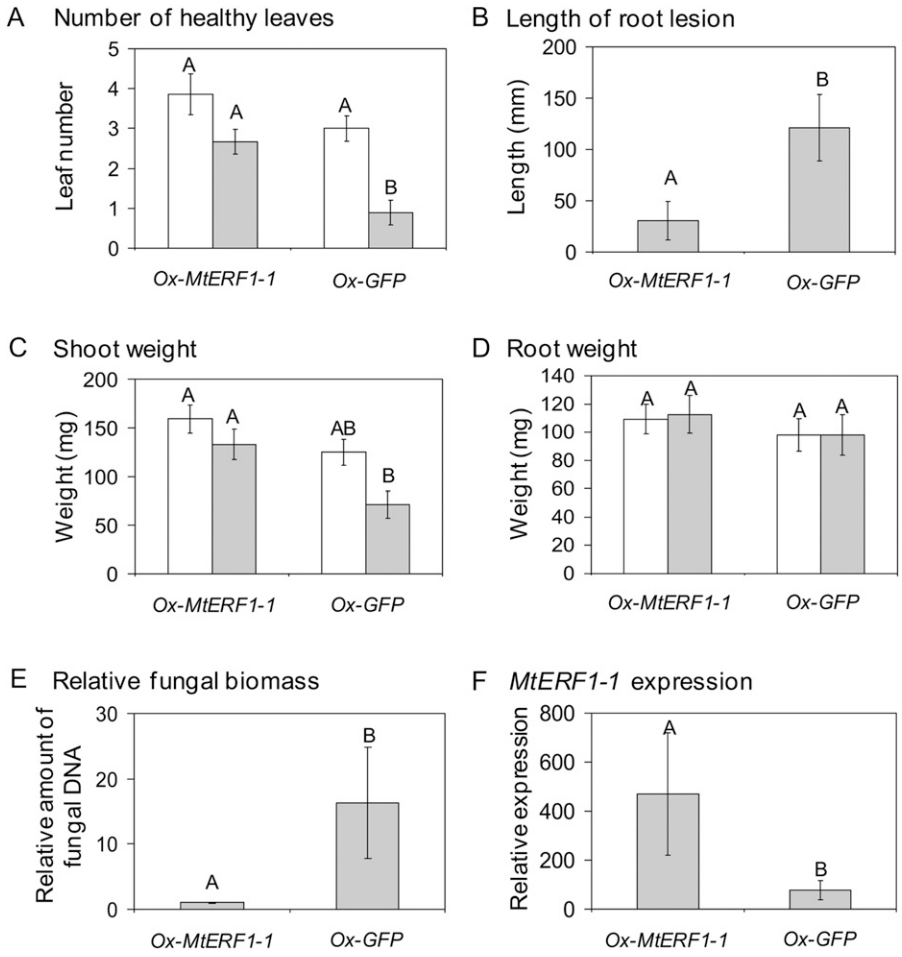

Figure 6. Overexpression of *MtERF1-1* in roots of composite plants inoculated with *R. solani* AG8. A, The number of healthy leaves per plant. B, Length of root lesion per plant. C and D, Shoot (C) and root (D) weights per plant. E, Relative fungal biomass as the relative abundance of *P. medicaginis* DNA to plant DNA measured by quantitative PCR. F, Relative expression of *MtERF1-1* in roots. *Ox-MtERF1-1*, Composite plants with transgenic roots overexpressing *MtERF1-1*; *Ox-GFP*, composite plants with transgenic roots overexpressing *GFP* as a control. White bars indicate noninoculated controls, and gray bars indicate plants inoculated with *R. solani* AG8. In each graph, columns not connected by the same letter are significantly different according to the Tukey-Kramer HSD test for multiple comparisons (A, C, and D) or a *t* test for pair comparisons (B, E, and F). $n \geq 6$.

GFP in the number of galls (Fig. 7C), suggesting that MtERF1-1-independent defense mechanisms are required to confer resistance to this pathogen.

Overexpression of *MtERF1-1* in Transgenic Roots Does Not Affect Plant Growth or Development

Severe stunting phenotypes have been reported for plants overexpressing some *ERF* genes (Solano et al., 1998; Oñate-Sánchez et al., 2007). However, it was noted that plants with roots transformed with the *MtERF1-1* overexpression construct did not show altered growth and development when compared with *Ox-GFP* or wild-type plants. Composite plants were grown to maturity, and the number of healthy leaves on *Ox-MtERF1-1* and *Ox-GFP* plants confirmed that the growth of the plants was not significantly affected by overexpression of *MtERF1-1* (Fig. 8, A and B).

Ethylene and specific ERFs have been linked to the regulation of nodulation in *Medicago* (Penmetsa and Cook, 1997; Oldroyd et al., 2001; Middleton et al., 2007; Penmetsa et al., 2008). Therefore, it was of interest to see if overexpression of *MtERF1-1* affected nodule development. Inoculation of composite plants with a *GFP*-expressing isolate of *S. meliloti* 1021 revealed that nodulation of the composite plants with *MtERF1-1*-overexpressing roots occurred at frequencies similar to the *Ox-GFP* plants and contained *GFP*-expressing bacteria (Fig. 8, C and D). To determine if overexpression of *MtERF1-1* in the roots of the *skl* mutant could complement the hypernodulation phenotype, we inoculated A17 and *skl* overexpressing either *GFP* or *MtERF1-1* with the *GFP*-expressing *S. meliloti*. The *Ox-GFP skl* plants showed the typical *skl* hypernodulation phenotype of large numbers of coalescing nodules; however, overexpression of *MtERF1-1* in *skl* led to clearly distinguishable individual nodules (Supplemental Fig. S2).

DISCUSSION

Necrotrophic fungal pathogens are a major constraint to agricultural production worldwide. One of the diseases causing substantial losses is bare patch or root/hypocotyl rot caused by *R. solani*. For example, Tachibana et al. (1971) determined that for soybean (*Glycine max*), *R. solani* could reduce yield by as much as 48%, and *R. solani* sheath blight is a major constraint to rice (*Oryza sativa*) production worldwide (Venu

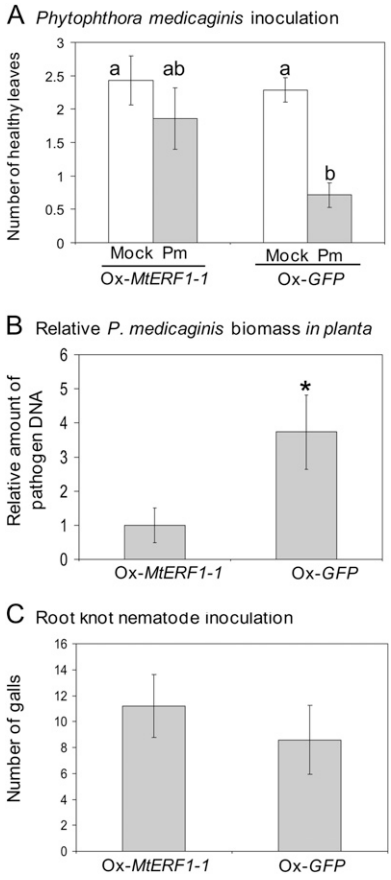

Figure 7. Performance of composite plants inoculated with other root pathogens. A, The number of healthy leaves on composite plants with and without inoculation with the oomycete *P. medicaginis* (Pm; $n = 7$). Columns not connected with the same letter are significantly different according the Tukey-Kramer HSD test ($P < 0.05$). B, Relative *P. medicaginis* biomass in planta as the relative abundance of *P. medicaginis* DNA to plant DNA measured by quantitative PCR. The asterisk indicates that the relative amount of pathogen DNA within *Ox-GFP* roots is significantly greater than that in *Ox-MtERF1-1* roots ($P < 0.05$, as indicated by *t* test). C, The number of galls caused by the root knot nematode ($n \geq 12$).

et al., 2007). Traditional methods of disease control such as crop rotation are ineffective against some races of *R. solani* due to the polyphagous habit of the pathogen, allowing disease development at all phases of the rotation. This is confounded by an absence of strong genetic resistance in most crop species and the inefficacy of fungicides.

The identification of moderate resistance in A17 and strong susceptibility in *skl* to *R. solani* AG8 (Penmetsa et al., 2008) opens the way to study mechanisms of resistance and susceptibility in a tractable model host species. The increased susceptibility in *skl* suggests that the ethylene pathway is important in conferring moderate resistance. These findings in *M. truncatula* are consistent with the findings of Hoffman et al. (1999), who observed enhanced susceptibility to another *R. solani* strain (2B-12) in transgenic ethylene-insensitive soybean. The ability of this pathway to protect plants from *R. solani* was also evident from the reduced seedling damping off of *M. truncatula* A17 when treated with exogenous ethylene (Fig. 1A), suggesting that overactivation of this pathway may be a viable option for increasing resistance to this pathogen, for which no strong natural resistance has been identified.

The activation of defense responses by ethylene has previously been associated with the activity of transcription factors binding the GCC box in defense promoters, resulting in the induction of antimicrobial genes such as *PDF1.2* and chitinases in Arabidopsis (Ohme-Takagi and Shinshi, 1995; Fujimoto et al., 2000; Brown et al., 2003). When transgenic hairy roots containing the 4×GCC::*luciferase* construct were challenged with *R. solani*, an early induction of the reporter gene suggested that an ethylene-mediated response was induced at the initial stages of infection, while an additional induction peaking at 14 h suggested a secondary phase of activity of the GCC element. The lack of response in roots of *skl* suggests that either (1) a functional ethylene signaling pathway is required for *R. solani*-induced gene expression from the GCC box promoter element in *M. truncatula*, even though jasmonate is also able to activate this pathway in wild-type plants, or (2) the jasmonate pathway is not involved in the response to *R. solani* infection. Consistent with the former explanation, in Arabidopsis both the ethylene and jasmonate pathways must be functional for *Alternaria brassicicola* induction of *PDF1.2* gene expression (Penninckx et al., 1998).

The molecular pathways associated with the response to *R. solani* were further explored by studying the expression of several defense-related genes typically associated with the three common defense signaling pathways: salicylate, jasmonate, and ethylene. Of the typically salicylate-related defense genes, *PR10* showed increased expression only in *skl* following *R. solani* inoculation, while *PR1* did not show significant regulation in either A17 or *skl* at either time point, suggesting that salicylate-mediated gene expression may not be playing a central role in the moderate resistance to *R. solani* observed in A17. Interestingly, enhanced expression of *PR10* has previously been correlated with increased susceptibility of *M. truncatula* to another necrotrophic root rot pathogen, *Aphanomycetes euteiches* (Colditz et al., 2007). The jasmonate-inducible but not ethylene-inducible *VSP* (Gao et al., 2007) was not significantly regulated in A17 by *R. solani* infection. Interestingly, *VSP* was significantly down-regulated by exogenous ethylene and showed a trend toward reduced expression in A17 at 12 h after *R. solani* inoculation, the same time when the ethylene-inducible genes *BGL2* and *HEL1* were up-regulated. The up-regulation of the ethylene- and salicylate-responsive gene *BGL2* and the ethylene responsive *HEL1* only in A17 is consistent with ethylene mediating the *R. solani*-responsive expression of these genes. This expression

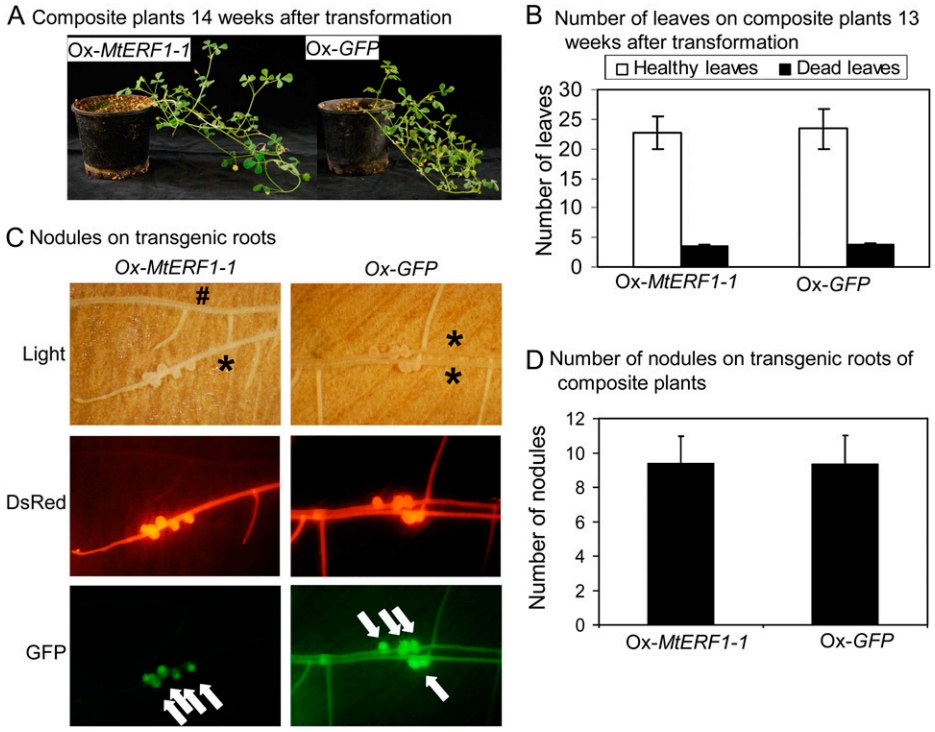

Figure 8. Performance of A17 composite plants overexpressing *MtERF1-1* and *GFP*. A, Composite plants 14 weeks after transformation. B, Number of leaves (healthy and dead) on composite plants 13 weeks after transformation. Means show no significant difference ($P > 0.05$) between *Ox-GFP* and *Ox-MtERF1-1* according to *t* test ($n = 16$). C, Fluorescence microscopy of transgenic roots inoculated with *GFP* expressing *S. meliloti*. *M. truncatula* overexpression constructs contain *DsRed* as a visible marker. The hash mark indicates nontransgenic root, asterisks indicate transgenic roots, and arrows indicate the location of root nodules containing *GFP* expressing *S. meliloti*. D, Number of nodules on transgenic roots of composite plants. A minimum of 19 composite plants were scored.

pattern was the opposite of the gene encoding the ethylene synthesis enzyme ACC oxidase, which was only significantly increased in *skl* following *R. solani* AG8 inoculation at the time points studied. ACC is involved in the production of ethylene, and this is expected to be active in both A17 and *skl*, since the *skl* mutation affects signal transduction downstream of ethylene perception rather than production (Penmetsa et al., 2008). The induction in *skl* may be related to a lack of perception of the ethylene produced and thus a lack of self-regulation of ethylene synthesis in the *skl* mutant background. These findings are consistent with the situation in rice, where silencing *OsEIN2* increased the expression of ACC oxidase and another ethylene synthesis-related gene, *ACC synthase*, and ethylene production increased 3.5-fold over wild-type plants (Jun et al., 2004). Similarly, Guzman and Ecker (1990) demonstrated that blocking the ethylene signaling pathway in Arabidopsis enhanced the rate of ethylene biosynthesis. Together, these results suggest that the salicylate- and jasmonate-mediated signaling pathways may not play a major role in regulating defense gene expression following *R. solani* challenge and further support the association of the ethylene pathway with the moderate resistance in A17 that is absent from *skl*. The importance of ethylene-mediated defenses, often closely associated with jasmonate-mediated defenses, is highlighted by its implication in resistance to a number of other necrotrophic pathogens (for review, see Broekaert et al., 2006). Compared with other pathogens, very little is known about the mechanisms of plant resistance to the important diseases caused by *R. solani*. Moreover, the very limited success of traditional approaches to providing resistance to *R. solani* illustrate the importance of implementing alternative approaches to resistance based on a thorough understanding of plant mechanisms contributing to moderate resistance. Thus, the significant role of ethylene in regulating resistance to *R. solani* warranted further investigation.

Given the involvement of ERFs in the GCC box-mediated regulation of ethylene-responsive defense genes in Arabidopsis (Brown et al., 2003; McGrath et al., 2005; Oñate-Sánchez et al., 2007; Pre et al., 2008), we studied the expression of homologs of GCC box-binding ERFs in A17 and *skl* following *R. solani* AG8 inoculation. We focused our studies on genes of subgroups B-3 and B-1, as these have previously been associated with the response to pathogens (Zhou et al., 1997; Oñate-Sánchez and Singh 2002; Berrocal-Lobo and Molina, 2004; McGrath et al., 2005; Oñate-Sánchez et al., 2007; Pre et al., 2008). The up-regulation of *MtERF1-1*, *MtERF1-2*, and *MtERF5-2* in A17 following *R. solani* AG8 inoculation, coupled with either no response or a substantially reduced response of these genes in *skl* (Fig. 5), suggest that they may be important for the moderate resistance to *R. solani* AG8 present in A17 but absent from *skl*. Two other B-3 ERF genes, *MtERF1-3* and *MtERF1-4*, showed a similar up-regulation in both A17 and *skl*, suggesting that an EIN2-independent mechanism may contribute to the pathogen-mediated regulation of these genes. *MtERF1-4* was not induced by exogenous ethylene (Supplemental Table S2), further supporting the contribution of ethylene-independent regulation of this

gene. The activation of these genes in *skl* following inoculation also suggests that they are not sufficient for the moderate resistance observed in A17. The repression of the B-3 gene *MtERF5-1* and the absence of a response of the B-1 genes *MtERF4-1*, *MtERF4-2*, and *MtERF7* in either A17 or *skl* demonstrate that the up-regulation of the observed genes is not due to a comprehensive up-regulation of the entire *ERF* family or even the B-3 subgroup.

The association of several B-3 subgroup ERFs with resistance to *R. solani* was supported by quantitative increases in resistance in composite A17 and *skl* plants with roots overexpressing one of these genes, *MtERF1-1* (Fig. 6; Supplemental Fig. S1). However, *skl* plants overexpressing *MtERF1-1* did not show a significant reduction in lesion length compared with the wild type. One possible explanation could be that overexpression of *MtERF1-1* in A17 leads to the activation of additional defenses in an EIN2-dependent manner. The mutation of *EIN2* in *skl* would prevent these additional defenses being recruited. In contrast, overexpression of *MtERF4-1* had no effect on the performance of A17 plants with *R. solani* infection (Supplemental Fig. S1), consistent with our observation that *MtERF4-1* RNA levels were not induced by *R. solani*. However, overexpression of *MtERF4-1* in *skl* reduced the impact of *R. solani* on the number of healthy leaves and lesion size. One possibility is that MtERF4-1 may be associated with an ethylene-independent defense response, the activation of which in the absence of a functional ethylene response may impede infection by *R. solani* but may be masked by the functional ethylene response in the A17 background.

Phytophthora root rot is another necrotrophic root disease of importance to the legume industry (Irwin et al., 1995). Penmetsa et al. (2008) reported an increased susceptibility of *skl* mutants to *P. Medicaginis*, suggesting that the ethylene-associated defense pathway is also important for resistance to this pathogen. Given the ability of MtERF1-1 to increase resistance to *R. solani*, where the *skl* mutation increased susceptibility, the ability of MtERF1-1 to alter susceptibility to *P. medicaginis* was also examined (Fig. 7A). The increase in resistance in the A17 composite plants overexpressing *MtERF1-1* to *P. medicaginis* supports the importance of this gene in defense against a diverse range of necrotrophic root-infecting pathogens. *P. medicaginis* and *R. solani* are both filamentous necrotrophic pathogens causing similar root/hypocotyl rot symptoms in mature plants; however, biotrophic root knot nematodes utilize a very distinct mechanism of infection involving the manipulation of the host to form specific feeding sites in the root (Williamson and Gleason, 2003). The absence of enhanced resistance to root knot nematode in *Ox-MtERF1-1* roots suggests a degree of specificity in the defenses mediated by MtERF1-1.

In addition to an involvement in the defense response, both ethylene and ERFs have been linked to the regulation of symbiosis with nitrogen-fixing bacteria (Penmetsa and Cook, 1997; Oldroyd et al., 2001; Middleton et al., 2007; Penmetsa et al., 2008). An example of this is the hypernodulation phenotype of the *skl* mutant (Penmetsa and Cook, 1997; Penmetsa et al., 2008), and an ERF protein, ERN, has been shown to be essential for nodulation (Middleton et al., 2007). Interestingly, overexpression of *MtERF1-1* in the roots of composite A17 plants did not have any obvious effect on the nodulation of these plants, with an equivalent number of bacteria containing nodules produced on *Ox-MtERF1-1* and control A17 roots (Fig. 8, C and D; Supplemental Fig. S1). Furthermore, the fluorescence from the *GFP*-expressing *S. meliloti* strain used was clearly located in the nodules, confirming that the nodules did house the symbiotic bacteria. Overexpression of *MtERF1-1* in *skl*, however, led to individual distinguishable nodules, unlike the *Ox-GFP skl* roots, where large numbers of coalescing nodules were observed (Supplemental Fig. S1). In wild-type plants, a subtle effect on nodule number may be masked by the existing ethylene regulation of nodulation. These results suggest that under normal regulation of nodulation, the overexpression of *MtERF1-1* does not interfere with root symbiosis with rhizobia in a significant manner; however, the role of individual ERFs in defense versus nodulation may not be completely distinct. In addition, our results suggest that deployment of root-specific overexpression of *MtERF1-1* in biotechnological strategies to increase resistance to pathogens in legumes may not be detrimental in terms of plant development or nodulation. This is in contrast to other studies, where constitutive overexpression of *ERF* genes throughout the plant resulted in severe stunting, inhibition of cell enlargement, spontaneous lesion development in the absence of a pathogen, and even loss of seed production (Solano et al., 1998; Gurr and Rushton, 2005; Oñate-Sánchez et al., 2007). One possible explanation for the absence of pleiotropic developmental effects is the root-specific overexpression provided by the hairy root transformation technique. Alternatively, the altered hormone sensitivity in *Agrobacterium rhizogenes*-transformed roots or the formation of additional nontransformed lateral roots in mature plants may be partially countering the detrimental effects in these tissues. Studies are ongoing to further evaluate the efficacy of root-specific expression of *MtERF1-1* and other ERF genes.

CONCLUSION

The overexpression of defense-associated transcription factors such as MtERF1-1, particularly in a root-specific manner, appears to be a viable strategy to increase resistance to a range of pathogens. Taken together, these results further support the value of investigating the molecular responses of moderately or quantitatively resistant hosts in order to design strategies to provide resistance to intractable pathogens where strong natural resistance could not be achieved using conventional methods.

MATERIALS AND METHODS

Plant Material and Growth

Included in this study were *Medicago truncatula* genotype A17, the reference genotype for the development of genomic resources, and mutant line *skl*, an ethylene-insensitive mutant of A17 with a loss-of-function mutation in the homolog of the Arabidopsis (*Arabidopsis thaliana*) *EIN2* gene (Penmetsa et al., 2008). Seeds of A17 and *skl* were scarified, bleached, and germinated in the dark on moist filter paper for 2 d before planting into vermiculite. Plants were grown in growth cabinets with a 16-h/8-h photoperiod at 200 μmol m^{-2} s^{-1} and a constant temperature of 22°C ±2°C.

Ethylene Treatment

Seedlings used to test the effect of exogenous ethylene in pot assays were germinated on moist filter paper until radicles were approximately 10 to 20 mm. Half the seedlings were transferred to moist filter paper in an air-tight container and sealed, and the remaining seedlings were transferred to an air-tight container and ethylene added to 200 μL L^{-1}. After 24 h, the seedlings were removed and planted for pot assays.

Two-week-old plants used for assessment of ethylene-responsive gene expression were transferred to an air-tight container and ethylene added to 200 μL L^{-1}. Mock plants were transferred to an identical air-tight container, and an equivalent volume of air was added. Plants were harvested and immediately frozen in liquid nitrogen 12 h after ethylene treatment.

Infection of *M. truncatula* with *R. solani* in Pot Assays

Cultures of *Rhizoctonia solani* isolate AG8 (ZG1-1; WAC10335) were prepared and stored as described by Lichtenzveig et al. (2006) with a few modifications. Flasks were shaken at 35 rpm at ambient temperature for 7 d prior to filtration and collection of fungal mycelium. Mycelium was homogenized in 100 mL of sterile water, pelleted, and resuspended in sterile water to give an equivalent of 2.22 mg dry weight mL^{-1}. Pots half-filled with a 50:50 mixture of fine and medium vermiculite were wetted with nutrient solution containing 4 mL L^{-1} Nitrosol (Envirogreen). Five milliliters of inoculum or water alone was added to the pots, and the seedlings were added. The seedlings were subsequently covered with additional vermiculite mixture. Each pot contained four seedlings, and at least four replicate pots were planted for each genotype. Vermiculite moisture content was maintained at 100% for the duration of the experiment. After 1 week, seedling survival was scored, and number of healthy leaves (i.e. leaves without symptoms of disease such as chlorosis or necrosis) and the root and shoot weights were assessed 3 weeks after inoculation.

For gene expression experiments, plants were grown in vermiculite prewetted with nutrient solution containing 4 mL L^{-1} Nitrosol for 2 weeks, removed from vermiculite, and immersed in either sterile water for control plants or a *R. solani* AG8 solution (2.22 mg dry weight equivalent mL^{-1} sterile water) for 1 h before being replanted into vermiculite. Root tissue of plants was sampled at 12 and 24 h after inoculation. All samples were frozen in liquid N_2 and stored at −80°C until RNA isolation.

Infection of *M. truncatula* Composite Plants with *R. solani* or *P. medicaginis* in Plate Assays

Composite plants with hairy roots were generated using *Agrobacterium rhizogenes* strain Arqua1 according to Chabaud et al. (2006). Selection of transformed roots was performed based on fluorescence of the *DsRed* reporter gene in the pEarleyGateRedroot plasmid (Penmetsa et al., 2008; Figs. 6 and 7) or resistance to kanamycin from the pBIN19-based plasmid, pCH184 (Supplemental Fig. S1). PCR using primers that bind to the cauliflower mosaic virus 35S promoter (5′-CACTGACGTAAGGGATGACGC-3′) and the *MtERF* open reading frame (*MtERF1-1*, 5′-CCCTCAGGAACCACTTGCT-3′; *MtERF4-1*, 5′-AAAGGGAACCGATCCATGA-3′) was performed on genomic DNA extracted from root systems of representative plants to confirm the presence of the constructs (data not shown). Genomic DNA extractions were performed according to Kazan et al. (1993). Relative pathogen biomass in root tissue was determined as described by Anderson et al. (2004a) with quantitative PCR conditions according to Gao et al. (2007) using 100 ng per reaction of genomic DNA extracted from roots as the template. The primers B36F and B36R (González García et al., 2006) or p990F and p1050R (Vandemark and Barker, 2003) were used to quantify *R. solani* and *Phytophthora medicaginis*, respectively. Plant DNA was quantified using the *M. truncatula* ribosomal DNA primers (Supplemental Table S3). The relative amount of fungal DNA is shown as the ratio of the relative abundance for each sample to that in the *Ox-MtERF1-1* roots. The specificity of the primers for the intended target was confirmed and the ability of the system to reliably detect changes in relative pathogen biomass was confirmed using quantitative PCR with a series of templates containing known plant DNA-to-pathogen DNA ratios (data not shown). The *R. solani* AG8 inoculation experiments for Supplemental Figure S1 used *A. rhizogenes*-transformed roots without a transgene as controls and consisted of eight replicate plants for each root genotype/treatment combination. The *R. solani* AG8 inoculation experiments for Figure 6 used pEarleyGateRedroot with *GFP* insert as the control and consisted of at least six replicate plants per treatment/genotype combination (Fig. 6). The *P. medicaginis* inoculation experiments used roots of accession SA30199 transformed with the pGFP-EarleyGateRedroot construct as a control and consisted of at least seven replicate plants for each root genotype/treatment combination. The entire experiments were repeated with similar results.

Infection of *M. truncatula* Composite Plants with Root Knot Nematode in Pouch Assays

Composite plants were generated as described, and the composite plants with transgenic roots were transferred to growth pouches (Mega International) containing Fahraeus solution. Composite plants were inoculated with 200 J2-stage root knot nematodes (*Meloidogyne javonica*) per pouch. Two weeks after inoculation, the number of galls produced on each plant was counted.

Nodulation of *M. truncatula* Composite Plants

Composite plants were transferred from Fahraeus medium containing 1 mM NH_4NO_3 to growth pouches containing nitrogen-free Fahraeus medium and nodulated according to the "inoculation/nodulation of plants in growth pouches" protocol described by Journet et al. (2006). The *GFP*-expressing strain of *Sinorhizobium meliloti* 1021 is described by Cheng and Walker (1998) and was kindly supplied by Ulrike Mathesius (Australian National University).

Analysis of Promoter Activity in Composite Plants

Composite plants were generated according to Chabaud et al. (2006) and grown on Fahraeus medium. The construct containing the tetramer of the GCC box promoter element was created by replacing the *OCS* tetramer of the construct described by Perl-Treves et al. (2004) with the sequence 5′-CCGGATCCTCCAGCCGCCAGCACAGCCGCCCGGGCAGCCGCCAGCACAGCCGCCACAAGCTTGT-3′ while maintaining the −58/+8 GSTF8 minimal promoter. Plants were inoculated with *R. solani* AG8 inoculum prepared as described above, and bioluminescence was measured using an EG&G Berthold Molecular Light Imager using a 5-min exposure after a 10-min delay for fluorescence decay.

RNA Isolation, cDNA Synthesis, and Quantitative PCR

RNA isolation, cDNA synthesis, and quantitative reverse transcription-PCR were performed according to Gao et al. (2007). The cycle number at which the fluorescence passed the threshold (CT) for all selected genes were normalized to the CT value of Actin (TC147263). Expression of Actin remained stable among various control and pathogen-infected tissues (data not shown). *M. truncatula* tentative consensus (TC) sequences with homology to ERF and defense genes were identified by tBLASTX queries of the Dana-Farber Cancer Institute *Medicago* Gene Index (http://compbio.dfci.harvard.edu/tgi/plant.html). TCs with ESTs originating from defense-related EST libraries were preferentially selected. Information on the libraries contributing the ESTs to the TCs is available in the Dana-Farber Cancer Institute *Medicago* Gene Index. Primers used for quantitative reverse transcription-PCR are presented in Supplemental Table S3.

Phylogenetic Analysis of Arabidopsis and *Medicago* ERF Genes

A phylogenetic tree of selected Arabidopsis and *Medicago* ERF protein sequences was created using the neighbor-joining method with a bootstrap

consensus tree inferred from 1,000 replicates (Felsenstein, 1985; Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches (Fig. 4). Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

All statistical analyses were done using the software JMP7 (SAS).

Sequence data from this article can be found in the Dana-Farber Cancer Institute *Medicago* Gene Index under the accessions numbers listed in Supplemental Table S3.

Supplemental Data

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

Supplemental Figure S1. Overexpression of *MtERF1-1* and *MtERF4-1* in A17 and *skl* composite plants.

Supplemental Figure S2. Nodulation of *skl* roots transformed with *MtERF1-1* or *GFP* overexpression constructs.

Supplemental Table S1. ANOVA corresponding to Figures 3 and 5.

Supplemental Table S2. Expression of *M. truncatula* ERF and defense genes in response to exogenous ethylene treatment.

Supplemental Table S3. Genes and primer sequences.

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