Integration of Ethylene and Jasmonic Acid Signaling Pathways in the Expression of Maize Defense Protein Mir1-CP

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In plants, ethylene and jasmonate control the defense responses to multiple stressors, including insect predation. Among the defense proteins known to be regulated by ethylene is maize insect resistance 1-cysteine protease (Mir1-CP). This protein is constitutively expressed in the insect-resistant maize (*Zea mays*) genotype Mp708; however, its abundance significantly increases during fall armyworm (*Spodoptera frugiperda*) herbivory. Within 1 h of herbivory by fall armyworm, Mir1-CP accumulates at the feeding site and continues to increase in abundance until 24 h without any increase in its transcript (*mir1*) levels. To resolve this discrepancy and elucidate the role of ethylene and jasmonate in the signaling of Mir1-CP expression, the effects of phytohormone biosynthesis and perception inhibitors on Mir1-CP expression were tested. Immunoblot analysis of Mir1-CP accumulation and quantitative reverse-transcriptase polymerase chain reaction examination of *mir1* levels in these treated plants demonstrate that Mir1-CP accumulation is regulated by both transcript abundance and protein expression levels. The results also suggest that jasmonate functions upstream of ethylene in the Mir1-CP expression pathway, allowing for both low-level constitutive expression and a two-stage defensive response, an immediate response involving Mir1-CP accumulation and a delayed response inducing *mir1* transcript expression.

Ethylene (ET) and jasmonate (JA) are widely studied phytohormones observed to accumulate in plants in response to wounding and pathogen and herbivore attack, as well as other biotic and abiotic stresses (Creelman and Mullett 1997; Wang et al. 2002; Wasternack et al. 2006; Howe and Jander 2008). Both hormones have been shown to mediate various defense responses either individually or coordinately, including induced systemic resistance (Dong 1998; Maleck and Dietrich 1999; Adie et al. 2007; Shinshi 2008). The coordinated crosstalk between ET and JA has been demonstrated to converge at the transcriptional activation of *ERF1* (Lorenzo et al. 2003) or *CE1* (Nakano et al. 2006). In addition to integrating with each other, ET and JA act coordinately with other plant hormones, including salicylic acid (SA) and abscisic acid (ABA), through a complex network to induce a myriad of defense responses (O'Donnell et al. 1996; Lorenzo and Solano 2005; Lorenzo et al. 2003; Robert-Seilaniantz et al. 2007; López et al. 2008;

Spoel and Dong 2008). However, the mode of interaction of JA and ET varies depending on the elicitor triggering the signal transduction, the target gene expressed, and the plant species.

JA or its volatile methyl ester (methyl jasmonate [MEJA]) has been reported to stimulate ET production in several plants, including tomato (O'Donnell et al. 1996), tobacco (Xu et al. 1994), and *Arabidopsis* (Penninckx et al. 1998). Although observed to occur at a much lower frequency, ET has also been reported to stimulate JA production (O'Donnell et al. 1996). In contrast to these sequential activations, concomitant activation of JA and ET response pathways mediate the induction of the defensin gene in *Arabidopsis* (Penninckx et al. 1998). Regardless of the sequence of activation, ET and JA appear to interact synergistically in the majority of defense responses (Xu et al. 1994; O'Donnell et al. 1996; Laudert and Weiler 1998; Lorenzo et al. 2003; Zhao et al. 2004). However, ET has also been shown to function antagonistically to suppress JA-induced gene expression in nicotine biosynthesis (Shoji et al. 2000) and in wounded leaves of *Arabidopsis* (Rojo et al. 1999). Similarly, the phytohormone SA has been shown to be antagonistic to both JA and ET (Doares et al. 1995; Iverson et al. 2001; Spoel et al. 2003). The balance of these two phytohormones is critical in the defense response and their coordination is potentially unique in every plant species and, additionally, may be specific within a species for each pathogen or insect (Pieterse and Dicke 2007).

Successful defense, which may be mediated by these signal molecules, depends on both constitutive and induced defense mechanisms (Mauricio et al. 1997; Buell 1998). Constitutive defenses are present whether or not plants are attacked by pathogens or herbivores whereas induced defenses are produced only after attack or are produced in greater amount after attack (Mauricio et al. 1997; Buell 1998). Both types of mechanisms are costly for the plant due to the diversion of nitrogen and carbon from vegetative and reproductive growth to effective defense responses (Baldwin 1998; Baldwin and Preston 1999). Thus, plants must maintain a balance between vegetative growth and constitutive defense to maintain maximum fitness; inducible defenses are assumed to compromise plant fitness less because they only require plant resources after pathogen or herbivore assault (Agarwal 1998). By balancing constitutive and induced defense responses, the plant may combat insect predation without sacrificing vegetative growth and reproduction. In addition, the early signaling events triggered at the plant–insect interface (which eventually lead to the defense responses) add to this balance by aiding herbivore recognition (Maffei et al. 2007).

Although the ET- and JA-regulated defense pathways have been extensively studied in the model plant *Arabidopsis* and other dicots, far less is known about their role in regulating direct herbivore defenses in monocots, especially maize. This study examines the interaction of these two phytohormones in regulating herbivore defense in an insect-resistant maize inbred line, Mp708. This line was developed to withstand herbivory by the fall armyworm (FAW) (*Spodoptera frugiperda*) and other lepidopteran caterpillars. The germplasm used in breeding Mp708 originated in Antigua, where farmers probably selected it for resistance to several insect pests for many generations. Multiple trait analysis and quantitative trait loci (QTL) mapping have shown that several genes known to confer resistance to several lepidopteran insects and related pests have been successfully bred into Mp708 (Brooks et al. 2007). Thus, Mp708 possesses typical maize fitness traits and the additional trait of maintaining readiness for resisting insect attack. It appears to achieve this, in part, by constitutive expression of the maize insect resistance-1-cysteine protease (Mir1-CP), whose locus lies in one of the resistance QTL to FAW (Jiang et al. 1995; Brooks et al. 2007). Mir1-CP is encoded by a single-copy gene, *mir1*, located on chromosome 6 of the maize genome (Pechan et al. 1999). Mir1-CP is constitutively expressed at low levels prior to insect feeding and increases upon herbivore attack (Pechan et al. 2000). When infested with FAW larvae, increased Mir1-CP accumulation occurs at insect feeding site (Pechan et al. 2000). Once ingested, the proteolytic activity of Mir1-CP damages the insect's peritrophic matrix, impairing nutrient utilization (Chang et al. 1999; Pechan et al. 2002, 2004; Mohan et al. 2006, 2008). Although Mir1-CP accumulates in the leaves and whorl region by as early as 1 to 4 h in response to herbivory (Pechan et al. 2002), a corresponding increase in *mir1* transcript levels has not been observed during these early time points (R. Shivaji and A. A. Luthe, *unpublished data*). It has been proposed that the accumulation of Mir1-CP in the whorl during the first 24 h of foliar feeding by FAW is due to its importation from the roots (López et al. 2007). This was supported by three observations: i) immunolocalization data indicating that Mir1-CP is present in maize

vascular tissues (López et al. 2007), ii) root removal that prevents Mir1-CP accumulation in the whorl (López et al. 2007) and iii) immunoblot data showing that Mir1-CP is in the xylem sap (O. Pechanova and D.S. Luthe, *unpublished data*). It has been proposed that herbivore feeding in the whorl caused the transmission of a signal to roots, resulting in the below-ground accumulation of Mir1-CP and its subsequent export to the caterpillar feeding site (López et al. 2007).

It has previously been established that ET synthesis and perception are essential for the accumulation of Mir1-CP and its transcript in response to herbivory in Mp708 (Harfouche et al. 2006). Blocking ET synthesis or inhibiting ET perception reduced the accumulation of Mir1-CP and its transcript *mir1* in response to insect herbivory (Harfouche et al. 2006) and increased plant susceptibility to FAW, as shown by the high relative larval growth rate and increased foliar damage (Harfouche et al. 2006). However, ET signaling often depends on a complex relationship with JA. With the establishment of the role of ET signaling and the suggested systemic induction of Mir1-CP accumulation in roots in response to foliar herbivory, we predicted a possible role of JA in the accumulation of Mir1-CP. Thus, the present study was conducted to investigate the roles of JA and ET in the observed increased accumulation of Mir1-CP in the absence of increased *mir1* transcript levels during the first 24 h of herbivory. Therefore, we employed a pharmacological approach to investigate the role of JA signaling and its interaction with ET in regulating Mir1-CP accumulation.

RESULTS

Exogenous ET or MEJA induces *mir1* transcript and Mir1-CP accumulation in leaves.

Previous studies with ET synthesis and perception inhibitors have shown that ET has a role in the regulation of Mir1-CP accumulation (Harfouche et al. 2006). To further investigate the possible role and interaction of JA, maize plants were treated with exogenous ET or MEJA and leaf samples were collected 0, 6, 12, and 24 h after treatment. Immunoblot analyses with total protein extracts showed that exogenous ET increased

Fig. 1. Maize insect resistance 1-cysteine protease (Mir1-CP) accumulation in response to exogenous **A**, ethylene (ET); **B**, methyl jasmonate (MEJA); and **C**, fall armyworm (FAW) feeding. Plants were sprayed with **A**, 3 mM 2-chloroethylphosphonic acid (CEPA), which releases ET or **B**, MEJA, and samples were collected from the whorl region after 0, 6, 12, and 24 h. **C**, Samples were also collected from untreated control plants after 0, 6, 12, and 24 h of feeding by FAW. Immunoblot analysis was carried out using antibodies developed against Mir1-CP. Protein isolated from callus generated from Mp708 embryos was used as positive control and 15 μ g of total protein extract was loaded in each lane. The molecular mass of Mir1-CP is 33 kDa, as indicated by the arrow in the left margin.

Mir1-CP accumulation 6 h after treatment (Fig. 1A). Accumulation of Mir1-CP appeared to peak at 6 h and slightly lower levels were detected at both the 12- and 24-h time points. Similarly, immunoblot analysis of MEJA-treated plants revealed increased Mir1-CP accumulation at 6 h. Unlike during ET treatment, levels of Mir1-CP appeared to increase with time during the MEJA treatment (Fig. 1B), mimicking the plant response to FAW feeding (Fig. 1C). Clearly, both ET and MEJA treatments positively contribute to the accumulation of Mir1-CP. However, when quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) examinations of the *mir1* transcript levels were performed under these same conditions, no increase was detected until 24 h after ET or MEJA treatment (Fig. 2A and B). To validate the observed difference in transcript and protein expression of Mir1-CP, transcript levels of the maize proteinase inhibitor *mpi* also were measured.

Maize proteinase inhibitor (MPI) is a well-characterized defense protein that accumulates by as early as 4 h after mechanical wounding, insect feeding, or pathogen infection (Cordero et al. 1994). Previous examinations of *mpi* have established that its transcript levels increase in response to MEJA (Cordero et al. 1994). Although the effects of ET on *mpi* transcript levels have not been previously reported, ET has been reported to increase transcript levels of several related proteinase inhibitors (Margossian et al. 1988). Therefore, relative *mpi* transcript abundance also was measured in the ET- and MEJA-treated samples to determine if they were regulated in a manner similar to those of *mir1*. As described by Cordero and associates (1994), *mpi* transcript levels increased in Mp708 in response to MEJA, as well as in response to ET, by as early as 6 h (Fig. 2C

and D), unlike the *mir1* transcript levels, which remained unchanged until 24 h. These comparative qRT-PCR studies of *mir1* and *mpi* transcript abundance show that there are temporal differences in their accumulation and substantiate our findings of unchanged *mir1* transcript levels in response to MEJA and ET treatment during early time points of the study.

JA biosynthesis inhibitors reduce Mir1-CP accumulation in maize leaves in response to larval damage.

To confirm the observed role of JA signaling pathway in the induction of Mir1-CP accumulation, varying concentrations (1, 3, and 5 mM) of the lipoxygenase inhibitor ibuprofen (IBU) were applied to maize plants to block the octadecanoid pathway leading to JA biosynthesis. Several similar studies in rice, barley, soybean, and maize have been performed to investigate JA- and MEJA-induced genes using IBU (Doherty et al. 1998; Staswick et al. 1991; Nojiri et al. 1996; Ortel et al. 1999; Oikawa et al. 2001). At 24 h post IBU treatment, plants were infested with six to eight FAW larvae. Samples were collected after 24 h of insect predation and analyzed by immunoblotting to determine Mir1-CP expression patterns. Relative to the control plants, Mir1-CP accumulation was reduced at all IBU concentrations, with the greatest inhibition seen with 5 mM IBU (Fig. 3).

Blocking hormone signaling reduced *mir1* transcript levels and increased insect predation.

To determine whether JA and ET act synergistically, production of *mir1* message and larval performance during whorl predation were studied in the presence of hormone inhibitors (Fig.

Fig. 2. Effects of exogenous ethylene (ET) and methyl jasmonate (MEJA) on the accumulation of **A** and **B**, *mir1* and **C** and **D**, *mpi* transcripts. cDNA was isolated from leaf tissue collected from the whorl region of Mp708 plants sprayed with 3 mM 2-chloroethylphosphonic acid, which releases ET or MEJA. Quantitative reverse-transcriptase polymerase chain reaction analysis was performed using ubiquitin expression levels to normalize the target gene expression. The data represented are mean values with error bars ($\pm$ standard deviation, $n = 6$).

Fig. 3. Effect of ibuprofen (IBU) on maize insect resistance 1-cysteine protease (Mir1-CP) accumulation in response to fall armyworm (FAW) feeding. Plants were treated with 0, 1, 3, or 5 mM IBU for 24 h and then infested with FAW larvae for another 24 h before sample collection. Immunoblot analysis was carried out using antibodies raised against Mir1-CP. Protein isolated from callus generated from Mp708 embryos was used as a positive control and 15 μ g of total protein extract was loaded in each lane.

4). Although the effect of ET inhibitors alone on *mir1* transcript levels have been previously studied (Harfouche et al. 2006), their effect in association with JA inhibitors has not been tested. To investigate any probable interaction of the ET and JA pathways in *mir1* induction, plants were pretreated with either 1-methylcyclopropane (MCP, commercially available as Ethylbloc) to block ET perception, IBU to block the JA pathway, or both for 24 h prior to FAW larval infestation. One set of plants was treated with 1 mM SA for 24 h. SA has been reported to inhibit the biosynthesis of JA and its signaling pathway in tomato plants by potentially inhibiting the enzymatic activity of allene oxide

synthase (Peña-Cortés et al. 1993; Doares et al. 1995). In the same study, SA was shown to significantly inhibit the induction of proteinase inhibitor genes in tomato in response to JA. However, in a different study, JA and SA have been observed to produce similar inducing effects on a different lipoxygenase *ZmLOX10* (Nemchenko et al. 2006; Gao et al. 2008), indicating that JA and SA are not always antagonistic.

After 24 h of FAW feeding, *mir1* transcript levels in the inhibitor-treated plants were significantly reduced relative to control samples (Fig. 4A). The *mir1* transcript levels decreased three- to fourfold in response to MCP treatment, similar to earlier studies conducted by Harfouche and associates (2006). Blocking ET perception, the JA biosynthesis pathway, or both equally reduced *mir1* transcript levels, indicating that both signaling pathways are involved in its accumulation. Critically, blocking either of these hormone pathways rendered the maize plants susceptible to FAW larval feeding, as seen by the enhanced larval growth rate and increased leaf damage (Fig. 4B and C). Although treatment with both inhibitors did not significantly reduce transcript levels more than the application of a single hormone, the significant increase in FAW growth rate and leaf predation (Fig. 4B and 4C) clearly indicate that ET and JA are needed for the insect defense response shown by the maize genotype Mp708.

ET can induce Mir1-CP accumulation in the absence of the octadecanoid pathway to signaling.

To evaluate the relationship between JA and ET in the signaling of Mir1-CP accumulation, the effect of exogenous ET in the absence of JA signaling was examined (Fig. 5). Maize plants were pretreated with 5 mM IBU or 1 mM SA for 24 h before the exposure to exogenous ET. Because the results with SA were similar to those of IBU, they are not reported here. After 24 h of IBU treatment, plants were exposed to exogenous ET for 6 or 24 h. Controls of ET, IBU, and FAW feeding as well as untreated plants were collected at the same time. Additionally, to compare the expression of Mir1-CP protein in the inhibitor-treated plants in response to FAW damage, a set of IBU pretreated plants was subjected to 24 h of larval feeding and collected. When the octadecanoid pathway was blocked, Mir1-CP levels decreased compared with control plants, as seen in the IBU-treated plants. In fact, 24 h of FAW herbivory on IBU-treated plants failed to induce Mir1-CP (Fig. 5A) and *mir1* transcript accumulation (Fig. 5B). However, a subsequent application of ET for 6 h partially reversed the effects of IBU and resulted in increased Mir1-CP expression (Fig. 5A) but the same treatment did not result in any significant increase in *mir1* transcript levels until 24 h (Fig. 5C). This indicates that ET can stimulate the accumulation of Mir1-CP in the absence of JA signaling and probably functions down-

Fig. 4. Effect of hormone inhibitors on **A**, the relative abundance of feeding-induced *mir1* transcripts; **B**, larval growth rate; and **C**, foliar damage in the maize genotype Mp708. Fall armyworms (FAW) were allowed to feed for 24 h on plants treated with the jasmonate (JA) pathway inhibitors ibuprofen (IBU) or salicylic acid (SA), ethylene perception inhibitor 1-methylcyclopropane (MCP), as well as on plants treated simultaneously with MCP and IBU or MCP and SA. The control was a set of untreated plants subjected to larval infestation for 24 h. **A**, cDNA was synthesized from leaf tissue harvested from the whorl region and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed to detect *mir1* transcripts. Ubiquitin was used as the reference gene for normalization of qRT-PCR data. **B**, The larvae were weighed before and after foliar predation and the relative growth rate (RGR) was calculated according to Waldbauer's equation (1968); $RGR = (\text{total final weight} - \text{total initial weight}) / \text{average weight per day}$. **C**, Foliar damage after herbivory is represented as length of the damaged area, as indicated by the numbers and arrows. The data represented are mean values with error bars ($\pm$ standard deviation, $n = 6$).

stream from JA to regulate Mir1-CP accumulation. However, this is not necessarily the case for *mir1* transcript levels, which did not increase in response to ET treatment in the presence of IBU until 24 h of ET treatment. This suggests that prolonged ET treatment can overcome the effects of the IBU block of the JA pathway and cause the accumulation of Mir1-CP and its transcripts.

MEJA treatment has no effect on *mir1* transcript and Mir1-CP protein levels in ET-perception-blocked Mp708 plants.

To investigate whether JA alone can induce *mir1* transcripts or Mir1-CP in the absence of ET signaling, plants pretreated

with MCP for 24 h were subsequently treated with MEJA for 24 h. Exogenous MEJA application was not capable of increasing the accumulation of *mir1* or Mir1-CP in the MCP pretreated plants (Fig. 6A and B), even though JA treatment increased their abundance in control plants. These results show that blocking ET signaling prevents the accumulation of both Mir1-CP and its transcripts. This block cannot be overcome with JA treatment. If ET and JA were acting coordinately, we would expect JA treatment of MCP-inhibited plants to restore Mir1-CP and *mir1* accumulation just as ET treatment of IBU-inhibited plants did in the experiments shown in Figure 5. However, this was not the case, and suggests that JA acts upstream from ET signaling.

DISCUSSION

Plants, which exhibit a sessile lifestyle, encounter numerous microbial and insect pests. Although these pests are armed with a diverse array of strategies to damage plants, plants, in turn, are equipped with myriad defense mechanisms to protect themselves from these attacks (Walling 2000). These various defenses can divert energy resources, reducing the overall fitness of the plant. Therefore, elaborate regulatory mechanisms to balance static (constitutive) defenses and active (induced) defenses and minimize fitness costs while optimizing resistance are needed (Agarwal 1998; Pieterse and Dicke 2007). One such defense strategy is exhibited by the insect-resistant maize genotype Mp708.

The caterpillar-resistant maize genotype Mp708 expresses the defense protein Mir1-CP in response to lepidopteran larval predation. Low levels of Mir1-CP are constitutively expressed in Mp708 plants but higher levels of the protein accumulate at the wound site in response to feeding by lepidopteran larvae (Pechan et al. 2000). Although Mir1-CP accumulation increases

Fig. 5. Effect of jasmonate (JA) inhibitors on **A**, ethylene (ET)-induced maize insect resistance 1-cysteine protease (Mir1-CP) accumulation and **B** and **C**, *mir1* transcript levels. Plants pretreated with 5 mM ibuprofen (IBU) for 24 h were further subjected to treatment with ET (3 mM 2-chloroethylphosphonic acid) for 6 h or fall armyworm (FAW) feeding for 24 h before sample collection. **A** and **B**, A set of plants for each single treatment—IBU (24 h), ET (6 h), or FAW feeding (24 h)—was also collected. Control plants not treated with hormone or inhibitor were treated with sterile water throughout the experiments. Tissue was collected from the whorl region. **A**, Immunoblot analysis of Mir1-CP accumulation was carried out using total protein extract and **B** and **C**, quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was carried out using cDNA to quantify *mir1* transcript abundance. Ubiquitin was used as the reference gene for relative quantification of qRT-PCR data. The data represented are mean values with error bars ($\pm$ standard deviation, $n = 6$).

Fig. 6. Methyl jasmonate (MEJA)-induced accumulation of **A**, maize insect resistance 1-cysteine protease (Mir1-CP) and **B**, *mir1* transcripts when ethylene perception was inhibited. Plants were treated with 1-methylcyclopropane (MCP) in buffer or with buffer alone for 24 h and then were treated with either MEJA or sterile water for an additional 24 h. Plants pretreated with releasing buffer followed by 24 h of sterile water treatment were used as control plants. **A**, Tissue was collected from the whorl region and immunoblot analysis was carried out with 15 μ g of total protein loaded in each lane and **B**, *mir1* transcript abundance was quantified using (qRT-PCR) analysis. Ubiquitin was used as the reference gene for normalization of qRT-PCR data. The data represented are mean values with error bars ($\pm$ standard deviation, $n = 6$).

within a few hours of insect infestation (Pechan et al. 2000), transcript levels have not been found to increase commensurably. To understand this apparent discrepancy between protein and transcript levels and to elucidate the signal transduction pathway of this protein, we investigated the role of two defense-signaling phytohormones in the regulation of Mir1-CP expression.

In a prior study, it was shown that ET biosynthesis and perception were necessary for Mir1-CP accumulation (Harfouche et al. 2006) and disruption of this pathway resulted in increased susceptibility to FAW. If blocking ET synthesis and perception prevents Mir1-CP accumulation, then one would expect treatment with exogenous ET to enhance its accumulation. The results reported here clearly show that this is the case, and treating plants with exogenous ET increased the abundance of both Mir1-CP transcripts and protein. However, appearance of the protein occurred faster (6 h) than the message (24 h). Similarly, exogenous MEJA application also increased Mir1-CP accumulation. Alternatively, plants pretreated with JA pathway inhibitors and challenged with FAW accumulated less Mir1-CP, in a dose-dependent manner, confirming the involvement of the JA pathway in Mir1-CP accumulation (Fig. 3). Blocking ET and JA pathways singly or simultaneously resulted in an equal reduction of *mir1* transcript levels (Fig. 4A) and increased FAW larvae consumption of foliage and growth rates (Fig. 4B and C).

ET has been shown to function antagonistically to suppress JA-induced gene expression in nicotine biosynthesis (Shoji et al. 2000) and JA-responsive genes *JR1* and *JR2* in wounded

leaves of *Arabidopsis* (Rojo et al. 1999). However, concomitant activation of ET and JA pathways is seen in expression of *PDF1.2* in *Arabidopsis* during pathogen infection (Penninckx et al. 1998). In addition to acting coordinately, it is possible that sequential activation of one hormone by the other is possible. Several instances have been reported where JA signaling induced ET production, which subsequently mediated defense gene expression. One example of this is the production of defensive phenolic compounds by polyphenolic parenchyma cells in conifer phloem (Hudgins and Franseschi 2004). ET functioning upstream of JA appears to be far less common but is still a mechanism that must be considered (O'Donnell et al. 1996).

In the case of Mir1-CP expression, it appears that ET functions downstream from JA. We propose this model (Fig. 7) because of the following observations. When plants were treated with exogenous ET, both Mir1-CP and its transcripts accumulated in response to the treatment. However, when pretreatment of Mp708 plants with MCP blocked ET perception, there was no significant accumulation of Mir1-CP or its transcripts in response to FAW feeding. Furthermore, the application of exogenous MEJA to plants with blocked ET perception could not overcome the block in the pathway and there was no accumulation of Mir1-CP or its transcripts (Fig. 6). If MEJA acts downstream of ET, one would expect that MEJA treatment would trigger the accumulation of Mir1-CP and its transcripts even when ET perception is blocked; however, this was not the case. In the reciprocal experiment, plants were pretreated with

Fig. 7. Proposed signal transduction pathway for the expression and accumulation of maize insect resistance 1-cysteine protease (Mir1-CP) and its transcripts in insect-resistant maize genotype Mp708. In the figure, positive regulation or induction is represented by '+' and a negative regulation or inhibition is represented by '-'.

IBU to block the JA synthesis pathway. When this pathway was blocked, FAW feeding could not induce the accumulation of Mir1-CP or its transcripts (Fig. 5). However, exposing these plants to ET for 6 to 24 h reversed this inhibition and Mir1-CP and its transcripts accumulated in the absence of FAW feeding (Fig. 5). These results, indicating that ET can overcome the block imposed by IBU treatment but that MEJA cannot overcome a block in the ET signaling pathway, suggest that ET acts downstream of JA. This appears to rule out the possibility of JA and ET acting simultaneously and implies that JA functions upstream of ET in this maize genotype.

ET-dependent accumulation of Mir1-CP does not appear to depend on increased *mir1* transcript levels. This suggests that post-transcriptional or post-translational regulation accounts for the rapid increase in Mir1-CP abundance. We propose that the constitutively expressed *mir1* transcripts await a secondary signal mediated by ET for eventual expression of Mir1-CP. Similar regulation is observed in the E17 gene of tomato plants, which is both transcriptionally and post-transcriptionally regulated by ET (Lincoln and Fischer 1988a and b). In the investigation of E17, it was reported that basal levels of ET are sufficient to activate gene expression whereas increased levels of ET are required for post-transcriptional modification, resulting in subsequent transcript accumulation (Lincoln and Fischer 1988a and b).

In light of these observations, we propose a possible signal transduction pathway model detailing the interaction of the two major phytohormones, ET and JA, and the inhibitors studied in the regulation and induction of Mir1-CP (Fig. 7). The signaling pathway is initiated by herbivory and the possible release of an unknown elicitor, which may be derived from the plant, the insect, or both. We propose that the elicitor stimulates the JA pathway and transduces responses downstream via ET. ET regulates both *mir1* transcript and Mir1-CP accumulation. Therefore, the defense response involving Mir1-CP production is two-pronged, with a constitutively maintained active pathway for the expression of relatively high basal levels of *mir1* transcripts and a rapid induction and accumulation of Mir1-CP mediated by ET. A similar constitutively active JA pathway has been shown to be responsible for the expression of several defense-related genes, including *VSP1*, *VSP2*, *THI2.1*, *PDF1.2*, and *CHI-B*, in the *CEVI* mutants of *Arabidopsis* (Ellis and Turner 2001). This two-stage response of Mir1-CP accumulation in response to herbivory to some extent exemplifies the involvement of early wound-response genes activated within 30 min to 2 h (Ryan 2000) and late wound-response genes activated approximately 24 h after insect infestation (Howe 2004). *LapA* is one such late wound-response gene acting downstream from JA in tomato during wound response (Howe 2004; Fowler et al. 2009).

The existence of a second mode of regulation provides a rationale for the previous results observing Mir1-CP accumulation at the wound site within 1 h of larval infestation (Pechan et al. 2002). This rapid accumulation of the defense protein is many times faster than that of other plant defense proteins, which require both transcription and translation for expression and can require 8 to 12 h for maximum expression (Ryan 2000). Therefore, we speculate that the insect-resistant maize genotype Mp708 constitutively expresses *mir1*, which is then post-transcriptionally or post-translationally regulated by ET, leading to the accumulation of Mir1-CP at the feeding site.

This molecular model of Mir1-CP regulation can be superimposed on a spatial model for defense signaling in Mp708 that appears to involve above- and below-ground communication that has previously been reported for maize (Erb et al. 2008). Immunolocalization indicated that low levels of Mir1-CP are present in the vascular elements of the maize whorl and

roots prior to herbivory (López et al. 2007). After 24 h of FAW feeding in the whorl, Mir1-CP levels increase in both of these organs. If the roots are excised prior to FAW feeding, far less Mir1-CP accumulates in the whorl (López et al. 2007). These observations lead to a model in which FAW feeding sends a signal, possibly JA, to the roots that results in the accumulation of Mir1-CP. Then, in turn, Mir1-CP may be exported to the whorl during the initial stages of FAW feeding. This hypothesis of Mir1-CP translocation from the roots appears to explain the lag between the rapid accumulation of Mir1-CP and the delayed increase in its transcripts. Although it has not yet been established, we suspect that JA or its conjugate could be responsible for the long-distance signaling that triggers *mir1* transcript accumulation not only at the feeding site but also in parts of the plant systemic to the wound site, as observed by López and associates (2007). This would allow for subsequent translocation of the protein to the wound site. Reciprocal grafting experiments conducted in tomato plants support the idea that JA or its derivatives probably act as phloem-mobile signal aiding in systemic signaling (Schillmiller and Howe 2005; Howe and Jander 2008). Unfortunately, there currently are no techniques that can be used for successfully grafting maize plants.

Determination of the molecular components mediating this defense response and characterization of the interactions among these signaling pathways is essential for enhancing resistance to herbivores. This study helps to better understand the signaling events leading to insect defense in maize and exemplifies the strategic potency of the plants to survive herbivory.

MATERIALS AND METHODS

Plant growth and FAW rearing.

The insect-resistant maize (*Zea mays*) inbred line Mp708 (Williams et al. 1990) was used for this study. All plants were grown at the Plant Science Research Center, Mississippi Agricultural and Forestry Experiment Station (Mississippi State University) under greenhouse conditions. Seed were soaked in sterile water for 5 to 10 min and washed thoroughly before sowing. Four seeds were placed together in potting soil (Bacto Premium Potting Soil; Michigan Peat Company, Houston, TX, U.S.A.) in each pot and were collected together as one single biological replicate. Three such biological replicates were analyzed for each treatment of the experiment and each experiment was repeated twice. Plants were maintained in a greenhouse at a maximum day temperature of 33.1°C and a minimum night temperature of 26.3°C and watered as needed. Plants in mid-whorl (V8) developmental stage (four to five weeks old) (Ritchie et al. 1986) were selected for FAW infestation or hormone treatment. FAW (*S. frugiperda*) larvae were provided by the United States Department of Agriculture–Agricultural Research Service (USDA-ARS) Insect Rearing Laboratory (Starkville, MS, U.S.A.). FAW were reared in 30-ml plastic insect diet cups (Bio-Serv, Frenchtown, NJ, U.S.A.) for 5 days under a photoperiod of 16 h of light and 8 h of darkness at 26.6°C on an artificial diet (Davis 1976, 1989). This corresponded to the third instar larvae stage, when each larva weighed approximately 10 to 12 mg. As described below, this stage was used for plant infestation experiments.

Hormone and hormone inhibitor applications.

The effect of exogenous ET was studied by spraying plants with 50 ml of 3 mM 2-chloroethylphosphonic acid (CEPA), commercially available as Ethephon (C0143; Sigma-Aldrich, St. Louis). The concentration of CEPA (3 mM) used in these studies is an intermediate dosage compared with the 1- and 10-mM concentrations used in studies conducted in apple seedlings

(Poupard et al. 2003). ET perception was blocked using MCP, commercially available as Ethylbloc (Floralife, Waterloo, SC, U.S.A.). The Ethylbloc solution was prepared at a final concentration of 5 mg/liter by dissolving 450 mg of the Ethylbloc powder in 9 ml of the releasing buffer supplied by the manufacturer (Harfouche et al. 2006). A beaker containing this solution was placed within a 3-by-1-by-1-inch plexiglass chamber that enclosed a pot containing four plants, as described by Harfouche and associates (2006). MEJA was supplied as vapors to study the expression of Mir1-CP in response to induced JA signaling. MEJA (Bedoukian Research Inc., Danbury, CT, U.S.A.) was diluted 10-fold with ethanol to make a 10% MEJA stock (vol/vol) stored at -20°C and a fresh working solution of 0.01% (vol/vol) was prepared with sterile water as needed. A beaker containing 50 ml of 0.01% MEJA solution was placed within the plexiglass chamber; the plants were exposed to the MEJA for a period of 6, 12, or 24 h; and tissue samples were collected as described previously. To block the JA biosynthesis pathway, 1, 3, and 5 mM IBU solutions (I1892; Sigma-Aldrich) or 1 mM SA in the form of sodium salicylate (13316; Sigma-Aldrich) solution were sprayed on the leaves (Molina and Olmedo 1993).

Insect infestation and sample collection.

After the plants were exposed to the respective hormone treatments, they were either harvested immediately or subjected to insect infestation. For insect infestation, seven to eight larvae, each weighing approximately 10 to 12 mg, were carefully placed in the whorls of the plants, and the plants were covered with the previously described plexiglass chambers. After 24 h, the FAW larvae were collected and relative growth rate was calculated as $\text{RGR} = (\text{total final weight} - \text{total initial weight}) / \text{average weight per day}$ (Waldbauer 1968). Tissue from the yellow-green midwhorl region was collected after hormone, hormone inhibitor, and FAW treatments; immediately frozen in liquid nitrogen; and stored at -80°C for later analyses. Four biological replicates were performed and collected for each treatment and each experiment was performed twice. In FAW-infested plants, whorl tissue within a 1-cm radius of insect damage was also collected and processed. All plants, unless otherwise mentioned, were maintained intact in soil until time of collection.

Protein extraction and immunoblot analysis.

For protein extraction, frozen whorl tissue from each treatment and control were thoroughly ground in liquid N_2 using a mortar and pestle. A total of $2\times$ Laemmli sodium dodecyl sulfate (SDS) sample buffer (Laemmli 1970) was added to the ground sample and homogenized. Thawed homogenates were boiled in water for 5 min, vortexed, and centrifuged at $13,000 \times g$ for 5 min to remove cellular debris. The supernatants containing the dissolved proteins were collected and stored at -20°C for further analyses. Total protein concentration in each sample was determined by RC DC protein quantification assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.) using bovine serum albumin as standard. The isolated protein samples were separated on a 12% polyacrylamide gel using a discontinuous buffer

system (Laemmli 1970). A total of 15 μg of protein was loaded in each lane except for the positive control. For positive control, 2 μg of total protein extract from callus generated from Mp708 embryos was used. Mp708 callus tissue has been shown to constitutively overexpress Mir1-CP (Jiang et al. 1995). All gels were run in duplicate at a constant voltage of 50 V for 45 min followed by 70 V until the tracking dye reached the bottom of the gel. After SDS polyacrylamide gel electrophoresis was complete, proteins were transferred to nitrocellulose membranes using an ABN Polyblot system (American Bio-netics, Hayward, CA, U.S.A.). Following transfer, the membrane-bound proteins were immunodetected using primary monoclonal antibodies against Mir1-CP prepared as described by Pechan and associates (2000), using a secondary HRP-conjugated goat anti-mouse antibody. The enhanced signals from immunoreacting proteins were detected by chemiluminescence using Supersignal West Femto Maximum Sensitivity Substrate (34096; Pierce, Rockford, IL, U.S.A.).

RNA extraction, quantification, and analysis.

Using a ball-mill tissue grinder (Genogrinder 2000; Spex Centriprep Inc., Metuchen, NJ, U.S.A.), 100 mg of each frozen tissue sample was pulverized for 2 min at 1,100 strokes/min in polyET vials prechilled with liquid nitrogen in an aluminum block within a cryo-station (Spex Centriprep Inc.). To the ground sample, 1 ml of Purazol (Bio-Rad Laboratories Inc.) was added before an additional 30 s of homogenization on the Genogrinder. The homogenized samples were processed for total RNA using the Aurum Total RNA fatty and fibrous tissue packs (Bio-Rad Laboratories Inc.) as per the manufacturer's protocol. Briefly, after application of the supernatant, on-column DNase I digestion is performed to remove genomic DNA contamination and the resulting RNA is eluted using the RNA elution buffer provided with the extraction kit. Aliquots of purified RNA samples were stored for further analyses at -80°C .

All RNA samples were quantified using a Nanodrop Spectrophotometer ND-1000 purchased from NanoDrop Technologies, Inc. (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) and RNA quality was visually inspected by 1.0% agarose gel electrophoresis. In total, 500 ng of RNA was used as template for first-strand cDNA synthesis using the Thermoscript RT-PCR system (Invitrogen Corporation, Carlsbad, CA, U.S.A.). Differential mRNA expression was quantified and analyzed by real-time PCR performed on a Roche Lightcycler 2.0 PCR instrument (Roche Applied Science, Indianapolis, IN, U.S.A.) using the Lightcycler Fast start DNA Master^{PLUS} SYBR Green I kit (Roche Applied Science). The final reaction mix consisted of 1 μl of cDNA sample, corresponding to 25 ng of total RNA, and 9 μl of the PCR master mix to make a final reaction volume of 10 μl . The qRT-PCR was performed individually using specific primer pairs for each target gene studied (Table 1). Two technical replicates were run for each gene along with the reference gene with the following cycle parameters: preincubation for 10 s at 95°C , followed by 45 cycles of amplification consisting of denaturation at 95°C for 10 s, annealing at 60°C for 10 s (64°C for 5 s for *mir1*), and extension at 72°C for 5 s (9 s for *mir1*). The specificity of the amplified product was

Table 1. Primer pairs used for target genes studied

Gene	Accession number	Primer sequence (5'-3')	Product size (bp)
Mir1	AF019145	CTACTGGATCGTGAAGAAGCTCGTG	211
	...	CTCCCTCCTAAGCAAGAACCATC	...
MPI	X78988	ATGAGCTCCACGGAGTGC	222
	...	TCAGCCGATGTGGGGCGTC	...
Ubiquitin	U29162	TGATAATGTGAAGGCCAAGATCCAG	277
	...	GGTCTGGGGGAATCCCCTCCTTGTC	...

checked by analyzing the melting curves and by agarose gel electrophoresis. A nontemplate negative control was run with each set of samples to ensure that there was no significant interference of primer dimers with the product amplification or fluorescence detection. Standard curves were derived from the analysis of different sequential fold dilutions of the most highly concentrated cDNA samples for each particular gene, and efficiencies were calculated (Pfaffl 2001; Rasmussen 2001). The expression (concentration) of target genes in each treatment sample was quantified relative to that of the reference gene using the mathematical model proposed by Pfaffl (2001). Statistical analysis for these data was done by a mixed model analysis of variance using the MIXED procedure of SAS.

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LITERATURE CITED

- Adie, B., Chico, J. M., Rubio-Somoza, I. and Solano, R. 2007. Modulation of plant defenses by ethylene. *J. Plant Growth Regul.* 26:160-177.
- Agarwal, A. A. 1998. Induced responses to herbivory and increased plant performance. *Science* 279:1201-1202.
- Baldwin, I. T. 1998. Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proc. Natl. Acad. Sci. U.S.A.* 95:8113-8118.
- Baldwin, I. T., and Preston, C. A. 1999. The eco-physiological complexity of plant responses to insect herbivores. *Planta* 208:137-145.
- Brooks, T. D., Bushman, B. S., Williams, W. P., McMullen, M. D., and Buckley, P. M. 2007. Genetic basis of resistance to FAW (Lepidoptera: Noctuidae) and southwestern corn borer (Lepidoptera: Crambidae) leaf-feeding damage in maize. *J. Econ. Entomol.* 100:1470-1475.
- Buell, C. R. 1998. *Arabidopsis*: A weed leading the field of plant-pathogen interactions. *Plant Physiol. Biochem.* 36:177-186.
- Chang, Y.-M., Luthe, D. S., Davis, F. M., and Williams, W. P. 1999. Influence of whorl region from resistant and susceptible corn genotypes on FAW (Lepidoptera:Noctuidae) growth and development. *J. Econ. Entomol.* 93:477-483.
- Cordero, M. J., Ravent, D., and Segundo, B. S. 1994. Expression of a maize proteinase inhibitor gene is induced in response to wounding and fungal infection: Systemic wound-response of a monocot gene. *Plant J.* 6:141-150.
- Creelman, R. A., and Mullet, J. E. 1997. Biosynthesis and action of jasmonates in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:355-381.
- Davis, F. M. 1976. Rearing the southwestern corn borer and FAWs at Mississippi State. *Tech. Bull. Miss. Agric. For. Exp. Stn.* 75:54-67.
- Davis, F. M. 1989. Rearing the southwestern corn borer and FAW at Mississippi State. Toward insect resistant maize for the Third World. Pages 27-36 in: *Proc. Int. Symp. Methodologies for Developing Host Plant Resistance to Maize Insects*. International Maize and Wheat Improvement Center (CIMMYT), El Batán, Mexico.
- Doares, S. H., Narváez-Vásquez, J., Conconi, A., and Ryan, C. A. 1995. Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. *Plant Physiol.* 108:1741-1746.
- Doherty, H. M., Selvendran, R. R., and Bowles, D. J. 1988. The wound response of plants can be inhibited by aspirin and related hydroxyl-benzoic acids. *Physiol. Mol. Plant Pathol.* 33:377-384.
- Dong, X. 1998. SA, JA, ethylene and disease resistance in plants. *Curr. Opin. Plant Biol.* 1:316-323.
- Ellis, C., and Turner, J. G. 2001. The *Arabidopsis* mutant *cev1* has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. *Plant Cell* 13:1025-1033.
- Erb, M., Ton, J., Degenhardt, J., and Turlings, T. C. 2008. Interactions between arthropod-induced aboveground and belowground defenses in plants. *Plant Physiol.* 146:867-874.
- Fowler, J. H., Vásquez, J. N., Aromdee, D. N., Pautot, V., Holzer, F. M., and Walling, L. L. 2009. Leucine aminopeptidase regulates defense and wound signaling in tomato downstream of jasmonic acid. *Plant Cell* 21:1239-1251.
- Gao, X., Stumpe, M., Feussner, I., and Kolemiets, M. 2008. A novel plasmalipoxigenase of maize (*Zea mays*) *ZmLOX6* encodes for a fatty acid hydroperoxide lyase and is uniquely regulated by phytohormones and pathogen infection. *Planta* 227:491-503.
- Harfouche, A. L., Shivaji, R., Stocker, R., Williams, W. P., and Luthe, D. S. 2006. Ethylene signaling mediates a maize defense response to insect herbivory. *Mol. Plant-Microbe Interact.* 19:189-199.
- Howe, G. A. 2004. Jasmonates as signals in the wound response. *J. Plant Growth Regul.* 23:223-237.
- Howe, G. A., and Jander, G. 2008. Plant immunity to insect herbivores. *Annu. Rev. Plant Biol.* 59:41-66.
- Hudgins, J. W., and Franceschi, V. R. 2004. Methyl jasmonate-induced ethylene production is responsible for conifer phloem defense responses and reprogramming of stem cambial zone for traumatic resin duct formation. *Plant Physiol.* 135:2134-2149.
- Iverson, A. L., Iverson, L. R., and Eshita, S. 2001. The effects of surface applied jasmonic and salicylic acids on caterpillar growth and damage to tomato plants. *Ohio J. Sci.* 5:90-94.
- Jiang, B., Siregar, U., Willeford, K. O., Luthe, D. S., and Williams, W. P. 1995. Association of a 33-kilodalton cysteine proteinase found in corn callus with the inhibition of FAW larval growth. *Plant Physiol.* 108:1631-1640.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Laudert, D., and Weiler, E. W. 1998. Allene oxide synthase: A major control point in *Arabidopsis thaliana* octadecanoid signaling. *Plant J.* 15:675-684.
- Lincoln, J. E., and Fischer, R. L. 1988a. Diverse mechanisms for the regulation of ethylene-inducible gene expression. *Mol. Gen. Genet.* 212:71-75.
- Lincoln, J. E., and Fischer, R. L. 1988b. Regulation of gene expression by ethylene in wild-type and *rin* Tomato (*Lycopersicon esculentum*) fruit. *Plant Physiol.* 88:370-374.
- López, L., Camas, A., Shivaji, R., Ankala, A., Williams, P., and Luthe, D. S. 2007. Mirl-CP, a novel defense cysteine protease accumulates in maize vascular tissues in response to herbivory. *Planta* 226:517-527.
- López M. A., Bannenberg, G., and Castresana, C. 2008. Controlling hormone signaling is a plant and pathogen challenge for growth and survival. *Curr. Opin. Plant Biol.* 11:420-427.
- Lorenzo, O., and Solano, R. 2005. Molecular players regulating the jasmonate signaling network. *Curr. Opin. Plant Biol.* 8:532-540.
- Lorenzo, O., Piqueras, R., Sanchez-Serrano, J. J., and Solano, R. 2003. Ethylene response factor 1 integrates signals from ethylene and jasmonate pathways in plant defense. *Plant Cell* 15:165-178.
- Maffei, M. E., Mithöfer, A., and Boland, W. 2007. Before gene expression: Early events in plant insect interaction. *Trends Plant Sci.* 12:310-316.
- Maleck, K., and Dietrich, R. A. 1999. Defense on multiple fronts: How do plants cope with diverse enemies? *Trends Plant Sci.* 4:215-219.
- Margossian, L. J., Federman, A. D., Giovannoni, J. J., and Fischer, R. L. 1988. Ethylene-regulated expression of a tomato fruit ripening gene encoding a proteinase inhibitor I with a glutamic residue at the reactive site. *Proc. Natl. Acad. Sci. U.S.A.* 85:8012-8016.
- Mauricio, R., Rausher, M. D., and Burdick, D. S. 1997. Variation in the defense strategies of plants: Are resistance and tolerance mutually exclusive? Variability in ecology. *Ecology (Wash. D.C.)* 78:1301-1311.
- Mohan, S., Ma, P. K. W., Pechan, T., Bassford, E. R., Williams, W. P., and Luthe, D. S. 2006. Degradation of the *Spodoptera frugiperda* peritrophic matrix by an inducible maize cysteine protease. *J. Insect Physiol.* 52:21-28.
- Mohan, S., Ma, P. K.W., Williams, W. P., and Luthe, D. S. 2008. A naturally occurring plant cysteine protease possesses remarkable toxicity against insect pests and synergizes *Bacillus thuringiensis* toxin. *PLoS One* 3:e1786. Published online.
- Molina, A., and Olmedo, F. G. 1993. Developmental and pathogen induced expression of three barley genes encoding lipid transfer proteins. *Plant J.* 4:983-991.
- Nakano, T., Suzuki, K., Ohtsuki, N., Tsujimoto, Y., Fujimura, T., and Shinshi, H. 2006. Identification of genes of the plant-specific transcription-factor families cooperatively regulated by ethylene and jasmonate in *Arabidopsis thaliana*. *J. Plant Res.* 119:407-413.
- Nemchenko, A., Kunze, S., Feussner, I., and Kolomiets, M. 2006. Duplicate maize 13-lipoxygenase genes are differentially regulated by circadian rhythm, cold stress, wounding, pathogen infection and hormonal treatments. *J. Exp. Bot.* 57:3767-3779.
- Nojiri, H., Sugimori, M., Yamane, H., Nishimura, Y., Yamada, A., Shibuya, N., Kodama, O., Murofushi, N., and Omori, T. 1996. Involvement of

- jasmonic acid in elicitor induced phytoalexin production in suspension cultured rice cells. *Plant Physiol.* 110:387-392.
- O'Donnell, P. J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H. M. O., and Bowles, D. J. 1996. Ethylene as a signal mediating the wound response of tomato plants. *Science* 274:1914-1917.
- Oikawa, A., Ishihara, A., Hasegawa, M., Kodama, O., and Iwamura, H. 2001. Induced accumulation of 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc) in maize leaves. *Phytochemistry* 56:699-675.
- Ortel, B., Atzorn, R., Hause, B., Feussner, I., Miersch, O., and Wasternack, C. 1999. Jasmonate-induced gene expression of barley (*Hordeum vulgare*) leaves—the link between jasmonate and abscissic acid. *Plant Growth Regul.* 29:113-122.
- Pechan, B., Jiang, B., Steckler, D., Ye, L., Lin, L., Luthe, D. S., and Williams, P. W. 1999. Characterization of three distinct cDNA clones encoding cysteine proteinases from maize (*Zea mays* L.) callus. *Plant Mol. Biol.* 40:111-119.
- Pechan, B., Ye, L., Chang, Y. M., Mitra, A., Lin, L., Davis, F. M., Williams, W. P., and Luthe, D. S. 2000. A unique 33-KD cysteine protease accumulates in response to larval feeding in maize genotypes resistant to FAW and other Lepidoptera. *Plant Cell* 12:1031-1040.
- Pechan, T., Cohen, A., Williams, W. P., and Luthe, D. S. 2002. Insect feeding mobilizes a unique plant defense proteinase that disrupts the peritrophic matrix of caterpillars. *Proc. Natl. Acad. Sci. U.S.A.* 99:13319-13323.
- Pechan, T., Ma, P. W. K., and Luthe, D. S. 2004. Heterologous expression of maize (*Zea mays* L.) Mir-1 cysteine protease in eukaryotic and prokaryotic expression systems. *Protein Expr. Purif.* 34:134-141.
- Peña-Cortés, H., Albrecht, T., Prat, S., Weiler, E. W., and Willmitzer, L. 1993. Aspirin prevents wound induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. *Planta* 191:123-128.
- Penninckx, I. A. M. A., Thomma, B. P. H. J., Buchala, A., Mettraux, J. P., and Broekaert, W. F. 1998. Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. *Plant Cell* 10:2103-2113.
- Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:2002-2007.
- Pieterse, C. M. J., and Dicke, M. 2007. Plant interactions with microbes and insects: From molecular mechanisms to ecology. *Trends Plant Sci.* 12:564-569.
- Poupard, P., Parisi, L., Campion, C., Ziadi, S., and Simoneau, P. 2003. A wound- and ethephon-inducible PR-10 gene subclass from apple is differentially expressed during infection with a compatible and an incompatible race of *Venturia inaequalis*. *Physiol. Mol. Plant Pathol.* 62:3-12.
- Rasmussen, R. 2001. Quantification on the LightCycler. Pages 21-34 in: *Rapid Cycle Real-time PCR, Methods and Applications*. S. Meuer, C. Wittwer, and K. Nakagawara, eds. Springer Press, Heidelberg, Germany.
- Ritchie, S. W., Hanway, J. J., and Benson, G. O. 1986. How a corn plant develops. Iowa State University of Science and Technology Cooperative Extension Service Special Report 48 (Revised), Ames, IA, U.S.A.
- Robert-Seilanianz, A., Navarro, L., Bari, R., and Jones J. D. G. 2007. Pathological hormone imbalances. *Curr. Opin. Plant Biol.* 10:372-379.
- Rojo, E., Leon, J., and Sanchez-Serrano, J. J. 1999. Cross-talk between wound signalling pathways determines local versus systemic gene expression in *Arabidopsis thaliana*. *Plant J.* 20:135-142.
- Ryan, C. A. 2000. The systemin signaling pathway: Differential activation of plant defensive genes. *Biochim. Biophys. Acta.* 1477:112-121.
- Schilmiller, A. L., and Howe, G. A. 2005. Systemic signaling in the wound response. *Curr. Opin. Plant Biol.* 8:369-377.
- Shinshi, H. 2008. Ethylene-regulated transcription and crosstalk with jasmonic acid. *Plant Sci.* 175:18-23.
- Shoji, T., Nakajima, K., and Hashimoto, T. 2000. Ethylene suppresses jasmonate-induced gene expression in nicotine bio-synthesis. *Plant Cell Physiol.* 41:1072-1076.
- Spoel, S. H., and Dong, X. 2008. Making sense of hormone crosstalk during plant immune response. *Cell Host Microbe* 3:348-351.
- Spoel, S. H., Koornneef, A., Claessens, S. M. C., Korzelius, J. P., Pelt, J. A. V., Muller, M. J., Buchala, A. J., Métraux, J. P., Brown, R., Kazan, K., Loon, L. C. V., Dong, X., and Pieterse, C. M. J. 2003. NPR1 modulates cross-talk between salicylate and jasmonate dependent defense pathways through a novel function in the cytosol. *Plant Cell* 15:760-770.
- Staswick, P. E., Huang, J. F., and Rhee, Y. 1991. Nitrogen and methyl jasmonate induction of soybean vegetative storage protein genes. *Plant Physiol.* 96:130-136.
- Waldbauer, G. P. 1968. The consumption and utilization of food by insects. *Adv. Insect Physiol.* 5:229-288.
- Walling, L. L. 2000. The myriad plant responses to herbivores. *J. Plant Growth Regul.* 19:195-216.
- Wang, K. I., Li, H., and Ecker, J. R. 2002. Ethylene biosynthesis and signaling networks. *Plant Cell* 14:S131-S151.
- Wasternack, C., Stenzel, I., Hause, B., Hause, G., Kutter, C., Maucher, H., Neumerkel, J., Feussner, I., and Miersch, O. 2006. The wound response in tomato—role of jasmonic acid. *J. Plant Physiol.* 163:297-306.
- Williams, W. P., Buckley, P. M., and Windham, G. L. 1990. Registration of Mp708 germplasm line of maize. *Crop Sci.* 30:757.
- Xu, Y., Chang, P. F. L., Liu, D., Narasimhan, M. L., Raghothama, K. G., Hasegawa, P. M., and Bressan, R. A. 1994. Plant defense genes are synergistically induced by ethylene and methyl jasmonate. *Plant Cell* 6:1077-1085.
- Zhao, J., Zheng, S. H., Fujita, K., and Sakai, K. 2004. Jasmonate and ethylene signaling and their interaction are integral parts of the elicitor signaling pathway leading to β -thujaplicin biosynthesis in *Cupressus lusitanica* cell cultures. *J. Exp. Bot.* 55:1003-1012.