Loss of Function of FATTY ACID DESATURASE7 in Tomato Enhances Basal Aphid Resistance in a Salicylate-Dependent Manner[1][W][OA]

Carlos A. Avila, Lirio M. Arévalo-Soliz[2], Lingling Jia[3], Duroy A. Navarre, Zhaorigetu Chen, Gregg A. Howe, Qing-Wei Meng, Jonathon E. Smith, and Fiona L. Goggin*

Department of Entomology (C.A.A., L.M.A.-S., L.J., Z.C., F.L.G.) and Department of Plant Pathology (J.E.S.), University of Arkansas, Fayetteville, Arkansas 72701; Agricultural Research Service, United States Department of Agriculture, Washington State University, Prosser, Washington 99350 (D.A.N.); Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824 (G.A.H.); and College of Life Sciences, State Key Laboratory of Crop Biology, Shandong Agricultural University, Tai'an 271018, China (Q.-W.M.)

We report here that disruption of function of the ω-3 FATTY ACID DESATURASE7 (FAD7) enhances plant defenses against aphids. The *suppressor of prosystemin-mediated responses2* (*spr2*) mutation in tomato (*Solanum lycopersicum*), which eliminates the function of FAD7, reduces the settling behavior, survival, and fecundity of the potato aphid (*Macrosiphum euphorbiae*). Likewise, the antisense suppression of *LeFAD7* expression in wild-type tomato plants reduces aphid infestations. Aphid resistance in the *spr2* mutant is associated with enhanced levels of salicylic acid (SA) and mRNA encoding the pathogenesis-related protein P4. Introduction of the *Naphthalene/salicylate hydroxylase* transgene, which suppresses SA accumulation, restores wild-type levels of aphid susceptibility to *spr2*. Resistance in *spr2* is also lost when we utilize virus-induced gene silencing to suppress the expression of *NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1* (*NPR1*), a positive regulator of many SA-dependent defenses. These results indicate that FAD7 suppresses defenses against aphids that are mediated through SA and *NPR1*. Although loss of function of FAD7 also inhibits the synthesis of jasmonate (JA), the effects of this desaturase on aphid resistance are not dependent on JA; other mutants impaired in JA synthesis (*acx1*) or perception (*jai1-1*) show wild-type levels of aphid susceptibility, and *spr2* retains aphid resistance when treated with methyl jasmonate. Thus, FAD7 may influence JA-dependent defenses against chewing insects and SA-dependent defenses against aphids through independent effects on JA synthesis and SA signaling. The Arabidopsis (*Arabidopsis thaliana*) mutants *Atfad7-2* and *Atfad7-1fad8* also show enhanced resistance to the green peach aphid (*Myzus persicae*) compared with wild-type controls, indicating that FAD7 influences plant-aphid interactions in at least two plant families.

Fatty acid desaturases (FADs), which introduce double bonds into the aliphatic tails of fatty acids, influence plant susceptibility to a wide variety of stresses. They promote drought and salt tolerance and also mediate plant adaptation to temperature extremes (Upchurch, 2008). Several FADs are up-regulated in response to chilling and confer cold tolerance in a variety of plant species by increasing the production of trienoic fatty acids, which enhance membrane fluidity (Kodama et al., 1994; Berberich et al., 1998; Khodakovskaya et al., 2006; Wang et al., 2006; Zhou et al., 2010). Conversely, FAD activity and trienoic fatty acid levels decrease at high temperatures, and Arabidopsis (*Arabidopsis thaliana*) double mutants that are deficient in two chloroplast-localized ω-3 FADs (FAD7 and FAD8) display enhanced heat tolerance (Murakami et al., 2000). FADs also influence resistance to numerous biotic stresses. For example, the Arabidopsis *Atfad7fad8* mutant shows increased vulnerability to the bacterial pathogen *Pseudomonas syringae*, whereas suppression of the homologous *OsFAD7* and *OsFAD8* genes in rice (*Oryza sativa*) results in enhanced resistance to the rice blast fungus *Magnaporthe grisea* (Yaeno et al., 2004; Yara et al., 2007). Thus, FADs appear to act as a sort of rolling fulcrum that can shift the balance between resistance to some stresses and susceptibility to others.

The influence of ω-3 FADs on biotic stress is due in part to their critical role in the biosynthesis of the

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[2] Present address: Department of Molecular Virology and Microbiology, Baylor College of Medicine, One Baylor Plaza, Mail Stop BCM:280, Houston, TX 77030.

[3] Present address: Bristol-Myers Squibb Company, 5 Research Parkway, Wallingford, CT 06492.

* Corresponding author; e-mail fgoggin@uark.edu.

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defense hormone jasmonate (JA; Fig. 1). Jasmonoyl-L-Ile, which is a receptor-active form of JA (Howe and Jander, 2008; Fonseca et al., 2009; Sheard et al., 2010), activates many plant responses to wounding, insect attack, and certain pathogens. JA levels are enhanced in transgenic rice that overexpress *FAD7* and are depleted by inhibition of ω-3 FAD activity in Arabidopsis and potato (*Solanum tuberosum*) plants (McConn et al., 1997; Martín et al., 1999; Song et al., 2004). Furthermore, wounding and other stresses up-regulate *FAD7*, suggesting that FADs may play a role in regulating JA accumulation (Nishiuchi et al., 1997). In addition to influencing the availability of precursors for JA synthesis, FADs also modulate salicylate signaling. Salicylic acid (SA) is a

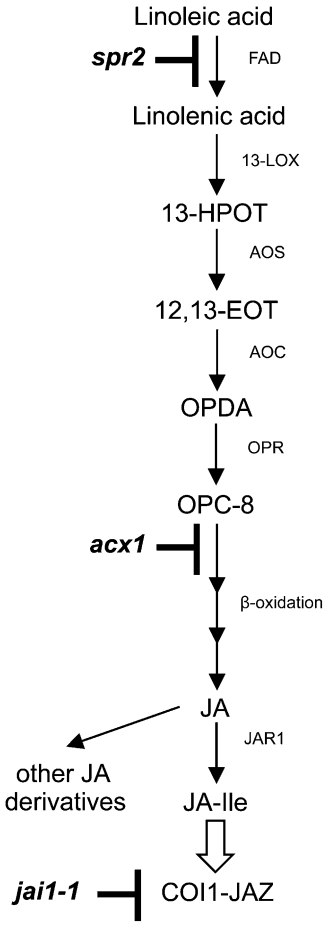

Figure 1. JA synthesis and perception in plants. Mutations in tomato that block JA synthesis or perception are represented in boldface. Abbreviations are as follows: AOC, allene oxide cyclase; AOS, allene oxide synthase; COI1, coronatine insensitive 1; EOT, epoxy-9,11,15-octadecatrienoic acid; JA-Ile, jasmonic acid-isoleucine conjugate; JAR1, jasmonic acid resistant 1; JAZ, jasmonate ZIM domain protein; HPOT, hydroperoxy-octadecatrienoic acid; LOX, lipoxygenase; OPC, 3-oxo-2(2′(Z)-pentenyl)-cyclopentane-1-octanoic acid; OPDA, 2-oxo-phytodienoic acid; OPR, 12-oxo-phytodienoic acid reductase. Black arrows represent biosynthetic steps, whereas the white arrow represents recognition of JA-Ile by the COI1/JAZ coreceptor (Sheard et al., 2010). This figure was modified from Schaller (2001).

β-hydroxy-benzoic acid that is required for basal resistance, systemic acquired resistance, and effector-triggered immunity against many pathogens (Vlot et al., 2009) and can also contribute to plant defenses against aphids (Li et al., 2006). The *SUPPRESSOR OF SA INSENSITIVITY2* (*SSI2*) gene, which encodes a stearoyl acyl carrier protein FAD that converts stearic acid (C18:0) to oleic acid (C18:1), inhibits SA signaling. Accumulation of SA is enhanced as a result of decreased levels of oleic acid in the Arabidopsis *ssi2* mutant (Kachroo et al., 2001, 2004; Shah et al., 2001) and in rice and soybean (*Glycine max*) plants in which *SSI2* homologs have been silenced (Kachroo et al., 2008; Jiang et al., 2009). In soybean, SA accumulation is also enhanced by transient suppression of FAD3, an ω-3 FAD localized in the endoplasmic reticulum (Singh et al., 2011), and in Arabidopsis, high constitutive levels of SA have been reported in the *Atfad3fad7fad8* triple mutant (Mène-Saffrané et al., 2009). Thus, there is growing evidence that certain FADs inhibit SA accumulation.

Because loss of function of ω-3 FADs impairs JA accumulation, mutants deficient in these enzymes have been utilized to study JA-dependent defenses against chewing insects. Compared with wild-type Arabidopsis, a triple mutant with defects in *FAD3*, *FAD7*, and *FAD8* was shown to be highly susceptible to the fungus gnat *Bradysia impatiens* (McConn et al., 1997). This susceptibility reflects the plant's inability to synthesize JA, because treating the triple mutant with exogenous methyl jasmonate (MeJA) restored insect resistance. Loss of function of a *FAD7* homolog in tomato (*Solanum lycopersicum*) also impairs plant defenses against chewing insects. The *suppressor of prosystemin-mediated responses2* (*spr2*) mutant in tomato carries a point mutation in *LeFAD7* that introduces a premature stop codon and is predicted to result in a total loss of function of the protein (Li et al., 2003). The foliage of this mutant has enhanced levels of linoleic acid (C18:2) and only approximately 10% of the linolenic acid (C18:3) content observed in wild-type tomato plants (Li et al., 2003). The *spr2* mutation inhibits JA-dependent responses to the wound signal systemin and nearly eliminates the expression of the JA-responsive *PROTEINASE INHIBITOR II* (*PI-II*) gene, a well-characterized marker of induced resistance to insects (Howe and Ryan, 1999; Li et al., 2003). Furthermore, tobacco hornworm larvae (*Manduca sexta*) consume much more foliage and grow two to three times larger on *spr2* plants than on wild-type controls, and *M. sexta* adults preferentially oviposit on *spr2* (Li et al., 2003; Sánchez-Hernández et al., 2006). Thus, although there is considerable functional redundancy among FAD7, FAD8, and FAD3 in Arabidopsis and genes homologous to *FAD8* and *FAD3* are present in tomato (Yu et al., 2009; ITAG, 2011), FAD7 appears to play a dominant role in regulating induced resistance in tomato.

Whereas the contribution of the octadecanoid pathway to induced resistance against chewing insects and cell-content feeders is well established, its role in plant interactions with piercing-sucking herbivores requires

further characterization (Thompson and Goggin, 2006). Aphids and whiteflies extract phloem sap through slender mouth parts that cause far less mechanical injury than the mandibles of chewing insects (Walling, 2008), and to date, they have not been reported to induce detectable levels of JA (Heidel and Baldwin, 2004; De Vos et al., 2005). The decoy hypothesis posits that these phloem-feeding insects limit the induction of JA-dependent defenses by inducing SA, which can interact antagonistically with JA signaling (Zhu-Salzman et al., 2004; de Vos et al., 2007). Although recent evidence suggests that SA accumulation may not be required for the repression of JA by whiteflies (Zhang et al., 2009), there is strong evidence that whitefly nymphs down-regulate genes associated with JA signaling (Kempema et al., 2007; Zhang et al., 2009). Furthermore, the development of whitefly nymphs on Arabidopsis is promoted by mutations that impair JA perception (*coronatine insensitive1* [*coi1*]) or constitutively activate SA signaling (*cim10*; Zarate et al., 2007), and the *spr2* mutation in tomato results in increased whitefly oviposition, although it does not affect nymphal development (Sánchez-Hernández et al., 2006). Thus, there is strong evidence that JA contributes to basal resistance against whiteflies but that whiteflies are also adapted to inhibit JA signaling in their hosts.

Further work is needed to test the decoy hypothesis in plant-aphid interactions. Aphids can in some cases up-regulate genes associated with JA signaling (Thompson and Goggin, 2006; Gao et al., 2007; Kusnierczyk et al., 2007; Kuśnierczyk et al., 2008), and several studies suggest that JA-dependent defenses hinder aphid infestation. Artificial JA treatment enhances aphid resistance in several plant species (Omer et al., 2001; Bruce et al., 2003, 2008; Zhu-Salzman et al., 2004; Cooper and Goggin, 2005); furthermore, aphid population growth on Arabidopsis is enhanced by the *coi1* mutation, which inhibits JA perception, and is suppressed by the *cev1* mutation, which promotes constitutive JA and ethylene signaling (Ellis et al., 2002; Mewis et al., 2005). On the other hand, there is evidence that SA also contributes to plant defenses against aphids, in contrast to its putative role as a decoy response in interactions between whiteflies and Arabidopsis. Although analyses of aphid population growth on Arabidopsis mutants with altered SA signaling have given equivocal results (Thompson and Goggin, 2006; de Vos et al., 2007), in tomato, SA induction by aphids has been shown to be an important component of effector-mediated immunity and may also contribute to basal defense against aphids (Li et al., 2006). Therefore, further work is needed to elucidate the relative contributions of JA and SA to plant defenses against aphids. In addition to the impact of FADs on JA synthesis and SA signaling, there are also other routes through which this group of enzymes may influence plant-aphid interactions. At least two FADs are known to influence plant defenses against aphids either directly or indirectly. In zonal geranium (*Pelargonium* × *hortorum*), a $\Delta^9$14:0 FAD mediates resistance to aphids and mites through its role in the synthesis of toxic anacardic acids (Schultz et al., 1996). In Arabidopsis, loss of function of another FAD, SSI2, enhances aphid resistance, and petiole exudates from *ssi2* mutants have antibiotic effects on aphids (Pegadaraju et al., 2005; Louis et al., 2010). Although the *ssi2* mutant has high constitutive levels of SA, aphid resistance in *ssi2* does not appear to require this hormone because resistance is retained in the *ssi2 Naphthalene/salicylate hydroxylase* (*NahG*) double mutant, and *NahG* inhibits SA accumulation (Pegadaraju et al., 2005). Instead, Pegadaraju and coworkers (2005) propose that aphid resistance in this mutant is due to hypersenescence. These studies indicate that FADs may influence a plant's susceptibility to aphids through a diversity of mechanisms. Therefore, the goal of this study was to further investigate the role of FADs in plant interactions with aphids.

In contrast to previous observations that ω-3 FADs are required for resistance to chewing insects, we report here that loss of function of FAD7 in the *spr2* mutant in tomato confers resistance to the sap-feeding potato aphid, *Macrosiphum euphorbiae*. To our knowledge, this is the first report of an ω-3 FAD inhibiting insect resistance, and this finding suggests that FAD7 can mediate tradeoffs between plant defenses against different herbivores. Aphid resistance in *spr2* does not appear to depend upon impaired JA signaling, because exogenous MeJA fails to restore aphid susceptibility in *spr2*. Furthermore, aphid resistance is not observed in other mutants blocked in JA synthesis or perception. Instead, aphid resistance in the *spr2* mutant requires SA accumulation and is dependent upon NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1 (NPR1). Aphid resistance is also observed in Arabidopsis mutants deficient in FAD7 and a double mutant deficient in FAD7 and FAD8, indicating that the impact of FAD7 on aphid resistance may be conserved in other species.

RESULTS

Aphid Infestations Are Reduced on *spr2* but Not on Other JA Mutants

Population growth of *M. euphorbiae* was significantly lower on the *spr2* mutant ($P < 0.0001$; Fig. 2A), which carries a loss-of-function mutation in *LeFAD7*, than on wild-type tomato plants (cv Castlemart) or on *jasmonic acid insensitive1* (*jai1-1*), a mutant line impaired in JA-Ile perception due to a deletion mutation in *LeCOI1* (Fig. 1). Aphids in this assay were not confined to cages, and so final aphid numbers were influenced by aphid host acceptance as well as by aphid survival and fecundity on the different plant genotypes. Suppression of the JA pathway in *spr2* and *jai1-1* was confirmed by the absence of wound-induced proteinase inhibitor accumulation in these plants (data not shown). To determine whether the effect of *spr2* on aphid performance is due to the inhibition of JA synthesis, we also examined aphid performance on

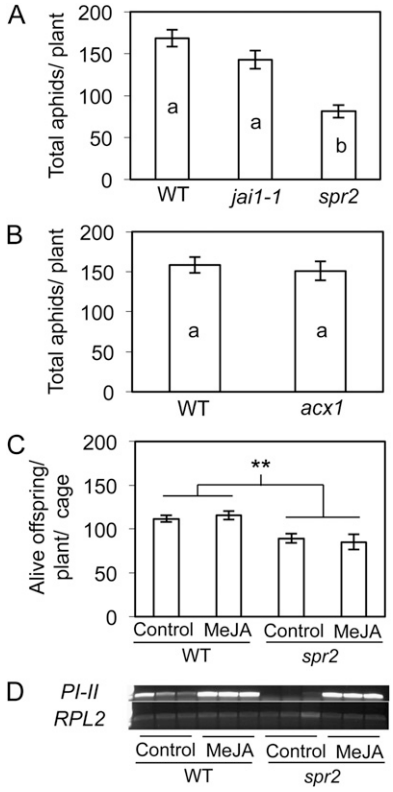

Figure 2. Aphid infestations are reduced on *spr2* but are unaffected by MeJA treatment or by other mutations that impair JA signaling. A and B, Wild-type (WT; cv Castlemart) and mutant (*spr2*, *jai1-1*, and *acx1*) tomato plants were inoculated with 15 aphids per plant, which were not confined to cages and were free to leave the plants. The total number of remaining aphids and their progeny per plant were counted 5 d after inoculation and analyzed by one-way ANOVA. Mean separations were performed using Student's *t* test. Values ± SE labeled with different letters differ significantly at $\alpha = 0.05$. C, Wild-type and *spr2* plants were treated with MeJA (75 μM) and inoculated with aphids 24 h after treatment (five aphids per cage; three clip cages per plant; 10 plants per treatment group). Live offspring were counted 6 d after inoculation, and the average numbers of offspring per cage per plant were analyzed by two-way ANOVA. ** Significant main effect of genotype at $P < 0.0001$. D, Expression of JA-responsive *PI-II* was monitored by RT-PCR 24 h after MeJA treatment. Expression of constitutive *RPL2* is presented as a loading control. $n = 10$ for A, $n = 8$ for B, and $n = 10$ for C.

acx1, which carries a mutation that results in a loss of function of acyl-CoA oxidase 1A, thereby blocking the β-oxidation step of JA synthesis (Li et al., 2005; Fig. 1). Like *spr2*, both *jai1-1* and *acx1* are deficient in JA-dependent defenses and are highly susceptible to chewing insects (Li et al., 2001, 2003, 2004, 2005; Chen et al., 2005a; Sánchez-Hernández et al., 2006). Neither *jai1-1* nor *acx1*, however, had a significant effect on aphid population growth compared with wild-type plants (Fig. 2, A and B; $P < 0.05$). Furthermore, application of MeJA did not impact aphid population growth on *spr2*, even though, as expected, it up-regulated the defensive gene *PI-II* (Fig. 2, C and D). These findings support prior assertions that JAs are not required for plant defenses against aphids (Bhattarai et al., 2007) and indicate that the effects of *spr2* on aphids are likely independent of its effects on JA synthesis.

Loss of Function of FAD7 Confers Aphid Resistance in Both Tomato and Arabidopsis

The fact that *spr2* is resistant to aphids suggests that FAD7 suppresses aphid resistance. To further demonstrate the role of FAD7 in aphid resistance, aphid population growth was measured on a previously described tomato line that has low linolenic acid (C18:3) and high linoleic acid (C18:2) content as a result of antisense silencing of *LeFAD7* (Liu et al., 2006, 2010). Offspring production of potato aphids caged on the antisense line was significantly lower ($P = 0.0013$) than on the wild-type control (cv L402; Fig. 3A). Moreover, loss of function of FAD7 has similar effects on aphid infestations on Arabidopsis. Unlike its homolog in tomato, *AtFAD7* in Arabidopsis shows considerable functional redundancy with *AtFAD8* (Gibson et al., 1994; McConn et al., 1994); therefore, the *Atfad7-1fad8-1* double mutant was tested in addition to the *Atfad7-2* single mutant. Plants were challenged with the green peach aphid (*Myzus persicae*) because Arabidopsis is not a host for the potato aphid. Seven days after inoculation, both *Atfad7-2* and *Atfad7-1fad8-1* plants had approximately 42% fewer aphids than the wild-type control (ecotype Columbia; $P = 0.0083$; Fig. 3B). The alternative mutant allele *Atfad7-1*, which is in the Co-

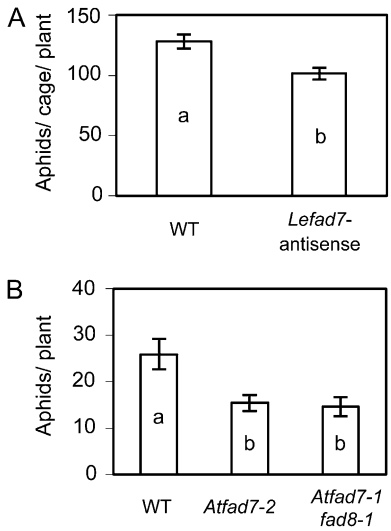

Figure 3. Loss of function of FAD7 confers aphid resistance in both tomato and Arabidopsis. A, Adult potato aphids were confined to individual leaflets of intact tomato plants using clip cages (five aphids per cage; three cages per plant; 12 plants per genotype), and the total number of aphids was recorded after 6 d. Cultivar L402 was used as the untransformed wild-type (WT) control. B, Adult green peach aphids were confined on individual Arabidopsis plants using sleeve cages (two aphids per plant; 18 plants per genotype), and the total number of aphids per plant was recorded after 7 d. Aphid numbers were analyzed by one-way ANOVA, and mean separations were performed using Student's *t* test. Values ± SE labeled with different letters differ significantly at $\alpha = 0.05$.

lumbia-*glabra1* background, also conferred aphid resistance (data not shown).

Loss of Function of FAD7 Reduces Aphid Infestations by Decreasing Aphid Settling, Survival, and Fecundity

Host plant defenses against aphids may act by deterring aphids from settling on the plants (i.e. antixenosis) or by reducing their survival and/or offspring production (i.e. antibiosis). To determine if loss of function of FAD7 results in antixenotic effects, adult potato aphids were placed between intact *spr2* and wild-type tomato plants on choice arenas that allowed them to go back and forth between plants (Fig. 4A). Because aphid reproduction is an indicator of host acceptance and is typically initiated shortly after identifying a suitable host plant, offspring production as well as adult position were monitored at 1, 4, and 24 h after release. At 1 h, adults were distributed roughly equally between the two genotypes (Fig. 4B). However, at 4 and 24 h, after the aphids had a more lengthy opportunity to feed on the plants, adults preferentially congregated (Fig. 4B) and reproduced (Fig. 4C) on wild-type plants, and 24 h after inoculation, total aphid numbers were almost five times higher on wild-type plants than on *spr2* plants (pairwise *t* test, $P < 0.001$). This suggests that, compared with wild-type plants, *spr2* mutants either lack important cues that promote host acceptance or produce deterrents that actively repel aphids. To measure the potential antibiotic effects of *spr2* independent of any effect on host preference, adult females were individually caged on *spr2* and wild-type plants, and adult survival was monitored daily until all aphids on the mutant genotype were dead (14 d). Offspring were also counted and removed daily to assess reproduction. The mortality rate was more than 50% higher on *spr2* plants than on wild-type plants (Fig. 5A), and the average number of days that adult aphids survived on this mutant (5 ± 0.5 d) was significantly lower ($P < 0.05$) than on wild-type controls (8 ± 1 d). Aphid fecundity, as measured by the number of offspring divided by the number of surviving adult females, also significantly declined over time on *spr2*, whereas fecundity remained stable on wild-type plants (Fig. 5B). As a result of these decreases in fecundity and longevity, lifetime offspring production (Fig. 5C) was more than 50% lower on *spr2* than on wild-type plants ($P < 0.01$). Therefore, choice and no-choice tests demonstrate that loss of function of FAD7 has both antixenotic effects that inhibit aphid settling and antibiotic effects that reduce survival and fecundity.

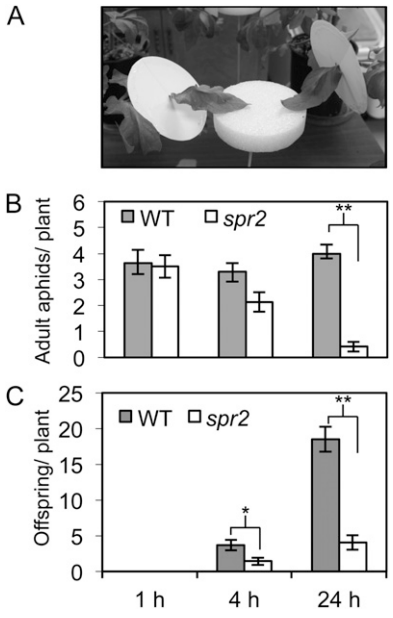

Figure 4. Loss of function of FAD7 reduces aphid host acceptance. A, Wingless adult aphids (10 adults per arena) were placed on choice arenas between paired 6-week-old plants of *spr2* and the wild-type (WT) control (cv Castlemart). The majority of aphids moved off the choice arena onto the plants within minutes of release. Aphids were free to move back and forth between the two plants. B and C, The number of adults on each plant (B) and the offspring they produced (C) were counted at 1, 4, and 24 h after aphids were placed on the arenas. Marked pairwise comparisons denote significant differences according to paired *t* tests at $\alpha = 0.05$ (*) or $\alpha = 0.001$ (**). Error bars indicate ± SE ($n = 10$ pairs).

Loss of Function of FAD7 Enhances *P4* Expression and Local SA Induction in Response to Aphid Feeding

The pathogenesis-related gene *P4* in tomato (GenBank gene identifier 544185**)** is homologous to *PR1a* in tobacco (*Nicotiana tabacum*) and Arabidopsis and is up-regulated in response to exogenous SA and its analog benzothiadiazole (Van Kan et al., 1995; Fidantsef et al., 1999; Schuhegger et al., 2006) as well as in response to the ethylene mimic ethephon (Van Kan et al., 1995; Chao et al., 1999). The proteinase inhibitor gene *PI-II* (GenBank gene identifier 543955) is transcriptionally activated in response to JAs and also ethylene (Farmer et al., 1992; Ohtsubo et al., 1999). Reverse transcription-quantitative PCR (RT-qPCR) was used to assess the effects of aphid feeding on the transcript abundance of *P4* and *PI-II* in locally infested foliage of *spr2* and wild-type tomato plants (cv Castlemart) 48 h after aphid inoculation. Aphid feeding on wild-type plants up-regulated the expression of *P4* from 6- to 39-fold (Fig. 6A; Supplemental Fig. S1) and had a relatively modest effect on *PI-II*, causing either a slight up-regulation (Fig. 6B) or no significant change in expression (Supplemental Fig. S1). This is consistent with prior reports that the induction of *PI-II* by aphids is relatively weak and transient compared with the induction of P4 (Fidantsef et al., 1999; Martinez de Ilarduya et al., 2003). As predicted by previous observations (Li et al., 2006), *PI-II* expression was negligible in *spr2* (Fig. 6B). In contrast, *P4* expression was approximately four to five times higher in *spr2* plants when compared with the respective wild-type treatments (Fig. 6A), suggesting that SA signaling might be enhanced in this mutant. SA and JA are known to interact antagonistically under certain circumstances (for

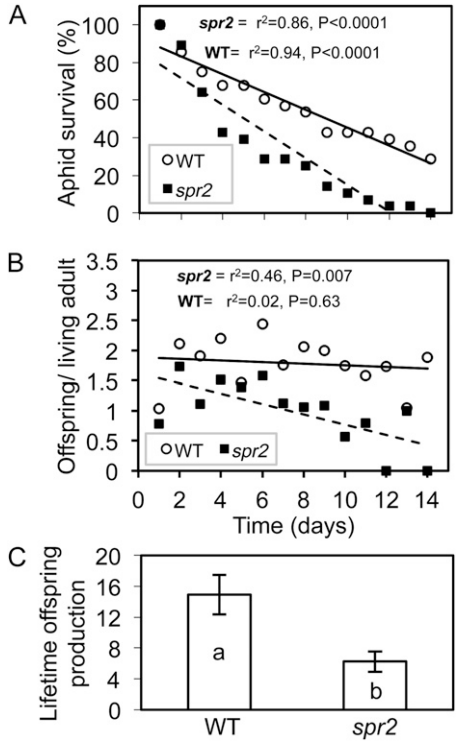

Figure 5. Loss of function of FAD7 decreases aphid survival and fecundity. Newly emerged adult female aphids were caged on *spr2* or wild-type (WT; cv Castlemart) plants (one aphid per cage; two cages per plant; 14 plants per genotype), and the cages were monitored daily to track the survival (A) and daily offspring production (B) of each aphid as well as their lifetime totals for offspring production (C). Regression analyses were performed to estimate aphid mortality rates and changes over time in daily fecundity. Lifetime offspring production was analyzed by one-way ANOVA, and values ± SE having different letters are significantly different at $\alpha = 0.05$.

review, see Pieterse et al., 2009), so it is possible that by suppressing JA synthesis, the *spr2* mutation relieves the SA pathway from repression by JA. However, unlike *spr2*, the *jai1-1* mutation did not dramatically enhance *P4* accumulation in response to aphids (Supplemental Fig. S1).

To explore the possibility of enhanced SA signaling in *spr2*, local accumulation of SA in response to aphid feeding was measured in *spr2* and wild-type plants 24 and 48 h after aphid infestation. In locally infested tissue, the total, free, and bound SA levels significantly increased in response to aphid feeding in the *spr2* mutant but not in wild-type tomato (Fig. 6, C and D). These results indicate that loss of function of FAD7 enhances local SA induction and *PR* gene expression in response to aphids.

Aphid Resistance Conferred by Loss of Function of FAD7 Requires SA Accumulation and Is Dependent upon *NPR1*

To determine if SA has a causal role in aphid resistance in *spr2*, this mutant tomato line was crossed to a transgenic line that carries *NahG*, a bacterial gene encoding salicylate hydroxylase, which degrades SA to catechol (Gaffney et al., 1993). The *NahG* transgene has been shown to reduce SA accumulation in plants and abrogate SA-dependent defenses (Gaffney et al., 1993). Segregating plants from the (*spr2* × *NahG*)F_2 generation were screened by PCR to select four phenotypic bulks that varied in the presence or absence of *NahG* (*NahG*$^+$ or *NahG*$^-$) and of a functional copy of *LeFAD7* (wild type or *spr2*). When all four bulks were challenged with the potato aphid, offspring production (Fig. 7A) and survival (Fig. 7B) were reduced on the *spr2* single mutant bulk (*spr2*/*NahG*$^-$) compared with the wild-type bulk (WT/*NahG*$^-$), providing further evidence that aphid resistance in the *spr2* mutant line is due to the presence of the mutation at the *Lefad7* locus. Furthermore, in the double mutant bulk (*spr2*/*NahG*$^+$), the presence of *NahG* compromised aphid resistance, restoring offspring production to wild-type levels (Fig. 7A) and significantly reducing offspring mortality (Fig. 7B). Li et al. (2006) have also shown that the *NahG* transgene causes a modest increase in aphid longevity in wild-type tomato, although overexpression of *NahG* in Arabidopsis did not alter short-term aphid population growth (Pegadaraju et al., 2005), possibly because of the shorter duration of the bioassay on Arabidopsis (2 d) compared with tomato (17 d). Infiltration of 1 mM catechol to *spr2* did not influence aphid population growth or mortality (Supplemental Fig. S2), suggesting that the effects of *NahG* on aphids were due to SA depletion rather than catechol accumulation.

The foliar fatty acid content of the four bulks was also compared by gas chromatography to confirm that this was not impacted by *NahG*. Consistent with previous reports (Li et al., 2003), all plants that were homozygous for the *spr2* mutation had higher C16:2 and C18:2 fatty acid content and lower C16:3 and C18:3 content compared with plants that carried the wild-type *FAD7* allele (Supplemental Fig. S3). The fatty acid profile of the double mutants [(*spr2*/*NahG*$^+$)F_4] was also equivalent to that of the *spr2* single mutant plants [*spr2* or (*spr2*/*NahG*$^-$)F_4], indicating that the observed differences in aphid resistance between these two bulks were not due to changes in fatty acid content. Therefore, the effects of *NahG* on aphid resistance in the *spr2* background can be attributed to suppressed SA accumulation, which was confirmed by HPLC in all bulks that carried the *NahG* transgene (Fig. 7C).

We also examined the contribution of a tomato ortholog of *NPR1*, a positive regulator of many SA-dependent defenses (Zhang et al., 1999; Dong, 2004). The *NPR1* transcript was up-regulated by aphid feeding in both wild-type and *spr2* plants (Fig. 8A). Virus-induced gene silencing (VIGS) was performed in both genotypes, and the ability of our silencing construct to suppress *NPR1* transcript accumulation was confirmed by RT-qPCR (Fig. 8B). Whereas silencing of *NPR1* did not significantly influence aphid population growth on wild-type plants, aphid numbers were nearly 70% higher on *spr2* plants that received the *NPR1* silencing construct than on mutant plants infil-

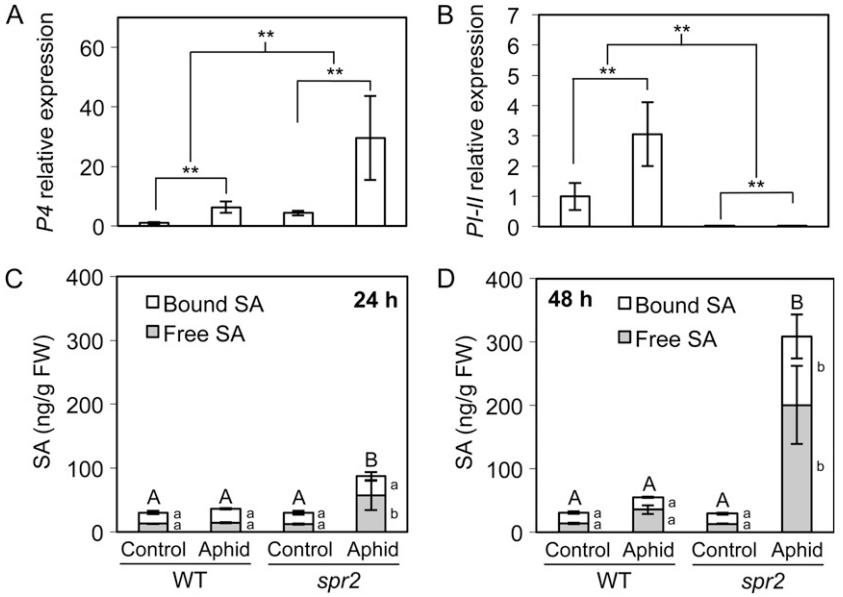

Figure 6. Loss of function of FAD7 enhances the expression of the SA-responsive gene *P4* and local SA accumulation in response to aphid feeding but suppresses the expression of the JA-responsive gene *PI-II*. A and B, Wild-type (WT; cv Castlemart) and *spr2* tomato plants were challenged with the potato aphid (100 aphids confined to a single leaf with a sleeve cage) or mock inoculated with empty cages, and *P4* and *PI-II* transcript abundance was analyzed 48 h after inoculation. Expression values were calculated by RT-qPCR relative to the wild-type mock-inoculated control, normalized using the *RPL2* gene, and analyzed by two-way ANOVA. Error bars represent ± SE ($n = 4$). ** $P < 0.001$. C and D, Wild-type (cv Castlemart) and *spr2* tomato plants were challenged with potato aphids or mock inoculated with empty cages (60 aphids confined to the three terminal leaflets with a sleeve cage; five plants per genotype per time point). At 24 and 48 h after inoculation, total, free, and bound SA were quantified by HPLC in infested or mock-inoculated leaflets. Values were analyzed by ANOVA, and mean separations were performed using Student's *t* test. Bars of the same pattern ± SE with different lowercase letters are significantly different at $\alpha = 0.05$; bars with different uppercase letters show significant differences in total SA content (free + bound). FW, Fresh weight.

trated with the control vector (Fig. 8C). Total SA levels were higher in *spr2* than in wild-type plants but were not significantly altered by silencing of *NPR1* (Fig. 8D); therefore, we hypothesize that silencing *NPR1* compromised aphid resistance by suppressing defenses downstream of this regulator.

DISCUSSION

Although JA mediates induced resistance against many chewing insects and cell-content feeders, our results suggest that, in tomato, JA does not contribute to antibiotic defenses against a phloem feeder, the potato aphid. Mutations that block JA synthesis (*spr2* and *acx1*) or perception (*jai1-1*) in tomato fail to enhance aphid population growth (Fig. 2), despite the fact that these mutations improve host suitability for other herbivores (Li et al., 2003, 2004, 2005). Instead, the mutant line *spr2* reduces the settling behavior, survival, and fecundity of the potato aphid. This contrasts sharply with a prior report that oviposition by another phloem-feeding insect, *Bemisia tabaci*, is enhanced on *spr2* (Sánchez-Hernández et al., 2006). Our results support prior assertions that plant responses to whiteflies and aphids differ (Kempema et al., 2007) and indicate that mechanisms of effective basal host plant resistance may vary even within a single feeding guild of insects such as phloem feeders.

Several lines of evidence establish that aphid resistance is due to the loss of function of FAD7, independent of the host genetic background. We observed the same aphid-resistant phenotype in the FAD7 antisense suppression line generated in tomato cv L402 (Fig. 3A) and in the Arabidopsis FAD mutants (Fig. 3B) that we observed in the *spr2* line, which was developed by chemical mutagenesis in tomato cv Castlemart (*spr2*). Moreover, the *spr2* aphid-resistant phenotype was recovered in the segregating tomato (*spr2* × *NahG*)F_2 population when plants were selected based on the presence or absence of the *fad7* mutation (Fig. 7, A and B), directly linking the loss of function of FAD7 to aphid resistance. Aphid resistance in *spr2* does not appear to be the result of impaired JA signaling, since aphid performance did not change in the *jai1-1* or *acx1* mutant and application of MeJA did not compromise resistance in *spr2* (Fig. 2). The presence of aphid resistance in the Arabidopsis *Atfad7-1* and *Atfad7-2* mutants also lends strong support for the hypothesis that the impact of FAD7 on aphids is not mediated by JA. At 22°C to 23°C, these mutants retain approximately 50% to 75% of wild-type levels of C18:3 (Browse et al., 1986; McConn et al., 1994; McConn and Browse, 1996), which is likely in excess of the

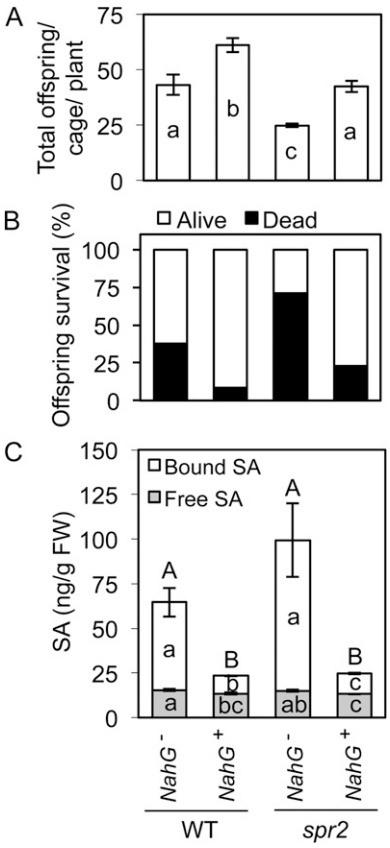

Figure 7. Aphid resistance conferred by loss of function of FAD7 is compromised by the *NahG* transgene. The *spr2* and *NahG* tomato lines were crossed, the F1 progeny were self-pollinated, and the F_2 generation was screened by PCR for the presence or absence of the *NahG* transgene, the wild-type (WT) *LeFAD7* allele, and the *spr2* mutation in *LeFAD7*. Four phenotypic bulks were selected: (1) WT/*NahG*$^-$ = plants carrying at least one copy of the wild-type *LeFAD7* allele and lacking the *NahG* transgene; (2) WT/*NahG*$^+$ = plants carrying the wild-type *LeFAD7* allele and *NahG*; (3) *spr2*/*NahG*$^-$ = plants homozygous for the *spr2* mutation in *LeFAD7* but lacking *NahG*; and (4) *spr2*/*NahG*$^+$ = double mutant plants homozygous for the *spr2* mutation and carrying *NahG*. All four bulks were inoculated with potato aphids (five aphids per cage; three cages per plant; 14–17 plants per bulk), and 6 d after inoculation, total offspring (dead and alive) were counted to measure adult fecundity (A) and offspring survival (B). One day after the aphids were counted, total, free, and bound SA content was measured in six randomly selected samples per bulk (C). The average number of total, dead, and living aphids per cage per plant was Box Cox transformed (Box and Cox, 1964) to stabilize variances, all values were analyzed by one-way ANOVA, and means were separated using Student's *t* tests. Bars of the same pattern ± SE with different letters differ significantly at $\alpha = 0.05$. Uppercase letters above the bars in C denote significant differences in total (free + bound) SA content. FW, Fresh weight.

amount needed to produce JA in response to stress; moreover, the absence of male sterility in these lines also suggests the ability to synthesize JA (McConn and Browse, 1996). Together, these findings demonstrate that FAD7 inhibits aphid resistance in both tomato and Arabidopsis and that its impact on aphids is likely independent of JA.

Aphid resistance in plants with impaired FAD7 function could potentially be due to altered fatty acid metabolism. The most abundant fatty acids in tomato foliage are C18:3, C18:2, C16:3, and C16:0 fatty acids, in descending order of abundance. Compared with wild-type plants, the *spr2* mutant is characterized by high C18:2 (approximately four times the wild-type level), very low C18:3 (less than 10% of the wild-type level), and undetectable C16:3 levels as well by slight increases in C18:1 and C16:2 fatty acids (Supplemental Fig. S3; Li et al., 2003). Potentially, aphid resistance in

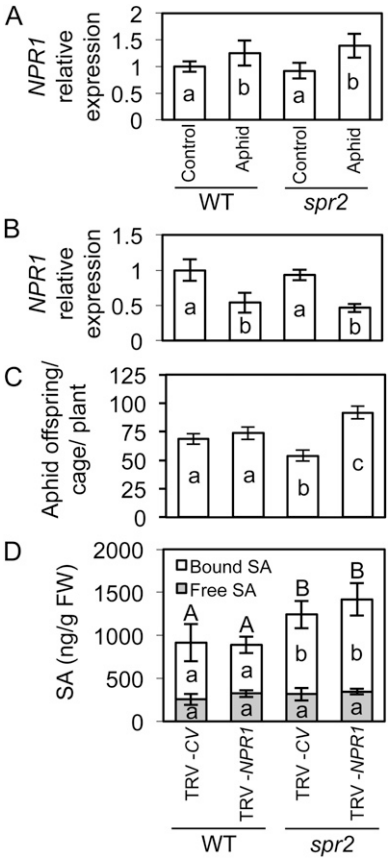

Figure 8. *NPR1* is up-regulated by aphid feeding and contributes to aphid resistance in the *spr2* mutant. A, Expression of the *NPR1* gene was measured 48 h after inoculation in *spr2* and wild-type (WT; cv Castlemart) plants infested with aphids by RT-qPCR using *RPL2* as the reference gene. B to D, VIGS using TRV was performed to suppress the expression of *NPR1* in tomato, and a construct of similar size that does not silence any endogenous genes in tomato was used as a control vector (TRV-CV). Silencing of *NPR1* was corroborated by RT-qPCR using *RPL2* as the reference gene (B). Plants were challenged with the potato aphid (four aphids per cage; four cages per plant; eight plants per treatment group), and the total number of live adults and offspring was recorded 6 d after inoculation (C). Local total, free, and bound SA was measured 1 d after aphid count (D). Values were analyzed by ANOVA, and mean separations were performed using Student's *t* test. Values ± SE with different letters are statistically different at $\alpha = 0.05$. Uppercase letters above bars in C denote significant differences in total (free + bound) SA content. ($n = 7$, 8, 6, and 8 respectively). FW, Fresh weight.

spr2 may be due to (1) an increase in oleic acid (C18:1) or its derivatives; (2) an increase in dienoic fatty acids (primarily C18:2) and their derivatives; or (3) a decrease in trienoic fatty acids (C18:3 and C16:3) and their products. It is unlikely that these changes would have direct nutritional consequences for aphids, because phloem sap appears to contain primarily C16:0 rather than dienoic or trienoic fatty acids (Madey et al., 2002) and aphids can survive and reproduce on artificial diets entirely lacking in fatty acids (Douglas and Simpson, 2003). However, fatty acids have been proposed to participate in defense signaling either directly or indirectly (Kachroo and Kachroo, 2009) and are also precursors for the synthesis of azelaic acid and numerous oxylipins that contribute to plant immunity (Blée, 2002; Jung et al., 2009). For example, a study in potato demonstrated that aphids strongly induce the production of 9-hydroperoxy-octadecadienoic acid, a derivative of linoleic acid synthesized by 9-lipoxygenases (Gosset et al., 2009). Potentially, increases in 9-hydroperoxy-octadecadienoic acid or other oxylipin derivatives of linoleic acid may contribute to aphid resistance in *spr2*. Alternatively, fatty acid desaturation could influence plant defenses against aphids by altering the composition of the plant's cuticle, which has recently been shown to play a role in defense signaling (Kachroo and Kachroo, 2009; Xia et al., 2009, 2010).

Another possibility to consider is that FAD7 enzymatic activity might influence aphid host selection behavior or performance through its influence on plant volatile profiles. Emission of volatile terpenes is more than 2-fold lower in *spr2* than in wild-type plants, probably as a result of reduced JA levels (Sánchez-Hernández et al., 2006). Typically, terpenoids have repellent effects on aphids and other insects (Aharoni et al., 2003; Bleeker et al., 2009), but it is conceivable that one or more terpenes that are reduced in *spr2* could contribute to aphid attraction. The *spr2* mutation in tomato also alters the profile of C6 volatile organic compounds (VOCs) generated from linoleic acid (C18:2) and linolenic acid (C18:3) via the hydroperoxide lyase pathway (HPL), resulting in a dramatic increase in hexanal and hexanol production and a decrease in (*Z*)-3-hexenal and (*Z*)-3-hexanol (Canoles et al., 2006; Sánchez-Hernández et al., 2006). Similar shifts in volatile profiles were also observed in *Atfad7* Arabidopsis plants (Zhuang et al., 1996), although overall production of C6 volatiles is reported to be extremely low in the Columbia ecotype (Duan et al., 2005; Chehab et al., 2008). HPL appears to contribute to aphid resistance in potato (Vancanneyt et al., 2001), and several VOCs have direct antibiotic effects on aphids in vitro (Hildebrand et al., 1993). However, several lines of evidence suggest that HPL-derived VOCs are not essential to aphid resistance in plants with impaired FAD7 function. In Arabidopsis, we observed aphid resistance in *Atfad7* mutants developed in a Columbia background (Fig. 3B), even though this ecotype carries a mutation that inhibits HPL activity and C6 volatile production (Duan et al., 2005). Overexpression of HPL in Arabidopsis also does not alter the host preference, population increase, or weight gain of the green peach aphid, even though it results in a more than 40-fold increase in C6 volatile production (Chehab et al., 2008). Furthermore, in tomato, antisense suppression of lipoxygenase C had no detectable effect on aphid host acceptance or population growth, despite dramatic reductions in VOC emissions (H. Klee and F. Goggin, unpublished data). Therefore, it is unlikely that plant volatile profiles alone are responsible for the effects of FAD7 on aphids.

While inhibition of FAD7 enzymatic activity clearly alters the production of C6 volatiles and many other fatty acid-derived compounds with roles in signaling and defense, it is also possible that the effects of FAD7 on aphid resistance may be independent of fatty acid metabolism. This would be consistent with the fact that *Atfad7-2* and *Atfad7-1fad8-1* confer aphid resistance even though these mutations cause relatively modest changes in fatty acid content at moderate temperatures (Browse et al., 1986; McConn and Browse, 1996). Instead, the FAD7 protein itself may influence aphid resistance, possibly through interactions with other chloroplast-localized proteins.

Whether through its desaturase activity or through other protein functions, our data indicate that wild-type FAD7 suppresses local SA-dependent defenses against aphids in tomato. Aphid resistance in the tomato *spr2* mutant is associated with higher than normal levels of local SA (Fig. 6, C and D) and mRNA encoding the pathogenesis-related protein P4 (Fig. 6A) in aphid-infested foliage. The *NahG* transgene, which suppresses SA accumulation, restores wild-type levels of aphid susceptibility to *spr2* (Fig. 7, A and B). Resistance in *spr2* is also compromised when we utilize VIGS to suppress the expression of *NPR1* (Fig. 8C). *NPR1* is a key regulator of SA-dependent defenses (Zhang et al., 1999; Dong, 2004). Interestingly, silencing *NPR1* in *Nicotiana attenuata* also reduces free fatty acid levels and thereby inhibits the induction of 13-hydroperoxy-octadecatrienoic acid and JA in response to wounded or simulated insect herbivory (Kallenbach et al., 2010). Further work is needed to explore the potential interaction between *NPR1* on fatty acid metabolism. Additional studies are also necessary to determine if aphid resistance in Arabidopsis FAD mutants is also SA dependent, particularly in light of recent conflicting reports about the potential impacts of FAD7 on SA signaling in this species (Chaturvedi et al., 2008; Xia et al., 2010). However, our findings are consistent with other studies indicating that SA can contribute to plant defenses against aphids in other tomato genotypes. Suppression of SA accumulation increases aphid longevity in the wild-type cv Moneymaker and compromises aphid resistance in another cultivar (Motelle) that carries the *Mi-1.2* aphid resistance gene (Li et al., 2006). Application of the SA analog benzothiadiazole also reduces aphid population growth on the Moneymaker cultivar (Cooper et al.,

2004; Boughton et al., 2006; Li et al., 2006). Furthermore, tobacco mosaic virus infection reduces plant susceptibility to aphids in wild-type tomato but not in transgenic plants impaired in SA accumulation, which suggests that the SA-mediated defense responses against pathogens in tomato are also effective against aphids (Rodriguez-Saona et al., 2010).

There are several potential routes through which loss of function of FAD7 may enhance SA accumulation in tomato in response to aphid infestation. SA synthesis from chorismate occurs in the plastid (Wildermuth et al., 2001), where FAD7 is also localized; thus, it is possible that the FAD7 protein or a metabolite whose abundance is affected by FAD7 activity modulates a plastid component involved in SA biosynthesis. Although aphid resistance appears to be independent of JA itself, it is also conceivable that SA signaling might be enhanced by a decrease in the abundance of intermediates in JA synthesis [e.g. 13-hydroperoxy-octadecatrienoic acid, 12,13-epoxy-9,11,15-octadecatrienoic acid, 2-oxo-phytodienoic acid, or 3-oxo-2(2′(*Z*)-pentenyl)-cyclopentane-1-octanoic acid 8; Fig. 1], because the SA and JA pathways can interact antagonistically under certain conditions (Bostock, 2005). Alternatively, FAD7 could potentially influence SA signaling indirectly by influencing the accumulation of reactive oxygen species (Yaeno et al., 2004; Mène-Saffrané et al., 2009). Mène-Saffrané et al. (2009) propose that trienoic fatty acids serve as important antioxidants and that constitutive SA levels are enhanced in the Arabidopsis *Atfad3fad7fad8* mutant as a result of increased accumulation of reactive oxygen species. On the other hand, Yaeno and coworkers (2004) propose that linolenic acid promotes the reactive oxygen burst in response to pathogens by activating NADPH oxidase and that decreased linolenic acid levels in the *Atfad7fad8* mutant result in decreased accumulation of hydrogen peroxide and superoxide in response to *P. syringae*. Clearly, further work is needed to investigate how ω-3 FADs impact reactive oxygen species accumulation and how this, in turn, may influence SA signaling.

CONCLUSION

Plants must defend themselves against a broad array of pests, including insect herbivores that utilize a diversity of feeding strategies to exploit their hosts. Plant defenses vary with the nature of the attacker and are coordinated by a highly conserved group of plant hormones, including JA and SA. Our study demonstrates that loss of function of FAD7 in the *spr2* mutant in tomato enhances basal resistance to the potato aphid, a phloem-feeding herbivore with piercing-sucking mouth parts. Although JA synthesis and JA-dependent defenses against caterpillars and whiteflies are compromised in this mutant, decreased JA levels in these plants are not directly responsible for aphid resistance. Instead, resistance is linked to enhanced SA accumulation and requires SA signaling mediated by the *NPR1* gene. Thus, loss of function of FAD7 enhances defenses against aphids and represses defenses against other insects through independent effects on SA and JA signaling. Our results provide novel insights into the contribution of FADs to plant defenses against aphids and to tradeoffs in resistance to different insects between and within feeding guilds.

MATERIALS AND METHODS

Plant and Insect Materials

Eight tomato (*Solanum lycopersicum*) genotypes were used in this study: the mutant lines *spr2*, *jai1-1*, and *acx1* and the corresponding wild-type background cv Castlemart; the transgenic line *Lefad7-antisense* and the untransformed control cv L402; and *NahG* and its untransformed wild-type control, cv Moneymaker. In addition, crosses were performed between *spr2* and *NahG* (described further below). All genotypes were grown in LC1 Sunshine potting mix (Sungro Horticulture) supplemented with 15-9-12 Osmocote Plus slow-release fertilizer (Scotts-MiracleGro). The *NahG* transgenic line was kindly provided by Dr. Jonathan Jones (Sainsbury Laboratory). Plants were maintained under stable greenhouse conditions (approximately 21°C–27°C, 16-h/8-h light/dark photoperiod) and watered with a dilute nutrient solution containing 1,000 μL L^{-1} $CaNO_3$ (Hydro Agri North America), 500 μL L^{-1} $MgSO_4$ (Giles Chemical), and 500 μL L^{-1} 4-18-38 Gromore fertilizer (Gromore). Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (CS60000) and mutants *Atfad7-2* (CS8042) and *Atfad7-1fad8-1* (CS8036) were obtained from the Arabidopsis Biological Resource Center. The *Atfad7gl1-1* and *Atgl1-1* mutants were kindly donated by Dr. Jyoti Shah (University of North Texas). The plants were maintained in a Conviron growth chamber (Controlled Environments; 23°C, 65% relative humidity, 16-h/8-h light/dark photoperiod) and grown in LC1 Sunshine potting mix supplemented with 15-9-12 Osmocote Plus fertilizer, fertilized weekly with 24-8-16 MiracleGro all-purpose plant food (Scotts-MiracleGro). The potato aphid (*Macrosiphum euphorbiae*) was maintained in Conviron growth chambers (20°C, 16-h/8-h light/dark photoperiod) on a combination of tomato seedlings (cv UC82), potato (*Solanum tuberosum*), and jimson weed (*Datura stramonium*). The green peach aphid (*Myzus persicae*) was maintained at room temperature (approximately 23°C, 16-h/8-h light/dark photoperiod) on cabbage (*Brassica oleracea* var Capitata) seedlings.

JA Treatment

Wild-type tomato (cv Castlemart) and *spr2* plants each were sprayed with 75 μM MeJA or water and covered with a plastic bag for 2 h to allow MeJA penetration. Twenty-four hours after treatment, 10 plants per treatment group were used to measure population growth of the potato aphid (described below), and tissue from three additional plants was collected to confirm the induction of JA-dependent defenses through semiquantitative RT-PCR analysis of *PI-II* expression (see below).

Gene Expression Analysis

Semiquantitative RT-PCR

To analyze *PI-II* expression in MeJA-treated plants, leaf samples were flash frozen in liquid nitrogen 24 h after treatment and stored at −80°C until RNA extraction. Total RNA was extracted from each leaf sample using TRIzol reagent (Invitrogen), and RNA concentration and quality were assessed using a Nanodrop spectrophotometer (Thermo Scientific). Total RNA was DNase treated with TURBO DNA-free (Ambion) followed by RT of 0.5 μg of RNA using oligo$(dT)_{18}$ primers and SuperScript II reverse transcriptase (Invitrogen) in a 20-μL reaction volume. Semiquantitative PCR was performed using 50 ng of cDNA as template and 0.2 μM final concentration of each primer under the following conditions: a 5-min initial denaturation at 95°C; 22 amplification cycles (denaturation at 95°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 45 s); and a final extension at 72°C for 5 min using GoTaq green master mix (Promega). The endogenous, constitutively expressed gene *Ribosomal Protein L2* (*RPL2*) was used as a loading control. Primers were *PI-II* (GenBank accession no. AY129402 for mRNA sequence) forward (5′-CCCACGTTCAGAAGGAAGTC-3′) and reverse (5′-TGAACGGGGACATCTTGAAT-3′) and *RPL2* (GenBank accession no. X64562) forward (5′-GAGGGCGTACTGAGAAACCA-3′) and reverse (5′-CTTTTGTCCAGGAGGTGCAT-3′).

PCR products were visualized on a 1% agarose gel stained with GelRed dye (Biotium).

RT-qPCR

To measure the impact of potato aphid infestation on *P4*, *PI-II*, and *NPR1* expression, wild-type (cv Castlemart) and *spr2* plants were inoculated with aphids, which were confined to a single leaf on each plant using large organza sleeve cages (100 aphids per cage). Control plants were mock inoculated with empty cages, and leaf tissue was collected 48 h after inoculation (four plants per treatment group). *NPR1* expression was also analyzed in a second set of plants with a lower inoculum level (25 aphids per cage; three plants per treatment group), and because *NPR1* expression did not differ between the two inoculum levels, data from these two sets of plants were pooled for analysis (seven plants per treatment group). RNA extraction and RT were performed as described above. The RT reaction products were diluted to 40 μL, and a 2-μL aliquot (a 25-ng RNA equivalent) was used as the template for real-time PCR. For each of the four biological replicates, two technical replicates were included in the quantitative PCR experiments. Mock reactions lacking reverse transcriptase were also included for each RNA sample to confirm the absence of DNA contamination. Real-time qPCR was carried out using the QuantiTect SYBR Green PCR kit (Qiagen) scaled for a 20-μL reaction volume, with final primer concentrations of 0.5 μM. The Mx3000P real-time PCR system (Stratagene) was used for PCR and fluorescence detection. The PCR conditions were as follows: 15 min of activation at 95°C; 40 amplification cycles (denaturation at 94°C for 15s, annealing at 59°C for 30s, and extension at 72°C for 30s); and a final data acquisition step to generate disassociation curves (95°C for 1 min and 55°C for 30s). Dissociation curves were examined for all samples to confirm that each primer set generated a single amplification product. Primer pairs used for RT-qPCR were as follows: *P4* (GenBank accession no. M69247 for mRNA sequence) forward (5′-CAACTCAAGAGCGGGTAGTTG-3′) and reverse (5′-CCACACATTTTTCCACCAACAC-3′) and *LeNPR1* (GenBank accession no. AY640378.1) forward (5′-CTCCAGGCGGTAAGGAAA-3′) and reverse (5′-CAAATAGGCGAGCACACTGA-3′); see above for *RPL2* and *PI-II* primer sets. To estimate the efficiency of amplification for each of these primer sets, RT-qPCR was performed on serial dilutions of a set of cDNA standards, and the PCR efficiencies were calculated using the $E = 10^{[-1/Ct\ slope]}$ methodology (Rasmussen, 2000). Relative gene expression was calculated using the methodology of Pfaffl (2001). Data for our genes of interest were normalized to the expression levels of *RPL2*, and relative gene expression for each treatment group was calculated relative to the untreated wild-type control group in each experiment. For statistical analysis, the relative expression values for each treatment group were $\log_2$ transformed to stabilize variances. Data were analyzed by two-way ANOVA, and means for significant effects at $\alpha = 0.05$ were separated using Student's *t* test with JMP version 8.0 (SAS Institute).

SA Quantification

To measure the influence of potato aphid infestation on SA accumulation, *spr2* and wild-type (cv Castlemart) tomato plants were challenged with potato aphids by introducing 60 aphids into an organza sleeve cage enclosing the terminal three leaflets of a single leaf, selected from the sixth or seventh node position up from the oldest true leaf (one cage per plant; five plants per treatment group per time point). Control plants were mock inoculated with empty cages. The inoculated leaflets (approximately 250 g of tissue per plant) were collected 24 and 48 h after inoculation (local tissues), and a similar amount of tissue was also sampled from an upper unwounded leaf (eighth or ninth node) of each plant to measure the systemic SA accumulation. SA was extracted and quantified by HPLC as described previously (Branch et al., 2004). In brief, SA was measured using an Agilent 1100 HPLC device with fluorometric detection. The column was a 4.6- × 75-mm Agilent RR XDB C18 used with an isocratic mobile phase composed of 75% 20 mM formate, pH 3.8, 20% methanol, and 5% acetonitrile at a flow rate of 0.75 mL min^{-1} at 35°C. Bound SA was measured after converting to free SA by acid hydrolysis. Recovery rates were determined using *o*-anisic acid as an internal standard and were typically greater than 60%. Free and bound SA levels were analyzed using three-way ANOVA, and means separations were performed using Student's *t* test with JMP version 8.0.

Development and Characterization of Tomato *spr2*/*NahG* Double Mutants

Crosses were performed using *spr2* as the maternal parent and *NahG* as the pollen donor. The *spr2* flowers were emasculated 1 d prior to anthesis and crossed manually with *NahG* pollen the next day. Then, the (*spr2* × *NahG*)F1 hybrid plants were self-pollinated to obtain the (*spr2* × *NahG*)F_2 population. Since the *spr2* mutation in LeFAD7 and the *NahG* transgene were found in different genetic backgrounds (cv Castlemart and cv Moneymaker, respectively), a modified bulked segregant analysis approach was performed on the F_2 generation to determine the effects of *NahG* and the *spr2* mutation on SA levels and aphid resistance. This method compensates for segregation at other loci and is a rapid alternative to the development of near-isogenic lines (Michelmore et al., 1991). Segregating plants from the (*spr2* × *NahG*)F_2 generation were screened by PCR for the presence or absence of the wild-type *LeFAD7* allele, the *spr2* mutation, and the *NahG* transgene. Four phenotypic bulks of at least 14 plants each were selected: (1) plants carrying at least one copy of the wild-type *LeFAD7* allele and lacking the *NahG* transgene; (2) plants carrying the wild-type *LeFAD7* allele and *NahG*; (3) plants homozygous for the *spr2* mutation in *LeFAD7* but lacking *NahG*; and (4) double mutant plants homozygous for the *spr2* mutation and carrying *NahG*. Since plants within each phenotypic bulk had the same genotype at the *LeFAD7* and *NahG* loci but had random variations at other unlinked loci, comparisons among bulks eliminate the potential effects of genetic background, and differences in aphid resistance and SA levels among bulks could be attributed to *LeFAD7*, *NahG*, and the interaction between these genes. Approximately 400 (*spr2* × *NahG*)F_2 plants were PCR screened for the presence of the *LeFAD7* wild-type allele or the *spr2* mutation using single-nucleotide polymorphism primers (wild-type *LeFAD7* allele, forward [5′-ATATTGGGCGGAGATGTGAA-3′] and reverse [5′-AACCACATTCTGATAGAACC-3′]; *spr2* mutation, forward [5′-CTAACTAAAATGGCAAGTTGA-3′] and reverse [5′-TACCCTCAATGCCCAACAAT-3′]). Then, selected plants were PCR screened for the presence of the *NahG* transgene (forward [5′-GTAGCCATGTGCTGGAAGGT-3′] and reverse [5′-CCTCACTGGAAAGGTGAGGA-3′]). DNA was isolated using the REDExtract-N-Amp plant PCR kit (Sigma), and touchdown PCR (Korbie and Mattick, 2008) was performed to increase amplification sensitivity and specificity using the following conditions: initial denaturation = 95°C for 5 min; phase I = 95°C for 45 s, 65°C to 56°C for 45 s (reducing 1°C per cycle), and 72°C for 45 s; phase II = 95°C for 45 s, 55°C for 45 s, and 72°C for 45 s (20 cycles); and final extension at 72°C for 5 min. PCR products were visualized on 1% agarose gels. For each of the four bulks selected through PCR screening, six plants were used for SA measurement as described above and 14 to 17 plants were used for an aphid bioassay (described below).

Silencing of *NPR1*

VIGS

The tobacco rattle virus (TRV) vector pYL156 was kindly provided by Dr. Dinesh-Kumar (Yale University). An insert of 414 bp corresponding to nucleotides 1,124 to 1,537 of the *LeNPR1* gene (GenBank accession no. AY640378.1) was cloned (forward primer, 5′-ATATA**GAATTC**CTGCTCCAAAGGATCGGTTA-3′; reverse primer, 5′-ATATA**CTCGAG**CAGACAAGTCATCAGCATCCA-3′) and inserted into pYL156 using *Eco*RI and *Xho*I sites. A construct (TRV-CV) carrying a 396-bp insert corresponding to nucleotides 544 to 939 of the GUS reporter gene (GenBank accession no. S69414.1) was used as a control vector (Hartl et al., 2008; Wu et al., 2011), and another construct that carries a 408-bp DNA fragment corresponding to nucleotides 1,175 to 1,583 of the *Phytoene Desaturase* (*PDS*) gene (GenBank accession no. M88683.1) was used as a visual reporter to monitor the onset of VIGS. The *NPR1* silencing construct TRV-*NPR1*, TRV-CV, and TRV-*PDS* were introduced into 2-week-old *spr2* and wild-type tomato plants by agroinfiltration as described by Wu et al. (2011) and maintained at 20°C/16 h of light. Plants were used for an insect bioassay (described below) 21 d after agroinfiltration, when widespread bleaching symptoms were observed in plants infiltrated with TRV-*PDS*. One day after the bioassay was scored, aphids were gently removed from the plants using paint brushes and leaf tissue was collected for RNA extraction and SA quantification by HPLC (performed as described above).

Confirmation of Gene Silencing

Leaf tissue for every cage was flash frozen in liquid nitrogen and stored at −80°C. RNA was isolated from eight randomly selected samples for each treatment group, and RT-qPCR was performed following the methods described above. Primers for gene expression analysis were designed so that they would not overlap with the inserts in the VIGS constructs (*LeNPR1*

forward [5′-CTCCAGGCGGTAAGGAAA-3′] and reverse [5′-CAAATAGGC-GAGCACACTGA-3′]).

Aphid Performance Bioassays

Potato Aphid Performance on Tomato

All insect bioassays were performed when tomato plants were between 4 and 5 weeks old, and, unless otherwise specified, they were conducted in growth chambers (23°C, 16-h/8-h light/dark photoperiod). Four types of bioassays were used to measure different aspects of aphid performance. Uncaged population growth assays were conducted to compare aphid infestations on wild-type plants, *spr2*, *jai1-1*, and *acx1* (Fig. 2, A and B). Each plant was inoculated with 15 aphids that were not confined to cages and were able to move from leaf to leaf on the same plant or to reject the host by dropping off the plant. Total aphid numbers per plant were counted 5 d after inoculation and reflected the net effects of the host plants on aphid host acceptance, survival, and fecundity. Clip-cage assays were also used to measure differences in short-term aphid population growth in response to MeJA treatment (Fig. 2C), antisense suppression of *FAD7* (Fig. 3A), the separate and combined effects of *spr2* and *NahG* (Fig. 7, A and B), and VIGS silencing of *NPR1* (Fig. 8). A fixed number of young adult aphids were confined to a single leaflet using a clip cage (four to five per cage and three to four cages per plant; exact numbers for each assay are reported in the figure legends), and aphid reproduction and survival were assessed 6 d after inoculation. Values for the individual cages (subreplicates) were averaged to obtain single data points for each replicate plant. This assay design allowed comparison of aphid population growth in the absence of host choice. Aphid numbers for both of these assay types were analyzed by ANOVA, and where appropriate, mean separations were performed with Student's *t* tests using JMP version 8.0. A third assay type allowed precise measurement of the longevity and daily offspring production of individual adult aphids on wild-type and *spr2* plants (Fig. 5). Newly emerged wingless adults (less than 24 h within emergence to adulthood) were confined to clip cages (one aphid per cage; two subreplicate cages per plant), and every 24 h adult survival was monitored and offspring were counted and removed from the cages. Plants were maintained in a greenhouse (21°C–27°C, 16-h/8-h light/dark photoperiod) due to the large amount of space required for this assay and were monitored for 14 d, until all aphids on the *spr2* genotype were dead. The average aphid life spans (days lived), total offspring production, and daily fecundity (total offspring production per days lived) were analyzed by one-way ANOVA. Additionally, regression analyses were performed to estimate the aphid daily survival rate and fecundity with JMP version 8.0. A fourth assay type was utilized to compare aphid host preference between wild-type and *spr2* plants (Fig. 4). Choice tests were performed by placing 10 newly emerged wingless adult aphids on a Styrofoam choice arena (15 cm diameter) between paired leaflets (sixth or seventh node position) on intact wild-type and *spr2* plants (10 replicate pairs). The leaflets were isolated from the rest of the plant by placing 15-cm round barriers made of glossy photo paper and coated with Tanglefoot tangle-trap insect trap coating (Contech Enterprises) around the base of the leaflet petiole (Fig. 4A). Aphids typically moved from the arena onto a leaflet within minutes of release and would then either remain on the leaflet and begin reproduction or move to the other leaflet via the arena. The number of aphids on each leaflet or remaining on the arena was counted at 1, 4, and 24 h after release and analyzed by paired *t* tests with JMP version 8.0. Aphids that were on the plastic arena between the plants or that crawled down the plastic platform without settling on a leaflet were not included in the analysis.

Green Peach Aphid Population Growth on Arabidopsis

Newly emerged wingless adults were confined to plastic sleeve cages that cover the entire plant (two aphids per plant; 18 plants per genotype) at growth stage 5.10 (Boyes et al., 2001). Seven days after infestation, the total number of aphids was counted and analyzed by one-way ANOVA. Means were separated using Student's *t* test with JMP version 8.0.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Loss of JA sensitivity in the *jai1-1* mutant does not enhance the expression of SA-responsive gene *P4* but suppresses the expression of the JA-responsive gene *PI-II*.

Supplemental Figure S2. Effect of catechol on aphid performance.

Supplemental Figure S3. Impact of *spr2* and *NahG* on foliar fatty acid profiles.

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