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Antagonistic Interaction between Abscisic Acid and Jasmonate-Ethylene Signaling Pathways Modulates Defense Gene Expression and Disease Resistance in Arabidopsis

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The plant hormones abscisic acid (ABA), jasmonic acid (JA), and ethylene are involved in diverse plant processes, including the regulation of gene expression during adaptive responses to abiotic and biotic stresses. Previously, ABA has been implicated in enhancing disease susceptibility in various plant species, but currently very little is known about the molecular mechanisms underlying this phenomenon. In this study, we obtained evidence that a complex interplay between ABA and JA-ethylene signaling pathways regulate plant defense gene expression and disease resistance. First, we showed that exogenous ABA suppressed both basal and JA-ethylene–activated transcription from defense genes. By contrast, ABA deficiency as conditioned by the mutations in the *ABA1* and *ABA2* genes, which encode enzymes involved in ABA biosynthesis, resulted in upregulation of basal and induced transcription from JA-ethylene responsive defense genes. Second, we found that disruption of *AtMYC2* (allelic to *JASMONATE INSENSITIVE1* [*JIN1*]), encoding a basic helix-loop-helix Leu zipper transcription factor, which is a positive regulator of ABA signaling, results in elevated levels of basal and activated transcription from JA-ethylene responsive defense genes. Furthermore, the *jin1/myc2* and *aba2-1* mutants showed increased resistance to the necrotrophic fungal pathogen *Fusarium oxysporum*. Finally, using ethylene and ABA signaling mutants, we showed that interaction between ABA and ethylene signaling is mutually antagonistic in vegetative tissues. Collectively, our results indicate that the antagonistic interactions between multiple components of ABA and the JA-ethylene signaling pathways modulate defense and stress responsive gene expression in response to biotic and abiotic stresses.

INTRODUCTION

In their natural environment, plants are continuously threatened by various biotic and abiotic stresses. Their survival under such conditions is dependent on the ability to perceive external signals and respond in a timely manner. Our current understanding of plant signaling pathways involved in biotic and abiotic stresses is still rudimentary. However, the emerging picture from several studies supports the notion that plant signaling pathways are composed of intricate networks with frequent cross talk that allow the plant to activate an appropriate spectrum of responses depending on the type of stimuli present. This leads to the production of proteins with direct roles in alleviating the damaging effects of stressful conditions.

The plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene are the major endogenous low molecular weight signal molecules involved in regulating defense responses in plants. In *Arabidopsis thaliana*, an intact JA-ethylene signaling pathway is thought to be necessary for resistance to necrotrophic pathogens, such as *Botrytis cinerea* and *Erwinia carotovora*. By contrast, the SA signaling pathway is believed to mediate the resistance to biotrophic pathogens, such as *Erysiphe orontii*, *Peronospora parasitica*, and *Pseudomonas syringae* (Thomma et al., 2001; Rojo et al., 2003). Thus, it is conceivable that to mount an effective defense response, the plant activates the particular signaling pathways that are most likely to enhance resistance to a class of invader. Simultaneously, other signaling pathways with minimal or no significant effects on the invading pathogen may be suppressed to avoid depletion of valuable physiological resources. An example of such antagonistic interaction occurs between the SA and the JA signaling pathways in Arabidopsis. Mutations that impair SA signaling or biosynthesis result in elevated expression of the JA-ethylene responsive antifungal defensin *PDF1.2* (Spoel et al., 2003). By contrast, mutations that constitutively activate the SA-signaling pathway

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suppress *PDF1.2* expression and render the plants susceptible to pathogens (Petersen et al., 2000). However, antagonism between the SA and JA signaling pathways does not apply in all cases, and some defense response genes require intact JA, ethylene, and SA signaling pathways after pathogen challenge (Campbell et al., 2003).

The plant hormone abscisic acid (ABA) regulates interacting signaling pathways involved in plant responses to several abiotic stresses, such as drought, salt, and cold, as well as plant growth and development. The ABA-dependent signaling pathway regulates stress-inducible gene expression through several positive and negative regulators (reviewed in Shinozaki et al., 2003). Genetic analysis of Arabidopsis mutants compromised in ABA biosynthesis or signaling has identified a complex interplay between ABA and various other phytohormone signaling pathways. One of the relatively better characterized genetic interactions occurs among ABA, ethylene, and the sugar signaling pathways (Gazzarrini and McCourt, 2001; Finkelstein and Gibson, 2002; Leon and Sheen, 2003). Genetic analyses have also demonstrated that the ABA signaling pathway interacts antagonistically with the ethylene signaling pathway and vice versa to modulate plant development (Beaudoin et al., 2000; Ghassemian et al., 2000). First, it was suggested that one of the functions of ABA is to inhibit overproduction of ethylene as ABA-deficient maize (*Zea mays*) (Spollen et al., 2000) and tomato (*Lycopersicon esculentum*) (Sharp et al., 2000) mutants overaccumulate ethylene and display stunted growth phenotypes. In addition, the maintenance of shoot growth by ABA involves, in part, suppression of ethylene synthesis (LeNoble et al., 2004). By contrast, ethylene negatively regulates ABA signaling in controlling seed dormancy (Beaudoin et al., 2000). For instance, the ethylene insensitive mutant *ein2* (allelic to the *enhanced response to ABA3* or *era3*) shows enhanced ABA sensitivity during seed germination (Ghassemian et al., 2000). Mutations in the genes (e.g., *ETHYLENE RECEPTOR1* [*ETR1*], *EIN2*, and *EIN3*) involved in positive regulation of ethylene signaling result in a glucose (*glo*) and an ABA-oversensitivity phenotype with greater growth inhibition in response to exogenous glucose and ABA than in wild-type plants, whereas mutations affecting negative regulators of ethylene signaling (e.g., CONSTITUTIVE TRIPLE RESPONSE1 [CTR1]) result in reduced ABA sensitivity and a glucose insensitivity (*gin*) phenotype (Zhou et al., 1998; Ghassemian et al., 2000; Cheng et al., 2002). An antagonistic interaction between the ABA and the JA signaling pathways has also been observed in the *jasmonic acid resistant1* (*jar1*) and *jasmonic acid insensitive4* (*jin4*) mutants, which show hypersensitivity to ABA inhibition of germination (Staswick et al., 1992; Berger et al., 1996). Another study found that ABA and JA antagonistically regulate the expression of salt stress–inducible transcripts in rice (*Oryza sativa*) (Moons et al., 1997).

Although this seemingly antagonistic interaction between the ABA-sugar and the ethylene signaling pathways is relatively well known (reviewed in Finkelstein and Gibson, 2002; Finkelstein et al., 2002; Leon and Sheen, 2003), our current understanding on how this interaction influences other ethylene-mediated plant responses, such as defense gene expression and pathogen resistance, is very limited. Also largely unknown is how the ABA signaling pathway interacts with the JA signaling pathway that in itself partially overlaps with the ethylene signaling pathway in regulating defense gene expression and resistance to necrotrophic pathogens. An increased understanding of the potential interactions between ABA and biotic stress responses is of vital importance for engineering plants for disease resistance without compromising ABA-regulated abiotic stress response pathways.

Here, using various ABA biosynthesis or signaling mutants, we first demonstrated that ABA signaling antagonizes the JA-ethylene responsive defense gene expression and fungal disease resistance in Arabidopsis. Furthermore, using ethylene signaling mutants we demonstrated a mutually antagonistic interaction between ABA and ethylene signaling that modulates stress responsive gene expression during vegetative growth and propose potential points for such interaction. Collectively, our results suggest that the complex interplay between biotic and abiotic stress pathways provides a means of managing and prioritizing diverse stress responses and could have important implications on engineering of biotic and abiotic stress resistance in plants.

RESULTS

ABA Antagonizes JA-Ethylene Responsive Defense Gene Expression in Arabidopsis

Recent results from several studies showed that ABA negatively influences disease resistance phenotypes in various plant species (Rezzonico et al., 1998; McDonald and Cahill, 1999; Audenaert et al., 2002; Mohr and Cahill, 2003). Similarly, water stress reduces disease tolerance to some pathogens (Wildermuth and Morgan, 2004). We hypothesized that the enhanced susceptibility phenotype could be attributable to ABA's suppression of JA-ethylene responsive defense gene expression. To test this hypothesis, we first studied *PDF1.2* expression, a known marker gene positively regulated by the JA-ethylene signaling pathway, in plants treated with methyl jasmonate (MJ), ethylene, ABA, or a combination of MJ and ABA or ethylene and ABA. To specifically quantify and compare the changes in defense gene transcript levels after various treatments and/or in mutant backgrounds, we used real-time quantitative RT-PCR (RT-Q-PCR) in all gene expression studies reported in this article. The use of the highly sensitive method of RT-Q-PCR permitted the measurement of basal (uninduced) transcript levels of the genes studied here as well as their induction or suppression by external stimuli. In particular, it should be noted that the quantification of reductions in transcript levels observed after treatments are not readily measured by traditional RNA gel blotting. In these analyses, we quantified target gene expression relative to the expression measured from the reference genes (e.g., three β-*actin* genes or β-*tubulin*) in the same sample. The transcript abundance of the reference genes measured as cycle threshold value did not show any significant change after treatments/inoculations (see Methods for details and also Hoth et al., 2002; Campbell et al., 2003; Schenk et al., 2003). Normalization of gene expression using three β-*actin* genes or β-*tubulin* also showed a strong correlation with expression normalized relative to that of the 25S rRNA. The high abundance of the 25S rRNA transcripts requires severalfold dilution of cDNAs before use in RT-Q-PCR

reactions and therefore cannot be conveniently assayed on the same sample as the assay for the target gene (J. Anderson and K. Kazan, unpublished data).

As expected, treatments with MJ and ethylene significantly induced *PDF1.2* (30-fold and 15-fold, respectively) in treated wild-type plants relative to the mock-treated plants, whereas ABA treatment caused 10-fold reduction at the basal transcript levels of *PDF1.2* relative to those in mock-treated wild-type plants (Figure 1A). In addition, we noted that, in the presence of ABA, neither ethylene nor MJ were able to induce *PDF1.2* expression in wild-type plants (Figure 1A). Interestingly, water stress also significantly reduced (sixfold) *PDF1.2* transcript levels over those measured in plants not exposed to water stress (Figure 1A).

We next examined the effect of ABA on MJ-responsive expression of *PDF1.2* using Arabidopsis plants transformed with the *PDF1.2promoter*:*GUS* construct (Manners et al., 1998). The expression of *PDF1.2* expression is known to increase with plant age (He et al., 2002), and this explains the relatively high background GUS expression observed in mock-treated *PDF1.2promoter*:*GUS* plants. However, this attribute proved to be useful for testing whether ABA treatment could reduce background GUS activity in these plants. Indeed, both histochemical staining and enzyme activity assays showed significantly reduced GUS expression after ABA treatment as well as MJ-ABA cotreatment in these plants, whereas as reported previously by Manners et al. (1998), the MJ-treatment significantly induced GUS activity in *PDF1.2promoter*:*GUS* plants as evidenced by saturated dark blue color on the leaves (Figures 1B and 1C). Collectively, these results suggested that ABA acts antagonistically to MJ and ethylene in suppressing *PDF1.2* expression.

To test whether the suppression caused by ABA was specific to *PDF1.2* or whether other JA-ethylene responsive defense genes also show a similar response to ABA, we quantified the transcript levels of three other MJ-ethylene responsive defense genes, namely *CHI* (basic chitinase), *HEL* (hevein-like protein or PR4), and *LEC*, an MJ- and ethylene-responsive lectin-like protein (Schenk et al., 2000) after MJ or MJ-ABA cotreatment. As expected, MJ treatment strongly induced the transcript levels of *PDF1.2*, *CHI*, *HEL*, and *LEC* in wild-type plants relative to the expression of these genes in mock-treated plants (Figure 2A). We next examined the effect of MJ-ABA cotreatment on defense gene induction. These experiments again showed that MJ, when applied together with ABA, was not able to fully induce the expression of these defense genes (Figure 2A). Consistent with the known ethylene inducibility of these genes, treatments of wild-type plants with ethylene produced results that are similar to those by MJ (data not shown).

It can be argued, however, that endogenous ABA levels attained in the plant after ABA treatment do not represent physiologically relevant ABA concentrations; thus, the observed reductions in the transcript levels of defense genes after ABA treatment could be misleading. We therefore studied expression of these defense genes in the *abscisic acid deficient2* (*aba2-1*) and *aba1-2* mutants (Koornneef et al., 1982). The *aba2-1* mutant contains significantly reduced levels of endogenous ABA because of a mutation in the *SDR1* gene, which encodes a short-chain dehydrogenase/reductase involved in ABA biosynthesis (Cheng et al., 2002; González-Guzmán et al., 2002). We observed 125-, 10-, 30-, and 20-fold higher basal transcript levels of *PDF1.2*, *CHI*, *HEL*, and *LEC*, respectively, in untreated *aba2-1* plants than in untreated wild-type plants (Figure 2B). ABA treatment reduced the transcript levels of these defense genes in both the wild type and the *aba2-1* mutant. However, the *aba2-1* mutant had significantly higher transcript levels of all four genes examined when compared with those in ABA-treated wild-type plants as demonstrated by the *aba2-1*/wild type normalized expression ratios (Figure 2B). In addition, MJ treatment induced the transcript levels of most of these defense genes to slightly higher levels (approximately twofold) in the *aba2-1* mutant background than in wild-type plants (Figure 2B). We further confirmed that basal and MJ-activated *PDF1.2* transcript levels in the *aba1-2* (in Landsberg *erecta* background) mutant (Koornneef et al., 1982), which also shows an ABA-deficient phenotype because of a mutation in the *ZEP* (zeaxanthin epoxidase) gene (Marin et al., 1996), were higher than those in wild-type plants (data not shown). Overall, these results indicated that ABA, whether endogenously synthesized or exogenously applied, could play a role in antagonizing JA-ethylene responsive defense gene expression in Arabidopsis.

A Positive Regulator of ABA Signaling Negatively Regulates JA-Ethylene Responsive Defense Gene Expression

We next examined whether a positive regulator of ABA signaling pathway could have a negative effect on JA-ethylene responsive defense gene expression. The role of AtMYC2 as a positive regulator of ABA signaling has been previously established (Abe et al., 1997, 2003). To examine the potential role of this gene in plant defense, we first studied the regulation of *AtMYC2* during plant defense responses. We conducted time-course inoculation experiments with the soil-borne pathogenic fungus *Fusarium oxysporum* and quantified the transcript abundance of *AtMYC2* by RT-Q-PCR. These experiments showed that *AtMYC2* is induced at early time points after inoculation with *F. oxysporum* relative to the expression measured in mock-inoculated plants (Figure 3A). Similarly, MJ treatment significantly induced the expression from the *AtMYC2* gene relative to the expression detected in mock-treated plants (Figure 3B). As a positive control for pathogen and MJ treatments, we also measured expression from the *PDF1.2* gene in the same samples. Consistent with the previous reports (Schenk et al., 2003), a significant *PDF1.2* induction was detected at 12 and 24 h onwards after treatment with MJ or inoculation with *F. oxysporum*, respectively (Figures 3A and 3B). Interestingly, ethylene treatment significantly suppressed *AtMYC2* expression relative to the untreated plants at 6 h (2 ± 0.5-fold) and 24 h (5 ± 0.2-fold) after treatment. Altered expression of *AtMYC2* transcripts during plant defense suggests a role for this gene in biotic stress in addition to its known role in ABA-mediated responses to abiotic stress.

To determine potential function(s) of AtMYC2 in plant defense, we characterized two Arabidopsis lines carrying independent T-DNA insertions in the *AtMYC2* gene (Alonso et al., 2003a). Importantly, while this manuscript was in preparation, two other articles describing a series of *AtMYC2* mutant alleles were

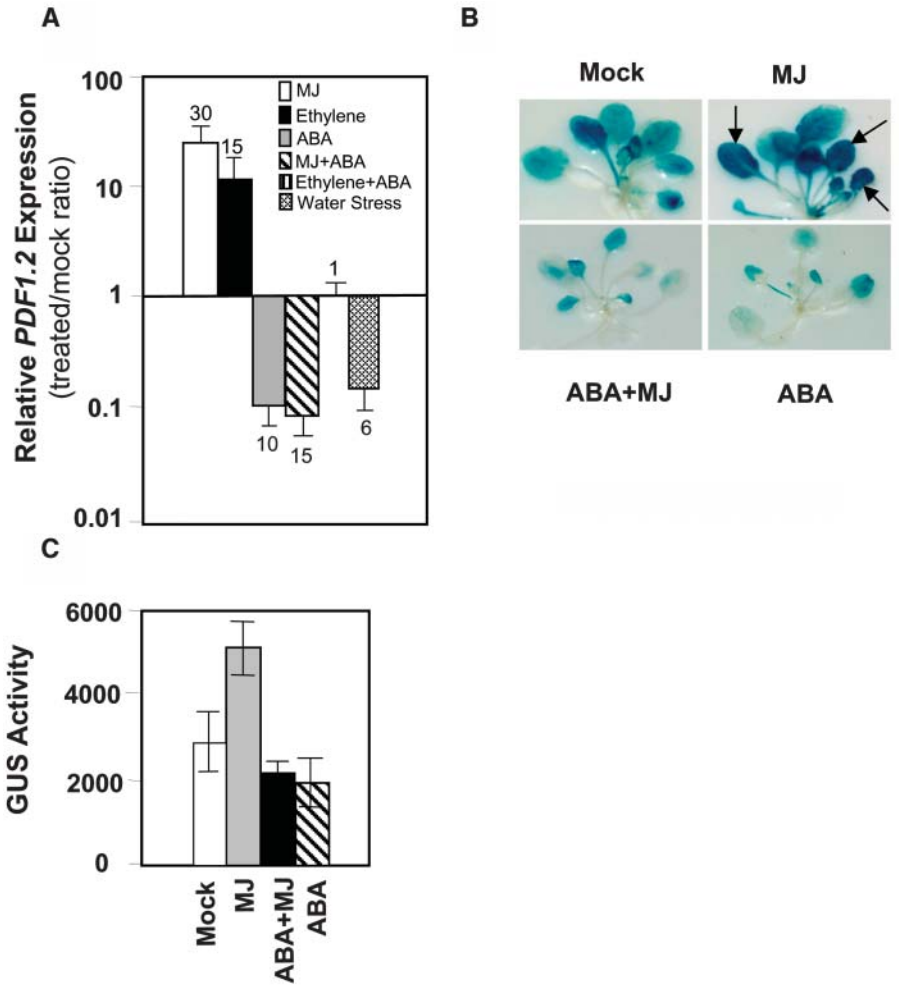

Figure 1. Exogenous ABA Suppresses *PDF1.2* Expression in Arabidopsis.

(A) Fold changes in relative transcript abundance of the *PDF1.2* in MJ-, ethylene-, ABA-, MJ+ABA-, ethylene+ABA- (second to last column), and water stress–treated wild-type (Columbia-0 [Col-0]) plants. Total RNA was isolated from plants 48 h after each treatment, converted to cDNA, and used as template in RT-Q-PCR assays. Transcript levels of *PDF1.2* were normalized to the expression of β-*actin* genes measured in the same samples and expressed logarithmically relative to the normalized transcript levels in mock-treated wild-type plants. Average data with error bars from two independent experiments are presented. The numbers on each bar show fold increase or fold decrease caused by each treatment in *PDF1.2* transcript levels relative to those in mock-treated plants.

(B) and **(C)** Three- to four-week-old homozygous plants transformed with the *PDF1.2promoter*:*GUS* construct were either mock treated or treated with ABA, MJ, or combination of ABA and MJ for 48 h. Histochemical GUS staining was performed overnight on 10 seedlings for each experiment **(B)**. The leaves that display strong GUS activity after MJ treatment as evidenced by saturated blue color are indicated by arrows. GUS activity was also measured fluorometrically using at least 10 seedlings for each treatment **(C)**. Average data from two separate experiments are shown. Error bars indicate standard deviation.

concomitantly published. Of these, Lorenzo et al. (2004) reported the map-based cloning of the *JASMONATE INSENSITIVE1* (*JIN1*/*JAI1*) locus, which is found to be identical to the *AtMYC2* locus. Lorenzo et al. (2004) have characterized eight other mutant alleles of the *JIN1*/*JAI1*/*AtMYC2* locus generated either by ethyl methanesulfonate mutagenesis (*jin1-1* to *jin1-6*) or T-DNA insertions (*jin1-7* to *jin1-8*). A second article by Boter et al. (2004) also described two insertional *AtMYC2* mutants. One of the T-DNA mutants (SALK_040500) characterized by Boter et al. (2004) corresponds to *jin1-7* of Lorenzo et al. (2004), and the second one (SALK_083483) is common between Boter et al. (2004) and this study. Following these studies, we renamed the two-*AtMYC2* mutant alleles that we have characterized in this study as *jin1-9* (SALK_017005) and *jin1-10* (SALK_083483).

PCR amplification followed by DNA sequencing of the T-DNA–plant genomic DNA junction in one of such lines (SALK_017005) confirmed that the T-DNA is inserted into the 320th codon of *AtMYC2* that encodes the amino acid Ile. Thus, the T-DNA insertion in this line truncates the basic helix-loop-helix domain necessary for dimerization and DNA binding and NH_2-terminal

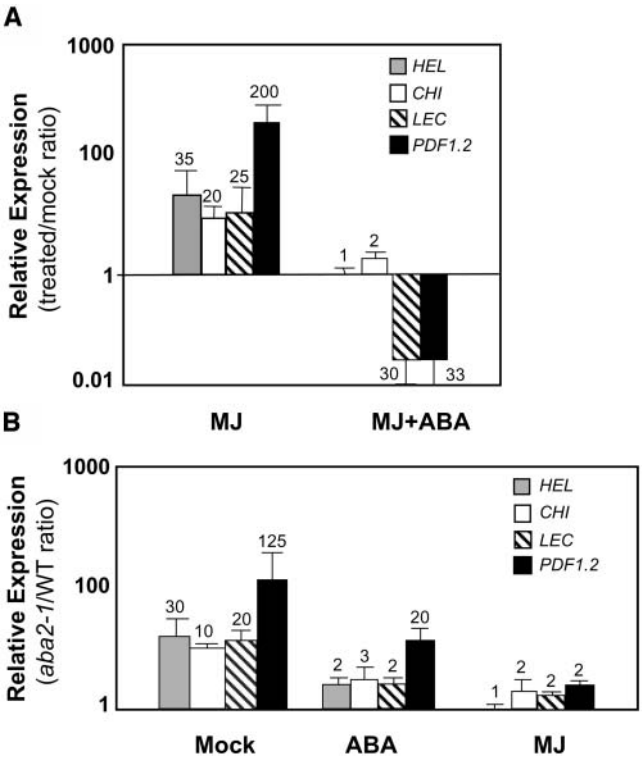

Figure 2. ABA Suppresses JA-Ethylene Responsive Defense Gene Expression in Arabidopsis.

(A) Fold changes (induction or suppression) in relative transcript abundance of *PDF1.2*, *CHI*, *HEL*, and *LEC* in MJ- and MJ+ABA-treated wild-type (Col-0) plants relative to the mock-treated wild-type plants.
(B) Fold changes in relative transcript abundance of *PDF1.2*, *CHI*, *HEL*, and *LEC* in mock-, MJ-, or ABA-treated *aba2-1* mutant relative to the expression of the same genes in similarly treated wild-type plants. Total RNA was isolated from plants 48 h after each treatment, converted to cDNA, and used as template in RT-Q-PCR assays. Transcript levels in treated wild-type plants were normalized to the expression of β-*actin* genes measured in the same samples and expressed logarithmically relative to the similarly normalized transcript levels in mock-treated wild-type plants. The numbers on each bar show fold increase or fold decrease caused by each treatment in the transcript levels of genes relative to those in mock-treated plants **(A)**. Transcript levels in treated *aba2-1* plants were normalized relative to the expression of β-*actin* genes measured in the same samples and expressed logarithmically relative to the similarly normalized transcript levels in treated wild-type plants. The numbers on each bar show fold increase or fold decrease caused by each treatment in the transcript levels of genes in the *aba2-1* background relative to those in similarly treated wild-type plants **(B)**. Average data with error bars from two independent experiments are shown in both **(A)** and **(B)**.

domain necessary for either *trans*-activation or -repression. Given the importance of the major domains disrupted in AtMYC2, the abnormal transcript resulting from the T-DNA insertion, if expressed stably, is unlikely to be functional. Indeed, the basal transcript abundance of the *AtMYC2* sequence in homozygous plants of the *myc2* mutant was strongly suppressed in untreated and MJ-treated plants compared with the *AtMYC2* levels in similarly treated wild-type plants (data not shown). This result provides further evidence that the T-DNA insertion interferes with the transcript accumulation as well as disrupting the coding sequence of the *AtMYC2* gene.

There was no detectable effect of the insertion on normal plant growth and development in the *jin1-9/myc2* mutant. However, we found significantly higher basal transcript levels of the JA-ethylene responsive defense genes in untreated *jin1-9/myc2* plants than untreated wild-type plants grown and sampled at the same time. Basal transcript levels of *PDF1.2*, *CHI*, and *HEL* were fivefold, twofold, and twofold higher, respectively, in the untreated *jin1-9/myc2* mutant than in untreated wild-type plants (Figure 4A). To further confirm that the enhanced defense gene expression phenotype observed in this line was because of the disruption of *AtMYC2*, we examined the basal transcript level of *PDF1.2* in homozygous plants of *jin1-10/myc2* mutant. The T-DNA in this line is inserted into the coding region of *AtMYC2*, similarly truncating the AtMYC2 protein between N-terminal and

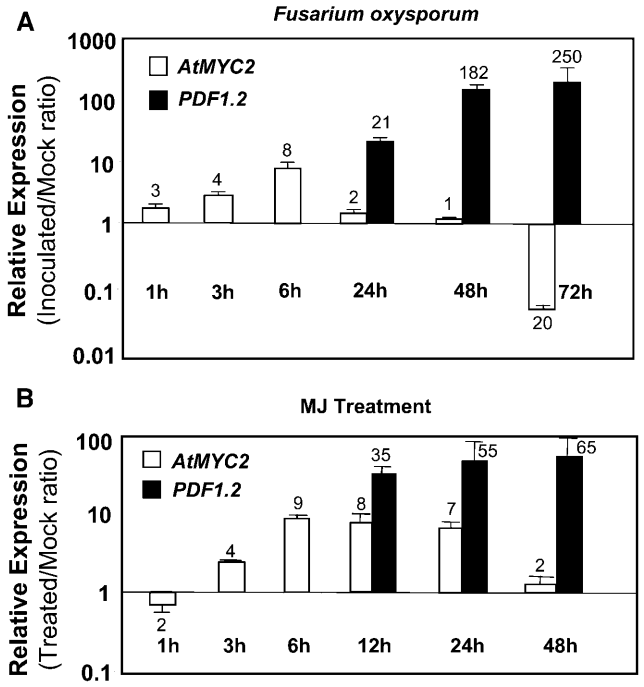

Figure 3. *AtMYC2* Is Induced during Plant Defense Responses in Arabidopsis.

AtMYC2 and *PDF1.2* expression in wild-type (Col-0) plants were examined in time-course studies after inoculation with *F. oxysporum* **(A)** and after treatment with MJ **(B)**. Total RNA was extracted from leaf tissue of 3- to 4-week-old plants (8 to 10 leaf stage) for each time point, converted to cDNA, and subjected to RT-Q-PCR analysis. The *AtMYC2* and *PDF1.2* transcript levels in treated/inoculated plants were normalized to the expression of β-*actin* measured in the same samples and expressed logarithmically relative to the similarly normalized expression levels in mock-inoculated/mock-treated plants. Each bar represents average data with error bars from two independent experiments. The numbers on each bar show fold increase or fold decrease caused by each treatment at transcript levels of *AtMYC2* and *PDF1.2* relative to those in mock-treated/inoculated plants.

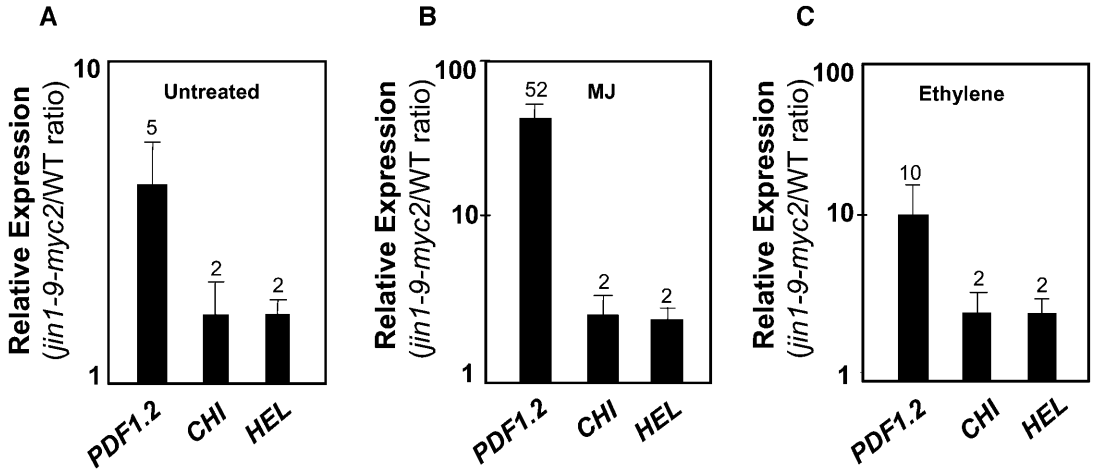

Figure 4. JA-Ethylene Responsive Defense Genes Show Elevated Levels of Basal and Activated Transcription in the *jin1-9/myc2* Mutant.

Relative expression ratios of *PDF1.2*, *CHI*, and *HEL* transcripts in untreated **(A)**, MJ- **(B)**, and ethylene-treated **(C)** plants of the wild type (Col-0) and the homozygous plants of the *jin1-9/myc2* mutant. Total RNA was extracted from leaf tissue of 3- to 4-week-old plants 24 h after MJ and ethylene treatments, converted to cDNA, and subjected to RT-Q-PCR analysis. The transcript levels in the *jin1-9/myc2* mutant were normalized to the expression of β-*tubulin* measured in the same samples and expressed logarithmically relative to the normalized expression levels in similarly treated wild-type plants. Each bar represents average data with error bars from two independent experiments. The numbers on each bar show fold increase in defense gene transcript levels in the *jin1-9/myc2* mutant relative to those in mock-treated plants.

basic helix-loop-helix domains (data not shown). Similarly to *jin1-9/myc2*, untreated homozygous plants of the *jin1-9/myc2* mutant showed threefold higher basal transcription from the *PDF1.2* gene; thus, all subsequent experiments were conducted on homozygous plants of the *jin1-9/myc2* mutant. The molecular phenotypes of *jin1/myc2* mutants reported in this article were consistent with those reported by Lorenzo et al. (2004) and Boter et al. (2004) (see also below).

Disruption of AtMYC2 Increases Defense Gene Induction by MJ and Ethylene

To determine whether JA-ethylene responsive defense genes are hypersensitive to induction in the *jin1/myc2* mutant, we treated the wild-type and the *jin1-9/myc2* mutant with MJ and ethylene and examined the transcript levels of selected JA-ethylene responsive genes in treated plants 24 h after treatment. MJ and ethylene treatments of the *jin1-9/myc2* mutant resulted in 52- and 10-fold higher induction of *PDF1.2* in the *myc2* mutant background than in wild-type plants treated similarly (Figures 4B and 4C). *CHI* and *HEL* transcript levels were also higher in MJ- and ethylene-treated *jin1-9/myc2* plants than in similarly treated wild-type plants (Figures 4B and 4C). Although increases in transcript levels of *CHI* and *HEL* in the *jin1-9/myc2* mutant after MJ and ethylene treatments were relatively small (approximately twofold increase over that measured in similarly treated wild-type plants), the changes observed were highly reproducible in independent experiments. The enhanced responsiveness observed in defense gene induction in the *jin1-9/myc2* mutant after MJ-ethylene treatment suggests that AtMYC2, as a positive regulator of ABA signaling, might play a role in negative regulation of JA-ethylene responsive defense genes in Arabidopsis.

AtMYC2 Overexpression in Arabidopsis

To determine whether the overexpression of AtMYC2 could inhibit defense gene expression, which would further support its putative function as a negative regulator, we first used transient protoplast transformation assays. Such assays have been extensively used to dissect plant signaling pathways (Hwang and Sheen, 2001) and similarly allowed us to test the individual and combinatorial expression of various gene constructs in a convenient way. As explained in Methods, all transformations included the *35S:GFP* construct for constitutive expression of the green fluorescent protein (GFP), which was used for normalization of transformation efficiency. As a positive control, we first used the *35S:ERF1* construct, which resulted in a 10- and 99-fold increase in *ERF1* (data not shown) and *PDF1.2* (Figure 5A) transcript levels, respectively, relative to the expression detected in control protoplasts transformed with the vector only. The increase observed in *PDF1.2* transcript levels is consistent with previous evidence that ERF1 is a positive regulator of the *PDF1.2* gene (Solano et al., 1998). Overexpression of AtMYC2 resulted in an eightfold increase and a fourfold reduction in the transcript levels of *AtMYC2* (data not shown) and *PDF1.2* (Figure 5A), respectively, relative to the transcript levels of these genes measured in vector-transformed protoplasts. However, when both AtMYC2 and ERF1 are simultaneously overexpressed by the 35S promoter of *Cauliflower mosaic virus* in Arabidopsis protoplasts, no suppression of *PDF1.2* transcripts was evident (Figure 5A). Taken together, these results suggest that the negative regulatory effect of AtMYC2 on *PDF1.2* occurs mainly upstream of ERF1 and possibly not directly via an interaction of AtMYC2 and the *PDF1.2* promoter.

Subsequently, we generated transgenic Arabidopsis plants containing the *35S:AtMYC2* construct. After screening 13

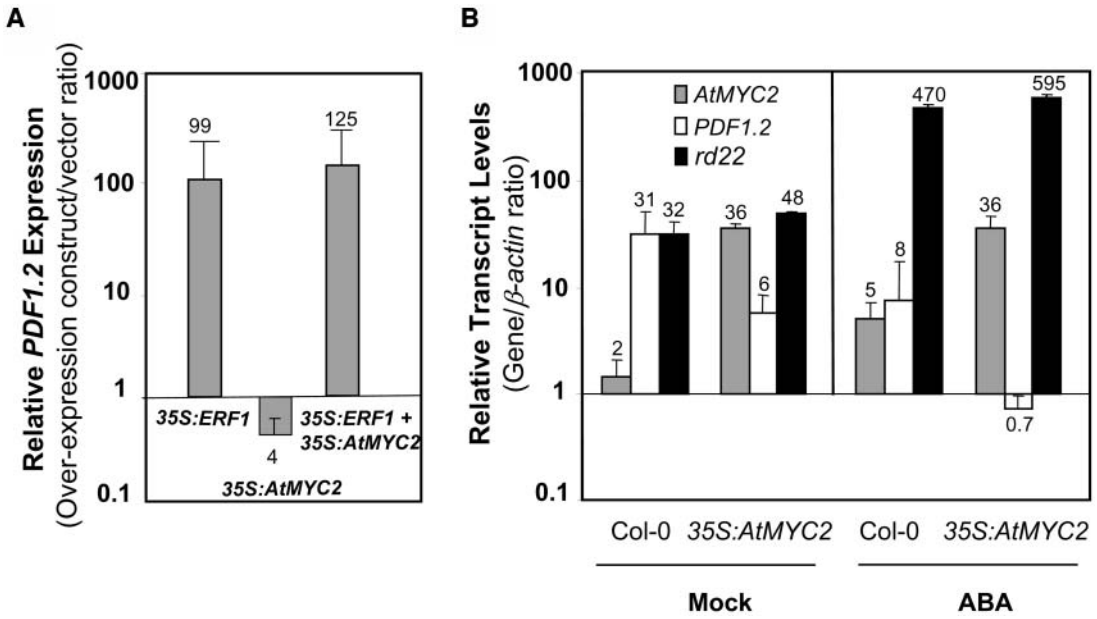

Figure 5. Transient or Stable Overexpression of AtMYC2 in Arabidopsis Suppresses *PDF1.2* Expression.

(A) Transient overexpression of ERF1 in Arabidopsis protoplasts activates expression from the *PDF1.2*, whereas transient overexpression of AtMYC2 in Arabidopsis protoplasts suppresses the basal transcription from *PDF1.2* as detected by RT-Q-PCR 48 h after transformation. AtMYC2 overexpression is not sufficient in suppressing *PDF1.2* transcript levels when coexpressed with ERF1 in Arabidopsis protoplasts. Total RNA was isolated from the *35S*:*ERF1*, *35S*:*AtMYC2*, and *35S*:*ERF1*+*35S*:*AtMYC2* transformed protoplasts, converted to cDNA, and subjected to RT-Q-PCR analysis. The *PDF1.2* transcript levels in the transformed samples were normalized to the expression of β-*actin* measured in the same samples and expressed logarithmically relative to the normalized expression levels measured in vector-only transformed protoplasts. Each bar represents average data with error bars from two independent experiments. The numbers on each bar show fold increase or fold decrease in *PDF1.2* transcript levels caused by overexpression relative to the *PDF1.2* levels in vector-transformed protoplasts.

(B) The stable overexpression of AtMYC2 in Arabidopsis activates *rd22* expression while suppressing *PDF1.2* in plants either mock-treated or treated with ABA for 11 h. Total RNA was isolated from transformed protoplasts or treated plants, converted to cDNA, and subjected to RT-Q-PCR analysis. The *AtMYC2*, *rd22*, and *PDF1.2* transcript levels in untreated and ABA-treated wild type and the *35S*:*AtMYC2* plants were normalized to the expression of β-*actin* (multiplied by 1000 for clarity) measured in the same sample and expressed logarithmically. Each bar represents average data with error bars from two independent experiments. The numbers on each bar show relative transcript abundance of *AtMYC2*, *PDF1.2*, and *rd22*, relative to the β-*actin* transcript levels measured in the same samples.

independently generated transgenic lines for elevated AtMYC2 expression, we identified two transgenic lines that contained increased transcript levels of *AtMYC2* as high as 18-fold over those found in wild-type plants. Because AtMYC2 is a positive regulator of ABA responsive *rd22* (Yamaguchi-Shinozaki and Shinozaki, 1993; Abe et al., 2003), we first confirmed that AtMYC2 overexpression could indeed induce *rd22*. The expression data, which show transcript levels of *AtMYC2*, *rd22*, and *PDF1.2* relative to those of β-*actin* measured in the samples, are presented in Figure 5B. The *rd22* transcript abundance in untreated *35S*:*AtMYC2* plants was indeed slightly higher (1.5-fold) than that in untreated wild-type plants. Furthermore, we found that induction of *rd22* by ABA was also stronger in ABA-treated *35S*:*AtMYC2* plants than in ABA-treated wild-type plants (Figure 5B), confirming previous results by Abe et al. (2003). In contrast with *rd22*, quantification of *PDF1.2* transcript abundance showed approximately fivefold lower *PDF1.2* expression in untreated *35S*:*AtMYC2* plants than in untreated wild-type plants (Figure 5B). Furthermore, although ABA treatment reduced the transcript levels of *PDF1.2* in both wild-type and the *35S*:*AtMYC2* plants, the reduction observed in *PDF1.2* transcript levels in the *35S*:*AtMYC2* plants was significantly greater than that in ABA-treated wild-type plants. Overall, these results are consistent with a positive and negative regulatory role of AtMYC2 on *rd22* and *PDF1.2*, respectively. The differences observed between the *35S*:*AtMYC2* and wild-type plants in the transcript levels of *PDF1.2* and *rd22* at 11 h after ABA treatment were no longer detectable at 24 and 48 h after ABA treatment possibly because of response saturation at later time points. Similarly, no reduction was evident in the *PDF1.2* transcript levels after MJ treatment in the *35S*:*AtMYC2* plants relative to the treated wild-type plants (data not shown).

AtMYC2 Function Is Dispensable for Suppression of *PDF1.2* by Exogenous ABA

Although the data presented here strongly suggest that AtMYC2 is a negative regulator of plant defense gene expression, the exact location where AtMYC2 mediates an interaction between ABA and JA-ethylene signaling pathways is not clear. It is

possible that AtMYC2, acting downstream from ABA, directly mediates the antagonistic interaction between these two pathways. If this were the case, then one would expect that *PDF1.2* could not be suppressed or attenuated by exogenous ABA in the *jin1-9/myc2* mutant. To test this possibility, we treated the wild type and the *jin1-9/myc2* mutant with ABA and measured the transcript levels of *AtMYC2* and *PDF1.2* in treated plants. These experiments showed that exogenous ABA could still suppress the *PDF1.2* transcript accumulation in the *jin1-9/myc2* mutant plants (Figure 6). The extent of suppression (fold reduction in basal transcript abundance) of *PDF1.2* in wild-type plants 24 h after treatment was not significantly different from that in *jin1-9/myc2*. This result suggests that AtMYC2 function is dispensable for the antagonistic effect of ABA on the JA-ethylene defense pathway and that multiple control points may exist for cross talk between these pathways.

ABA Signaling Pathway Negatively Regulates Resistance to *F. oxysporum*

The antagonistic interaction between ABA and JA-ethylene signaling pathways could also influence resistance to the necrotrophic pathogens. To determine whether the increased levels of the JA-ethylene responsive defense gene expression

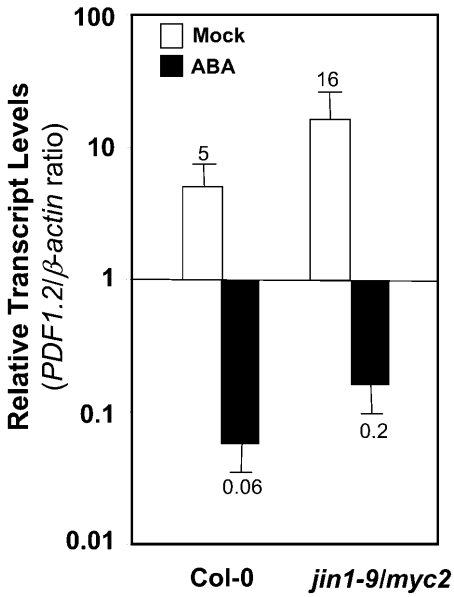

Figure 6. AtMYC2 Function Is Dispensable for Suppression of *PDF1.2* by Exogenous ABA.

Total RNA was isolated from mock- or ABA-treated wild-type and *jin1-9/myc2* plants 24 h after treatment, converted to cDNA, and subjected to RT-Q-PCR analysis. The *PDF1.2* transcript levels in mock- and ABA-treated wild-type and the *jin1-9/myc2* mutant were normalized to the expression of β-*actin* genes measured in the same samples (multiplied by 1000 for clarity). Each bar represents average data with error bars from two independent experiments. The numbers on each bar show relative transcript abundance of *PDF1.2* relative to the β-*actin* transcript levels measured in the same samples.

in *jin1-9/myc2* could provide enhanced pathogen resistance, we conducted inoculation experiments using the necrotrophic fungal pathogen *F. oxysporum*. This pathogen was chosen because it is known to influence the expression of *AtMYC2* and *PDF1.2* (this study) and is thought to be sensitive to JA-ethylene mediated defense (Berrocal-Lobo and Molina, 2004). The root dip inoculation assay in soil-grown plants by *F. oxysporum* is the preferred method to study infection by this soil-borne pathogen (Narasimhan et al., 2003). In inoculated plants, *F. oxysporum* penetrates through the roots and moves upward into the shoot via the vascular system. In three separate inoculation experiments, we inoculated the roots of 140 plants for each of the *jin1-9/myc2* and wild-type plants. As shown in Figure 7A, infection by this pathogen caused stunting of shoots and chlorosis, especially in the lower leaves of infected plants. Ten days after inoculation, the number of plants showing severe wilting symptoms and the total number of chlorotic leaves were counted to assess the extent of disease severity. The number of plants that showed a strong wilting symptom was significantly lower in the *jin1-9/myc2* mutant (29%) than in wild type-plants (81%; $P < 0.01$) (Figure 7B). We also noted that the total number of leaves that displayed chlorosis after inoculation was significantly higher in wild-type plants (181 leaves out of 140 plants) than in the *myc2* mutant (68 leaves out of 140 plants), which translated to ~2.7-fold reduction in disease development ($P < 0.01$) (Figure 7C). To further compare the level of disease tolerance between the wild type and the *jin1-9/myc2* mutant, we estimated fungal biomass by measuring the amount of fungal RNA in plant tissue by combining all 30 plants from the wild type and the *jin1-9/myc2* mutant from the inoculation experiment 3 by RT-Q-PCR, using primers specific for a transcribed spacer region of *F. oxysporum* rRNA. These analyses showed the presence of eightfold more fungal rRNA in wild-type plants than in the *jin1-9/myc2* mutant, further supporting the conclusion that the *jin1-9/myc2* mutant sustained significantly less fungal growth than the wild-type (Figure 7D).

We next tested whether enhanced defense gene expression observed in the *aba2-1* mutant leads to increased disease resistance; we inoculated wild-type and *aba2-1* plants with *F. oxysporum*. These inoculation experiments showed that the number of plants showing wilt symptoms at 10 d after inoculation was significantly reduced in *aba2-1* (58%) plants as compared with wild-type plants (80%; $P < 0.05$) (Figures 7E and 7F). This result further suggested that ABA deficiency could indeed positively affect disease resistance against *F. oxysporum*.

The Effect of *abi1-1* and *abi2-1* Mutations on *PDF1.2*

Because ABA itself and a positive regulator of ABA signaling AtMYC2, both appear to antagonize JA-ethylene responsive expression of defense genes and disease resistance, we explored other known mutations in ABA signaling for effects on *PDF1.2* expression. The *ABI1* and *ABI2* genes encoding homologous Ser/Thr protein phosphatases 2C act in a negative regulatory loop of the ABA signaling pathway (Merlot et al., 2001). ABA signaling mutants, *aba insensitive1* (*abi1-1*) and *abi2-1*, show a dominant-negative phenotype on ABA inhibition of seed germination (Gosti et al., 1999). Studies of genetic revertants of

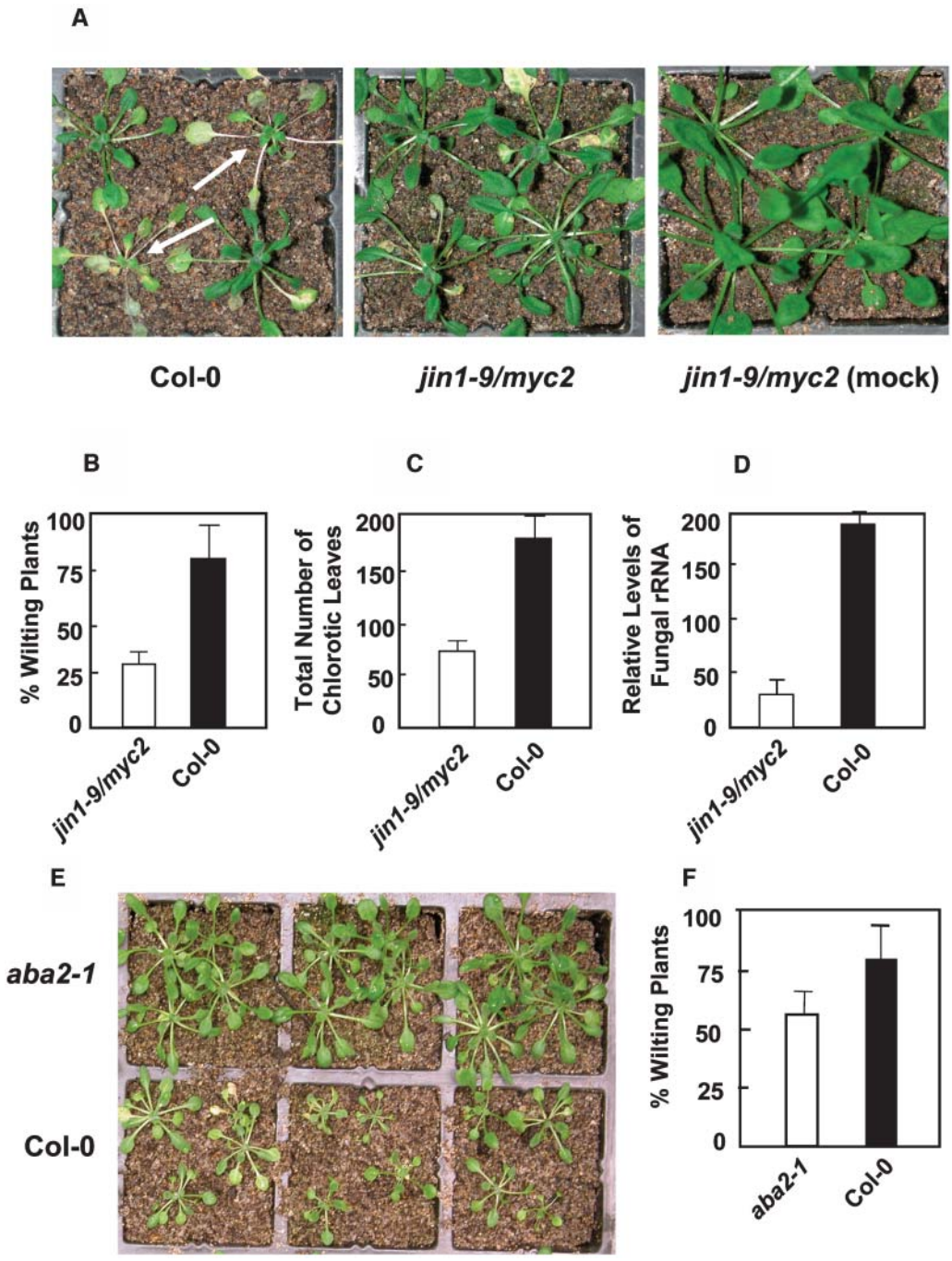

Figure 7. The *jin1-9/myc2* and *aba2-1* Mutants Show Enhanced Resistance to the Root-Infecting Fungal Pathogen *F. oxysporum*.

(A) to **(D)** In three separate experiments, 140 each of the 3-week-old wild-type (Col-0) and *myc2* plants were inoculated with *F. oxysporum* **(A)**. The percentage of plants showing strong wilting phenotype (arrows) **(B),** and the total numbers of necrotic leaves **(C)** were scored 10 d after inoculation. The amount of fungal RNA present in the inoculated tissue is estimated **(D)** using all 30 plants of each of the inoculated wild-type and *myc2* plants in experiment 3 (10 d after inoculation) by RT-Q-PCR with primers specific to *F. oxysporum* rRNA.

(E) and **(F)** In two separate experiments, 60 plants of each of the wild-type (Col-0) and *aba2-1* plants were inoculated with root-infecting fungal pathogen *F. oxysporum* **(E)**. The plants showing strong wilting symptoms were counted 10 d after inoculation to assess the disease severity **(F)**.

these mutant alleles, which show ABA hypersensitivity in seed germination assays, have lead to a proposed function for the *ABI1* and *ABI2* genes as negative regulators of ABA signaling (Gosti et al., 1999). Previous studies also showed that ABI1 inhibits both ABA-inducible and ABA-repressible gene expression when overexpressed transiently in maize mesophyll protoplasts (Sheen, 1996, 1998).

Therefore, we studied the *PDF1.2* transcript levels in mock- or MJ-treated ABA signaling mutants *abi1-1* and *abi2-1*. We observed a strong reduction at the *PDF1.2* transcript levels in untreated *abi1-1* and *abi2-1* mutants relative to the corresponding wild-type Landsberg *erecta* (L*er*) plants grown and sampled simultaneously. However, the response to MJ measured as the fold induction in the *abi1-1* and *abi2-1* mutants and the wild type were identical (Figure 8A). The regulation of *PDF1.2* expression observed in these mutants confirms that there is an interaction between ABA signaling and *PDF1.2* transcript accumulation. However, the effects observed on *PDF1.2* expression were not consistent with the expectation that the *abi1-1* and *abi2-1* mutants would have a dominant negative effect on ABA signaling and therefore may abolish ABA's inhibition of *PDF1.2* expression. Indeed, increased *PDF1.2* transcript levels were found in the *abi8* mutant (Brocard-Gifford et al., 2004). However, we found that ABA treatment could still suppress *PDF1.2* expression in both L*er* and *abi1-1* and *abi2-1* mutants, indicating that ABA antagonism on *PDF1.2* was still functional in these mutants (Figure 8A). ABA is known to act through multiple signaling pathways, and these may interact differentially with the JA and ethylene pathways. For example, AtMYC2, a key regulator of the JA response, and ABI1 are thought to be on separate branches (Lorenzo et al., 2004). Our

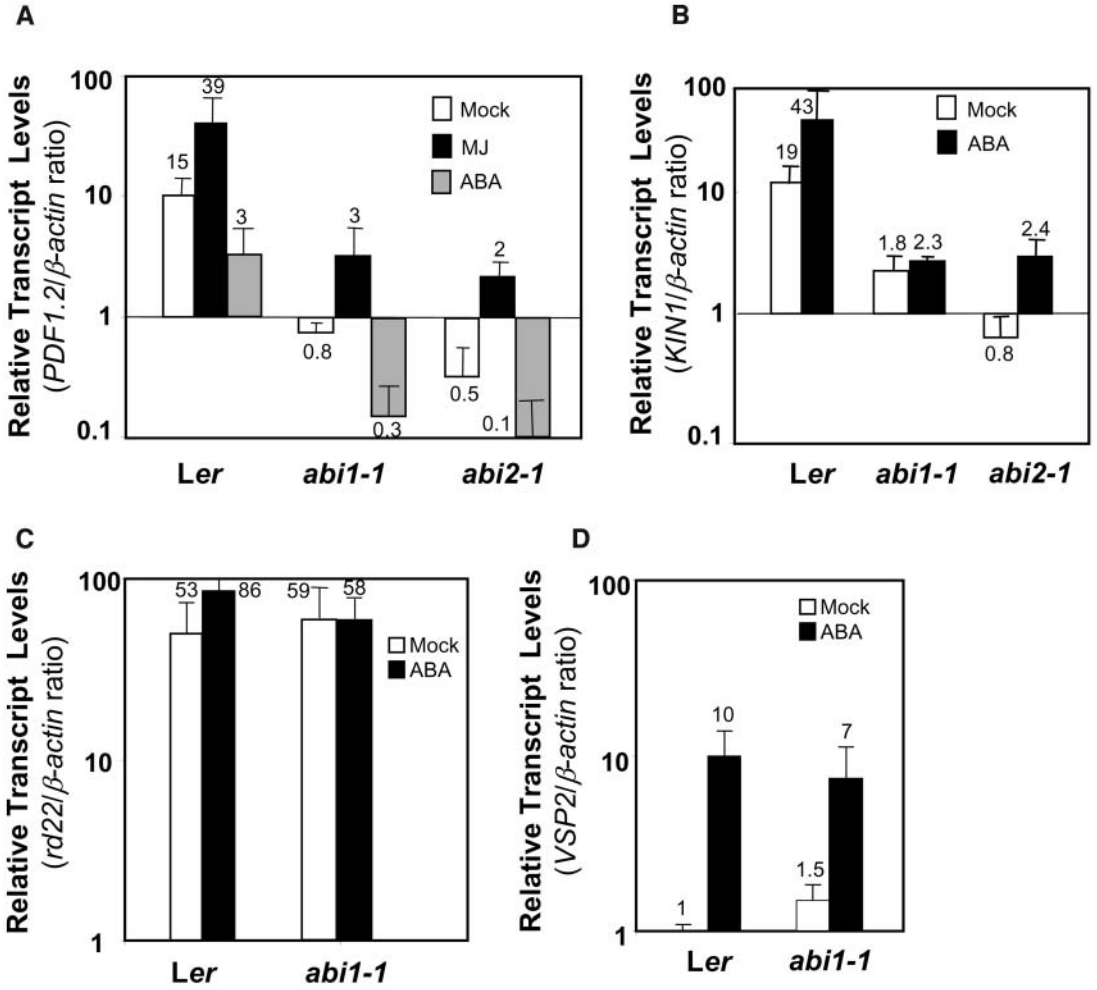

Figure 8. Analysis of Expression from ABA-Regulated Genes in *abi1-1* and *abi2-1* Mutants.

Quantification of the relative abundance of the *PDF1.2*, *KIN1*, *rd22*, and *VSP2* transcripts in mock- or MJ- and/or ABA-treated wild-type (L*er*) and the *abi1-1* and *abi2-1* mutants. Total RNA was isolated from plants 48 h after each treatment, converted to cDNA, and used as template in RT-Q-PCR assays. The *PDF1.2*, *KIN1*, *rd22*, and *VSP2* transcript levels were normalized to the expression of β-*actin* (multiplied by 1000 for clarity) measured in the same samples and expressed logarithmically. Each bar represents average data with error bars from two independent experiments. The numbers on each bar show transcript levels of *PDF1.2*, *KIN1*, *VSP2*, and *rd22* relative to the β-*actin* transcript levels measured in the same samples.

results are consistent with a dominant role for AtMYC2 in cross talk with the JA pathway when compared with that for ABI1, which appears to be more involved in regulating net expression levels of *PDF1.2* rather than MJ induction.

To further explore the pathways that *abi1-1* and *abi2-1* mutations affect, we measured the transcript levels of three ABA-responsive genes, *KIN1*, *rd22*, and *VEGETATIVE STORAGE PROTEIN2* (*VSP2*), in the same cDNA samples used to measure *PDF1.2* expression. We included *KIN1* encoding a cold- and ABA-inducible protein in these studies because ABI1 and ABI2 are proposed to be the regulators of the ABA-responsive element–dependent ABA signaling branch, which positively regulates *KIN1* expression (Goh et al., 2003; Chini et al., 2004). This branch of ABA signaling is thought to act independently from the ABA signaling pathway regulated by AtMYC2 (Shinozaki et al., 2003). Indeed, consistent with the dominant-negative phenotype predicted for *abi1-1* and *abi2-1*, *KIN1* transcript levels were dramatically reduced in mock-treated *abi1-1* and *abi2-1* plants relative to those in wild-type plants (Figure 8B). Although exogenous ABA treatment slightly induced *KIN1* transcript levels in the *abi2-1* mutant, the *KIN1* transcript levels were still significantly lower after ABA treatment than those in untreated wild-type plants and thus could have been undetectable using traditional RNA gel blot assays.

No significant differences were observed in *rd22* transcript levels between control plants of wild-type L*er* and *abi1-1* (Figure 8C) and *abi2-1* (data not shown) mutants. In addition, in contrast with the wild type, no significant increases were detected in *rd22* transcript levels in the *abi1-1* (Figure 8C) and *abi2-1* (data not shown) mutants treated with ABA. Overexpression of AtMYC2 increases *rd22* expression and ABA induction (Figure 5B; Abe et al., 2003), and Lorenzo et al. (2004) reported that AtMYC2 expression was inducible by ABA in the *abi1-1* mutant. Therefore, it is likely that *abi1-1* mutation acts in a branch downstream from AtMYC2 in influencing the ABA-insensitivity phenotype of *rd22* in this mutant.

In contrast with the effects on *KIN1* and *rd22* gene expression, *VSP2* transcript levels did not show any significant change in untreated and ABA-treated plants of L*er* and the *abi1-1* mutant, suggesting that expression of *VSP2*, which encodes an ABA- and MJ-inducible vegetative storage protein (Berger et al., 1996), is independent from *abi1-1* (Figure 8D). Taken together, these results are consistent with the earlier studies that multiple ABA signaling pathways may be operating in vegetative tissues of Arabidopsis and only some of these pathways are affected by the *abi1* and *abi2* mutations (Finkelstein, 1993).

Finally, to determine whether the reduced *PDF1.2* expression observed in *abi1-1* and *abi2-1* mutants might increase susceptibility to *F. oxysporum*, we conducted pathogen inoculation experiments on wild-type L*er* and *abi1-1* and *abi2-1* plants. However, inoculation of the *abi1-1* and *abi2-1* mutants with *F. oxysporum* did not reveal any altered (e.g., enhanced susceptibility) disease resistance as compared with inoculated wild-type plants. However, we noted that the L*er* ecotype is much more susceptible to *F. oxysporum* than Col-0, with plants dying more rapidly and at lower spore concentrations (data not shown); therefore, it may be difficult to evaluate an increased susceptibility phenotype in this genetic background.

Ethylene Signaling Antagonizes ABA Responsive Gene Expression in Vegetative Tissues

The interaction among JA, ethylene, and ABA signaling is just emerging from this and other recent studies (Boter et al., 2004; Lorenzo et al., 2004). However, there is a well established interaction between ethylene and ABA signaling in relation to the regulation of seed germination (Beaudoin et al., 2000; Ghassemian et al., 2000). The ethylene insensitive mutants *etr1-1*, *ein2-1/era3*, and *ein3* show increased sensitivity to ABA in germination assays (Beaudoin et al., 2000; Ghassemian et al., 2000; Yanagisawa et al., 2003) as well as reduced *PDF1.2* transcript levels (Penninckx et al., 1996) and increased pathogen susceptibility (Thomma et al., 1999; Berrocal-Lobo et al., 2002; Geraats et al., 2002). By contrast, the mutations in the *CTR1* gene, a negative regulator of ethylene signaling, constitutively activate the ethylene pathway while making seed germination less sensitive to ABA (Beaudoin et al., 2000; Ghassemian et al., 2000). However, it is possible that the antagonistic relationship between ethylene and ABA signaling may be different in vegetative tissues than in seeds (Fedoroff, 2002). To study the nature of interactions between JA-ethylene and ABA signaling pathways in vegetative tissues, we measured *KIN1*, *VSP2*, *rd22*, and *AtMYC2* transcript levels in mock- and ABA-treated wild-type, JA signaling mutant *jar1-1*, and ethylene signaling mutants *etr1-1*, *ein2-1/era3*, and *ein3-1*. The transcript levels of all four genes in mock-treated *etr1-1*, *ein2-1/era3*, and *ein3-1* mutants were significantly higher than those in wild-type plants (Figure 9A). In addition, we observed threefold to 4.5-fold higher expression from all four genes in the ethylene mutants after ABA treatment than in similarly treated wild-type plants. A typical result from these experiments is presented for *KIN1* in Figure 9B. In contrast with results obtained with ethylene-signaling mutants, the reduced JA sensitivity as conditioned by the *jar1-1* mutation did not show any effect on the ABA-responsive gene expression in these experiments (Figures 9A and 9B). Taken together, these results suggested that ethylene signaling acts antagonistically to ABA signaling in vegetative tissues.

To further test the interaction between ethylene and ABA signaling, we measured *ABI1* transcript levels in the *ein2-1/era3* mutant. The average results from three independent experiments showed that the *ABI1* transcript levels in the untreated *ein2-1/era3* mutant were approximately threefold lower than that observed in untreated wild-type plants (Figure 9C). This result suggests a putative link between reduced levels of negative regulators of ABA signaling and previously reported features of the *ein2-1/era3* mutant, namely, increased sensitivity to ABA (Beaudoin et al., 2000; Ghassemian et al., 2000), reduced *PDF1.2* expression (Penninckx et al., 1996; see also Figure 9D), and enhanced disease susceptibility (Thomma et al., 1999). This result is also consistent with the view that the interaction between the ABA and ethylene signaling pathway is mutually antagonistic in vegetative tissues in Arabidopsis.

The *PDF1.2* transcript levels measured in mock-, MJ-, and ethylene-treated wild-type and mutant plants were consistent with the known features of these mutants (Figure 9D), such that we observed slightly higher *PDF1.2* transcript levels in the MJ-treated *ein3-1* and *jar1-1* mutants than those in *ein2-1/era3*, but

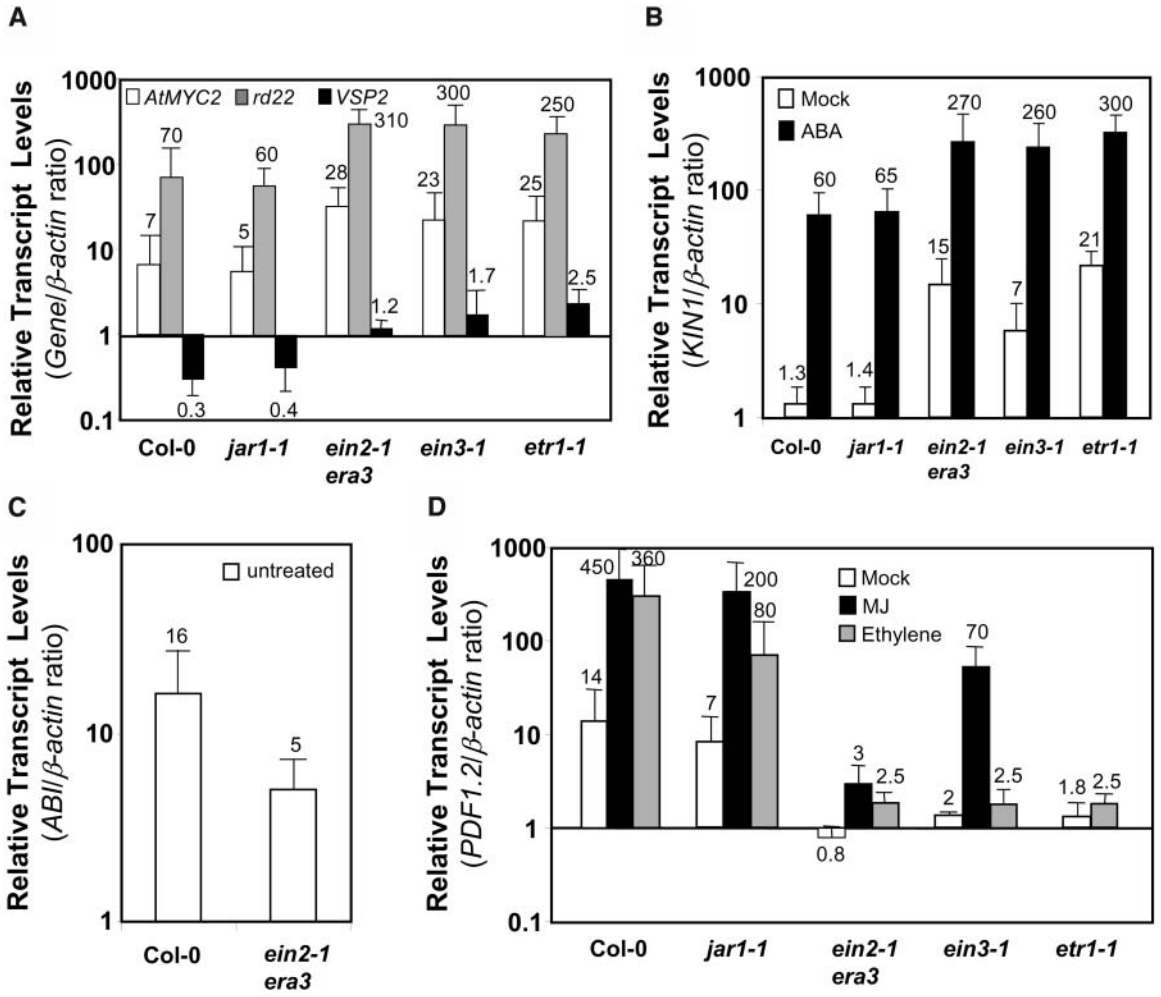

Figure 9. Ethylene Insensitivity Enhances ABA-Responsive Gene Expression in Vegetative Tissues.

Relative transcript levels of *KIN1*, *rd22*, *VSP2*, *ABI1*, and *PDF1.2* in mock- and/or ABA-, MJ-, and/or ethylene-treated wild-type and *jar1-1*, *ein2-1*, *etr1-1*, and *ein3-1* mutant plants. Total RNA was isolated from plants 48 h after each treatment, converted to cDNA, and used as template in RT-Q-PCR assays. The *KIN1*, *rd22*, *VSP2*, *PDF1.2*, and *ABI1* transcript levels were normalized to the expression of β-*actin* (multiplied by 1000 for clarity) measured in the same samples and expressed logarithmically. Each bar represents average data with error bars from two independent experiments. The numbers on each bar show relative transcript levels of each gene relative to the β-*actin* transcript levels measured in the same samples.

all were lower than those in wild-type plants. The partial MJ induction of *PDF1.2* in these mutants detected by RT-Q-PCR is possibly because of the leaky nature of these mutants (e.g., Alonso et al., 2003b).

A Complex Interplay among ABA, JA, and Ethylene Signaling Pathways Regulates the Expression of Diverse Stress Response Gene Classes

It is evident from the evidence presented so far that ABA, JA, and ethylene signaling pathways interact to regulate diverse stress responses. The nature of the interaction between these pathways appears to depend on the type of stress experienced by the plant. To further resolve these signaling interactions, we conducted independent experiments to examine transcript levels of the *VSP2*, *rd22*, and *PDF1.2* genes as markers for wound, drought, and biotic stress responses, respectively, after treatments with either MJ-, ABA-, ethylene-, ABA and MJ-, or ABA and ethylene-treated wild-type plants. Overall, expression detected from *PDF1.2* was consistent with the known MJ, ethylene, and ABA regulated expression patterns of this gene (Figure 10). However, as reported previously (Manners et al., 1998), the MJ-induced expression of *PDF1.2* was significantly stronger than ethylene induced expression of *PDF1.2. rd22* was induced by ABA but strongly suppressed by ethylene (Figure 10). Also, as expected, MJ and ABA induced *VSP2*, whereas ethylene suppressed expression of this gene relative to that in a mock-treated plant (Figure 10).

We also examined the expressions from these genes in wild-type plants treated with either ABA and MJ, or ABA and ethylene.

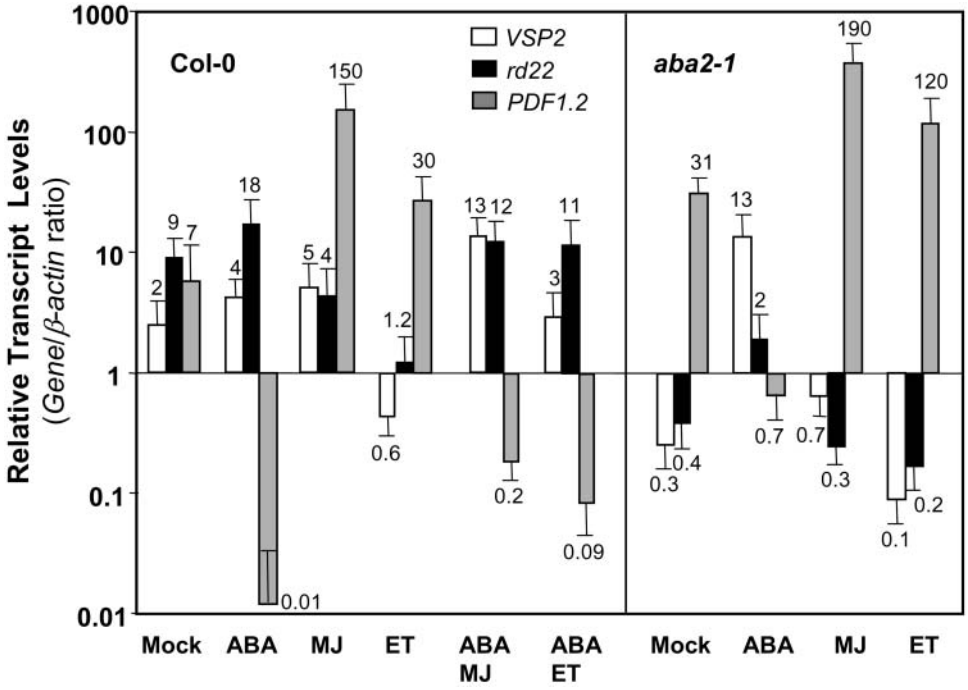

Figure 10. ABA, JA, and Ethylene Signaling Pathways Interact for Regulation of Stress Responsive Gene Expression in Arabidopsis.

Relative transcript levels of *VSP2*, *rd22*, and *PDF1.2* in mock-, ABA-, MJ-, ethylene-, or combination of ABA-MJ and ABA-ethylene (Col-0 only)-treated wild-type and *aba2-1* mutant plants. Total RNA was isolated from plants 48 h after each treatment, converted to cDNA, and used as template in RT-Q-PCR assays. The *VSP2*, *rd22*, and *PDF1.2* transcript levels were normalized to the expression of β-*actin* (multiplied by 1000 for clarity) measured in the same samples and expressed logarithmically. Average data from two independent experiments with error bars are presented. The numbers on each bar show transcript levels of each gene relative to the β-*actin* transcript levels measured in the same samples.

As expected, neither MJ nor ethylene was able to induce *PDF1.2* when applied together with ABA. In addition, ABA was also the dominant signal for the induction of *rd22* as this gene was still slightly inducible by ABA in the presence of MJ or ethylene. Furthermore, the combined treatment of MJ with ABA induced *VSP2* expression more strongly than either of the individual MJ or ABA treatments. The induction of *VSP2* after ABA-ethylene cotreatment was lower than that after ABA treatment but higher than that in mock-treated plants (Figure 10).

Finally, we measured the expression of *PDF1.2*, *rd22*, and *VSP2* in mock-, ABA-, MJ-, and ethylene-treated *aba2-1* mutant. In these independent experiments, the expression of *PDF1.2* was consistent with those reported earlier in Figure 2B, although differences in the actual magnitude of *aba2-1*/wild-type expression ratios of *PDF1.2* were observed. These could be because of differences in plant growth and development between the experiments and also sensitivity of the RT-Q-PCR technique used to measure transcript levels. As expected, in mock-treated *aba2-1* plants, the *rd22* transcript levels were lower than those in mock-treated wild-type plants. In addition, induction of *rd22* in this mutant by ABA was less, whereas suppression by MJ and ethylene were only slightly more than those in mock-treated *aba2-1* plants. Analysis of the *VSP2* transcript levels in the mock-treated *aba2-1* mutant showed reduced *VSP2* transcript levels relative to the mock-treated wild-type plants. Similarly, the *VSP2* transcript levels were lower in the MJ-treated *aba2-1* mutant than that in MJ-treated wild-type plants. Furthermore, the suppression of *VSP2* by ethylene was stronger in the *aba2-1* mutant than in wild-type plants. This suggests that ABA is required for full induction of *VSP2* by MJ and also attenuation of suppression of this gene by ethylene. Here, we did not examine the antagonistic interaction between JA and ethylene signaling pathways in regulating *VSP2* because expression of this gene was previously reported to be higher in untreated and MJ-treated ethylene signaling mutants than similarly treated wild-type plants (Rojo et al., 1999).

Collectively, the results from treated wild-type as well as those from treated and untreated ethylene, JA, and ABA signaling mutants suggest the presence of a complex interplay among these three signaling pathways. The simple models presented in Figure 11 summarize the signaling interactions among JA, ethylene, and ABA pathways in regulating the wound-, dehydration-, and pathogen-responsive gene expression in Arabidopsis.

DISCUSSION

ABA Antagonizes Defense Gene Expression and Disease Resistance in Arabidopsis

In this article, we studied the effect of ABA and various components of ABA signaling pathway on JA-ethylene responsive

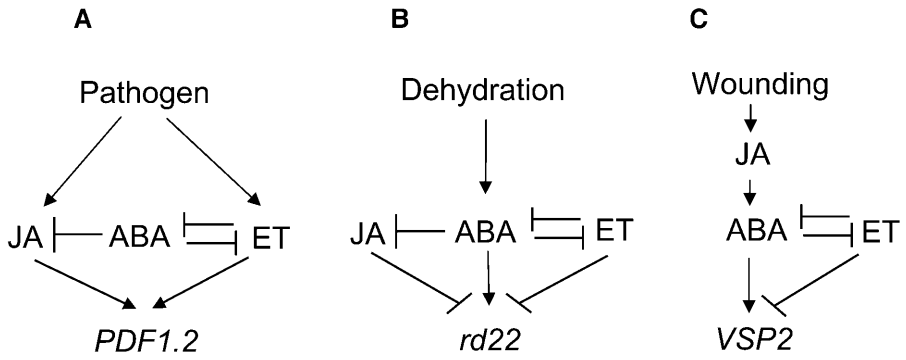

Figure 11. Proposed Model of Genetic Interactions among ABA, JA, and Ethylene Signaling Pathways for Modulation of Stress-Responsive Gene Expression in Arabidopsis.

Arrows indicate positive regulation, and blunt ends indicate negative regulation.

defense gene expression and disease resistance in Arabidopsis. We first demonstrated that endogenous and exogenous ABA strongly reduced the transcript levels of JA-ethylene responsive defense genes. In addition, exogenous MJ or ethylene was not able to reverse the suppression caused by the ABA treatment in wild-type plants. Second, we showed that AtMYC2, a positive regulator of ABA signaling, negatively regulated expression from *PDF1.2*. Finally, we observed that both *jin1-9/myc2* and *aba2-1* mutants with substantially higher transcript levels of JA-ethylene regulated defense genes showed enhanced resistance to the necrotrophic fungal pathogen *F. oxysporum*. Taken together, these data strongly suggest a novel role for ABA in modulating defense gene expression and disease resistance in Arabidopsis.

Previously, there have been several indirect observations linking ABA with disease resistance in plants. For instance, exogenously supplied ABA increases the susceptibility of various plant species to fungal pathogens (Henfling et al., 1980; Ward et al., 1989; McDonald and Cahill, 1999). ABA suppresses Phe-ammonia-lyase transcript accumulation after inoculation of soybean (*Glycine max*) with the incompatible fungal pathogen *Phytophthora megasperma* f. sp *glycinea* (Ward et al., 1989). ABA also downregulates the β-1,3-glucanase transcript levels in tobacco (*Nicotiana tabacum*) cell cultures (Rezzonico et al., 1998). One recent study indicated that a tomato mutant with reduced ABA levels showed enhanced resistance to the necrotrophic pathogen *B. cinerea*, whereas exogenous application of ABA restored the susceptibility to this pathogen in the mutant plants (Audenaert et al., 2002). Another recent study in tomato showed that ABA-deficient plants were more resistant to infection by the bacterial pathogen *P. syringae* pv *tomato* (*Pst*) (Thaler and Bostock, 2004). Finally, Mohr and Cahill (2003) demonstrated that the treatment of wild-type Arabidopsis plants with ABA or water stress increases the susceptibility to *Pst* and *P. parasitica*, whereas the ABA-deficient Arabidopsis mutant *aba1-1* showed reduced susceptibility to virulent isolates of *P. parasitica*.

The antagonistic interaction between biotic and abiotic stress responses has also been observed in other plant species, such as rice (Xiong and Yang, 2003). By contrast, other recent studies (Park et al., 2001; Mengiste et al., 2003; Chini et al., 2004) suggest that biotic and abiotic stress responses might also share common components. This further indicates the complexity of interplay among various signaling pathways during stress adaptation. The antagonistic interaction between the ABA and the JA-ethylene signaling pathways in regulating defense gene expression might be a strategy that plants employ to avoid simultaneous production of abiotic stress-related and biotic defensive proteins. Interestingly, our data also show that defense gene suppression mediated by ABA cannot be reversed by exogenous application of MJ and ethylene. This is consistent with the view that ABA action is a dominant process. One of the possible reasons for this may be that water stress affects plant survival in a more systemic and dramatic way than localized pathogen stresses, and plants have developed strategies to prioritize between these two stress responses. One would think that the antagonistic interaction between these two signaling pathways would compromise a plant's ability to tolerate both stresses should they occur simultaneously. However, simultaneous drought and necrotrophic pathogen attack may not happen very frequently in nature because these pathogens require relatively humid conditions for successful infection and under such conditions, water stress would not pose a significant threat.

ABA's involvement in plant disease resistance, however, seems to be complex and dependent on the type of the pathogen used. More recently, β-amino-butryic acid–induced resistance against leaf infecting necrotrophic pathogens *Alternaria brassicicola* and *Plectosphaerella cucumerina* was found to be compromised in the ABA-deficient mutant *aba1-5* and ABA-insensitive mutant *abi4-1*. Furthermore, exogenous application of ABA induced callose accumulation and resistance to these two leaf-infecting pathogens in wild-type plants (Ton and Mauch-Mani, 2004). Callose accumulation, which has recently been shown to be associated with increased disease susceptibility in Arabidopsis (Nishimura et al., 2003), and to our knowledge, there has been no evidence that callose confers enhanced resistance to the soil-borne fungal pathogen *F. oxysporum*. By contrast, antimicrobial proteins, such as defensins, chitinases, and lectins, upregulated in the *jin1/myc2* and *aba2-1* mutants show strong inhibitory activities against *F. oxysporum* (Lay et al.,

2003; Di Pietro et al., 2003; Madrid et al., 2003 and references therein). In addition, the signaling pathways conferring resistance to *F. oxysporum*, *A. brassicicola*, and *P. cucumerina* appear to be different. Resistance to *F. oxysporum* is mediated by JA-ethylene–dependent defense responses (Berrocal-Lobo and Molina, 2004), whereas these responses were found not to be effective against *A. brassicicola* and *P. cucumerina* (Ton and Mauch-Mani, 2004).

Negative Regulation of Defense Gene Expression and Disease Resistance by ABA Signaling

The data presented in this article showed that AtMYC2, a positive regulator of ABA signaling has a negative regulatory effect on defense gene expression in Arabidopsis. Importantly, while this manuscript was in preparation, Lorenzo et al. (2004) have reported the map-based cloning of the *JAI1/JIN1* locus, which turned out to be identical to the *AtMYC2* gene characterized here. The root elongation in the *jin1/myc2* mutant shows a JA-insensitive phenotype (Berger et al., 1996; Lorenzo et al., 2004), whereas defense gene expression shows hypersensitivity to induction by JA in this mutant (Boter et al., 2004; Lorenzo et al., 2004; this study), suggesting that AtMYC2 differentially regulates different stress responses. Indeed, AtMYC2 also positively regulates the wound responsive gene *VSP2* (Boter et al., 2004; Lorenzo et al., 2004). However, consistent with its negative regulatory role on defense gene expression, we showed here that the inactivation of AtMYC2 leads to heightened resistance to the necrotrophic fungal pathogen *F. oxysporum.* Similarly, Lorenzo et al. (2004) have reported that mutations in the *JAI1/JIN1/AtMYC2* locus resulted in enhanced disease resistance against two other necrotrophic pathogens, further supporting the notion that AtMYC2 is a negative regulator of plant defense in Arabidopsis.

Currently, the exact mechanism by which AtMYC2 regulates defense gene expression is not known. Our results clearly showed that transient or stable overexpression of AtMYC2 leads to the suppression of transcription from *PDF1.2*. In addition, suppression on *PDF1.2* was abolished when AtMYC2 and ERF1 were coexpressed in Arabidopsis protoplasts. Although these data may suggest that AtMYC2 possibly acts upstream from ERF1 in regulating defense gene expression, inactivation of AtMYC2 in the *jin1/myc2* mutants does not lead to constitutive upregulation of *ERF1* (Lorenzo et al., 2004). Although more experimental evidence is needed to determine the relative positions of ERF1 and AtMYC2 in defense signaling pathways, we speculate that AtMYC2's effect on defense gene suppression might not involve direct promoter binding for the following reasons. First of all, constitutive increases in *PDF1.2* expression (e.g., in the *cev1* mutants) do not necessarily accompany increases in *ERF1* transcript levels (Brown et al., 2003), suggesting that *PDF1.2* regulation may also be controlled by posttranscriptional or posttranslational processes. Secondly, the ABA-mediated suppression of *PDF1.2* was not compromised in the *jin1-9/myc2* mutant. This is in contrast with the role expected from a direct transcriptional repressor. Third, it is well established that the suppressive effects of c-MYC as negative regulators in animals involve interaction with other positive regulators of the target gene expression, whereas transcriptional activation by c-MYC occurs through direct DNA binding (reviewed in Wanzel et al., 2003). The same may well be the case for AtMYC2 in positively and negatively regulating different JA-ethylene and ABA-dependent responses, respectively.

It is possible that ABA and its positive regulators, such as AtMYC2, interfere with the signaling pathway regulating defense gene expression at a point upstream from ERF1, which integrates signals from both JA and ethylene pathways (Lorenzo et al., 2003). Because both JA and ethylene pathways are concomitantly required for expression of *PDF1.2* (Penninckx et al., 1998), interference with either pathway would result in altered *PDF1.2* expression. Our results, which showed that exogenous ABA could still suppress *PDF1.2* expression in the *jin1-9/myc2* mutant, suggest that ABA possibly interacts with the JA-ethylene pathway at multiple nodes, at least one of which is AtMYC2 independent. In fact, the *jin1/myc2* mutant plants have reduced sensitivity to JA (i.e., less suppression of root elongation in response to JA) but do not display any altered growth in response to ethylene (Lorenzo et al., 2004). It is therefore likely that ABA interacts with JA and ethylene pathways separately and that AtMYC2 is required for interaction with the JA pathway only.

ABA's antagonistic effect on ethylene signaling may also occur upstream from ERF1 but independently from AtMYC2. EIN3, a positive regulator of ethylene signaling acting upstream from ERF1, might be a likely target for ABA in suppressing the ethylene-responsive defense gene expression. Indeed, recent evidence has suggested that glucose interferes with ethylene signaling by destabilizing the EIN3 protein, whereas ethylene enhances the stability of EIN3 (Yanagisawa et al., 2003). Glucose is also known to suppress *PDF1.2* expression (Cheng et al., 2002). Interestingly, suppression of *PDF1.2* transcription by glucose is abolished in the ABA-deficient *aba2-1* mutant, suggesting that ABA is required for suppression of *PDF1.2* expression by glucose (Cheng et al., 2002). It is therefore possible that ABA antagonizes ethylene signaling by interfering with the EIN3 function possibly through protein degradation. Indeed, ABA treatment induces expression from several F-box genes (Hoth et al., 2002), and recent studies showed that EIN3 is degraded, in the absence of ethylene, by protein ubiquitination (Guo and Ecker, 2003; Potuschak et al., 2003). Further research is needed to elucidate the actual mechanisms involved in antagonistic effect of ethylene on ABA signaling pathway.

ABA and Ethylene Signaling Are Mutually Antagonistic in Vegetative Tissues

An antagonistic effect of ethylene on ABA signaling has been previously shown during seed germination as *etr1-1*, *ein2-1*, and *ein3-1* mutants compromised in reception or positive regulation of ethylene signaling show hypersensitivity to ABA for inhibition of germination (Beaudoin et al., 2000; Ghassemian et al., 2000; Yanagisawa et al., 2003). Our results presented here further extend these previous findings by showing that ABA-ethylene interaction is mutually antagonistic in vegetative tissues. First, we found higher transcript levels of ABA-responsive genes *VSP2*, *rd22*, and *KIN1* as well as those of the positive regulator of ABA signaling *AtMYC2* in mutants compromised in positive regulation

of ethylene signaling. In addition, transcript levels of the negative regulator *ABI1* were significantly reduced in the *ein2/era3* mutant. Interestingly, mutations in the *CTR1* gene encoding a negative regulator of ethylene signaling is known to reduce ABA sensitivity during germination, possibly because of constitutive activation of ethylene signaling (Beaudoin et al., 2000; Ghassemian et al., 2000). Consistent with this, *AtMYC2* and *VSP2* expression were found to be reduced in the *ctr1-1* mutant (Van Zhong and Burns, 2003). Therefore, the wild-type CTR1 allele, while negatively regulating ethylene signaling, positively influences ABA signaling. Similarly, it is possible that ABI1 and ABI2, as negative regulators of ABA signaling, may act to reduce ethylene sensitivity, a function that is analogous to that of CTR1 on the ABA pathway. Our results also showed that the *ein2-1/era3* mutant shows reduced transcript levels of *ABI1* and *PDF1.2* and increased transcript levels of ABA-responsive genes. It is therefore possible that the antagonistic effect of ethylene on the ABA pathway may be exerted partly through the activation of negative regulators of ABA signaling, such as ABI1 and ABI2. Indeed, a recent study showed that ethylene strongly induces *ABI1* and *ABI2* within 1 to 2 h of treatment (De Paepe et al., 2004), supporting the view that the antagonistic effect of ethylene on ABA signaling may involve negative regulators of ABA signaling.

Antagonistic effects of ethylene on ABA signaling may also require suppression of positive regulators of ABA signaling, such as AtMYC2. Our results presented here as well as those from Van Zhong and Burns (2003) showed that both endogenous ethylene itself and the ethylene signaling pathway suppress *AtMYC2* and the AtMYC2-regulated genes *VSP2* and *rd22*. Therefore, ethylene signaling most likely regulates *VSP2*, *rd22*, and *PDF1.2* expression through concerted action of the positive regulator ERF1 and negative regulator AtMYC2. However, further research is required to determine the relative positions of ERF1 and AtMYC2 in these signaling pathways.

The reduced *ABI1* expression and increased levels of ABA-inducible gene expression observed in this study in the ethylene signaling mutants suggest that ethylene insensitivity has the potential to increase abiotic stress tolerance in plants. Interestingly, antisense inhibition of the *AtPP2CA* gene, encoding an ABI1-related negative regulator of ABA signaling, was known to accelerate cold acclimation in Arabidopsis by increasing expression from cold- and ABA-responsive genes (Tahtiharju and Palva, 2001). The mutually antagonistic interactions observed between ethylene and ABA pathways might also better explain the enhanced disease tolerance observed in the ABA mutants studied here. In light of our results, it is tempting to speculate that the increased disease susceptibility observed in ethylene signaling mutants (e.g., *ein2* and *ein3*) may be resulted from not only disruption of the ethylene pathway but simultaneous activation of antagonistic ABA pathways in these mutants.

Finally, although exogenous MJ appeared to have some inhibitory effect on *rd22*, no antagonism of the JA pathway on the ABA pathway was evident in the *jar1-1* mutant. In fact, JA and ABA are known to act synergistically in a COI1-dependent but JAR1-independent manner for inhibition of seed germination (Ellis and Turner, 2002).

In Figure 11, we present three simple models to explain the signaling interactions among ABA, JA, and ethylene signaling pathways in regulating pathogen-, wound-, and dehydration-responsive gene expression. These models propose that in the absence of stress, the antagonistic interactions among signaling pathways help maintain low levels of expression from stress-responsive genes. Depending on the stress conditions experienced by the plant, however, one signaling pathway may become dominant over others. Consequently, a specific subset of stress-responsive genes is induced through activation of positive regulators of gene expression but also simultaneous suppression of negative regulators. For instance, induction of *rd22* and suppression of *PDF1.2* by ABA may require simultaneous activation of positive regulators of *rd22* and suppression of the ethylene signaling pathway that positively and negatively regulates *PDF1.2* and *rd22*, respectively. Similarly, induction of *PDF1.2* by ethylene may require coordinated activation of positive regulators of *PDF1.2* (e.g., *ERF1*) as well as suppression of negative regulation (e.g., AtMYC2) from the ABA pathway. Such cross-communication among plant hormone signaling pathways is probably achieved in a remarkably coordinated manner during adaptation to stress and would certainly enhance the plant's ability to respond to the stress factors in the most appropriate manner. Future research may reveal additional points of interactions between these signaling pathways.

METHODS

Plant Inoculations and Treatments

Arabidopsis thaliana plants were grown in plastic trays containing 30 (5 × 5 cm) cells at 24/18°C day and night temperatures, respectively, under an 8-h photoperiod (180 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in a controlled growth facility. Seeds sown directly in soil were stratified at 4°C for 3 d before transfer to a growth chamber. Inoculation or treatment of plants was conducted at the 8 to 10 rosette leaf stage. Inoculations with *Fusarium oxysporum* were done as described before (Schenk et al., 2000, 2003; Campbell et al., 2003). All inoculation experiments were arranged in a completely randomized split-plot design on trays containing 30 (5 × 5 cm) cells (one to three plants in each cell). The disease symptoms were scored 10 d after inoculation either by counting the number of chlorotic leaves per plant, total numbers of plants showing strong wilt-symptoms, or both. Furthermore, to determine the amount of fungal mass in inoculated plant tissue, total RNA from the inoculated wild type and the *myc2* mutant (30 plants of each) was isolated, converted to cDNA using random hexamers, and analyzed by RT-Q-PCR analysis using a primer set (5′-CGCCAGAGGACCCCTAAAC-3′ and 5′-ATCGATGCCAGAACCAAGAGA-3′) specific to the 18S transcribed internal spacer of *F. oxysporum* (National Center for Biotechnology Information accession no. AY237110) that demonstrated little or no sequence similarity in Arabidopsis. This relative expression was normalized to the levels of plant β-*actin* mix (see the next section), which did not show any significant difference between the RNA samples isolated from inoculated wild-type or insertion line plants.

For chemical treatments of plants, a solution containing 5% MJ (Aldrich, Milwaukee, WI) dissolved in 100% ethanol was prepared. Then, 200 μL of this was applied to a cotton ball and enclosed with each tray to be treated, to give a final concentration of 0.1 μM of MJ per liter of air. The whole tray was then sealed by two layers of opaque plastic bags and secured with masking tape. The control solution consisted of 0.1% (w/v) ethanol only and was applied to the plants (8 to 10 leaf stage) in the same manner as the MJ solution. SA (Sigma, St. Louis, MO) was dissolved in 100% ethanol, and this stock was then diluted with water to a final concentration of 5 mM. Plants were evenly sprayed with this

solution before sealing as described for the MJ treatment. Control plants were sprayed evenly with 0.1% ethanol solution only. ABA (*cis-trans* isomer; Sigma) was dissolved to 20 mM in 100% ethanol. This stock solution was then diluted with water to a final concentration of 100 μM and sprayed evenly over the plants before sealing as described for the MJ treatment. Control plants were sprayed evenly with 0.1% (w/v) ethanol solution. Ethylene was applied to a final concentration of 200 ppm, and the plants were sealed as explained above. Untreated controls were subjected to the same conditions but without addition of the inducers. For water stress treatment, plants were left without watering for 24 h while controls grown simultaneously were watered as normally. No wilting symptom was apparent in plants exposed to water stress by the time of sampling for RNA isolations.

RT-Q-PCR

Leaf samples taken from ~30 to 50 plants at time points after inoculation or treatments were ground to powder under liquid nitrogen for extraction of total RNA using an SV RNA isolation kit (Promega, Madison, WI) according to the manufacturer's instructions. For reverse transcription, total RNA (5 μg) was added to 1 μL of oligo(dT)$_{23}$, gene specific or both primers (10 μM), and the volume adjusted to 9.25 μL with sterile Milli-Q (Millipore, Billerica, MA) water. The solution was incubated at 70°C for 5 min then immediately transferred to ice before addition of 5.25 μL of reverse transcriptase master mix containing 3 μL 5× buffer, 1.5 μL 0.1 M DTT, and 0.75 μL 10 mM dNTPs and 0.5 μL (200 units/μL) Superscript RT II reverse transcriptase (Invitrogen, Carlsbad, CA). The reaction was incubated at 42°C for 90 min before heat inactivation of reverse transcriptase by incubation at 70°C for 15 min. The cDNA was then diluted in a total volume of 300 μL with sterile Milli-Q water. A second aliquot of total RNA was treated similarly using water in place of reverse transcriptase and these samples used as minus reverse transcriptase (−RT) controls for quantification of any genomic DNA contamination or nonspecific DNA amplification. Amplification of specific regions of targeted genes and real-time detection of amplicon production were undertaken in an ABI model 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA). RT-Q-PCR reactions contained 2.8 μL of primer mix (containing 1.5 μM of each forward and reverse primer), 2 μL cDNA template (derived from 6.6 ng/μL total RNA), 10 μL 2× SYBR-green master PCR mix (Perkin-Elmer Applied Biosystems), and 5.2 μL water to make a total volume of 20 μL. The following RT-Q-PCR primer sequences were used: for β-*actin* mix (β-*actin-2* At3a18780, β-*actin*-7 At5g09810, and β-*actin-8* At1g49240), a universal forward 5′-AGTGGTCGTACAACCGGTATTGT-3′ and individual reverse primers 5′-GATGGCATGAGGAAGAGAGAAAC-3′, 5′-GAGGAAGAGCATACCCCTCGTA-3′, and 5′-GAGGATAGCATGTGGAAGTGAGAA-3′ for β-*actin-2*, β-*actin-7*, and β-*actin-8*, respectively; for *PDF1.2* (At5g44420), forward 5′-TTTGCTGCTTTCGACGCAC-3′ and reverse 5′-CGCAAACCCCTGACCATG-3′; for *PR4* (At3g04720), forward 5′-TGCTACATCCAAATCCAAGCCT-3′ and reverse 5′-CGGCAAGTGTTTAAGGGTGAAG-3′; for *AtMYC2* (At1g32640), forward 5′-TCATACGACGGTTGCCAGAA-3′ and reverse 5′-AGCAACGTTTACAAGCTTTGATTG-3′; for *ERF1* (At1g27730), forward 5′-CGAGAAGCTCGGGTGGTAGT-3′ and reverse 5′-GCCGTGCATCCTTTTCC-3′; for *LEC* (At3g15356), forward 5′-GTTTCGTCTCTGGGTCATGGA-3′ and reverse 5′-GCAGCAACTTGTTATTCCTTGGA-3′; for *CHI* (At3g12500), forward 5′-ATCAGCGCTGCAAAGTCCTTC-3′ and reverse 5′-GTGCTGTAGCCCATCCACCTG-3′; *rd22* (At5g25610), forward 5′-CTGTTTCCACTGAGGTGGCTAAG-3′ and reverse 5′-TGGCAGTAGAACACCGCGA-3′; for *VSP2* (At5g24770), forward 5′-TCAGTGACCGTTGGAAGTTGTG-3′ and reverse 5′-GTTCGAACCATTAGGCTTCAATATG-3′; for *ABI1* (At4g26080), forward 5′-CGGCAAAACTGCACTTCCAT-3′ and reverse 5′-CACGAGCTCCATTCCACTGAA-3′; for *KIN1* (At5g15960), forward 5′-GCTGGCAAAGCTGAGGAGAA-3′ and reverse 5′-TTCCCGCCTGTTGTGCTC-3′; for β-*tubulin*, forward 5′-CGATTCCGTTCTCGATGTTGT-3′ and reverse 5′-AATGAGTGACACACTTGGAATCCTT-3′. Duplicate or triplicate assays were performed on all occasions using cDNA samples derived from two sets of independently grown plants for each experiment. Thermal cycling conditions consisted of 10 min at 95°C and 45 cycles of 15 s at 95°C and 1 min at 60°C before 2 min at 25°C. RT-Q-PCR reactions with minus RT controls as template were performed to correct for DNA contamination or amplification of nonspecific products. Controls with no added template were conducted for each primer pair to ensure primer dimer was not interfering with amplification detection. RT-Q-PCR results were captured and analyzed using the sequence detection software SDS version 1.7 (Perkin-Elmer Applied Biosystems). The SYBR green fluorescent signal was standardized to a passive reference dye (ROX) included in the SYBR green PCR master mix. The software generated plots of SYBR fluorescence (ΔRn) after successive cycles. The fluorescence threshold was set midway within the phase showing exponential rate of amplicon production. The cycle number at which the fluorescence passed the cycle threshold (C_T) for each reaction was calculated from this graph and exported to Microsoft Excel for further analysis. Amplification from a cDNA template was used for data analysis when fluorescence from plus RT reactions crossed the threshold level at or greater than five cycles before the minus RT (i.e., −RT is less than 1/32 of +RT). Relative expression levels in each cDNA sample were obtained by normalization to the reference gene either β-*tubulin* or β-*actin*, which produced similar results (Campbell et al., 2003; Schenk et al., 2003; J. Anderson and K. Kazan, unpublished data). The level of transcript abundance relative to reference gene (termed ΔC_T) was determined by subtraction of the C_T for reference gene from the candidate gene C_T according to the function $\Delta C_T = C_T(\text{test gene}) - C_T(\text{reference gene})$. To compare untreated and treated expression levels, the function $\Delta\Delta C_T$ was first determined using the equation $\Delta\Delta C_T = \Delta C_T(\text{treatment}) - \Delta C_T(\text{control})$ where control represents mock-treated plants. The induction ratio of treated/control was then calculated by the formula $2^{-\Delta\Delta Ct}$ in accordance with ABI Model 7700 sequence detection system user bulletin 2. After real-time PCR, amplification products for each primer set were subjected to melt-curve analysis to ensure that the fluorescence resulted from a single PCR product and did not represent primer dimer or nonspecific products. This was conducted by measuring fluorescence over a 20-min thermal gradient from 60 to 95°C and data analysis using the Dissociation Curves 1.0 software (Perkin-Elmer Applied Biosystems).

Plasmid Construction and Protoplast Transformation

Arabidopsis ecotype Col-0 plants were grown in tissue culture on MS medium for 2 weeks after germination under constant diffuse light. Leaves from 30 plants were cut into segments ~2 mm^2 and suspended in 500 mM mannitol. Protoplasts were produced from leaf segments and transformations performed according to Weigel and Glazebrook (2001). Briefly, protoplasts were assessed for viability by staining with fluorescein diacetate (0.5% FDA in W5) and subsequent observation using an ultraviolet microscope (Leica MZ6 stereomicroscope; Leica Microscopy and Scientific Instruments, Heerbrug, Switzerland). Viable protoplast concentration was calculated using a haemocytometer. Approximately 1.4×10^6 protoplasts were used for each transformation. All transformations used 20 μg of the *35S*:*GFP* construct as an indicator of transformation efficiency. The construct for overexpression of AtMYC2 was prepared by *Not*I digestion of the pJIT163 vector (Hellens et al., 2000) and ligation of the band corresponding to the double *35S* promoter/terminator cassette into the pGreenII vector (Hellens et al., 2000) previously linearized by digestion with *Hin*dIII, blunt ended using Klenow enzyme, and dephosphorylated using shrimp alkaline phosphatase (Roche, Nutley, NJ). The genomic sequence of *AtMYC2* was PCR amplified using the primers 5′-ATGACTGATTACCGGCTACA-3′ and 5′-GACCCCATAACTTTCTAAACTTC-3′ and ligated into the *Hin*dIII-digested and

blunt-ended overexpression plasmid pKEN (Brown et al., 2003). The construct for overexpression of *ERF1* was based on the pBI221 vector (Clontech, Palo Alto, CA) for the 35S promoter of *Cauliflower mosaic virus* driven constitutive expression of the inserted gene and were individually cotransfected with the *35S*:*GFP* vector using 20 μg of each plasmid. Control transformations were conducted using the pBI221 and *35S*:*GFP* constructs together. All transformations were replicated twice with the control transformation replicated three times. After transformations, the cells were incubated for 24 h at room temperature and samples examined using a haemocytometer and an ultraviolet microscope (Leica MZ6 stereomicroscope) for the presence of GFP fluorescence. The percentage of cells transformed with GFP was calculated for each transformation and the remaining cell pellet used for RNA extraction using the SV RNA extraction kit (Promega). The *35S*:*AtMYC2* construct was also introduced into Arabidopsis plants using the floral dip transformation procedure.

GUS Activity Assays

Promoter activity was assessed by quantitative GUS assays of the *uidA* gene using 4-methyl-umbelliferyl-β-D-glucuronide (Sigma) as a substrate. The assay was performed according to the procedure given by Brown et al. (2003) adapted for use with microtitre plates and the Fluoroskan Ascent fluorometer (Labsystems, Helsinki, Finland).

Mutants and T-DNA Insertion Lines

All the mutant lines (i.e., *aba2-1* [CS156], *abi1-1* [CS22], and *abi2-1* [CS23] and *ein2-1* [CS8844], *ein3-1* [CS8052], *etr1-1* [CS8058], and *jar1-1* [CS8072]) were obtained from the ABRC (The Ohio State University, Columbus, OH). All the mutant and wild-type plants used were in Arabidopsis Col-0 background except *abi1-1* and *abi2-1* mutants, and the corresponding wild-types which were in L*er* background. The *PDF1.2promoter*-GUS transformed plants were in C24 background. The locations of the T-DNA insertion in the *jin1/myc2* (SALK_017005 and SALK_083483) mutants were verified by PCR amplification of genomic DNA with a forward primer (5′-GCGTGGACCGCTTGCTGCAACT-3′) specific to the T-DNA insertion and a reverse primer specific to the *AtMYC2* gene (5′-GATCTGATTCTCCGGCGGTTT-3′ or 5′-CGGCGAG-CTCGAGTTTCACTT-3′ for SALK_017005 and SALK_083483, respectively). The amplified fragment was then sequenced to identify the exact location of the insert within the gene. To identify lines that were homozygous for the insertion, a second PCR was done using two gene-specific primers (5′-TGGCGCTCGAGGCTCTTACATC-3′ and 5′-GAAAGTCAA-ACCGAGGCTTCTTCG-3′ or 5′-GATCTGATTCTCCGGCGGTTT-3′ and 5′-AATTATCCGGGTCGGGTTGTG-3′). Only the wild-type allele was amplified by this PCR, whereas no amplification product was detectable from plants homozygous for the insertion. Kanamycin segregation ratios of plants identified as either wild-type or heterozygous for the T-DNA insertion at the *AtMYC2* locus suggested the presence of single T-DNA insertions in both T-DNA lines.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY237110.

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