Disruption of Ethylene Responses by *Turnip mosaic virus* Mediates Suppression of Plant Defense against the Green Peach Aphid Vector[1][OPEN]

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Plants employ diverse responses mediated by phytohormones to defend themselves against pathogens and herbivores. Adapted pathogens and herbivores often manipulate these responses to their benefit. Previously, we demonstrated that *Turnip mosaic virus* (TuMV) infection suppresses callose deposition, an important plant defense induced in response to feeding by its aphid vector, the green peach aphid (*Myzus persicae*), and increases aphid fecundity compared with uninfected control plants. Further, we determined that production of a single TuMV protein, Nuclear Inclusion a-Protease (NIa-Pro) domain, was responsible for changes in host plant physiology and increased green peach aphid reproduction. To characterize the underlying molecular mechanisms of this phenomenon, we examined the role of three phytohormone signaling pathways, jasmonic acid, salicylic acid, and ethylene (ET), in TuMV-infected Arabidopsis (*Arabidopsis thaliana*), with or without aphid herbivory. Experiments with Arabidopsis mutants *ethylene insensitive2* and *ethylene response1*, and chemical inhibitors of ET synthesis and perception (aminoethoxyvinyl-glycine and 1-methylcyclopropene, respectively), show that the ET signaling pathway is required for TuMV-mediated suppression of Arabidopsis resistance to the green peach aphid. Additionally, transgenic expression of NIa-Pro in Arabidopsis alters ET responses and suppresses aphid-induced callose formation in an ET-dependent manner. Thus, disruption of ET responses in plants is an additional function of NIa-Pro, a highly conserved potyvirus protein. Virus-induced changes in ET responses may mediate vector-plant interactions more broadly and thus represent a conserved mechanism for increasing transmission by insect vectors across generations.

Plants suffer from numerous pathogen and herbivore challenges in both natural and agricultural environments, often facing multiple simultaneous threats (Casteel and Hansen, 2014). For example, many plant pathogens depend on insect vectors for transmission, including over 75% of all described plant viruses (Nault, 1997). Thus, plants must recognize, prioritize, and mount the most appropriate response to both the insect that is feeding and the pathogen being transmitted. Despite constant attack, plants persist, largely due to a sophisticated surveillance system. Plants respond with an arsenal of defenses that may be morphological, biochemical, or molecular in nature (Jones and Dangl, 2006; Jander and Howe, 2008). Nevertheless, pathogens and insects successfully colonize plants by actively compromising plant perception and/or defense responses.

Recent studies show that synergisms exist between challengers, where both parties benefit during dual attack. For example, some virus infections can decrease plant defenses against insects, increasing plant palatability and vector fitness. Consequently, improved insect performance will increase the number of viruliferous vectors, promoting virus transmission to new hosts (Mauck et al., 2010; Casteel and Jander, 2013; Casteel et al., 2014; Li et al., 2014). Thus, vector-plant interactions represent a critical and synergistic relationship, ultimately determining survival and host range. Although numerous studies have examined virus-plant interactions, few have examined the molecular and genetic mechanisms mediating plant-virus-vector interactions and alterations in plant defenses (Li et al., 2014; Mauck et al., 2014).

While defenses vary widely across plant species, the phytohormones that regulate their production are somewhat conserved. Modulation of hormone composition, timing, and concentration specifies plant responses to an attack (Mur et al., 2006; Verhage et al., 2010) and represents an excellent target for compromising defenses. Numerous studies have demonstrated

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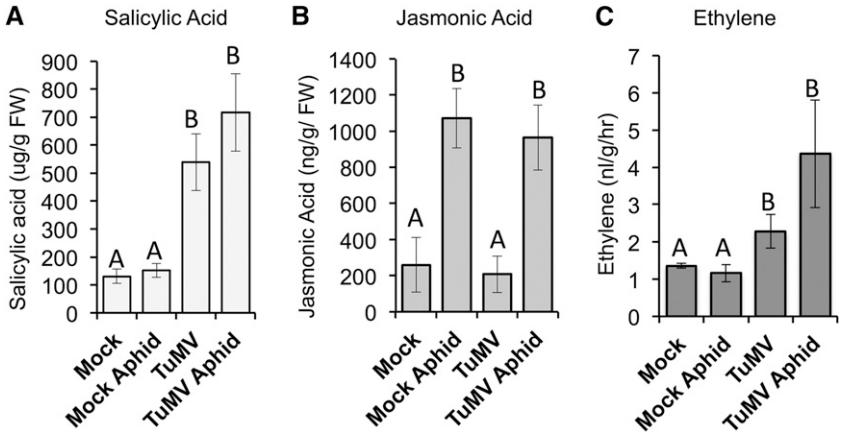

Figure 1. Concentrations of phytohormones in Arabidopsis plants with or without TuMV infection and aphid herbivory. Shown are SA (A), JA (B), and ET accumulation (C) in mock-inoculated or TuMV-GFP-infected Arabidopsis plants with or without 24 h of the feeding by the green peach aphid (mean ± SE of $n = 4–6$; letters represent significant differences, ANOVA and Tukey's HSD post hoc test). FW, Fresh weight.

that at least three phytohormones, jasmonic acid (JA), salicylic acid (SA), and ethylene (ET), have major roles in orchestrating plant defense responses (Bari and Jones, 2009; Erb et al., 2012; Pieterse et al., 2012). In general, SA signaling is critical for defense responses against a wide range of pathogens, including viruses (Glazebrook, 2005; Carr et al., 2010). Production of JA and ET, meanwhile, are involved in regulation of plant response to herbivores, necrotrophic pathogens, and nonpathogenic microbes (Glazebrook, 2005; Howe and Jander, 2008; Van der Ent et al., 2009). Virus infection can also alter JA and ET signaling (Carr et al., 2010; Lewsey et al., 2010; Wei et al., 2010; Mauck et al., 2014).

Together, Arabidopsis (*Arabidopsis thaliana*), the green peach aphid (*Myzus persicae*), and *Turnip mosaic virus* (TuMV) constitute an excellent model system for investigating the molecular and biochemical mechanisms that underlie plant-aphid-virus interactions. As a well-studied model plant, Arabidopsis provides numerous genetic resources that can be used to investigate responses to aphid feeding and virus infection. The green peach aphid is a broad-host-range aphid and the world's most prolific plant virus vector, transmitting more than 100 different viral species (Kennedy et al., 1962). The green peach aphid is the most common aphid pest on Arabidopsis in greenhouses and growth chambers (Bush et al., 2006), and we also have observed it feeding from Arabidopsis growing in nature. Due to the agricultural relevance of the green peach aphid, there is a large body of literature about the biology of this insect and its interactions with host plants, going back more than 100 years. More recently, several research groups have initiated projects to study plant defense against aphids using Arabidopsis and the green peach aphid as a model system (de Vos et al., 2007; Louis and Shah, 2013). TuMV is a positive-strand RNA virus that infects not only Arabidopsis but also hundreds of other species in more than 40 plant families (Walsh and Jenner, 2002). It is considered to be one of the most damaging viruses for vegetable crops worldwide (Tomlinson, 1987; Nguyen et al., 2013; Yasaka et al., 2015) and is transmitted by the green peach aphid and many other aphid species in both natural and agricultural settings (Shattuck, 1992). Largely due to its ability to systemically infect Arabidopsis (Sánchez et al., 1998; Martín Martín et al., 1999), TuMV has become a model for potyvirus-host interactions (Walsh and Jenner, 2002).

In this study, we investigate the role of phytohormone signals in TuMV's ability to suppress plant defense and enhance aphid fecundity during infection of host plants. First, we show that TuMV infection induces SA and ET accumulation in Arabidopsis. Next, using genetic and pharmacological analyses, we demonstrate that ET signaling is necessary for TuMV-initiated suppression of plant defense responses and enhanced aphid reproduction in plants. Further, we show that expression of the viral protein Nuclear Inclusion a-Protease (NIa-Pro) alters ET responses and that ET is also required

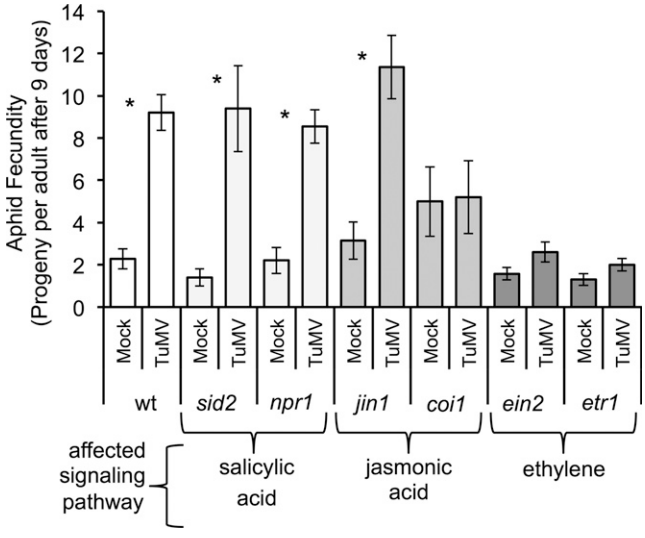

Figure 2. Involvement of plant defense signaling in TuMV-aphid-plant interactions. Number of progeny produced by a single aphid after 9 d on TuMV-GFP-infected or mock-inoculated Arabidopsis wild-type (WT) controls or hormone-signaling mutants (mean ± SE of $n = 15–30$, $^*P < 0.05$; two-tailed Student's t test comparing infected and uninfected plants of the same genotype).

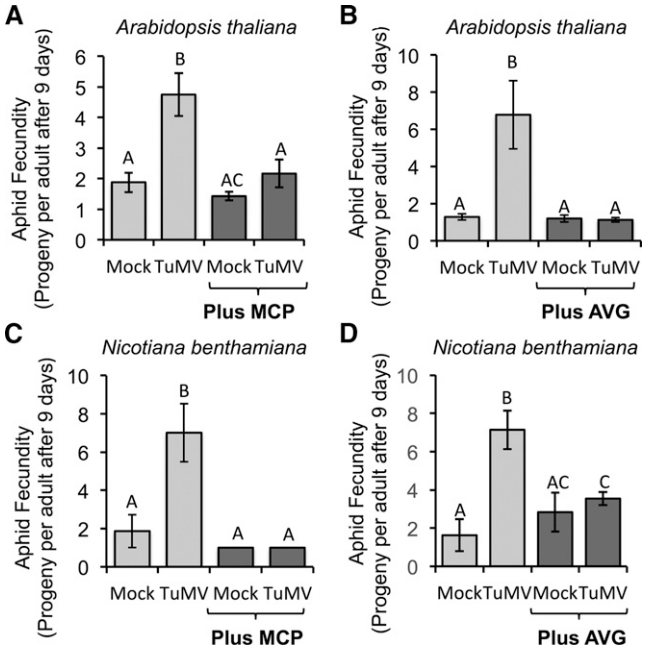

Figure 3. ET perception and biosynthesis is required for TuMV infection to increase green peach aphid fecundity on host plants. Number of progeny produced by a single aphid after 9 d on TuMV-GFP-infected or mock-inoculated Arabidopsis (A and B) and *N. benthamiana* (C and D) treated with MCP, which blocks ET perception, or AVG, which inhibits ET biosynthesis (mean ± SE of n = 15–18; letters represent significant differences, ANOVA and Tukey's HSD post hoc).

for NIa-Pro's role in suppressing aphid-induced defense in virus-infected plants. This molecular, biochemical, and genetic evidence reveals that TuMV may modulate ET responses not only to increase plant susceptibility to infection but also to increase vector performance.

RESULTS

SA, JA, and ET were quantified in Arabidopsis plants challenged with TuMV, aphids, and the combination of the two (Fig. 1). TuMV-GFP infection more than doubled SA and ET production in host plants relative to controls. Aphid feeding had no significant impact on SA or ET accumulation (Fig. 1, A and C) but induced JA accumulation more than 4-fold relative to control plants (Fig. 1B). There were no significant impacts of TuMV in combination with aphids on SA, JA, and ET.

To determine the biological relevance of these phytohormone changes, we surveyed Arabidopsis mutants that were compromised in individual signaling pathways for enhanced aphid fecundity on TuMV-infected plants. Aphids produced significantly more progeny on TuMV-infected wild-type Arabidopsis and mutants compromised in SA signaling (*salicylic acid induction deficient2* [*sid2-1*] and *nonexpressor of pathogenesis-related protein1* [*npr1*-2]; Fig. 2). This suggests that a functioning SA-signaling pathway is not required for TuMV's ability to enhance aphid fecundity. In contrast to SA-signaling mutants, aphid reproduction was not enhanced by TuMV-GFP infection of mutants insensitive to ET (*ethylene insensitive2* [*ein2-1*] and *ethylene receptor1* [*etr1-3*]; Fig. 2). Although aphid performance was not enhanced on the JA-insensitive mutant *coronatine insensitive1* (*coi1*) infected with TuMV-GFP compared with the mock-inoculated mutant, aphids generally performed better on *coi1* plants compared with wild-type controls without TuMV-GFP. This was not observed in another

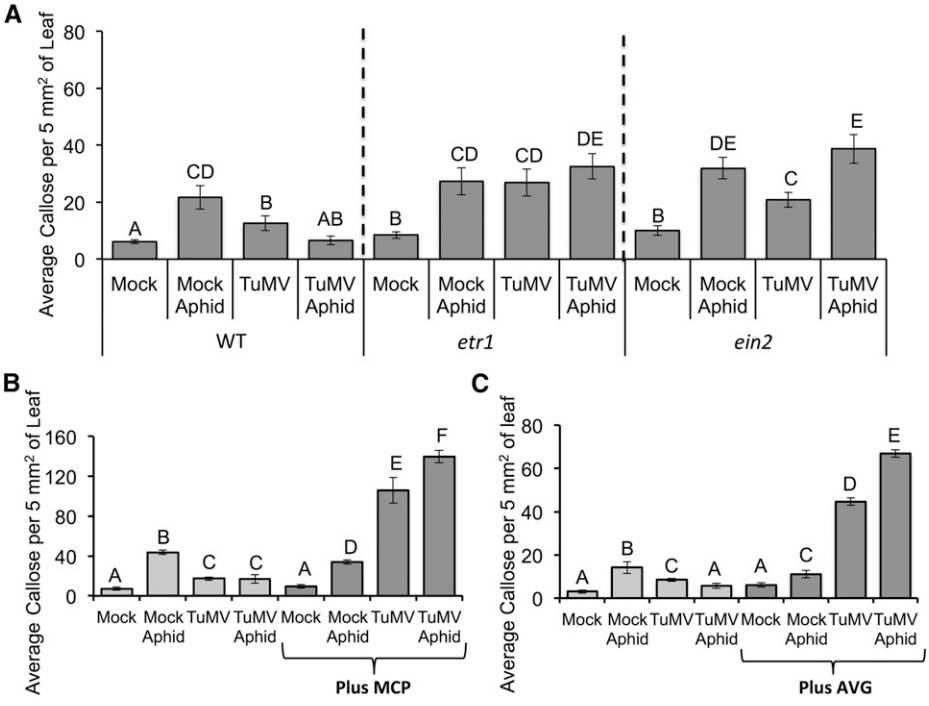

Figure 4. ET signaling is required for TuMV infection to reduce callose accumulation. A, Callose deposition in Arabidopsis wild-type (WT) controls or ET-insensitive mutants that were mock inoculated or TuMV-GFP infected with or without green peach aphid infestation. Callose deposition in Arabidopsis leaves that were mock inoculated or TuMV-GFP infected with or without green peach aphid infestation and treated with MCP (B), which blocks ET perception, or AVG (C), which inhibits ET biosynthesis (mean ± SE of n = 4–6; different letters indicate significant differences by ANOVA and Tukey's HSD post hoc).

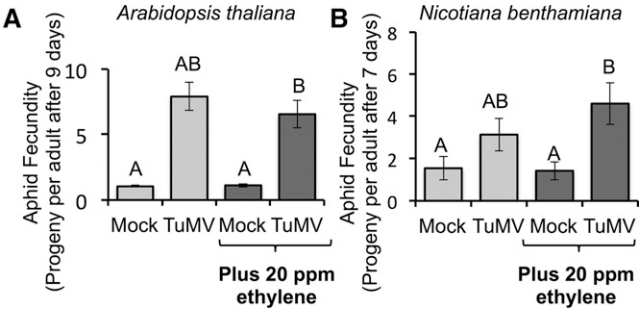

Figure 5. ET treatment does not enhance green peach aphid fecundity on host plants. Number of progeny produced by a single aphid after 7 d on TuMV-GFP-infected or mock-inoculated Arabidopsis (A) and *N. benthamiana* (B) treated with 20 μL L^{-1} ET (mean ± SE of n = 15–28; letters represent significant differences, ANOVA and Tukey's HSD post hoc test).

JA-insensitive mutant (*jasmonate insensitive1* [*jin1*]; Fig. 2). As reported previously, green peach aphids produced more progeny on plants with defects in JA signaling (Mewis et al., 2005). Together, these results indicate that induction of ET and components of jasmonic signaling may be critical to TuMV's ability to enhance aphid fecundity during infection of host plants, whereas the induction of SA is not.

To further investigate the role of ET signaling in virus-vector interactions, we used a pharmacological approach to inhibit ET signaling. TuMV-infected Arabidopsis plants were treated with aminoethoxyvinylglycine (AVG), which inhibits ET biosynthesis (Amrhein and Wenker, 1979), or with 1-methylcyclopropene (MCP), which blocks ET perception by binding to ET receptors (Sisler, 2006). Two chemical inhibitors were used in experiments, because, although AVG is easier to work with, it is known to also impact auxin signaling in host plants (Soeno et al., 2010). Aphids were then caged on these plants and allowed to develop and reproduce over time. TuMV-GFP infection did not enhance aphid fecundity when ET perception (MCP; Fig. 3, A and C) or biosynthesis (AVG; Fig. 3, B and D) was inhibited in Arabidopsis and *Nicotiana benthamiana*, further confirming the genetic approaches described above (Fig. 2).

Previously, we demonstrated that TuMV infection inhibits aphid-induced callose production in host plants (Casteel et al., 2014). Because ET plays a major role in bacteria-triggered callose deposition (Clay et al., 2009), it may be required for inhibition of aphid-induced callose deposition by TuMV. Consistent with this hypothesis, aphid-induced callose was inhibited in wild-type plants infected with TuMV, while aphids induced callose deposition significantly in ET-signaling mutants infected with TuMV-GFP (Fig. 4A). Next, we treated plants challenged with TuMV-GFP using the ET inhibitors MCP and AVG, as described above, and quantified aphid-induced callose accumulation. While TuMV-GFP infection inhibited aphid-induced callose deposition in wild-type plants, aphid feeding induced significant callose deposition in infected plants treated with ET biosynthesis or perception inhibitors (Fig. 4, B and C). Additionally, callose induction in TuMV-infected plants was generally higher in plants with compromised ET signaling compared with corresponding controls (Fig. 4), suggesting that induction of ET during virus infection (Fig. 1C) may play a major role in inhibition of virus-induced callose formation. To determine the role of ET induction in enhanced aphid fecundity, we treated mock- and TuMV-infected plants with 20 μL L^{-1} ET. Aphids were then added as previously described and fecundity quantified. Treatment of plants with ET did not enhance aphid fecundity in either treatment significantly (Fig. 5), suggesting that the amount of ET added is beyond the threshold required to increase aphid performance and aphids will not benefit further.

NIa-Pro is the major protease needed to process the TuMV polyprotein into individual functioning proteins (Urcuqui-Inchima et al., 2001). Recently, we demonstrated the additional ability of NIa-Pro to inhibit plant defenses and increase aphid performance (Casteel et al., 2014). Further experiments were designed to determine whether ET signaling is required for NIa-Pro's ability to enhance aphid performance and inhibit plant defenses (Fig. 6). Consistent with previous results, aphid-induced callose was inhibited and aphids were significantly more fecund on plants

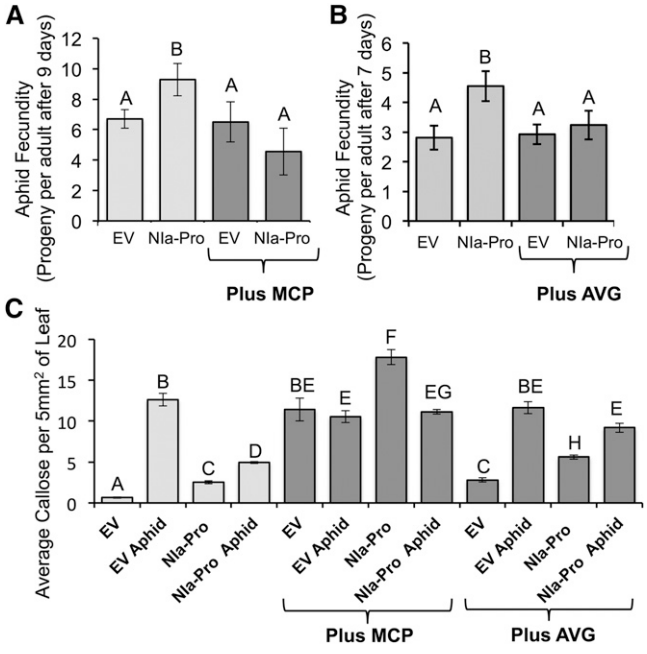

Figure 6. Expression of the TuMV protein NIa-Pro requires ET signaling to increase aphid growth and reduce callose accumulation. Number of progeny produced by aphids on Arabidopsis expressing NIa-Pro or the EV control treated with MCP (A), which blocks ET perception, or AVG (B), which inhibits ET biosynthesis (mean ± SE of n = 12–18; letters represent significant differences, ANOVA and Tukey's HSD post hoc). C, Callose accumulation on Arabidopsis expressing NIa-Pro or the EV control treated with MCP or AVG (mean ± SE of n = 4–6; letters represent significant differences, ANOVA and Tukey's HSD post hoc test).

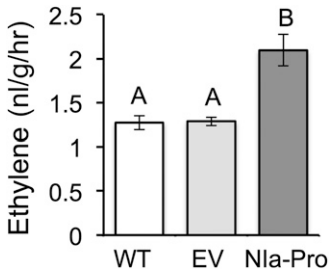

Figure 7. Expression of the TuMV protein NIa-Pro increases ET production. Concentrations of ET in wild-type (WT) Arabidopsis and transgenic plants expressing the EV control or NIa-Pro (mean ± SE of $n = 6$; letters represent significant differences, ANOVA and Tukey's HSD post hoc test).

expressing NIa-Pro compared with empty vector (EV) controls (Fig. 6, A–C). However, when ET signaling was inhibited in plants expressing NIa-Pro, aphid fecundity was not increased (Fig. 6, A and B). While inhibition of aphid-induced callose was prevented in transgenic plants expressing NIa-Pro treated with AVG, the same pattern was not observed with MCP (Fig. 6, B and C). Surprisingly, all plants treated with MCP had greater amounts of callose (Fig. 6B). However, this is consistent with a lack of significant difference in aphid fecundity observed on these plants (Fig. 6A). These results suggest that ET signaling is required for NIa-Pro's ability to enhance aphid performance.

To test the hypothesis that NIa-Pro expression alters ET production in plants, we conducted experiments with transgenic Arabidopsis expressing NIa-Pro. While there was no significant difference in ET production between wild-type plants and plants expressing the EV, plants expressing NIa-Pro produced greater amounts of constitutive ET compared with both controls (Fig. 7). Next, we examined ET-dependent and -independent changes in transcript abundance to determine the generality of the response. We measured accumulation of *EIN2*, a positive regulator of the ET-signaling pathway (Qiao et al., 2009), *EIN3*, a key transcription factor mediating ET-regulated gene expression (Guo and Ecker, 2003), *ETHYLENE RESPONSE FACTOR1* (*ERF1*), a transcription factor induced by ET production and targeted by *EIN3* (Solano et al., 1998), acting downstream of *EIN2* (Stepanova and Alonso, 2005), and an ET-inducible *PLANT DEFENSIN1.2* (*PDF1.2*; Penninckx et al., 1998). We did not observe a major modification of *EIN2* or *EIN3* transcript abundance in TuMV or aphid treatments (Fig. 8, A and B). However, *EIN2* transcript accumulation was increased in the plants expressing the EV and NIa-Pro with aphid feeding (Fig. 8A). *EIN2* and *EIN3* accumulation are not regulated by ET production as evident in previous studies (Alonso et al., 1999; Guo and Ecker, 2003). In contrast to *EIN2* and *EIN3*, *ERF1* and *PDF1.2* are induced by ET production (Brown et al., 2003; Lorenzo et al., 2003). *ERF1* transcript abundance is increased in plants by aphid feeding, TuMV infection, and NIa-Pro expression (Fig. 8C). Additionally, aphid feeding on plants expressing NIa-Pro increased *ERF1* transcript abundance the most compared with all other treatments (Fig. 7C). Surprisingly, *PDF1.2* was not induced by TuMV infection but was significantly increased in plants expressing NIa-Pro (Fig. 8D) compared with controls. However, *PDF1.2* was not induced in plants expressing NIa-Pro with aphid feeding compared with controls (Fig. 8D). Next, we measured the accumulation of *ORGAN SIZE RELATED1* (*OSR1*) and *AZELAIC ACID INDUCED1* (*AZI1*), two transcripts not related to ET signaling or ET-induced defense responses but robustly induced in response to ET treatment (Hall et al., 2012). TuMV infection increased *OSR1* accumulation, while aphid feeding induced *AZI1* accumulation compared with controls (Fig. 9). However, in contrast to the ET-induced transcripts related to plant defenses (*ERF1* and *PDF1.2*), NIa-Pro expression did not

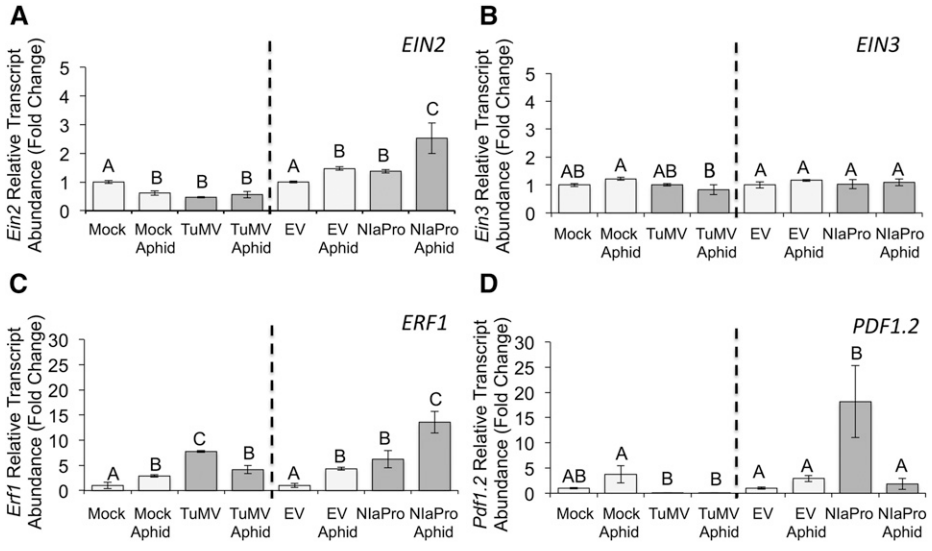

Figure 8. Expression of the TuMV protein NIa-Pro alters ET signaling. Relative *EIN2* (A), *EIN3* (B), *ERF1* (C), and *PDF1.2* (D) transcript abundance was measured by qRT-PCR in mock-inoculated and TuMV-GFP-inoculated plants with or without aphid feeding for 24 h and in leaves of plants expressing the EV or NIa-Pro with or without aphid feeding for 24 h (mean ± SE of $n = 3$; letters indicate significant differences by ANOVA and Tukey's HSD post hoc test, with transcript abundance in mock-inoculated and EV plants set to 1).

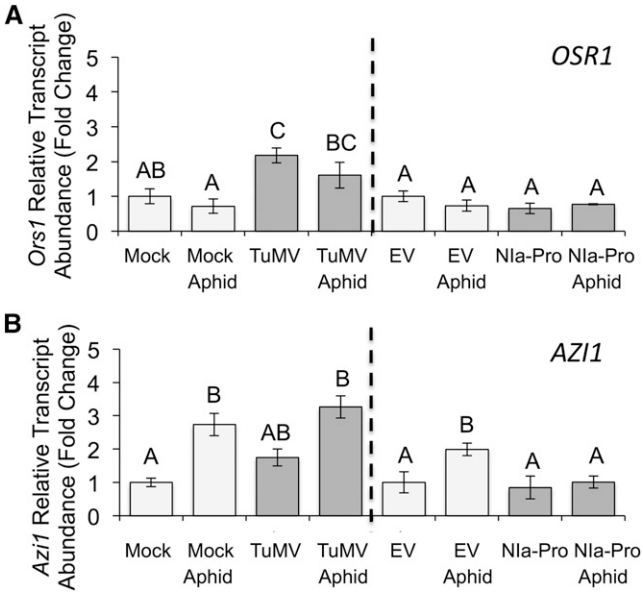

Figure 9. Expression of the TuMV protein NIa-Pro does not alter ET-induced transcripts. Relative *OSR1* (A) and *AZI1* (B) transcript abundance was measured by qRT-PCR in mock-inoculated and TuMV-GFP-inoculated plants with or without aphid feeding for 24 h and in leaves of plants expressing the EV or NIa-Pro with or without aphid feeding for 24 h (mean ± SE of $n = 3$; letters indicate significant differences by ANOVA and Tukey's HSD post hoc test, with transcript abundance in mock-inoculated and EV plants set to 1).

alter accumulation of *OSR1* and *AZI1* (Fig. 9). These results show that NIa-Pro expression interferes with a specific set of ET-induced defense responses.

Induction of ET by TuMV may indirectly benefit the virus by increasing vector performance and thus the number of inoculated vectors available for transmission. ET induction also may benefit the virus directly by increasing infection efficiency or performance. To determine whether ET induction is directly beneficial to TuMV, we challenged 3-week-old Arabidopsis mutants that were insensitive to ET (*ein2-1* and *etr1-3*) and mutants that constitutively induce ET (*ethylene-overproducer1-2* [*eto1-2*]; Fig. 10) with TuMV. Next, the number of infected plants was quantified after 5 d. Significantly greater numbers of ET-insensitive mutants were infected compared with wild-type controls (Fig. 10). However, in mutants that constitutively produce ET, there was no difference in infection rate (Fig. 10). These findings indicate that induction of ET signaling is important for successful TuMV infection of Arabidopsis.

DISCUSSION

Our results demonstrate that ET responses are critical for TuMV-vector synergisms. TuMV induces ET production (Fig. 1), and ET biosynthesis and perception are required for NIa-Pro to suppress plant defenses and increase insect performance on infected host plants (Figs. 2–4 and 6). Further, expression of NIa-Pro directly increases ET production (Fig. 7) and alters a specific set of ET-induced defense transcripts (Figs. 8 and 9). Additionally, plants with compromised ET signaling are more resistant to TuMV (Fig. 8). Taken together, these results suggest that TuMV may be inducing ET production to increase plant susceptibility. Alterations in ET also benefit aphids, increasing insect fecundity on infected plants and therefore the number of viruliferous aphids. Virus infection and aphid feeding have been shown to influence the production of ET (Love et al., 2005, 2007; Kim et al., 2008; Mantelin et al., 2009; Chen et al., 2013; Haikonen et al., 2013; Mandadi et al., 2014; Mauck et al., 2014), though ET's role in vector-virus-plant interactions has not yet been demonstrated. Virus-induced changes in ET responses may mediate vector-plant interactions more broadly and thus represent a conserved mechanism for increasing transmission by insect vectors across generations.

ET regulates plant responses to biotic and abiotic stress and mediates plant development and senescence (van Loon et al., 2006; Koyama, 2014). No generalized role of ET in plant-virus interactions has been established. Also, only a few systems have been investigated with significant variation across interactions (van Loon et al., 2006; Love et al., 2007; Kim et al., 2008; Endres et al., 2010; Haikonen et al., 2013; Mauck et al., 2014). However, ET may play an important role in antiviral defense. Recent studies found that Arabidopsis *ein2* and *etr1* mutations increased resistance to *Tobacco mosaic virus* and *Cauliflower mosaic virus* (Love et al., 2005, 2007; Chen et al., 2013), consistent with our results. Further, overexpression of *ERF5*, an ET response transcription factor, from tobacco (*Nicotiana tabacum*) conferred reduced susceptibility to *Tobacco mosaic virus*, indicating an important role of ET in plant-virus interactions. Increases in ET production following aphid feeding also have been reported in various plant-aphid interactions. However, ET production has been associated with both increased susceptibility and resistance to aphids (Miller et al., 1994; Argandoña et al., 2001; Mantelin et al., 2009; Lu et al., 2014; Wu et al., 2015). The role of ET in both plant-virus and plant-aphid interactions

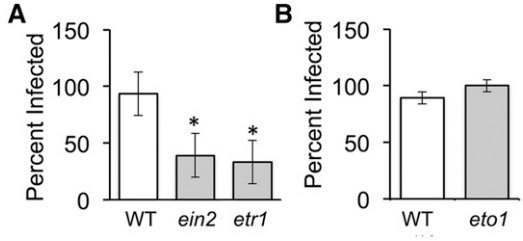

Figure 10. ET signaling is required for plant susceptibility to TuMV. Percentage of TuMV-GFP infection for Arabidopsis ET-insensitive mutants (A; *ein2-1* and *etr1-3*) and mutants that constitutively produce ET (B; *eto1-2*; $n = 30–48$, $^*P < 0.05$; χ^2 test relative to wild-type [WT] control).

may be mediated by the compatibility of the interactions, although additional studies are needed to confirm this.

NIa-Pro is the main protease for TuMV, cleaving the TuMV polyprotein into individual proteins (Urcuqui-Inchima et al., 2001). NIa-Pro possesses relatively strict substrate specificity, cleaving after Gln at Val-Xaa-His-Gln (Kang et al., 2001). A previous study demonstrated that the same consensus sequence site, Val-Xaa-His-Gln, exists in an amyloid-β peptide from animals and that NIa-Pro has activity against this site (Han et al., 2010). It is possible that NIa-Pro also cleaves a plant protein that possesses the NIa-Pro cleavage substrate. Yet NIa-Pro also has other functions, including non-specific DNAse activity (Anindya and Savithri, 2004; Rajamäki and Valkonen, 2009), and may possess additional unknown functions that are critical to aphid-virus-plant interactions.

Relatively few studies have identified the host plant genes that mediate virus-plant-vector interactions. However, two host proteins have been identified that may mediate plant interactions with *Tomato yellow leaf curl China virus* (TYLCCNV) and its whitefly vector, *Bemisia tabaci*. TYLCCNV-infected plants have reduced defense responses and impaired JA signaling, benefiting whitefly vectors and increasing attraction. TYLCCNV is transmitted with a β satellite pathogenicity factor, βC1, which mediates suppression of plant signaling and defense responses (Yang et al., 2008; Zhang et al., 2012). Recently, host proteins were identified that interact with βC1 and mediate suppression of plant signaling and defense responses. βC1 interacts with ASYMMETRIC LEAVES1, which suppresses JA signaling, and with the transcription factor MYC2, compromising activation of plant defense responses (Yang et al., 2008; Li et al., 2014). Future identification of host proteins that interact with NIa-Pro will shed light on this unique function.

While ET signaling is required for TuMV's ability to increase aphid fecundity, it does not appear that NIa-Pro is targeting a key regulator of ET signaling, as NIa-Pro alters a specific set of ET-induced defense transcripts (Figs. 8 and 9). Signaling by other hormones could also be involved. Virus infection alters the accumulation of many plant hormones, which can be viewed as either a disruption to a susceptible host or a coordination of responses by a resistant host (Alazem and Lin, 2014). Consistent with previous findings (Ellis et al., 2002), aphids produced more progeny on a *coi1* mutant, which lacks a receptor for JA-Ile conjugates, than on wild-type Arabidopsis. Additionally, TuMV-enhanced aphid fecundity was not observed on the *coi1* mutant, suggesting the involvement of JA signaling in virus-vector-plant interactions (Fig. 2). In contrast to *coi1*, TuMV-GFP infection in *jin1*, another JA-signaling mutant, still enhanced aphid fecundity (Fig. 2). These results suggest that NIa-Pro may target components of this pathway in addition to ET. Significant cross talk exists between the ET and JA pathways. However, changes may not be directly related to JA. For example, ET-dependent inhibition of root growth (Adams and Turner, 2010) and susceptibility to *Verticillium longisporum* (Ralhan et al., 2012) are altered in the *coi1* mutant independently of JA biosynthesis and related signaling. Thus, alterations in as yet unknown components of COI1 signaling may explain our observed results.

Although genetic resistance in the host plants is currently the best approach for TuMV management, such resistance is not available for all crop species or it may fail because it is not effective against all strains of the virus (Shattuck, 1992). Therefore, research on the interactions between TuMV, its aphid vectors, and host plants is necessary for the development of new strategies to combat viral infections in agricultural crops. Although further research on the interactions between green peach aphids, TuMV, and their host plants will be needed, the results presented here suggest that manipulation of ET signaling in response to virus infection may provide a means to limit the spread and transmission of TuMV in plants.

MATERIALS AND METHODS

Plants and Growth Conditions

Wild-type Arabidopsis (*Arabidopsis thaliana*) Columbia-0 and Arabidopsis mutants in the Columbia-0 background (*sid2-1*, *npr1-2*, *jin1-1*, *coi1-1*, *ein2-1*, *eto1-2*, and *etr1-3*) were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org). *Nicotiana benthamiana* seeds were obtained from Peter Moffett (Université de Sherbrooke). Plants were grown in Conviron growth chambers in 20- × 40-cm nursery flats using Cornell Mix (by weight, 56% peat moss, 35% vermiculite, 4% lime, 4% Osmocoat slow-release fertilizer [Scotts], and 1% Unimix [Scotts]) at 23°C and a 16-h-light/8-h-dark photoperiod, as previously described (Casteel et al., 2014). Seeds from *COI1/coi1-1* Arabidopsis were planted and grown as previously described (Rasmann et al., 2012). Plants were grown for 3 weeks and were used in experiments before flowering, unless otherwise noted. All experiments were conducted at least two times, with varying numbers of biological replicates per treatment per experiment.

TuMV Infection

TuMV-GFP was propagated from infectious clone p35TuMVGFP (Lellis et al., 2002). To prepare inoculum, fully infected *N. benthamiana* leaves were collected 3 weeks after inoculation and weighed. Leaves were then ground in 2 volumes of 20 mM sodium phosphate buffer (pH 7.2), filtered through organza mesh cloth, and frozen in aliquots at –80°C. For inoculations, one leaf from each plant was dusted with carborundum (Sigma) and rub inoculated with TuMV-GFP sap using a cotton stick applicator. A corresponding set of control plants was dusted with carborundum and mock inoculated with a cotton stick applicator that was soaked in uninfected *N. benthamiana* sap in 20 mM phosphate buffer, prepared in the same manner as the virus-infected sap (mock inoculation treatment throughout the article). Ten days after inoculation, a UV lamp (Blak Ray model B 100AP, UV Products) was used to identify fully infected leaves. For infection rate bioassays, a UV light was used after 5 d post inoculation to identify infected plants.

Insects

All experiments were conducted with a tobacco (*Nicotiana tabacum*)-adapted red strain of the green peach aphid (*Myzus persicae*) that was obtained from Stewart Gray (U.S. Department of Agriculture Plant Soil and Nutrition Laboratory). Aphids were reared on tobacco with a 16-h-light/8-h-dark photoperiod at 24°C (150 mmol m^{-2} s^{-1}).

Aphid Bioassays

To assess the effect of TuMV infection on aphid fecundity, one apterous adult aphid was placed in a plastic clip cage on the underside of a fully

infected or mock-inoculated *N. benthamiana* or the full leaf of Arabidopsis. After 24 h, all aphids except one nymph were removed. The single nymph was allowed to develop and progeny were counted after 7 to 9 d to determine fecundity.

JA and SA Analysis

Wild-type Arabidopsis was planted as described above, and, after 3 weeks of growth, one-half of the plants was infected with TuMV-GFP as described above. After 1 week, infected plants were identified by fluorescence under UV light. For aphid induction, 15 adult apterous aphids were caged on one leaf per plant on six plants with TuMV-GFP infection and six mock-inoculated plants. Corresponding sets of individual leaves received cages with no aphids as a control (six plants with TuMV-GFP infection and six mock-inoculated plants). Caged leaves were developmentally matched, and infected leaves were verified for full infection before caging based on GFP visualization. Twenty-four hours after aphid placement, each caged leaf was harvested individually. Harvested leaves were weighed and placed in tubes containing two steel balls before being flash frozen in liquid nitrogen and stored at –80°C until further use. One milliliter of extraction buffer (isopropanol:water:HCl [2:1:0.005, v/v]) was added to each sample. d_4-SA and d_5-JA (CDN Isotopes) were added as internal standards, and samples were homogenized in a paint shaker for 45 s. Samples were dissolved in 200 μL of methanol after extraction with dichloromethane and solvent evaporation, and 15 μL was analyzed using a triple-quadrupole liquid chromatography-tandem mass spectrometry system (Quantum Access, Thermo Scientific). Samples were separated on a C18 reversed-phase HPLC column (Phenomenex Gemini-NX, 3 μm, 150 × 2.00 mm;) using a gradient of 0.1% formic acid in water and 0.1% (v/v) formic acid in acetonitrile at a flow rate of 300 μL min^{-1}, as previously described (Rasmann et al., 2012).

ET Analysis

Four leaves were cut from each of 12 mock-inoculated plants and from 12 TuMV-GFP-infected plants and weighed. Only fully infected leaves were used, and all leaves were developmentally matched, as previously described. The four leaves from each individual plant were then placed in a gas-tight 10-mL glass jar with 1 mL of water. To allow wound-induced ET to dissipate, jars were left open for 2 h. After this period, 15 adult apterous aphids each were added to one-half of the jars for each treatment, and all jars were sealed. Twenty-four hours after the jars were sealed, a 1-mL sample of head space was injected using a loop injector and analyzed using a gas chromatograph equipped with a flame ionization detector (Agilent; column, GS-GAS PRO; custom-column 5″ cage; length, 30 m; diameter, 0.32 mm; limits from –80 to 260/300). Samples were compared to a standard of known concentration and values (nL h^{-1} g^{-1} fresh weight) representing averages from several independent plants of each treatment were calculated (n = 6 per line). ET production in Arabidopsis mutants and in the inhibition experiment below was verified by this method as well.

ET Inhibition Experiments

To examine the role of ET in virus-vector interactions, ET signaling was inhibited using MCP, which blocks ET perception by binding to ET receptors and AVG, which inhibits ET biosynthesis. For MCP treatment, 1 mg of MCP (0.14%) was dissolved in 100 mL of distilled water. Next, each flat of plants was enclosed in an airtight container and 20 mL of the MCP solution was immediately placed in each glass jar in the container with the plants. As a control, a similar experiment was set up with water. Plants were removed after 24 h. MCP treatment was done thrice: once the day after infecting, once the day before aphids were put on the plants, and once 4 d after aphids were added. For AVG treatment, 1 g of AVG was dissolved in 1 L of water with 0.05% (v/v) Silwet (Momentive Performance Materials). Plants were treated on the day after infecting and every 4 d until experiments were finished.

ET Induction Experiments

We challenged plants with TuMV, as previously described (Casteel et al., 2014). The plants were divided equally into two airtight chambers, and ET was injected to a concentration of 20 ppm. Four days later, aphids were added as described above and plants were treated again with ET. A final ET treatment was applied 4 d after aphids were added. At the end of the experiment, aphid progeny were counted.

Callose Staining

Arabidopsis leaves were collected 24 h after infestation with 25 aphids, depending on the experiment. Callose accumulation was visualized as previously described (Casteel et al., 2014). Briefly, leaves were cleared in 95% (v/v) ethanol overnight and stained with 150 mM K_2PO_4 (pH 9.5) and 0.01% (v/v) aniline blue for 2 h. The leaves were examined for UV fluorescence using a Leica fluorescence compound microscope (365-nm excitation, 396-nm chromatic beam splitter, and 420-nm barrier filter), and callose spots were quantified manually.

Arabidopsis Transgenic Plants

The transformation vectors harboring pMDC32 NIa-Pro or the pMDC32 EV were introduced into *Agrobacterium tumefaciens* and transferred into wild-type Arabidopsis plants by floral dip transformation (Clough and Bent, 1998). Positive transgenic lines were screened on kanamycin Murashige and Skoog agar plates and then confirmed by reverse-transcription PCR. Single leaves of 4-week-old Arabidopsis transformed with the pMDC32 EV, or constitutively expressing NIa-Pro, were used in experiments as described above.

Transcript Abundance Analysis

Total RNA was extracted from frozen tissue samples using the SV Total RNA Isolation system with on-column DNAse treatment (Promega). RNA integrity was verified using a 1.2% (v/v) agarose gel. After RNA extraction and DNAse treatment, 1 μg of total RNA was reverse transcribed with SMART MMLV reverse transcriptase (Clontech) using an oligo(dT)$_{12–18}$ primer. Transcript abundance was analyzed with real-time quantitative reverse transcription (qRT)-PCR, with ubiquitin10 (At4g05320, AAGAGATAACAGGAACGGAAACATA, GGCCTTGTATAATCCCTGATGAATAA) as the reference gene. Primers were synthesized following the recommendation of Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; *EIN2*, At5g03280, GCTCTGTTAGGGCTTCCTCCA, AGCCACTCTAACGCTTACTTGTT; *ERF1*, At3g23240, AAAGCAGCTTGATCGTAGGC, ATTCGACTAGAAACGGTATTAGGG; *EIN3*, At3g20770, TTGATCGTAATGGTCCTGCG, TCCTCTTTCCCATTAGGCCA; *OSR1*, At2g41230, GAACCTCTCGACCCCTGAT, TGACATGATCTTACTTGCACGA; *PDF1.2*, At5g44420, TTTCGACGCACCGGCAATG, TGCTGGGAAGACATAGTTGCATGA; and *AZI1*, At4g12470, GTCTATGCACTGCTCTGAGG, ACGATATTGTGCACTGGCAT). qRT-PCR was performed using the QuantStudio 6 Flex Real-Time PCR System in a 10-μL mixture containing SYBR Green PCR Master Mix (Applied Biosystems). The cycling conditions comprised 10-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 s, 55°C for 1 min, and 72°C for 40 s Following cycling, the melting curve was determined. Each assay was conducted in triplicate and included a no-template control. Cycle time values were automatically determined for all plates and genes using the QuantStudio 6 Flex Real-Time PCR System Software. To ensure comparability between data obtained from different genes, all samples were in the same plate. Analysis of qRT-PCR fluorescence data was then performed using the standard curve method.

Statistical Analysis

Aphid fecundity on phytohormone-signaling mutants was analyzed with Student's t tests comparing mock- and TuMV-infected treatments for each mutant. All remaining aphid fecundity and callose induction data were analyzed by ANOVA, with infection/NIa-Pro expression status and aphid feeding as main factors. Virus infection rate was analyzed with χ^2 tests. Phytohormone induction data were analyzed by ANOVA. Transcript abundance data were analyzed by ANOVA, followed by a Tukey's honestly significant difference (HSD) post hoc test. All analyses were performed in JMP 8 software (SAS Institute). Phytohormone experiments included four to six experimental units per treatment. All fecundity experiments were repeated at least twice and consisted of 12 to 30 experimental units per treatment. All callose experiments included four to six experimental units per treatment. TuMV-GFP infection experiments were repeated three times and consisted of 30 to 40 experimental units per treatment. For qRT-PCR, three biological replicates were analyzed per treatment for two separate experiments.

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