The ethylene response factor Pti5 contributes to potato aphid resistance in tomato independent of ethylene signalling

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Received 28 August 2014; Revised 23 October 2014; Accepted 28 October 2014

Abstract

Ethylene response factors (ERFs) comprise a large family of transcription factors that regulate numerous biological processes including growth, development, and response to environmental stresses. Here, we report that Pti5, an ERF in tomato [Solanum lycopersicum (Linnaeus)] was transcriptionally upregulated in response to the potato aphid Macrosiphum euphorbiae (Thomas), and contributed to plant defences that limited the population growth of this phloem-feeding insect. Virus-induced gene silencing of Pti5 enhanced aphid population growth on tomato, both on an aphid-susceptible cultivar and on a near-isogenic genotype that carried the Mi-1.2 resistance (R) gene. These results indicate that Pti5 contributes to basal resistance in susceptible plants and also can synergize with other R gene-mediated defences to limit aphid survival and reproduction. Although Pti5 contains the ERF motif, induction of this gene by aphids was independent of ethylene, since the ACC deaminase (ACD) transgene, which inhibits ethylene synthesis, did not diminish the responsiveness of Pti5 to aphid infestation. Furthermore, experiments with inhibitors of ethylene synthesis revealed that Pti5 and ethylene have distinctly different roles in plant responses to aphids. Whereas Pti5 contributed to antibiotic plant defences that limited aphid survival and reproduction on both resistant (Mi-1.2+) and susceptible (Mi-1.2–) genotypes, ethylene signalling promoted aphid infestation on susceptible plants but contributed to antixenotic defences that deterred the early stages of aphid host selection on resistant plants. These findings suggest that the antixenotic defences that inhibit aphid settling and the antibiotic defences that depress fecundity and promote mortality are regulated through different signalling pathways.

Key words: Basal resistance, ERF, EREBP, insect resistance, Macrosiphum euphorbiae, Mi-1.2.

Introduction

Aphids are a large and economically damaging group of insects that are specialized to feed on phloem sap and that can reduce crop yields by withdrawing photoassimilates, manipulating plant growth and resource allocation, and transmitting phytopathogenic viruses (Goggin, 2007). Plants limit losses to aphids through a variety of defences, including sensory cues that deter aphids from settling on the plant (i.e. antixenosis), and toxins or other resistance factors that suppress aphid fecundity and promote mortality (i.e. antibiosis) (Smith and Chuang, 2014). In some cases, host plant resistance depends upon single dominant resistance genes (so-called R genes) that act against specific pest biotypes, resulting in an incompatible interaction. For example, the Mi-1.2 gene in tomato [Solanum lycopersicum (Linnaeus)],
blocks infestation by some but not all populations of the potato aphid, _Macrosiphum euphorbiae_ (Thomas) (Rossi _et al._, 1998; Goggin _et al._, 2001). However, even in compatible interactions in which relevant R genes are absent and aphids can colonize the host, plants deploy defences that reduce the magnitude of infestations. These defences, present in compatible or ‘susceptible’ hosts, are described as basal resistance and provide a foundation upon which R gene-mediated resistance builds. Basal resistance encompasses not only direct defences with toxic or repellent effects on the pest but also any plant traits that interrupt the chain of events leading to susceptibility (Dangl and Jones, 2001; de Wit, 2007; Niks and Marcel, 2009; Anderson _et al._, 2014). Both basal and R gene-mediated resistance involve induced responses including hormone signalling and transcriptional reprogramming, and there is often extensive overlap in the signalling networks and transcription factors that contribute to these two forms of defence (Navarro _et al._, 2004).

This study explored the role of the Pti5 transcription factor in basal and _Mi-1.2_-mediated aphid resistance tomato, as well as the potential interactions of this ethylene response factor (ERF) with the hormone ethylene. Ethylene is a gaseous hormone that regulates plant responses to numerous biotic and abiotic stimuli including mechanical wounding, chewing insects, and pathogen infection (O’Donnell _et al._, 1996; Knoester _et al._, 1998; Solano and Ecker, 1998; von Dahl and Baldwin, 2007; Mantelin _et al._, 2009). Ethylene signalling activates the transcription of numerous pathogenesis-related (PR) proteins and other genes that contribute to defence, and many of these ethylene-inducible genes contain a conserved 11 bp promoter sequence (TAAGAGCCGCC) called the GCC box (Eyal _et al._, 1993). ERFs, formerly known as ethylene-responsive element binding proteins (EREBPs), are transcription factors that specifically bind to genes having the GCC box. The ERF family has important roles in regulating biological processes related to plant growth, development, metabolism, and response to biotic and abiotic stresses (Ohme-Takagi and Shinshi, 1995; Stockinger _et al._, 1997; Liu _et al._, 1998; Yamamoto _et al._, 1999; Gu _et al._, 2000; van der Fits and Memelink, 2000; Berrocal-Lobo _et al._, 2002; Nakano _et al._, 2006). Ethylene may regulate PR gene expression through ERF transcription factors (Eyal _et al._, 1993; Ohme-Takagi and Shinshi, 1995; Solano _et al._, 1998), and ERFs can act as either transcriptional activators or repressors of GCC box-mediated gene expression (Fujimoto _et al._, 2000; Yang _et al._, 2005).

ERFs have been characterized in several model plants including _Arabidopsis thaliana_ (Büttner and Singh, 1997; Solano _et al._, 1998; Fujimoto _et al._, 2000; Nakano _et al._, 2006), _tomato_ (Zhou _et al._, 1997), _Oryza sativa_ (Nakano _et al._, 2006), and _Nicotiana tabacum_ (Ohme-Takagi and Shinshi, 1995; Suzuki _et al._, 1998). In tomato, the Pti5 transcription factor containing the ERF binding domain was isolated based on its physical interaction with a serine–threonine kinase encoded by the _Pto_ gene (Pti=Pto interacting) (Zhou _et al._, 1997). _Pto_ acts in conjunction with the _Prf_ R gene to confer resistance against certain races of the bacterial pathogen _Pseudomonas syringae_ pv. _tomato_ (Salmeron _et al._, 1996; Pedley and Martin, 2003). _Pti5_ acts downstream of _Pto_ and _Prf_ to upregulate expression of defence genes including the GCC box-containing _PR_ genes encoding β-1,3-glucanase and osmotin (He _et al._, 2001). Furthermore, overexpression of _Pti5_ in a susceptible tomato background enhances basal resistance to _P. syringae_ pv. _tomato_, indicating that _Pti5_ contributes to pathogen resistance (He _et al._, 2001). Analysis of transcriptional responses in _melon_ (_Cucumis melo_) to infestation by the cotton-melon aphid (_Aphis gossypii_) also indicates that aphid feeding upregulates ERFs, and that the _Vat_ gene for aphid resistance may enhance this response (Anstead _et al._, 2010). Thus, ERFs could potentially play a role in R gene-mediated aphid resistance.

While the impact of ERFs on aphid resistance has not been tested previously, several earlier studies have examined the role of ethylene in plant–aphid interactions, and aphids have been shown to induce an ethylene burst in several different plant species (Dillwih _et al._, 1991; Anderson and Peters, 1994; Miller _et al._, 1994; Argandona _et al._, 2001; Mantelin _et al._, 2009). In barley, this ethylene burst was reported to be greater in an aphid-resistant genotype than in a susceptible cultivar, suggesting that ethylene may be associated with resistance (Argandona _et al._, 2001). However, the opposite trend was observed in alfalfa plants challenged with the spotted alfalfa aphid (_Theroaphis maculata_) and wheat plants challenged with the Russian wheat aphid (_Diuraphis noxia_); in these cases, ethylene generation in response to aphids was greater in susceptible genotypes than in resistant or tolerant lines (Dillwih _et al._, 1991; Miller _et al._, 1994). In tomato, artificially exposing plants to ethylene also rendered them more susceptible to the green peach aphid, _Myzus persicae_ (Boughton _et al._, 2006). Furthermore, Mantelin _et al._ (2009) found that inhibiting ethylene synthesis in tomato reduced host suitability for the potato aphid on a susceptible genotype but had no effect on a resistant tomato genotype that carries the _Mi-1.2_ R gene. These results indicate that ethylene signalling in response to aphids can actually facilitate the infestation process in some cases, but that the effects of ethylene vary among genotypes.

In this study, we investigated the role of the ERF _Pti5_ in plant–aphid interactions in resistant (_Mi-1.2_+) and susceptible (_Mi-1.2–_) tomato genotypes, and compared its effects with those of ethylene. We demonstrated that the potato aphid causes transcriptional upregulation of _Pti5_, and that this gene contributes to antibiotic defences against aphids in both susceptible and resistant plants. Ethylene was not required for upregulation of _Pti5_ by aphids and did not contribute to antibiosis in either genotype. Therefore, we concluded that _Pti5_ contributes to potato aphid resistance in tomato independent of ethylene signalling. Surprisingly, we also discovered that, even though ethylene promoted infestations on susceptible tomato plants, it contributed to the early stages of antixenotic defences in plants that carried the _Mi-1.2_ gene for aphid resistance. These results indicate that antibiosis and antixenosis depend upon different signalling pathways and further our understanding of the molecular basis of basal and R gene-mediated aphid resistance.
Materials and methods

Plant and insect materials

The following tomato (S. lycopersicum L.) genotypes were used in this study: three aphid-susceptible cultivars that lack the Mi-1.2 resistance gene (‘MoneyMaker’, ‘UC82B’, and ‘Castlemart’); a transgenic line (143-25) that was generated by transforming ‘MoneyMaker’ with Mi-1.2 (Milligan et al., 1998); a cultivar, ‘Motelle’, that is near-isogenic to ‘MoneyMaker’ but that carries a 650 kb introgressed region containing Mi-1.2 (Kaloshian et al., 1998); a transgenic line, ACD, that was generated by transforming the cultivar ‘UC82B’ with a gene from Pseudomonas chlorophis that encodes 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (ACD; Klee et al., 1991); two mutant tomato lines, acyl-CoA oxidase IA (acx1) and suppressor of proystemin-mediated response2 (spr2), that were generated in the ‘Castlemart’ genetic background (Li et al., 2003); and a transgenic line that was generated in a ‘MoneyMaker’ background and that expresses the naphthalene-salicylic hydroxylase (NahG) transgene from Pseudomonas putida (Gaffney et al., 1993). The aphid-resistant (Mi-1.2+) transgenic line 143-25 was used in initial gene expression experiments because it provides an isogenic control to the susceptible (Mi-1.2−) line ‘MoneyMaker’.

Tomato plants were grown in 0.2-l pots using LC1 Sunshine potting mix (Sungro Horticulture, MI, USA) supplemented with 15-9-12 nutrient solution containing 1000 ppm CaNO₃, USA). Seedlings were grown under uniform greenhouse conditions until impositional experiments. Plants were watered with a dilute Kelp solution (Great Lake, MI, USA) and were fertilized with 15-9-12 complete fertilizer in the greenhouse. For subsequent assays, ‘Motelle’ (Mi-1.2+) was utilized instead of 143-25 because levels of Mi-1.2-mediated resistance appear to decline as the Mi-1.2 transgene is passed through more than one generation (Goggin et al., 2004).

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Genetic crosses and genotype selection

The cultivar ‘Motelle’ (MT) and the transgenic line ACD were crossed to produce MT×ACD plants carrying both the Mi-1.2 gene and the ACD gene. The (MT×ACD) F₁ generation was PCR screened for the presence of both genes using the following sets of primers: ACD forward, 5′-CGATACGCTGGTTTCCATC-3′, and reverse, 5′-CGTCCTCTTCCGTAATCTCG-3′; and Mi-1.2 forward, 5′-CTAGAAGATCTGTGTTTGTCTAACAAGG-3′, and reverse, 5′-CTAAGAGGAATCTCATCACAGG-3′. Similar to Mi-1.2, the ACD transgene behaves as a single dominant gene inherited in a Mendelian fashion when transgenic tomato plants are crossed with other tomato lines (Reed et al., 1995); therefore, heterozygous F₁ plants were used for subsequent assays.

Gene expression analysis

In order to assess the effects of aphid feeding on gene expression in plants with and without the Mi-1.2 resistance gene, ‘MoneyMaker’ and 143-25 were mock infested with empty nylon sieve cages that each covered a single leaflet (control), or infested with potato aphids (25 aphids per sieve cage: two sieve cages per plant: three plants per treatment group). At 6 and 24 h after infestation (HAI), the aphids were gently removed with a paintbrush, and the leaf tissue was flash frozen with liquid nitrogen and stored at −80 °C. Total RNA was extracted from each sample using TRI Reagent (Molecular Research Center, OH, USA), and then was DNase treated with TURBO DNA-free (Ambion, Austin, TX, USA) followed by reverse transcription of 0.5 µg of RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a 20 µl reaction volume. The following primers were used then for quantitative PCR (qPCR): Pti5 (GenBank accession no. U89256) forward, 5′-GCGAGGTCTAGGGAGCTAC-3′, and reverse, 5′-TGGCA AGAATCTCCATGC-3′; E4 (GenBank accession no. S44989.1) forward, 5′-TGATGCTCAGGGCTCACTGG-3′, and reverse, 5′-ACTGCTCAACCTTCTCCAC-3′; Mi-1.2 (GenBank accession no. X64562) forward, 5′-GGAGGGCTATCAGGAAACCA-3′, and reverse, 5′-CTTTTGTCAGAGGTGCAT-3′. Real-time (RT)-qPCR was performed with a Quantitect SYBR Green PCR kit (Qiagen, CA, USA) in a 20 µl reaction. The StepOnePlus™ Real-Time PCR system (Applied Biosystems) was used for fluorescence detection. Two technical replicates per biological sample were run. The PCR conditions were as follows: 15 min of activation at 95 °C, 40 amplification cycles (94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s, with data acquisition at the end of each cycle), and a final data acquisition step to generate melting curves from 65 to 95 °C every 0.3 °C. Melting curves were performed for all samples to verify single amplification product. For each primer set, the amplification efficiency was calculated using the formula E=10⁻¹⁵·⁸⁷⁹² from data generated from serial dilutions of a set of bulked cdNA standards having equal aliquots from all the samples (Rasmussen, 2000). Relative gene expression was calculated using Pfaffl methodology (Pfaffl, 2001). Data were normalized to the expression levels of the endogenous control ribosomal protein L2 gene (RPL2), and gene expression for each treatment group was calculated relative to the wild-type (WT) control group in each experiment. For statistical analysis, the relative expression values for each treatment group were log-transformed to stabilize variances. Data were analysed by two-way analysis of variance (ANOVA) and means for significant effects at α=0.05 were separated using Student’s t-test with JMP® Pro 9.0 (SAS Institute, NC, USA).

Aphid bioassays

No-choice tests

Aphids were restricted to single leaflets using lightweight mesh clip cages (~3.5 cm diameter). Six-week old tomato plants were infested with four to five apterous young adult potato aphids per cage, with at least two cages per plant, and maintained at 20 °C and with a 16:8 h light:dark photoperiod. At 5–7 days after infestation (DAI), the total number of live aphids (adults and nymphs) was recorded in each cage. Each plant was treated as a biological replication, and separate cages were treated as subsamples. The average number of aphids per cage per plant was analysed by one- or two-way ANOVA, depending upon whether there were one or two independent variables in the experiment. If the ANOVAs revealed statistically significant main effects or significant interactions between independent variables, mean separations were performed using Student’s t-test to identify differences among treatment groups (α=0.05). All statistics were performed with JMP® Pro 9.0.

Choice tests

Eight-week-old ‘MoneyMaker’ and ‘Motelle’ plants were sprayed with 0.01% Tween 20 with or without 1 mM aminothio-oxyvinylglycine (AV; Valant USA Corporation, IL, USA). Six hours after treatment, when the foliage was fully dry, the plants were arranged in pairs on laboratory benches. For each possible pairing of plants, there were six replicate pairs per experiment. A choice arena consisting of a pedestal with a styrofoam platform approximately 10 cm in diameter was placed half way between the plant pairs as described by Avila et al. (2012). Each arena was in contact with one terminal leaflet of a mature leaf on each of the two plants in the pair. The leaf positions 6–10 (first true leaf=1) were used for this assay. Each leaflet used for the choice test was isolated from the rest of the plant using a circular plastic shield (11 cm in diameter) with Tanglefoot Pest Barrier (The Tanglefoot Company, MI, USA) around the edge. Twenty adult apterous potato aphids were released onto the centre of each platform at the start of the experiment. The number of aphids and offspring were counted on each leaflet at four time points after release (1, 6, 24, and 48 HAI). The data for each comparison were analysed by a matched-pairs one-sided t-test within each time point in JMP® Pro 9.0.
Silencing of Pti5

**Construction of TRV-Pti5** Virus-induced gene silencing (VIGS) was performed to suppress expression of Pti5 in tomato using the tobacco rattle virus (TRV) system. VIGS was performed as described previously (Wu et al., 2011; Avila et al., 2012, 2013). The TRV-Pti5 construct consisted of a 302 bp fragment corresponding to nt 379–680 of the tomato Pti5 gene (GenBank accession no. U89236), which was amplified using the following primers: forward, 5′-CGGTCGTAGCTAGTCGCTCTGTTCCAG-3′ and reverse 5′-CGGGATCCGGTACGCAAACTTCCAT-3′. The Pti5 fragment was cloned into the pYL156 vector to generate the TRV-Pti5 silencing construct. The pYL156-Pti5 vector was transformed into Escherichia coli strain DH10B, and the insert was confirmed by sequencing and then introduced into Agrobacterium tumefaciens strain GV3101. For the empty control vector (TRV-CV), we used pYL156 carrying a 396 bp insert from the β-glucuronidase reporter gene (GenBank accession no. S69414.1) to achieve a control vector that would have similar movement and proliferation in the plant compared with the experimental vector (Wu et al., 2011). Additionally, a construct that silences the phytoene desaturase gene (PDS, GenBank accession no. M88683.1) (Wu et al., 2011) was used in a separate set of plants as a visual reporter to monitor the onset of VIGS.

**Agro-infiltration and bioassays** The TRV vectors were infiltrated into tomato as described previously (Wu et al., 2011). Briefly, 2-week-old tomato plants were vacuum infiltrated with TRV-Pti5, TRV-CV, or TRV-PDS and kept at 20 °C in a growth chamber with a 16:8 light:dark photoperiod until ready for bioassay. No-choice aphid bioassays were performed as described above when monitor plants infiltrated with the TRV-PDS vector showed a widespread uniform bleaching phenotype (~4 weeks after infiltration; inoculum level of five aphids per cage; four cages per plant; the number of live aphids per cage scored at 4 and 7 DAI).

**Results**

**Aphid infestation induces Pti5 expression in tomato**

Gene expression of Pti5 was monitored by RT-qPCR at 6 and 24 h HAI in the aphid-susceptible tomato cultivar ‘MoneyMaker’ and the aphid-resistant transgenic line 143-25, which carries the Mi-1.2 transgene in a ‘Moneymaker’ genetic background (Fig. 1). No change in gene expression was observed at 6 HAI, but at 24 HAI, Pti5 transcripts were 3- to 4-fold higher in infested plants than in uninfested controls for both genotypes (P<0.05). No difference in Pti5 transcript abundance was observed between ‘MoneyMaker’ and 143-25 (P=0.7697), which suggested that Mi-1.2 does not influence constitutive or aphid-responsive levels of Pti5 expression.

The Pti5 gene contributes to antibiotic defences against aphids in both resistant and susceptible tomato genotypes

To investigate if Pti5 influenced plant defences against aphids, VIGS was performed to suppress Pti5 expression in the aphid-susceptible tomato ‘Moneymaker’ and the near-isogenic resistant line ‘Motelle’ carrying the Mi-1.2 gene. Plants were infiltrated either with a construct designed to silence Pti5 (TRV-Pti5) or with a control vector of comparable size (TRV-CV). Compared with plants that received the vector control, plants infiltrated with TRV-Pti5 had significantly lower abundance of Pti5 transcripts in the foliage (Fig. 2A; P=0.0013), and the efficacy of silencing was similar in the two genotypes. To measure antibiosis, plants were inoculated with caged adult aphids in a no-choice test, and insect survivorship and reproduction was monitored at 4 and 7 DAI. The aphid-susceptible cultivar ‘MoneyMaker’ had higher numbers of live adults (Fig. 2B; P=0.0098) and offspring (Fig. 2C; P=0.0001) than the aphid-resistant genotype ‘Motelle’ at 4 DAI. Regardless of the genotype tested, plants treated with TRV-Pti5 also supported higher numbers of surviving adults (Fig. 2B; P=0.0033) and live offspring (Fig. 2C; P=0.0063) than the TRV-CV-treated plants. The percentage increase in surviving adults (35% on ‘MoneyMaker’ and 35% on ‘Motelle’) and offspring (37% on ‘MoneyMaker’ and 40% on ‘Motelle’) that resulted from silencing of Pti5 was similar on the two genotypes, and there was no statistically significant interaction between VIGS treatment and plant genotype (P>0.1). This suggested that the effects of silencing by TRV-Pti5 was similar in the two genetic backgrounds. The same patterns of aphid population growth observed at 4 DAI persisted at 7 DAI (Fig. 2D, E), indicating that the effect of Pti5 silencing on aphid population growth remained stable for the time period tested.

In contrast to Pti5, ethylene signalling depresses basal resistance in susceptible tomato and does not contribute to antibiosis in resistant plants

Because Pti5 is classified as an ERF, we also investigated the influence of ethylene on defences that affect herbivore survival and reproduction (i.e. antibiosis) using the ACD transgene. Plants that express ACD are impaired in ethylene synthesis because this enzyme degrades ACC, an essential precursor for ethylene synthesis (Klee et al., 1991). We performed no-choice tests to compare the survival and offspring production of adult aphids on the transgenic line ACD (which carries the ACD gene in an aphid-susceptible ‘UC82B’ background), hybrids that carry both the ACD transgene and the Mi-1.2 Mi-1.2 transgene, or the empty control vector (ACD-CV). The empty vector that would have similar movement and proliferation in the plant compared with the experimental vector (Wu et al., 2011) was used in a separate set of plants as a visual reporter to monitor the onset of VIGS. The ACD transgene in the UC82B background increased antibiosis by 30% compared with the empty control vector (ACD-CV). This suggests that the effects of ethylene signalling on defences against aphids are distinct from those of Pti5, and that ethylene signalling may influence basal resistance but not antibiosis in susceptible tomato.
no significant impact on offspring production (P=0.4326) or adult aphid survival (P=0.3226) in a genetic background that carried the Mi-1.2 resistance gene (Fig. 3B). A similar trend was observed when we used a pharmacological approach to disrupt ethylene synthesis in ‘MoneyMaker’ and ‘Motelle’ by applying AVG to block the activity of 1-aminoacyclopropane-1-carboxylate synthase, which generates a necessary precursor for ethylene synthesis (Yu and Yang, 1979; Huai et al., 2001). AVG treatment lowered offspring production on the susceptible genotype ‘MoneyMaker’ but not on the resistant isolate ‘Motelle’, and as denoted by the statistically significant interaction between AVG treatment and plant genotype (Fig. 3C; two-way ANOVA, main effect of AVG treatment: P=0.4889; main effect of genotype: P<0.0001; and the interaction effect between AVG treatment and genotype: P=0.0401). AVG did not impact adult survival on either genotype (P>0.10). These results indicated that ethylene signalling promotes aphid infestation on susceptible tomato genotypes but not on resistant genotypes that carry the Mi-1.2 resistance gene.

**Ethylene signalling promotes aphid settling on susceptible plants but may contribute to antixenosis in plants that carry Mi-1.2**

We also tested the effect of ethylene on the plant’s ability to deter aphid settling during the host selection process (i.e. antixenosis or non-preference). To avoid the potential effects of multiple loci that are segregating in the ‘MT×ACD’ hybrids, we chose instead to manipulate ethylene signalling by applying AVG or a blank carrier solution to the near-isolines ‘MoneyMaker’ and ‘Motelle’. Aphid settling was tested in choice tests in which groups of 20 adult aphids were placed on platforms (choice arenas) between paired plants with and without AVG treatment. Aphids typically moved off the arena onto the foliage within minutes of introduction, and were free to move back and forth between the two plants by crossing the arena. At 1, 6, 24, and 48 HAI, we recorded the position of adults, as well their offspring production, which is a well-established marker of host plant acceptance (Powell et al., 2006). On the susceptible cultivar ‘MoneyMaker’, aphids had a significant preference to settle on plants treated with the control solution compared with plants treated with AVG (Fig. 4A, P<0.05 at all time points), and by 24 HAI, reproduction was also higher on the controls (Fig. 4B; matched-pairs test, 6 HAI: P=0.1146; 24 HAI: P=0.0304; and 48 HAI: P=0.0350. No nymphs were observed at 1 HAI). In contrast, on the resistant line ‘Motelle’, aphids preferred to settle (Fig. 4C) and reproduce (Fig. 4D) on plants treated with AVG than on plants treated with the blank carrier solution (adult settling: matched-pairs test, 1 HAI: P=0.0343; 6 HAI: P=0.1054; 24 HAI: P=0.0189; and 48 HAI: P=0.0272; offspring production: matched-pairs test, 6 HAI: P=0.0421; 24 HAI: P=0.0654; and 48 HAI: P=0.0351). These results indicated that ethylene signalling promotes aphid host acceptance on susceptible plants, but contributes to antixenosis (i.e. deterrent defences) in plants that carry the Mi-1.2 resistance gene.

We also performed additional choice tests to investigate whether ethylene influenced aphid host preferences between
When aphids were offered a choice between ‘MoneyMaker’ plants treated with AVG or ‘Motelle’ plants treated with AVG, they consistently preferred ‘MoneyMaker’ over ‘Motelle’ (Fig. 5B, \( P < 0.05 \) at all time points tested), indicating that inhibiting ethylene synthesis did not eliminate antixenosis in the resistant line. However, when aphids were offered a choice between AVG-treated or mock-treated plants, adult aphids were placed on choice arenas between paired plants and allowed to move back and forth between the plants (20 aphid per arena; six arenas per comparison). The percentage of adults that were located on treated plants versus controls (A, C) was recorded at 1, 6, 24, and 48 HAI, along with the number of offspring produced on each treatment group (B, D). Comparisons labelled with an asterisk (*) were statistically different at \( \alpha = 0.05 \) according to one-sided matched-pair comparisons. Error bars in (B) and (C) represent SEM (\( n = 14 \) for A and \( n = 6 \) for B and C).
Fig. 5. Aphid host preference between plants with and without Mi-1.2, in the presence or absence of an inhibitor of ethylene synthesis. The aphid-susceptible cultivar ‘MoneyMaker’ (MM) and the near-isogenic resistant line ‘Motelle’ (MT) were treated with AVG or mock treated with solvent (control), and choice assays were performed to determine whether AVG influenced the aphids’ ability to discriminate between the two genotypes (20 aphids per area; six arenas per comparison). The percentage of adults that were located on treated plants versus controls was recorded at 1, 6, 24, and 48 HAI. Comparisons labelled with an asterisk (*) were statistically different at α=0.05 according to one-sided matched-pair comparisons.

Together, these results suggested that, in resistant plants, ethylene signalling may contribute to antixenotic defences that act early (<24 h) in the plant–aphid interaction.

Induction of Pti5 expression by aphids is independent of ethylene, jasmonic acid (JA), or salicylic acid (SA) accumulation

Although several studies on ERF-like genes encoding GCC box-binding factors have focused on their role in ethylene signalling (Solano and Ecker, 1998; Suzuki et al., 1998), our results indicated that Pti5 and ethylene signalling have different roles in plant–aphid interactions. To further corroborate that induction of Pti5 acted independent of ethylene signalling, we monitored Pti5 gene expression at 48 HAI in the ethylene-deficient ACD transgenic line. Pti5 gene expression was induced by aphids to a similar extent in both ACD and the WT control ‘UC82B’, indicating that aphid induction was independent of ethylene levels. However, basal levels of the Pti5 transcript in uninfested plants were higher in ACD than in ‘UC82B’, suggesting that constitutive expression of Pti5 may be inhibited by ethylene (Fig. 6A, two-way ANOVA, main effect of genotype: P=0.0312; main effect of aphid infestation: P=0.168; effect of the interaction between genotype and aphid infestation: P=0.2140). The ethylene-induced marker gene E4 was downregulated in ACD plants as compared with ‘UC82B’ (Supplementary Fig. S1 at JXB online), confirming that ethylene signalling was impaired in the ACD transgenic plants.

Since induction of Pti5 by aphids did not require ethylene accumulation, we investigated the possible role of the alternative plant defensive hormones SA and JA. Transcript abundance of Pti5 in response to aphid infestation was measured in the NahG tomato line, which expresses salicylate hydroxylase, an enzyme that diminishes SA accumulation by degrading SA to catechol (Gaffney et al., 1993). Expression of Pti5 was upregulated at 48 HAI in both the NahG and WT background tomato cultivar ‘MoneyMaker’ in response to aphid infestation (Fig. 6B; P=0.0277). No significant interaction between genotype and aphid treatment was observed (P=0.7489), indicating that induction of Pti5 by aphids is also SA independent. However, constitutive transcript levels of Pti5 were ~40 times higher in NahG as compared with ‘MoneyMaker’ (P<0.0001). We hypothesized that high constitutive Pti5 expression in NahG plants resulted from the development of necrotic lesions in this genotype and not from a deficiency in SA. Several lines of evidence support our hypothesis: (i) constitutive expression of Pti5 is higher in older than younger leaves, suggesting that expression of Pti5 is regulated upon cell senescence (Gu et al., 2000); (ii) NahG plants develop necrotic lesions through the vegetative growth stage (Brading et al., 2000; Li et al., 2002); (iii) expression of senescence-related genes in NahG plants has been related to the appearance of necrotic lesions (Avila et al., 2013); and (iv) soil drenches of the SA analogue benzothiadiazole induce Pti5 transcripts in tomato, which argues against the idea that SA is a negative regulator of this gene (Harel et al., 2014).

We also measured transcript abundance of Pti5 in the JA-deficient mutant tomato lines acxl and spr2. The acxl mutation in tomato blocks the first β-oxidation step of JA synthesis reducing JA content by 95% (Li et al., 2005). The spr2 line carries a loss-of-function mutation in FATTY ACID DESATURASE 7 (FAD7) resulting in a 90% reduction in linolenic acid, a necessary precursor for JA synthesis (Li et al., 2003). Both mutations almost completely eliminate expression of the JA-responsive marker gene PROTEINASE INHIBITOR II (PI-II) (Howe and Ryan, 1999; Li et al., 2003; Li et al., 2005; Avila et al., 2012). Transcript abundance of

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**Fig. 6A**

Li FAD7 gene

**Fig. 6B**

MM-Culture MT-Culture

**Fig. 6C**

MM-Culture MT-Culture

**Fig. 6D**

MM-Culture MT-Culture

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**Fig. 6**. Aphid host preference between plants with and without Mi-1.2, in the presence or absence of an inhibitor of ethylene synthesis. The aphid-susceptible cultivar ‘MoneyMaker’ (MM) and the near-isogenic resistant line ‘Motelle’ (MT) were treated with AVG or mock treated with solvent (control), and choice assays were performed to determine whether AVG influenced the aphids’ ability to discriminate between the two genotypes (20 aphids per area; six arenas per comparison). The percentage of adults that were located on treated plants versus controls was recorded at 1, 6, 24, and 48 HAI. Comparisons labelled with an asterisk (*) were statistically different at α=0.05 according to one-sided matched-pair comparisons.
Pti5 was induced in both JA mutants in response to aphids at 48 HAI when compared with the WT background tomato cultivar ‘Castlemart’ (Fig. 6D; \( P < 0.05 \)). Similarly, there was no significant interaction between genotype and aphid treatment in either mutant (\( P > 0.10 \)), indicating that accumulation of JA is not required for aphid induction of Pti5 transcription. However, JA mutants showed a small decrease in constitutive Pti5 transcription levels compared with WT plants, although statistical significance was only reached in the \( acx1 \) mutant (\( acx1 \) genotype main effect \( P = 0.0390; \ spr2 \) genotype main effect \( P = 0.6736 \)). Thus, JA may potentially promote basal levels of Pti5 expression.

In summary, our results clearly demonstrated that ethylene, SA, and JA accumulation are not required for transcriptional upregulation of Pti5 by aphid infestation on tomato. While ethylene, SA, and JA are not the primary signals that trigger Pti5 expression in response to aphids, they may potentially fine-tune constitutive or inducible Pti5 expression levels; moreover, these phytohormone pathways may interact with Pti5 signalling downstream of Pti5 transcription. In fact, \( Mi \)-mediated aphid resistance is known to require SA (Martinez de Ilarduya et al., 2003); therefore, SA-dependent defences and Pti5-dependent defences may act synergistically.

**Discussion**

VIGS of Pti5 resulted in increased aphid infestations on tomato, indicating that this ERF contributes to plant defences against piercing/sucking insects. These results illustrated the overlap between plant responses to aphids and plant responses to microbes, since Pti5 and other ERFs are known for their roles in plant–pathogen interactions (Licauzi et al., 2013). As mentioned previously, Pti5 contributes to host plant resistance against the bacterial pathogen *Pseudomonas syringae* pv. *syringae* (Thara et al., 1999; He et al., 2001). Moreover, it is also transcriptionally upregulated by *Trichoderma harzianum*, a beneficial soil fungus that promotes induced systemic resistance against the grey mould fungus *Botrytis cinerea* and other pathogens (Harel et al., 2014). The Pti5 gene product may contribute to aphid resistance by upregulating expression of PR genes, as is seen in the defence response against *P. syringae* (Gu et al., 2000). In addition, ERFs are known to play other diverse roles in plant stress responses, including transcriptional regulation of secondary metabolite synthesis (Shoji et al., 2010), cell death (Ogata et al., 2013), reactive oxygen species signalling (Sewelam et al., 2013), adjustments in primary metabolism (Vogel et al., 2014), and crosstalk among hormone signalling pathways (Van der Does et al., 2013). In other words, the suppressive effects of Pti5 on aphids may be due to activation of direct defences and/or enhancement of defensive signalling by this transcription factor.

Silencing of Pti5 resulted in increased aphid infestations on a genotype that lacks the Mi-1.2 aphid resistance gene, which indicates that this gene contributes to basal levels of aphid resistance in susceptible hosts (Fig. 7). Suppression of Pti5 expression also increased aphid population growth on a resistant (\( Mi-1.2+ \)) cultivar, suggesting that this transcription factor might also contribute to \( R \) gene-mediated aphid resistance (Fig. 7). Previous studies have shown that transcriptional upregulation of Pti5 in response to *Pseudomonas syringae* is enhanced when the pathogen is recognized by the \( R \) gene \( Prf \) (Thara et al., 1999), and that Pti5 contributes to \( Prf \)-mediated resistance against *P. syringae* through a direct physical interaction with the \( Pto \) kinase (Zhou et al., 1997). However, transcriptional upregulation of Pti5 by aphid infestation was similar in magnitude in genotypes with and
Our results also indicated that ethylene is not required for transcriptional upregulation of \( Pti5 \) by aphids, despite this gene’s classification as an ERF. Aphid infestation induces \( Pti5 \) expression even in genotypes that express \( ACD \), a transgene that inhibits ethylene synthesis and suppresses expression of \( E4 \) and other ethylene-responsive genes. This is consistent with previous reports that \( Pti5 \) is not induced by exogenous ethylene (Thara et al., 1999; Gu et al., 2000), and that \( Pseudomonas syringae \) is able to upregulate this gene in a mutant tomato line with impaired ethylene perception, the Never-ripe (\( Nr \)) mutant (Thara et al., 1999). These findings highlight the fact that transcription factors are classified as ERFs based not on empirical evidence of ethylene responsiveness but on the presence of a conserved domain that can bind the GCC box, which is in turn common among genes that are upregulated by ethylene. In contrast to this classification scheme, a growing body of evidence has revealed that the ERF family does much more than mediate ethylene-responsive induction of GCC box-containing genes. For example, chromatin immunoprecipitation assays have shown that another ERF in tomato, Pti4, can bind to promoters that do not contain a GCC box, and microarray analysis has revealed that the majority of genes that were upregulated as a result of overexpression of \( Pti4 \) lacked this motif (Chakravarthy et al., 2003). The specificity of ERF binding can also vary in response to different stimuli; ERF1 in Arabidopsis binds to the GCC box in response to biotic stresses but interacts with the DRE/CRT motif when activated by abiotic stimuli (Cheng et al., 2013). Furthermore, ERFs can mediate plant responses to hormones other than ethylene, including jasmonates, abscisic acid, and cytokinins (Licauisi et al., 2013).

While upstream ethylene signalling is not required for \( Pti5 \) induction, it has been shown previously that ERFs can act as a point of convergence between ethylene and other signalling pathways (Lorenzo et al., 2003). However, our results suggest that ethylene and \( Pti5 \) play divergent rather than convergent roles in plant–aphid interactions (Fig. 7). In susceptible plants that lack \( Mi-1.2 \), the \( Pti5 \) gene contributes to basal resistance, whereas ethylene signalling promotes aphid infestation. In the absence of \( Mi-1.2 \), we found that suppressing ethylene synthesis with the \( ACD \) transgene depressed aphid population growth; likewise, Mantelin et al. (2009) showed that inhibiting ethylene synthesis by silencing ACC synthases decreased aphid life spans. Furthermore, we observed higher levels of constitutive \( Pti5 \) expression in the ethylene-deficient \( ACD \) plants than in WT controls, suggesting that ethylene suppresses basal \( Pti5 \) expression. Similarly, application of the ethylene precursor ACC inhibited induction of the jasmonate-responsive ERFs at the \( NtC2 \) locus in tobacco (Shoji et al., 2010). Thus, it is possible for ethylene and ERFs to act antagonistically, and this appears to be the case for ethylene and \( Pti5 \) in basal aphid resistance.

\( Pti5 \) and ethylene also play distinctly different roles in tomato genotypes that carry the \( Mi-1.2 \) aphid resistance gene. \( Pti5 \) is induced sometime after 6h and contributes to antibiotic defences that limit aphid survival or reproduction on the host. In contrast, ethylene does not help or hinder \( Mi-1.2 \)-mediated antibiosis, which is unaffected by the \( ACD \) transgene or AVG treatment (our results), the \( Nr \) mutation, or the pharmacological inhibitor 1-methylecyclopentene (Mantelin et al., 2009). Instead, we found that ethylene contributes to
Mi-1.2-mediated antixenotic defences that limit aphid settling on the host and that act early in the interaction (6 h and earlier). These results illustrate that ethylene can play different roles in different genetic backgrounds, promoting infestation on compatible hosts but contributing to resistance in backgrounds that carry a relevant R gene. This is consistent with the fact that ethylene-associated transcripts are responsive to aphid feeding on both resistant (Mi-1.2+) and susceptible (Mi-1.2−) cultivars (Anstead et al., 2010). In addition, our findings suggest that the antibiotic and antixenotic components of Mi-1.2-mediated aphid resistance are due to separate defence mechanisms regulated through different signalling pathways. Louis et al. (2012) also reported a divergence in molecular signalling between antibiotic and antixenotic components of basal aphid resistance in Arabidopsis. We have shown previously that, in addition to factors that inhibit ingestion from the phloem, Mi-1.2-mediated resistance also involves earlier-acting factors that deter sampling in the mesophyll or epidermis (Pallipparambil et al., 2010). Therefore, it is possible that these resistance factors localized outside the vascular tissue are involved in ethylene-dependent antixenosis. These results advance our understanding of the mechanisms of R gene-mediated aphid resistance and also expand the potential applications of ERFs, overexpression of which has been shown previously to enhance pathogen resistance and abiotic stress tolerance in multiple plant species (Xu et al., 2011).

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. Expression of E4 ethylene-responsive gene in the ACD transgenic line.

Acknowledgements

We thank Dr Stephanie Deibaugh-Chávez, Lingling Jia, Dr L. Milenka Arevalo-Soliz, Dr Carmen S. Padilla, and Dr Godshen Palliparambil for technical assistance and/or materials. We also thank Dr Harry Klee (University of Florida, FL, USA) for providing the ACD transgenic line, Valenta USA Corp. for AVG, and Dr Dinesh-Kumar (University of California, Davis, USA) and Dr Kyle Willis (USDA-ARS, Madison, WI, USA) for VIGS vectors. This research was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service (CSREES), grant no. 2006-35607-16612, the National Science Foundation IOS grant no. 2006-35607-33016612, the National Academy of Sciences, USA 94, 5961–5966. We thank Dr Stephanie Defibaugh-Chávez, Lingling Jia, Dr L. Milenka Arevalo-Soliz, Dr Carmen S. Padilla, and Dr Godshen Palliparambil for technical assistance and/or materials. We also thank Dr Harry Klee (University of Florida, FL, USA) for providing the ACD transgenic line, Valenta USA Corp. for AVG, and Dr Dinesh-Kumar (University of California, Davis, USA) and Dr Kyle Willis (USDA-ARS, Madison, WI, USA) for VIGS vectors. This research was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service (CSREES), grant no. 2006-35607-16612, the National Science Foundation IOS grant no. 2006-35607-33016612, the National Academy of Sciences, USA 94, 5961–5966.

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