Ethylene Signaling Mediates a Maize Defense Response to Insect Herbivory

Antoine L. Harfouche,¹ Renuka Shivaji,¹ Russell Stocker,² Paul W. Williams,³ and Dawn S. Luthe¹

¹Department of Biochemistry and Molecular Biology, Box 9650, Mississippi State University, MS 39762, U.S.A.;

²Department of Mathematics and Statistics, Box 9715, Mississippi State University, MS 39762, U.S.A.; ³USDA, ARS, Corn Host Plant Resistant Unit, Box 5367, Mississippi State, MS 39762, U.S.A.

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The signaling pathways that enable plants to mount defenses against insect herbivores are known to be complex. It was previously demonstrated that the insect-resistant maize (*Zea mays* L.) genotype Mp708 accumulates a unique defense cysteine proteinase, Mir1-CP, in response to caterpillar feeding. In this study, the role of ethylene in insect defense in Mp708 and an insect-susceptible line Tx601 was explored. Ethylene synthesis was blocked with either cobalt chloride or aminoethoxyvinylglycine. Alternatively, ethylene perception was inhibited with 1-methylcyclopropene. Blocking ethylene synthesis and perception resulted in Mp708 plants that were more susceptible to caterpillar feeding. In addition, fall armyworm (*Spodoptera frugiperda*) larvae that fed on inhibitor-treated Mp708 plants had significantly higher growth rates than those reared on untreated plants. In contrast, these responses were not significantly altered in Tx601. The ethylene synthesis and perception inhibitors also reduced the accumulation of Mir1-CP and its transcript *mir1* in response to herbivory. These results indicate that ethylene is a component of the signal transduction pathway leading to defense against insect herbivory in the resistant genotype Mp708.

In their natural habitat, plants encounter multiple biotic and abiotic challenges simultaneously. Each environmental stress activates multiple signal transduction pathways to ensure an effective spatial and temporal defense response (Dempsey and Klessig 1999; Genoud and Métraux 1999; Pieterse and van Loon 1999; Ryan 2000). Therefore, plants must be able to identify and prioritize each signaling pathway to mount the most efficacious defense to minimize damage and preserve vegetative growth and reproductive success (Karban and Baldwin 1997). To protect themselves from pathogen and herbivore attack, plants use constitutive and induced defenses. These defenses can influence herbivore settling, feeding, oviposition, growth and development, fecundity, and fertility, either singly or together (Baldwin and Preston 1999).

A novel insect defense mechanism in maize (*Zea mays*) genotypes that have genetic resistance to feeding by fall armyworm (FAW, *Spodoptera frugiperda*) and a number of other Lepidopteran species has previously been identified (Pechan et al. 2000). In the field, these resistant lines (Mp704, Mp708)

exhibit less foliar damage than the susceptible line (Tx601) and caterpillars that feed on the resistant plants are significantly smaller than those that feed on the susceptible lines (Williams et al. 1989). In response to caterpillar feeding, a unique 33-kDa cysteine proteinase (Mir1-CP), encoded by *mir1*, accumulates in the whorls of resistant lines Mp704 and Mp708. Ectopic expression of *mir1* in Black Mexican Sweet cells indicated that Mir1-CP inhibited caterpillar growth by as much as 80% (Pechan et al. 1999, 2000). When caterpillars ingest Mir1-CP, it attacks their peritrophic matrix, a structure that surrounds the food bolus and protects the intestinal microvilli from toxins and mechanical damage (Pechan et al. 2002). These studies indicate how Mir1-CP affects the digestive system of the insect herbivore, but information about the signaling pathways involved in Mir1-CP accumulation is virtually nonexistent.

The plant hormone ethylene plays important roles in plant defense (Dong 1998; Guo and Ecker 2004; Kende 1993; Wang et al. 2002). Insect herbivory is known to promote an increase in jasmonic acid (JA) accumulation and ethylene emission (Argandona et al. 2001; Schmelz et al. 2003). One of the primary effects of ethylene is to alter the expression of various target genes. In some plants, ethylene increases transcript levels of defense-related genes, including chitinase and β -1,3 glucanase (Shinshi et al. 1995; Wu and Bradford 2003), peroxidase (Ishige et al. 1993), chalcone synthase (Ecker and Davis 1987), pathogenesis-related (*PR*) genes (Lawton et al. 1994), ripening-related genes (Zegzouti et al. 1999), ethylene biosynthesis genes, as well as cysteine proteinase genes (Cervantes et al. 1994; Jones et al. 1995). However, in *Nicotiana attenuata*, ethylene suppresses defense against *Manduca sexta* by lowering the putrescine amino transferase transcripts in the root and nicotine levels in the shoot (Winz and Baldwin 2001).

In addition to herbivory, ethylene is involved in pathogen defense and its effect varies dramatically, depending on the type of pathogen and plant species. For example, in soybean, mutants with reduced ethylene sensitivity produce less severe chlorotic symptoms when challenged with the virulent strains *Pseudomonas syringae* pv. *glycinea* and *Phytophthora sojae*, whereas virulent strains of the fungi *Septoria glycines* and *Rhizoctonia solani* cause more severe symptoms (Hoffman et al. 1999). Similarly, in *Arabidopsis*, the ethylene-insensitive mutant *ein2* develops only minimal disease symptoms as the result of enhanced disease tolerance when infected by virulent *P. syringae* pv. *tomato* or *Xanthomonas campestris* pv. *campestris* (Bent et al. 1992). Ethylene also plays a role in gene-for-gene resistance between tomato and *P. syringae* pv. *tomato*. Transcription of *Pti4*, which is regulated by the activity of the resistance gene *Pto*, is rapidly induced by ethylene, and this

Corresponding author: Dawn S. Luthe; E-mail: dsluthe@ra.msstate.edu; Telephone: +1.662.325.7733; Fax: +1.662.325.8664.

Current address of A. L. Harfouche: Center for Environmental Stress Physiology, 625 Agricultural Mall Drive, Purdue University, West Lafayette, IN 47907, U.S.A.

induction precedes the expression of *PR* genes (Gu et al. 2000). Other research showed that ethylene may also function independently or with salicylic acid and JA in the activation of the hypersensitive response, which triggers a long-lasting response known as systemic acquired resistance to provide immunity against subsequent infections caused by a broad spectrum of pathogens. *Arabidopsis* plants with defects in ethylene perception (*ein2*) or JA signaling (*coi1*) fail to induce a subset of *PR* gene expression, including the plant defensin gene *PDF1.2*, a basic chitinase (*PR-3*), and an acidic hevein-like protein (*PR-4*), resulting in enhanced susceptibility toward certain pathogens (Penninckx et al. 1998). One of the more intriguing aspects of the induction of *PDF1.2* is that it requires both intact JA and ethylene signaling, whereas the majority of other responses mediated by these hormones are specific to only one of the signals. Likewise, it has been shown that both ethylene and JA are required for protease inhibitor (pin) production in response to wounding in tomato (O'Donnell et al. 1996). The pathway of ethylene synthesis is well established in higher plants (Bleecker and Kende 2000). Ethylene is formed from methionine via *S*-adenosyl-L-methionine (AdoMet) and the cyclic nonprotein amino acid 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is formed from AdoMet by the action of ACC synthase (ACS), and the conversion of ACC to ethylene is carried out by ACC oxidase (ACO) (Kende 1993). After synthesis, ethylene is perceived and its signal transduced through cellular machinery to trigger specific biological responses. Ethylene is perceived by a family of membrane-localized receptors that are homologous to bacterial two-component histidine kinases involved in sensing environmental changes. In *Arabidopsis*, five ethylene receptors exist, ETR1, ETR2, ERS1, ERS2, and EIN4 (Chang et al. 1993; Hua and Meyerowitz 1998; Hua et al. 1995; Sakai et al. 1998).

The physiological effects of ethylene can be blocked by biosynthesis inhibitors or antagonists. Chemical compounds such as aminoethoxyvinylglycine (AVG) and cobalt chloride (CoCl₂) inhibit the synthesis of ethylene. The former blocks the rate-limiting step of the ethylene biosynthesis pathway, which is the conversion of AdoMet to ACC by inhibiting ACS (Amrhein and Wenker 1979), whereas the latter blocks the conversion of ACC to ethylene by inhibiting ACO (Lau and Yang 1976). For ethylene to have an effect, it must first bind the ethylene receptors on the cell surface. 1-Methylcyclopropane (1-MCP) is a compound that blocks ethylene signaling by irreversibly binding to these receptors (Sisler and Serek 1997).

Because mutants in the ethylene signaling pathway are not readily available in maize, resistant (Mp708) and susceptible (Tx601) maize plants were treated with chemicals that block ethylene synthesis and perception, and their effects on herbivory, insect growth, and the accumulation of Mir1-CP and *mir1* mRNA were determined. The results indicate that blocking the ethylene pathway makes the resistant plants significantly more susceptible to herbivory and retards the accumulation of the *mir1* transcript and Mir1-CP.

RESULTS

Ethylene inhibitors increase leaf damage and larval growth in response to larval feeding in resistant but not in susceptible maize genotypes.

Initial experiments were conducted to determine if ethylene was involved in the defense signaling pathway leading to Mir1-CP accumulation in the resistant maize genotype Mp708 in response to FAW feeding. Plants were treated with the ethylene-generating compound, ethephon (Laudert and Weiler 1998), or were incubated in a chamber with a ripening banana (Chao et al. 1999). Neither treatment induced the accumulation of Mir1-CP

(data not shown). Other exploratory experiments indicated that blocking ethylene perception with 1-MCP reduced the accumulation of Mir1-CP and resulted in greater leaf feeding damage. To extend these preliminary observations, a pharmacological approach was used to block ethylene synthesis or perception in the resistant (Mp708) and susceptible (Tx601) maize genotypes, and the subsequent plant response to FAW feeding was determined. Plants were pretreated for 24 h with the ethylene synthesis inhibitors AVG or CoCl₂, which inhibit ACS and ACO activity, respectively. Plants were then infested with FAW larvae, and the effects of the treatments on leaf damage and larval relative growth rates (RGR) were determined. When compared with water-treated controls, a 24-h pretreatment with AVG or CoCl₂ significantly increased the level of leaf damage after 8, 24, and 48 h of larval feeding in Mp708 ($P < 0.0001$; Fig. 1A). There was no significant difference between AVG- and CoCl₂-treated plants ($P = 0.8983$). In addition, there was no interaction between treatment versus time ($P = 0.6988$), which indicated that the difference between water-treated controls and AVG- and CoCl₂-treated plants remained constant over time. Tx601 plants showed an interaction with time when leaf damage was evaluated. Therefore, the differences between water-treated controls and AVG- and CoCl₂-treated plants depended on the time. There were no significant increases in leaf damage when Tx601 was treated with the same inhibitors of ethylene biosynthesis at 8 h ($P = 0.9996$, $P = 1.000$) or 48 h ($P = 0.9999$, $P = 0.9907$) (Fig. 1C). However, at 24 h there was a small increase in leaf damage in the CoCl₂-treated plants relative to the control ($P = 0.0309$; Fig. 1C), and there was a significant increase in leaf damage between the AVG-treated plants and the control ($P = 0.0004$; Fig. 1C). Although the standard error bars overlap at this timepoint, Tukey's statistical adjustment indicates that these differences are significant.

Plants were also pretreated for 24 h with the ethylene antagonist 1-MCP. At 24 and 48 h after larval feeding, leaves of Mp708 treated with 1-MCP exhibited more leaf damage than controls ($P < 0.0001$; Fig. 1B). There was no significant increase in leaf damage in Tx601 at any of the feeding times ($P = 0.0603$; Fig. 1D). The results obtained with Mp708 suggested that blocking ethylene synthesis or perception increased plant susceptibility to FAW feeding. However, the same treatments did not significantly alter the response to insect feeding in Tx601, except for the AVG and CoCl₂ treatments at 24 h.

In addition to using leaf damage as a measure of susceptibility, the effect of treated plants on larval growth was also analyzed. RGR instead of the final weight was used to measure insect performance, because it takes into account the variability in the initial larval weights. The RGR of larvae that fed on Mp708 leaves treated with AVG, CoCl₂, and 1-MCP were significantly higher than those of larvae reared on control plants for 8, 24, and 48 h ($P < 0.0001$; Fig. 2A and B). In Tx601 there were no significant differences in larval growth rate (RGR) when larvae were reared on plants pretreated with AVG or CoCl₂ ($P = 0.2709$; Fig. 2C) or 1-MCP ($P = 0.7747$; Fig. 2D).

Because it is possible that the AVG and CoCl₂ taken up by the plant could alter insect growth directly, their effect on insect growth in an artificial diet at the same concentrations used for plant treatments was tested. When FAW larvae were reared on artificial diet containing AVG and CoCl₂ for 48 h, there were no significant differences in RGR between larvae reared on diets containing the ethylene synthesis inhibitors and the control ($P = 0.8113$; Fig. 3). This indicates that the differences in larval growth rate observed in Mp708 treated with AVG and CoCl₂ were not due to the chemicals but to physiological changes within the plant. The results from Figures 1 and 2 indicate that blocking ethylene synthesis and perception in the re-

sistant maize genotype Mp708 impairs its resistance, because both leaf damage and larval performance increased when the plants were treated. Ethylene biosynthesis and perception are required for resistance signaling in resistant Mp708. However, in the susceptible genotype Tx601, blocking ethylene synthesis or perception appeared to have little or no effect on susceptibility except at 24 h, when leaf damage was slightly higher in plants pretreated with ethylene synthesis inhibitors.

Reduced accumulation of Mir1-CP protein in insect-damaged maize leaves treated with ethylene inhibitors.

Because previous work (Pechan et al. 2000) demonstrated that the unique defense cysteine proteinase Mir1-CP rapidly accumulated in the whorls of resistant plants in response to FAW herbivory, the following experiments were conducted to determine if blocking ethylene synthesis or perception inhibited the accumulation of Mir1-CP. If Mir1-CP is a defense agent, blocking its accumulation should render the plants susceptible to FAW feeding. Immunoblot analyses were conducted using the tissues collected from treated and control plants from the experiments described in Figures 1 and 2 that were fed on

by FAW for 0, 8, 24, and 48 h. In Mp708, Mir1-CP was not detected in control plants or plants treated with AVG or CoCl₂ at 0 h, but its abundance increased in control plants as feeding progressed from 8 to 48 h (Fig. 4A). Although Mir1-CP was present in AVG- and CoCl₂-pretreated plants after 8, 24, and 48 h of larval feeding, the amount that accumulated was considerably less than the control. Inhibiting ethylene perception by 1-MCP also reduced Mir1-CP accumulation after 8, 24, and 48 h of larval feeding, relative to the control plants (Fig. 4B). As previously demonstrated (Pechan et al. 2000), Mir1-CP did not accumulate in Tx601 under any condition (data not shown). Overall, our results indicated that ethylene inhibitors, whether of biosynthesis or perception, reduced Mir1-CP accumulation and suggested that both ethylene signaling and herbivory are required for accumulation of Mir1-CP in Mp708.

Inhibition of ethylene biosynthesis and perception reduces but does not prevent insect-induced *mir1* gene expression.

To determine if *mir1* RNA and protein levels were correlated, tissues from the same samples used for immunoblot analyses were used to determine the relative abundance of *mir1* RNA using reverse transcriptase-polymerase chain reac-

Fig. 1. Ethylene inhibitors increase leaf damage caused by insects in maize leaves. **A**, Whorls of resistant Mp708 genotype were cut at their base and were fed through cut stems in water solution (Control) or in water supplemented with aminoethoxyvinylglycine (AVG) (200 μ M) or cobalt chloride (CoCl₂) (200 μ M), for 24 h. **B**, Whorls of resistant Mp708 genotype were placed in a sealed Plexiglas chamber, and methylcyclopropane (1-MCP) (5 mg/liter) was applied as a gas. Control plants were enclosed in Plexiglas chambers without 1-MCP. **C**, Whorls of susceptible Tx601 genotype were cut at their base and were fed through cut stems in water solution (Control) or in water supplemented with AVG (200 μ M) or with CoCl₂ (200 μ M) for 24 h. **D**, Whorls of susceptible Tx601 genotype were placed in Plexiglas chamber, and 1-MCP (5 mg/l) was applied as a gas. Control plants were enclosed in Plexiglas chambers without 1-MCP. Following the 24-h chemical treatment, yellow-green leaf sections were excised, placed in petri plates, and infested with fall armyworm larvae. Larvae were reared on leaves for 0, 8, 24, and 48 h. Leaf damage was assessed by taking the difference between initial and final weight of the leaves after each timepoint of larval feeding. The effects of CoCl₂, AVG, and 1-MCP are expressed as leaf damage (means $\pm$ standard error). Different letters indicate significant difference ($P < 0.05$) between treatments at each timepoint according to Tukey's multiple range test.

tion (RT-PCR). RT-PCR experiments revealed that *mir1* transcript began to appear after 8 h of larval feeding and continued to increase over the next 24 to 48 h (Fig. 5A and B) in Mp708. The *mir1* transcript was not detected in treated or untreated leaves prior to insect feeding (data not shown). However, in the presence of inhibitors, *mir1* transcript accumulation in response to larval feeding was less than in controls. Consistent with the results of Mir1-CP protein accumulation, the ACS inhibitor AVG and ACO inhibitor CoCl₂ resulted in lower levels of *mir1* transcripts as compared with untreated controls (Fig. 5A). No accumulation of *mir1* transcripts in response to larval feeding was detected in treated or control Tx601 (Fig. 5A and B).

The ethylene antagonist 1-MCP also decreased *mir1* transcript expression in response to larval feeding in Mp708 after 8, 24, and 48 h. Accumulation of *mir1* transcripts in Tx601 was not detected in any of the treatments (Fig. 5B). The lower levels of *mir1* RNA in response to both ethylene biosynthesis and perception inhibitors suggests that ethylene plays a role in regulating the accumulation of *mir1* in response to herbivory.

Quantitative RT-PCR was used to more accurately assess the effect of herbivory on *mir1* mRNA levels in Mp708 pretreated with AVG, CoCl₂, and 1-MCP. RNA was extracted from the

same samples used for immunoblot and RT-PCR analyses. Because no *mir1* mRNA was detected in Tx601, these experiments focused on measuring its relative abundance in Mp708. The abundance of the *mir1* transcript at each timepoint was normalized to that of *18S rRNA*. A calibrator sample at timepoint 24 h of larval feeding was then used to normalize the data. When ethylene biosynthesis enzymes were inhibited by feeding plants with AVG and CoCl₂ (Fig. 6A), ratios of *mir1/18S rRNA* showed that the *mir1* gene was induced in response to insect feeding. *mir1* transcript levels increased approximately twofold between 8 and 24 h of larval feeding and remained stable at 48 h. When plants were pretreated with 1-MCP in sealed plexiglass chambers, a 1.5-fold induction was obtained between 8 and 24 h of larval feeding and a 4.5-fold induction was reached after 48 h. This may be due to ethylene accumulation in the sealed chamber in response to larval feeding or the formation of new ethylene receptors that allowed the cells to regain sensitivity to ethylene (Fig. 6B). As expected, a comparison between ethylene inhibitor-treated and untreated controls revealed that CoCl₂ reduced *mir1* transcript levels approximately twofold relative to the control at each timepoint (Fig. 6A). When Mp708 plants were pretreated with AVG prior to insect feeding, *mir1* transcript levels decreased approxi-

Fig. 2. Ethylene inhibitors influence larval performance during plant-insect interactions in maize. **A**, The relative growth rate (RGR) of fall armyworm (FAW) larvae was tested on resistant Mp708 untreated control and cobalt chloride (CoCl₂)- and aminoethoxyvinylglycine (AVG)-treated plants. **B**, The RGR of FAW larvae was tested on resistant Mp708 untreated control and methylcyclopropane (1-MCP)-treated plants. **C**, The RGR of FAW larvae was tested on susceptible Tx601 untreated control and CoCl₂- and AVG-treated plants. **D**, The RGR of FAW larvae was tested on susceptible Tx601 untreated control and 1-MCP-treated plants. Third instar larvae were placed simultaneously on whorl sections of each treatment. Larvae were weighed after 0, 8, 24, and 48 h of feeding. Larval performance was measured by the RGR (means $\pm$ standard error). Columns with different letters are significantly ($P < 0.05$) different according to Tukey's multiple range test.

mately threefold in the treated plants compared with the control at 8 h and approximately fivefold at 24 and 48 h (Fig. 6A). Blocking ethylene perception with 1-MCP also reduced *mir1* transcript levels approximately threefold when compared with the control at each timepoint (Fig. 6B). None of the treatments completely inhibited *mir1* expression.

DISCUSSION

Ethylene mediates the insect defense response of Mp708 maize inbred.

Ethylene has profound effects on plant growth, development, and defense against biotic and abiotic stresses. There are numerous responses to ethylene throughout plant defense, including accumulation of hydrogen peroxide (Moeder et al. 2002) and defense-related genes (Gu et al. 2000; Knoester et al. 1999; Stotz et al. 2000; Thomma et al. 1999). Attack by pathogens or insects or mechanical wounding can induce ethylene production (Argandona et al. 2001; Kahl et al. 2000; Kende 1993; Krupnick et al. 2000; Watanabe et al. 2001). In some cases, stress-induced ethylene leads to defense responses (Abeles et al. 1992); in other cases however, ethylene suppresses herbivore defense responses (Kahl et al. 2001; Winz and Baldwin 2000).

If ethylene plays a crucial role in plant defense mechanisms, one would predict that treatment of plants with exogenous ethylene would enhance resistance to subsequent challenge with insects and pathogens or, conversely, that treatment with ethylene inhibitors would adversely affect their resistance level. This has been demonstrated for a number of plant-insect and plant-pathogen interactions in the eudicots but not in maize (Campbell et al. 2003; Dong et al. 2004; El-Kazzaz et al. 1983; Esquerré-Tugayé et al. 1979; Hudgins and Franceschi 2004; Marte et al. 1993; Thomma et al. 1999). This study was conducted to test the hypothesis that ethylene is a component in the signal transduction pathway leading to Mir1-CP accumulation in the maize inbred Mp708 that is resistant to insect herbivory. Because ethylene-response mutants were not available, pharmacological methods were used to inhibit the synthesis and perception of the hormone. The effects of three inhibitors, each blocking different steps in ethylene synthesis and perception, on plant resistance, larval growth, *mir1* mRNA, and Mir1-CP protein levels were then evaluated. Treating plants with the ACS inhibitor AVG or the ACO inhibitor CoCl_2 increased the amount of feeding damage on the whorl tissue and increased the RGR of larvae reared on treated tissues. The treatments reduced but did not completely inhibit insect-induced accumulation of Mir1-CP and *mir1*. Likewise, inhibiting ethylene perception with 1-MCP had similar effects on herbivory, larval growth, and the accumulation of Mir1-CP and *mir1* transcripts. Taken together, it appears that, in the insect-resistant maize genotype Mp708, both ethylene synthesis and perception are required for the processes that result in insect defense. It does not appear to be the case for the susceptible genotype Tx601, and we do not know if it is true for all maize lines.

To determine if conserved promoter sequence elements capable of mediating the ethylene-responsive expression of *mir1* were present, the 5' region of the *mir1* promoter sequence (3,197 bp) was examined, using PlantCARE software (Lescot et al. 2002). This analysis identified two GCC boxes (GCCGCC) and two ethylene motifs (ATTTCAAA) in the upstream region of the gene. Ethylene-responsive elements are commonly found in the promoter region of the ethylene-inducible defense genes. Previous studies by Ohme-Takagi and associates (2000) indicated that the ethylene response element binding proteins respond to extracellular signals and interact with the GCC box to modulate ethylene-responsive gene ex-

pression. In another study, transcriptome analysis suggested that the ethylene response factor 1 was important in the regulation of ethylene- and jasmonate-dependent defense responses (Lorenzo et al. 2003). Further experimental evidence will be required to establish definite links between these putative promoter elements and their one or more roles in the regulation of *mir1* expression in response to an ethylene signal.

Although ethylene is a signal in insect-plant defense, it is still not known if it acts directly or in combination with other signaling molecules. Previous studies have demonstrated a link between jasmonate and ethylene in the induction or suppression of defense-related genes (Lorenzo and Solano 2005). In conifers, the methyl-jasmonate (MJ)-induced resistance response to stem pests was tied to increased ethylene production (Hudgins and Franceschi 2004). The same study indicated that JA strongly induced ethylene production in stems, which activated polyphenolic parenchyma (PP) cells, resulting in the production of defensive phenolic compounds. In this case, the ethylene response inhibitor 1-MCP also significantly reduced JA-induced PP cell activation.

It is possible that one or more additional signaling pathways interact with ethylene to regulate the insect defense response in Mp708, because inhibiting ethylene synthesis and perception did not completely eliminate *mir1* gene and Mir1-CP expression. This may be due to residual amounts of ethylene that were sufficient to trigger the induction of *mir1* and Mir1-CP. In the case of 1-MCP treatment, it is possible that residual *mir1* transcript and Mir1-CP accumulation could be due to the synthesis of new ethylene receptors that might resensitize the plant to ethylene (Schaller and Kieber 2002).

Ethylene production is also stimulated by wounding, herbivore and pathogen attack, and other biotic or abiotic stresses (Wang et al. 2002). JA has been reported to stimulate ethylene production in many plants, such as tomato, *Arabidopsis*, and tobacco (Xu et al. 1994; O'Donnell et al. 1996; Pennincks et al. 1998). It is believed that crosstalk between ethylene and JA pathways enables plants to optimize their defense strategies more efficiently and economically (Baldwin 1998). Two pieces of preliminary data lead us to speculate that JA and ethylene may interact to regulate *mir1* and Mir1-CP accumulation in re-

Fig. 3. Inhibitors of ethylene biosynthesis do not affect larval growth on artificial diet. One larva per cup was allowed to feed for 48 h. Single cups with artificial food supplemented with or without cobalt chloride (CoCl_2) (200 μM) and aminoethoxyvinylglycine (AVG) (200 μM) were randomly assigned positions in 48-well trays. Larval weight was measured at time zero and, after 48 h of larval feeding, relative growth rate (RGR) was assessed. The effects of CoCl_2 and AVG were expressed as the RGR of larvae (means $\pm$ standard error). Columns with the same letter are not significantly ($P < 0.05$) different according to Tukey's multiple range test.

sponse to herbivory in Mp708. First, blocking the octadecanoid pathway with 1 mM ibuprofen renders the plant more susceptible to caterpillar feeding (A. L. Harfouche and D. S. Luthe, *unpublished data*), suggesting that it is also needed to elicit the defense response. Second, measurement of JA levels in uninfested Mp708 plants indicated that they were approximately fivefold higher than those of Tx601 (R. Shivaji, J. Engelberth, and D. S. Luthe, *unpublished data*). If JA levels are constitutively higher in Mp708, this may result in higher endogenous ethylene levels in the plant, which would explain why treatment with exogenous ethylene did not increase resistance or Mir1-CP expression in Mp708. By blocking the ethylene pathway with inhibitors, we may suppress a constitutively expressed signaling pathway. If endogenous JA and ethylene levels are higher in Mp708, the plant may be ready to respond to caterpillar attack and rapidly accumulate Mir1-CP and other components of its defensive system in response to herbivory. Mp708 plants are small-statured and poor-yielding, which suggests that their insect-defense pathway is always “on,” thereby limiting productivity. In some respects, the Mp708 phenotype

is similar to that of the *Arabidopsis* *cev1* mutant that has constitutively high production of JA and ethylene (Ellis et al. 2002).

The results of this study, which implicate ethylene in the regulation of *mir1* gene expression in Mp708, are in agreement with those of O'Donnell and associates (1996), who showed that ethylene and JA are required in the transduction pathway leading from wounding to regulate proteinase inhibitor (*pin*) gene expression in tomato. The results contrast with those in *N. attenuata* (Kahl et al. 2000; Winz and Baldwin 2001), in which herbivore-induced ethylene suppressed a direct defense in tobacco and made it more susceptible to herbivory. Currently, we do not know if or how JA and ethylene interact to regulate *mir1* gene expression in Mp708. It is possible that ethylene is downstream from JA or that the two pathways function in parallel. It will be interesting to determine how ethylene and JA influence each other in response to herbivory in Mp708 and other maize genotypes. Thus a more complete picture of interaction between JA and ethylene in insect defense response in maize awaits the analysis of JA signaling.

Fig. 4. Ethylene inhibitors reduced the accumulation of Mir1-CP protein induced by insect attack in maize. **A**, Western blot analysis of Mir1-CP protein expression in leaves that were excised from resistant Mp708 plants treated with cobalt chloride and aminoethoxyvinylglycine or untreated controls. **B**, Western blot analysis of Mir1-CP protein expression in leaves that were excised from resistant Mp708 plants treated with methylcyclopropane or untreated controls. Total proteins were extracted after 0, 8, 24, and 48 h of larval feeding and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15 µg of protein per lane). Blots were treated with monoclonal antibodies against Mir1-CP and followed by a secondary incubation using goat anti-mouse horseradish peroxidase-conjugated serum. The signal was detected using SuperSignal west femto maximum sensitivity substrate. The positive control (PC) was a protein extract from Mp708 callus.

MATERIALS AND METHODS

Plant growth and FAW rearing.

Maize (*Zea mays* L.) genotypes used in this study were the resistant inbred Mp708 (Williams et al. 1990) and the susceptible inbred Tx601 (Williams et al. 1989). Plants were grown under greenhouse conditions at the Plant Science Research Center, Mississippi Agricultural and Forestry Experiment Station (Mississippi State University) until they were four to five weeks old and in the mid-whorl (V8) developmental stage (Ritchie et al. 1986). FAW were reared on artificial diet (Davis 1989) at the U.S. Department of Agriculture-Agricultural Research Service, Corn Host Plant Research Unit, Insect-Rearing

Laboratory. Unless otherwise noted, larvae used for infestations were 5 days posthatch or approximately third instar.

Ethylene response inhibitor treatments.

CoCl₂ and AVG were purchased from Sigma-Aldrich (St. Louis). 1-MCP commercially named EthylBloc (0.43% [wt/wt] 1-MCP) was purchased from Floralife (Walterboro, SC, U.S.A.). Plants were taken to the laboratory, cut at the base of their stems, and stem-fed in a solution containing 200 μM AVG or 200 μM CoCl₂ for a period of 24 h. The concentration of 200 μM was selected because it gave the maximum inhibition of Mir1-CP accumulation in pilot dose-response experiments. Untreated controls were placed in water and sub-

Fig. 5. Inhibiting ethylene biosynthesis and perception decreases *mir1* mRNA expression in plant-insect defense response in maize. **A**, Effects of inhibitors of ethylene biosynthesis on *mir1* gene expression were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) experiments. First-strand cDNA synthesis was prepared from total RNA extracted from a leaf segment approximately 1 cm distal from the site of insect damage from leaves collected after 0, 8, 24, and 48 h of larval feeding. Samples were taken from Mp708 and Tx601 plants that were untreated controls or cobalt chloride- and aminoethoxyvinylglycine-treated and were challenged with fall armyworm (FAW) larvae in petri plates. *18S rRNA* was used as a constitutively expressed control. **B**, Effects of an inhibitor of ethylene perception on *mir1* gene expression were analyzed by RT-PCR experiments. First-strand cDNA synthesis was prepared from total RNA extracted from a leaf segment approximately 1 cm distal from the site of insect damage from leaves collected after 0, 8, 24, and 48 h of larval feeding. Leaves were excised from Mp708 and Tx601 plants treated with methylcyclopropane (1-MCP) and challenged with FAW larvae in petri plates. Control plants were enclosed in Plexiglas chambers without 1-MCP. *18S rRNA* was used as a constitutively expressed control.

jected to the same conditions but without inhibitors. To block ethylene perception, maize plants were placed in sealed 11-liter Plexiglas chambers, and 1-MCP gas was generated by dissolving 450 mg of EthylBloc powder containing 0.14% 1-MCP in 9 ml of EthylBloc releasing buffer in a glass beaker placed inside the sealed Plexiglas chamber. This resulted in a 1-MCP concentration of 5 mg/l, which is approximately 10-fold greater than the recommended dosage. Control plants were subjected to the same conditions, but only the releasing buffer was present. In preliminary experiments, plants were sprayed with ethephon at concentrations of 1, 10, and 50 mM (Laudert et al. 1998) or were treated with a ripening banana (Chao et al. 1999) in the same Plexiglas chambers described above.

Fig. 6. Inhibitors of ethylene biosynthesis and perception decrease insect-responsive defense gene expression in maize. **A**, Analysis of *mir1* transcript level in untreated control and cobalt chloride- and aminoethoxyvinylglycine-treated Mp708 plants after 8, 24, and 48 h of larval feeding by real-time quantitative PCR (RT-Q-PCR). **B**, Analysis of *mir1* transcript level in untreated control and methylcyclopropane-treated Mp708 plants. Total RNA was isolated from leaves at each timepoint of larval feeding, was converted to cDNA, and was used as a template in RT-Q-PCR assays. *mir1* transcript levels were normalized to the expression of *18S rRNA* (multiplied by 100 for clarity), which appeared to be constant in our conditions. *18S rRNA* reference gene was used as an external control. Average data with error bars from two independent experiments ($n = 4$) are presented. The numbers on each bar show transcript levels of *mir1* gene relative to the *18S rRNA* transcript levels.

Insect infestation.

Following the 24-h inhibitor treatment, yellow-green leaf sections (approximately 4 cm wide) were excised and weighed at time zero and after each timepoint of larval feeding. Eight Leaf sections (eight per petri plate) were placed on a layer of moistened filter paper and were infested with FAW larvae (five per plate). The first set of treatments consisted of water, CoCl₂, and AVG. The second set of treatments consisted of the buffer control and 1-MCP. A single experiment consisted of the first and the second set of treatments. Each experiment (biological replicate) was conducted twice. In the first experiment, there were two petri plates (two replicates) per treatment. In the second experiment there were four petri plates (four replicates) per treatment.

Larval growth, leaf damage assays, and harvest of plants.

Larval growth was measured as RGR under the following conditions: $RGR = \frac{\text{final weight} - \text{initial weight}}{\text{average weight} \times \text{number of days}}$ (Waldbauer 1968). Leaf damage was measured as the weight difference between time zero and the other timepoint. Data were analyzed using the general linear model procedure of the SAS Institute (Cary, NC, U.S.A.). When indicated, Tukey's multiple range test was used to test for differences among treatment means (at $P = 0.05$). Tissues (1 cm of leaf tissue immediately adjacent to the wounding site caused by insect feeding) from leaves subjected to the three types of ethylene inhibitors and controls were harvested at 0, 8, 24, and 48 h following larval feeding, were immediately placed in liquid nitrogen, and were stored at -80°C for protein and RNA gene expression analyses.

FAW feeding on artificial diet.

For bioassays, FAW larvae were placed in 30-ml plastic insect diet cups (Bio-Serv, Frenchtown, NJ, U.S.A.). Each cup contained artificial diet (Davis 1989) supplemented with or without CoCl₂ (200 μM) and AVG (200 μM). Cups were randomly assigned positions in 48-well trays. Each cup contained one larva and feeding was for 48 h. The general linear model procedure of the SAS Institute under replicated and randomized conditions was used for statistical analyses. When indicated, Tukey's multiple range test was used to test for differences among treatment means (at $P = 0.05$). Each treatment was replicated 16 times, and the experiment was repeated twice.

Protein extraction and expression analysis.

Protein extracts were prepared from insect-damaged leaf tissues of untreated controls or treated plants with ethylene inhibitors. Frozen samples (200 mg) were ground to a powder in liquid N₂ using a mortar and pestle. Just as the liquid N₂ evaporated, 0.4 ml of sodium dodecyl sulfate (SDS) sample buffer (Laemmli 1970) was added and the tissues were further ground. After thawing, the homogenates were heated at 95°C for 5 min and then were briefly centrifuged at $12,000 \times g$ to pellet the cellular debris. The resulting supernatant was stored at -20°C until analyzed. Prior to electrophoresis, sample protein concentrations were measured using a protein quantification assay, RCDC (Bio-Rad Laboratories, Hercules, CA, U.S.A.), with bovine serum albumin as a standard. One-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 5% (wt/vol) stacking and 12% (wt/vol) resolving gel bis-acrylamide concentrations, using the buffer system of Laemmli (1970) in a Mini-Protein II electrophoresis apparatus (Bio-Rad Laboratories), with 15 μg of protein loaded per lane. The positive control for immunoblot analyses was protein extracted from Mp708 callus, which has been shown to constitutively overproduce Mir-CP (Jiang et al.

1995). Constant voltage of 50 V was applied for 1 h, followed by 150 V until the tracking dye (bromophenol blue) reached the bottom of the gel. For all immunoblots, a duplicate SDS-PAGE gel was run and stained with Coomassie brilliant blue R 250 to confirm that equal amounts of protein were loaded in each lane. Following SDS-PAGE, immunoblotting of proteins was performed as described previously (Pechan et al. 2000). Immunolabeling of blots was performed using monoclonal antibodies against Mir1-CP, prepared according to Goding (1980), and a secondary hybridization using goat anti-mouse horseradish peroxidase-conjugated serum. Signal was detected using SuperSignal west femto maximum sensitivity substrate (Pierce Biotechnology, Rockford, IL, U.S.A.). Two repetitions were conducted for each treatment, and one representative result is shown.

RNA extraction and expression analysis.

Total RNA was extracted from 100 mg of treated and untreated leaves using NucleoSpin RNA plant kit (Macherey-Nagel, Düren, Germany), with a DNase I treatment applied directly onto the silica membrane of the Macherey-Nagel column as outlined by the manufacturer. The RNA samples were quantified spectrophotometrically at 260 nm. The RNA quality was also checked by 1.0% agarose gel electrophoresis, stained with 0.5 µg of ethidium bromide per milliliter. Total RNA (100 ng) was reverse-transcribed using Ready-To-Go RT-PCR beads two-step protocol according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). To each bead reaction was added 100 ng of RNA, 0.5 µg of oligo dT₍₁₂₋₁₈₎ or 2.5 µg p(dN)₆ cDNA synthesis primers, 0.5 µM of *mir1* (National Center for Biotechnology Information accession number AF019145) gene-specific primers (*mir1F*, 5'-CGCGTTCCGGTTCGTCATC-3' and *mir1R*, 5'-CCTCCTAAGCAAGAACC-3'), 4 mM MgCl₂, and RT-PCR grade water (Ambion, Austin, TX, U.S.A.) to a final volume of 50 µl. Primers were designed with Primers Select software (Lasergene; DNASTAR, Madison, WI, U.S.A.) and were synthesized by Invitrogen (Invitrogen Corp. Carlsbad, CA, U.S.A.). For the first step, only oligo dT₍₁₂₋₁₈₎ or p(dN)₆ first-strand cDNA synthesis primer was added to the dissolved beads, and reaction was incubated on a heat block at 42°C for 30 min when oligo dT₍₁₂₋₁₈₎ was used or at 20°C for 10 min then at 42°C for 30 min when random hexamer was used. Following RT reaction, the mix was incubated in a thermal cycler at 95°C for 5 min to inactivate the reverse transcriptase (M-MuLV) and to completely denature the template. In the second step, *mir1* gene-specific primers were added, and synthesis was performed at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 35 cycles. *18S rRNA* forward primer 5'-TCCTGAGTAACGAACGAGACC-3' and reverse primer 5'-CACGATGAAATTTCCCAAGAT-3' were used to amplify *18S rRNA* gene, which was used as a housekeeping gene, and amplification was performed at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 35 cycles. PCR reactions were carried out in a Mastercycler gradient (Eppendorf, Hamburg, Germany). To ensure that no false-positive PCR fragments would be generated from genomic DNA contamination or non-specific DNA amplification, a second aliquot of total RNA was treated similarly, omitting the reverse transcriptase step, and those samples were used as minus reverse transcriptase controls. PCR products were analyzed on a 1.8% agarose/ethidium bromide gel run in 1× tris-borate-EDTA buffer. Two repetitions were conducted for each treatment, and one representative result is shown. PCR products for *mir1* and *18S rRNA* genes were cloned into pCR2.1 plasmids using the TA cloning kit (Invitrogen Corp.). The cloned products were sequenced using the ABI Prism Big Dye terminator cycle se-

quencing ready reaction kit (Applied Biosystems, Foster City, CA, U.S.A.) and were compared against GenBank, using the BLAST program to confirm identity.

Real-time PCR was performed on a Roche Lightcycler 2.0 instrument. Oligonucleotide primers were synthesized and high-pressure liquid chromatography (HPLC)-purified by Invitrogen. Primer design and optimization concerning primer dimer, self-priming formation, and primer melting temperature was done with LightCycler Probe Design Software, v.1.0 (Roche Applied Science, Mannheim, Germany). Reverse transcription of mRNA was carried out in a 20-µl final volume from 1 µg total RNA, using 10 U Transcriptor reverse transcriptase (Roche) according to the manufacturer's instructions with either 10 µM oligo (dT)₁₅ or 3.2 µg random primer p(dN)₆ (Roche). Transcriptor reverse transcriptase was used in combination with 20 U of Protector RNase inhibitor and 1 mM of each PCR nucleotide mix (Roche). PCR was carried out with the LC fast start DNA Master^{PLUS} SYBR green I kit (Roche), using 2 µl of cDNA, corresponding to 100 ng of total RNA in a 20-µl final volume, and 0.5 µM of each HPLC-purified primer for target gene (*mir1*, maize insect resistance: forward primer, 5'-CTACTGGATCGTGAAGAAGACTCGTG-3' and reverse primer, 5'-CTCCCTCCTAAGCAAGAACCATC-3') and reference gene (*18S rRNA*: forward primer, 5'-TCCTGAGTAACGAACGAGACC-3' and reverse primer, 5'-CACGATGAAATTTCCCAAGAT-3'). For each sample, two reactions, one for *mir1* and one for the reference gene *18S rRNA*, were performed in separate capillaries. In the LightCycler 2.0 instrument (Roche), each reaction capillary was incubated for 10 min at 95°C before 45 cycles at 95°C for 10 s, specific annealing temperature for 5 s, and 72°C for 10 s. Finally, amplified products were denatured (95°C for 0 s), renatured (65°C for 15 s), and progressively denatured (95°C for 0 s, step over 0.1°C/s for the melting curve analysis). The PCR run was concluded with a 40°C incubation for 30 s. Both primer combinations (*mir1BF/mir1BR* and *18S rRNAF/18S rRNAR*) were tested (fusion curve, linearity, and efficiency of the primers). The primer combinations for *mir1* and *18S rRNA* have a PCR efficiency of 87%. Fluorescence was monitored at the end of the 72°C extension step in each cycle, and the second derivative maximum algorithm method was used to calculate crossing points (Cp). Cp is defined as the point at which the fluorescence rises appreciably above the background fluorescence. Amplification specificity was checked using melting curve following the manufacturer's instructions. A nontemplate control was run for each primer pair to assess the overall specificity and to ensure that primer dimers were not interfering with amplification detection. Melting curve analysis showed a single sharp peak for all samples, and agarose gel electrophoresis showed a single band at the expected size. The calculation method used was based on PCR efficiency-corrected relative quantification normalized to a calibrator. To determine *mir1* transcript levels, the ratio of *mir1* to *18S rRNA* in an unknown sample relative to the *mir1* to *18S rRNA* ratio in a calibrator sample was determined using LC relative quantification software, v.4.0 (Roche Applied Science). The calibrator is included in each run. Quantification using external standard curves for target and reference genes method was carried out with relative quantification software. Standard curves were achieved from fivefold dilutions of PCR product. Determination of the relative *mir1/18S* ratio was a function of the PCR reaction efficiency and the Cp of *mir1* and *18S rRNA* of the unknown and the calibrator sample. To calculate the calibrator normalized relative ratio, the amount of *mir1/18S* in each sample [$N_{T(S)}/N_{R(S)}$] was divided by the ratio of *mir1/18S* of the calibrator [$N_{T(C)}/N_{R(C)}$]. The normalized ratio was then calculated by the formula Normalized Ratio = $E_T^{CpT(C)-CpT(S)} \times E_R^{CpR(S)-CpR(C)}$, where E_T = efficiency

of the target gene *mir1*, E_R = efficiency of the reference gene *18S*, $CpT(C) - CpT(S) = Cp$ of target calibrator subtracted from Cp of target unknown sample, and $CpR(S) - CpR(C) = Cp$ of reference unknown sample subtracted from Cp of reference calibrator. This formula is in accordance with Roche Applied Science Technical Note No. LC 13/2001. Duplicate RNA extractions or technical replicates were done for each treatment for both experiments, therefore $n = 4$. Data were exported to Microsoft Excel for further analysis.

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