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Loss of function of FATTY ACID DESATURASE 7 in tomato enhances basal aphid resistance in a salicylate-dependent manner

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Abstract (249 words)
We report here that disruption of function of the ω-3-fatty acid desaturase FAD7 enhances plant defenses against aphids. The spr2 mutation in tomato (Solanum lycopersicum), which eliminates function of FAD7, reduces the settling behavior, survival and fecundity of the potato aphid (Macrosiphum euphorbiae). Likewise, antisense suppression of LeFAD7 expression in wild-type (WT) 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 NahG transgene, which suppresses SA accumulation, restores WT levels of aphid susceptibility to spr2. Resistance in spr2 is also lost when we utilize virus-induced gene silencing to suppress 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 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 WT 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 fad7-2 and fad7-1fad8-1 also show enhanced resistance to the green peach aphid (Myzus persicae) compared to WT controls, indicating that FAD7 influences plant-aphid interactions in at least two plant families.
INTRODUCTION (Characters 8,923)

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 upregulated 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 double mutants that are deficient in two chloroplast-localized \( \omega \)-3-fatty acid desaturases (FAD7 and FAD8) display enhanced heat tolerance (Murakami et al., 2000). FADs also influence resistance to numerous biotic stresses. For example, the Arabidopsis \( fad7fad8 \) mutant shows increased vulnerability to the bacterial pathogen \textit{Pseudomonas syringae}, whereas suppression of the homologous \( OsFAD7 \) and \( OsFAD8 \) genes in rice results in enhanced resistance to the rice blast fungus \textit{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 \( \omega \)-3-FADs on biotic stress is due in part to their critical role in the biosynthesis of the defense hormone jasmonate (JA) (Figure 1). Jasmonoyl-L-isoleucine, 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 over-express \( FAD7 \), and are depleted by inhibition of \( \omega \)-3-FAD activity in Arabidopsis and potato plants (McConn et al., 1997; Martin et al., 1999; Song et al., 2004). Furthermore, wounding and other stresses upregulate \( FAD7 \), 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 \( \beta \)-hydroxy-benzoic acid that is required for basal resistance, systemic acquired resistance (SAR), and effector-triggered immunity (ETI) against many pathogens (Vlot et al., 2009), and can also contribute to plant defenses against aphids (Li et al., 2006). The \textit{SUPPRESSOR OF SA INSENSITIVITY2} (\( SSI2 \)) gene, which encodes a stearoyl acyl carrier protein fatty acid desaturase 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; Shah et al., 2001; Kachroo et al., 2004), and in rice and soybean 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 fad3fad7fad8 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 jasmonate accumulation, mutants deficient in these enzymes have been utilized to study jasmonate-dependent defenses against chewing insects. Compared to wild-type (WT) 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 plants’ inability to synthesize JA, because treating the triple mutant with exogenous methyl jasmonate restored insect resistance. Loss of function of a FAD7 homolog in tomato 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 ~10% of the linolenic acid (C18:3) content observed in WT tomato plants (Li et al., 2003). The spr2 mutation inhibits JA-dependent responses to the wound signal systemin, and nearly eliminates 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 WT controls, and M. sexta adults preferentially oviposit on spr2 (Li et al., 2003; Sanchez-Hernandez 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 mouthparts that cause far less mechanical injury than the mandibles of chewing insects (Wallig, 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 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 repression of JA by whiteflies (Zhang et al., 2009), there
is strong evidence that whitefly nymphs downregulate 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 (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 (Sanchez-
Hernandez 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; Kusnierczyk et al., 2008), and several studies
suggest that JA-dependent defenses hinder aphid infestation. Artificial jasmonate treatment
enhances aphid resistance in several plant species (Omer et al., 2001; Bruce et al., 2003; Zhu-
Salzman et al., 2004; Cooper and Goggin, 2005; Bruce et al., 2008); 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 x hortorum), a Δ914:0 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 NahG double mutant, and NahG
inhibits SA accumulation (Pegadaraju et al., 2005). Instead, Pegadaraju and coworkers
propose that aphid resistance in this mutant is due to hypersenescence. These studies indicate that FADs may influence plants' susceptibility to aphids through a diversity of mechanisms. Therefore, the goal of the present 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 trade-offs between plant defenses against different herbivores. Aphid resistance in spr2 does not appear to depend upon impaired JA signaling because exogenous methyl jasmonate 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; Figure 2A), which carries a loss-of-function mutation in LeFAD7, than on WT tomato plants (Solanum lycopersicum cv. Castlemart), or on jai1-1 (jasmonic acid insensitive1), a mutant line impaired in JA-Ile perception due to a deletion mutation in LeCOI1 (Coronatine Insensitive1) (Figure 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 inhibition of JA synthesis, we also examined aphid performance on 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) (Figure 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; Li et al., 2003; Li et al., 2004; Chen et al., 2005; Li et al., 2005; Sanchez-Hernandez et al., 2006). Neither jai1-1 nor acx1, however, had a
significant effect on aphid population growth compared to WT plants (Figure 2A and B; P<0.05).

Furthermore, application of methyl jasmonate did not impact aphid population growth on spr2, even though, as expected, it upregulated the defensive gene PROTEINASE INHIBITOR II (PI-II) (Figure 2C 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; Liu et al., 2010). Offspring production of potato aphids caged on the antisense line was significantly lower (P=0.0013) than on the WT control (cv. L402) (Figure 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 ~42% fewer aphids than the WT control (ecotype Columbia, Col) (P=0.0083) (Figure 3B). The alternative mutant allele Atfad7-1, which is in the Columbia-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 (ie. antixenosis), or by reducing their survival and/or offspring production (ie. antibiosis). To determine if loss of function of FAD7 results in antixenotic effects, adult potato aphids were placed between intact spr2 and WT tomato plants on choice arenas that allowed them to go back and forth between plants (Figure 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 was monitored at 1, 4, and 24 hrs after release. At 1 hour, adults were distributed roughly equally between the two genotypes (Figure 4B). However, at 4 and 24 hours, after the aphids had a more lengthy opportunity to feed on the plants, adults preferentially congregated (Figure 4B) and reproduced (Figure 4C) on WT plants, and 24 hrs after inoculation, total aphid numbers were almost five times higher on WT than on spr2 plants (Pairwise t test, P<0.001). This suggests that, compared to WT 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 WT plants, and adult survival was monitored daily until all aphids on the mutant genotype were dead (14 days). Offspring were also counted and removed daily to assess reproduction. The mortality rate was more than 50% higher on spr2 plants than on WT (Figure 5A), and the average number of days that adult aphids survived on this mutant (5±0.5 days) was significantly lower (P<0.05) than on WT controls (8±1 days). 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 WT plants (Figure 5B). As a result of these decreases in fecundity and longevity, lifetime offspring production (Figure 5C) was more than 50% lower on spr2 than on WT 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.

**Loss of function of FAD7 enhances P4 expression and local SA induction in response to aphid feeding.** The pathogenesis related gene P4 in tomato (GB gene ID 544185) is homologous to PR1a in tobacco and Arabidopsis, and is upregulated in response to exogenous SA and its analog benzo thiadiazole (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 (GB gene ID 543955) is transcriptionally activated in response to jasmonates and also ethylene (Farmer et al., 1992; Ohtsubo et al., 1999). 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 WT tomato plants (cv. Castlemart) 48 hrs after aphid-inoculation. Aphid feeding on WT plants upregulated the expression of P4 from 6-39 fold (Figure 6A; Figure S1), and had a relatively modest effect on PI-II, causing either slight upregulation (Figure 6B) or no significant change in expression (Figure S1). This is consistent with prior reports that induction of PI-II by aphids is relatively weak and transient compared with 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 (Figure 6B). In contrast, P4 expression was ~4-5 times higher in spr2 plants when compared to the respective WT treatments (Figure 6A), suggesting that SA signaling might be enhanced in this mutant. SA and JA are known to interact antagonistically under certain circumstances [reviewed by (Pieterse et al., 2009)], and 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 (Figure 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 WT plants 24 and 48 hrs 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 WT tomato (Figure 6C 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 x NahG) F2 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 (WT or spr2). When all four bulks were challenged with the potato aphid, offspring production (Figure 7A) and survival (Figure 7B) were reduced on the spr2 single mutant bulk (spr2/NahG) compared to the WT 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 WT levels (Figure 7A) and significantly reducing offspring mortality (Figure 7B). Li and coauthors (Li et al., 2006) have also shown that the NahG transgene causes a modest increase in aphid longevity in WT tomato, although over-expression 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 days) compared with tomato (17 days). Infiltration of 1mM catechol to spr2 did not influence aphid population growth or mortality (Figure 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 to plants that carried the WT FAD7 allele (Figure S3). The fatty acid profile of the double mutants [(spr2/NahG*)F4] was also equivalent to...
that of the spr2 single mutant plants [spr2 or (spr2/NahG)F4], 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 (Figure 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
upregulated by aphid feeding in both WT and spr2 plants (Figure 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 (Figure 8B). Whereas
silencing of NPR1 did not significantly influence aphid population growth on WT plants, aphid
numbers were nearly 70% higher on spr2 plants that received the NPR1 silencing construct
than on mutant plants infiltrated with the control vector (Figure 8C). Total SA levels were higher
in spr2 than in WT plants, but were not significantly altered by silencing of NPR1 (Figure 8D);
therefore, we hypothesize that silencing NPR1 compromised aphid resistance by suppressing
defenses downstream of this regulator.

**DISCUSSION**

Although jasmonic acid (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,
acx1) or perception (jai1-1) in tomato fail to enhance aphid population growth (Figure 2), despite
the fact that these mutations improve host suitability for other herbivores (Li et al., 2003; Li et
al., 2004; Li et al., 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, B. tabaci, is enhanced on spr2 (Sanchez-Hernandez 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 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 cultivar L402 (Figure
3A), and in the Arabidopsis fatty acid desaturase mutants (Figure 3B) that we observed in the
spr2 line, which was developed by chemical mutagenesis in tomato cultivar Castlemart (spr2).
Moreover, the spr2 aphid-resistant phenotype was recovered in the segregating tomato (spr2 x NahG)F2 population when plants were selected based on the presence or absence of the fad7 mutation (Figure 7AB), directly linking 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 acxl mutants, and application of MeJA did not compromise resistance in spr2 (Figure 2). The presence of aphid resistance in the Arabidopsis fad7-1 and fad7-2 mutants also lends strong support for the hypothesis that the impact of FAD7 on aphids is not mediated by JA. At 22-23°C, these mutants retain approximately 50-75% of WT levels of C18:3 (Browse et al., 1986; McConn et al., 1994; McConn and Browse, 1996), which is likely in excess of the 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 (FA) in tomato foliage are C18:3, C18:2, C16:3, and C16:0 fatty acids, in descending order of abundance. Compared to WT plants, the spr2 mutant is characterized by high C18:2 (~4X WT), very low C18:3 (<10% of WT), and undetectable C16:3 levels, as well by slight increases in C18:1 and C16:2 FAs (Figure S3; Li et al., 2003). Potentially, aphid resistance in spr2 may be due to 1) an increase in oleic acid (C18:1) or its derivatives; 2) an increase in dienoic FAs (primarily C18:2) and their derivatives; or 3) to a decrease in trienoic FAs (C18:3, 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 FAs (Madey et al., 2002), and aphids can survive and reproduce on artificial diets entirely lacking in FAs (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 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 production of 9-hydroperoxy-octadecadienoic acid (9-HPOD), a derivative of linoleic acid synthesized by 9-lipoxygenases (LOXs) (Gosset et al., 2009). Potentially, increases in 9-HPOD 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; Xia et al., 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 two-fold lower in spr2 than in WT plants, probably as a result of reduced JA levels (Sanchez-Hernandez 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 (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; Sanchez-Hernandez et al., 2006). Similar shifts in volatile profiles were also observed in fad7 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 fad7 mutants developed in a Columbia background (Figure 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 FA-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 WT 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 (Figure 6CD) and mRNA encoding the pathogenesis-related protein P4 (Figure 6A) in aphid-
infested foliage. The NahG transgene, which suppresses SA accumulation, restores WT levels
of aphid susceptibility to spr2 (Figure 7A-B). Resistance in spr2 is also compromised when we
utilize virus-induced gene silencing to suppress expression of NPR1 (Figure 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 FA levels and thereby inhibits induction
of 13-HPOT 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 FA
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 WT genotype 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 (BTH) 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 WT 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. Salicylic acid 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 (eg. 13-HPOT, 12, 13-EOT, OPDA,
or OPC8; see Figure 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 (ROS) (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
fad3fad7fad8 mutant as a result of increased accumulation of ROS. 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 fad7fad8 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
ROS accumulation, and how this in turn may influence SA signaling.

CONCLUSIONS:
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 mouthparts. 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 fatty acid desaturases to plant defenses against aphids, and to trade-offs in
resistance to different insects between and within feeding guilds.

MATERIALS AND METHODS (2,654 words)
Plant and insect materials.
Eight tomato 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; NahG and its untransformed WT 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, Belevue, WA)
supplemented with 15-9-12 Osmocote Plus slow-release fertilizer (Scotts-MiracleGro Company,
Marysville, OH). The NahG transgenic line was kindly provided by Dr. Jonathan Jones,
Sainsbury laboratory, Norwich, UK. Plants were maintained under stable greenhouse conditions (~21-27°C; 16:8 L:D photoperiod) and watered with a dilute nutrient solution containing 1000 ppm Ca(NO₃)₂ (Hydro Agri North America, Tampa, FL), 500ppm MgSO₄ (Giles Chemical Corp, Waynesville, NC), and 500ppm 4-18-38 Gromore fertilizer (Gromore, Gardena, CA). Arabidopsis (Arabidopsis thaliana) ecotype Columbia (Col, CS60000), mutants Atfad7-2 (CS8042) and Atfad7-1fad8-1 (CS8036) were obtained from the Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH. The Atfadgl1-1 and Atgl1-1 mutants were kindly donated by Dr. Jyoti Shah, University of North Texas, Denton, TX. The plants were maintained in a Conviron growth chamber (Controlled Environments, Inc., Winnipeg, Canada; 23°C; 65% relative humidity; 16:8 L:D photoperiod) and grown in LC1 Sunshine potting mix supplemented with 15-9-12 Osmocote Plus fertilizer, fertilized weekly with 24-8-16 Miracle Gro® All purpose plant food (Scotts-MiracleGro Company, Marysville, OH). The potato aphid (M. euphorbiae) was maintained in Conviron growth chambers (20°C, 16:8 L:D 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 (~23°C, 16:8 L:D photoperiod) on cabbage (Brassica oleracea var Capitata) seedlings.


WT tomato (cv. Castlemart) and spr2 plants each were sprayed with 75 μM methyl jasmonate or water and covered with a plastic bag for 2 hrs 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 induction of JA-dependent defenses through semi-quantitative RT-PCR analysis of PI-II expression (see below).

Gene expression analysis.

Semi-quantitative RT-PCR: To analyze PI-II expression in MeJA-treated plants, leaf samples were flash-frozen in liquid nitrogen 24 hours after treatment and stored at -80°C until RNA extraction. Total RNA was extracted from each leaf sample using TRIzol reagent (Invitrogen Corp., Carlsbad, CA), and RNA concentration and quality was assessed using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington DE). Total RNA was DNase-treated with TURBO DNA-free (Ambion, Inc., Austin, TX) followed by reverse transcription of 0.5 μg RNA using oligo dT₁₈ primers and Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA) in a 20 μL reaction volume. Semi-quantitative PCR was performed using 50 ng of cDNA as template and 0.2 μM final concentration of each primer under the following conditions: 5 min
initial denaturation at 95°C; 22 amplification cycles (denaturation 95°C 45 sec, annealing 50°C 45 sec, extension 72°C 45 sec); and a final extension at 72°C 5 min using GoTaq® green master mix (Promega, Madison, WI). The endogenous, constitutively expressed gene Ribosomal protein L2 (RPL2) was used as a loading control. Primers were PI-II (GB accession number AY129402 for mRNA sequence) forward 5’-CCCACGTTCAAGGAAGTC-3’ and reverse 5’-TGAACGGGGACATCTTGAAT-3’; and RPL2 (GB X64562) forward 5’-GAGGGCGTACTGAGAAACCA-3’ and reverse 5’-CTTTTGTCCAGGAGGTGCAT-3’. PCR products were visualized on a 1% agarose gel stained with GelRed™ dye (Biotium, Hayward, CA).

RT-qPCR: To measure the impact of potato aphid infestation on P4, PI-II, and NPR1 expression, WT (cv. Castlemart), spr2 plants were inoculated with aphids, which were confined to a single leaf on each plant using large organza sleeve cages (100 aphids/cage). Control plants were mock-inoculated with empty cages, and leaf tissue was collected 48 hrs after inoculation (4 plants per treatment group). NPR1 expression was also analyzed in a second set of plants with a lower inoculum level (25 aphids/cage; 3 plants per treatment group); and because NPR1 expression did not differ between the two inoculum levels, data from these two sets of plants was pooled for analysis (7 plants/treatment group). RNA extraction and reverse transcription were performed as described above. The reverse transcription 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, Inc., Valencia, CA) scaled for a 20 μL reaction volume, with final primer concentrations of 0.5 μM. The Mx3000P Real-time PCR system (Stratagene, La Jolla, CA) was used for PCR and fluorescence detection. The PCR conditions were as follows: 15 min activation at 95°C; 40 amplification cycles (denaturation 94°C 15s, annealing 59°C 30s, extension 72°C 30s); and a final data acquisition step to generate disassociation curves (95°C 1 min, 55°C 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 (GB accession number M69247 for mRNA sequence) forward 5’-CAACTCAAGAGCGGGTAGTTG-3’ and reverse 5’-CCACACATTTTTCCACCAAC-3’; LeNPR1 (GB AY640378.1) forward 5’-CTCCAGGGGTAAAGAAA-3’ and reverse 5’-CAAATAGGGCAGCACACTGA-3’; and 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 $E = 10^{-\frac{1}{Ct \text{ slope}}}$ methodology (Rasmussen, 2000). Relative gene expression was calculated using Pfaffl methodology (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 WT control group in each experiment. For statistical analysis, the relative expression values for each treatment group were Log$_2$ transformed to stabilize variances. Data was analyzed by Two-Way ANOVA, and means for significant effects at $\alpha = 0.05$ were separated using Student's t-test with JMP® v8.0 (SAS Institute, Cary, NC).

**Salicylic acid (SA) quantification.**
To measure the influence of potato aphid infestation on SA accumulation, $spr2$ and WT (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 6$^{th}$ or 7$^{th}$ node position up from the oldest true leaf (1 cage/plant; 5 plants/treatment group/time point). Control plants were mock-inoculated with empty cages. The inoculated leaflets (~250 g of tissue per plant) were collected 24 and 48 hrs after inoculation (local tissues), and a similar amount of tissue was also sampled from an upper unwounded leaf (8$^{th}$ or 9$^{th}$ node) of each plant to measure the systemic SA accumulation. SA was extracted and quantified by high-performance liquid chromatography as previously described (Branch et al., 2004). In brief, SA was measured using an Agilent 1100 HPLC with fluorometric detection. The column was a 4.6x75mm Agilent RR XDB C18 used with an isocratic mobile phase comprised of 75% 20 mM formate pH 3.8, 20% methanol, 5% acetonitrile at a flow rate of 0.75 ml/min at 35 °C. SAG 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 Analysis of Variance (ANOVA), and means separations were performed using Student's t-test with JMP® v8.0.

**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 one day prior to anthesis and crossed manually with NahG pollen the next day. Then, the $(spr2 \times NahG)_{F1}$ hybrid plants were self pollinated to obtain the $(spr2 \times NahG)_{F2}$ 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 of 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 \((spr2 \times NahG) F_2\) generation were screened by PCR for the presence or absence of the WT \(LeFAD7\) 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 WT \(LeFAD7\) allele and lacking the \(NahG\) transgene; 2) plants carrying the WT \(LeFAD7\) allele and \(NahG\); 3) plants that are homozygous for the \(spr2\) mutation in \(LeFAD7\) but that lack \(NahG\), and 4) double mutant plants that are homozygous for the \(spr2\) mutation and carry \(NahG\). Since plants within each phenotypic bulk had the same genotype at the \(LeFAD7\) and \(NahG\) loci, but had random variation 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 four-hundred \((spr2 \times NahG) F_2\) plants were PCR screened for the presence of the \(LeFAD7\) WT allele or \(spr2\) mutation using single nucleotide polymorphism (SNP) primers (forward primer for the WT \(LeFAD7\) allele: 5' ATATTGGGCGGAGATGTGAA-3', reverse 5'-AACCACATTCTGATAGAACC-3'; forward primer for the \(spr2\) mutation: 5'-CTAACTAAAATGGCAAGTTGA-3', reverse 5'-TACCCTCAATGCCCAACAAT-3'; respectively). Then, selected plants were PCR screened for the presence of \(NahG\) transgene (forward 5'-GTAGCCATGTGCTGGAAGGT-3', reverse 5'-CCTCACTGGAAAGGTGAGGA-3'). DNA was isolated using the REDExtract-N-Amp plant PCR kit (Sigma, Saint Louis, MO) 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 sec, 65-56°C for 45 sec (reducing 1°C per cycle), and 72°C for 45 sec; phase II= 95°C for 45 sec, 55°C for 45 sec, and 72°C for 45 (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, 6 plants were used for SA measurement as described above, and 14-17 plants were used for an aphid bioassay (described below).

Silencing of \(NPR1\).

\textit{Virus induced gene silencing (VIGS):} The tobacco rattle virus (TRV) vector pYL156 was kindly provided by Dr. Dinesh-Kumar (Yale University, New Haven, CT). An insert of 414 bp corresponding to nt 1124-1537 of the \(LeNPR1\) gene (GB AY640378.1) was cloned (forward primer: ATATAGAATTTCCTGCTCACAAGGTATCGGTA; reverse primer: ATATACTCGAGCAGACAAGTCATCAGCATCCA) and inserted into pYL156 using EcoRI and \(XhoI\) sites. A construct (TRV-CV) carrying a 396 bp insert corresponding to nt 544-939 of the
β-glucuronidase reporter gene (GB S69414.1) was used as a control vector (Hartl et al., 2008; Wu et al., 2011), and a another construct that carries a 408-bp DNA fragment corresponding to nt 1175-1583 of the Phytoene desaturase (PDS) gene (GB 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 two-week-old spr2 and WT tomato plants by agroinfiltration as described by Wu and coworkers (Wu et al., 2011), and maintained at 20°C/16 hrs light. Plants were used for an insect bioassay (described below) 21 days 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'-CAAATAGGCGAGCACACTGA-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, were conducted in growth chambers (23°C, 16:8 L:D 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 WT plants, spr2, jai1-1, and acx1 (Figure 2A 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 five days 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 methyl jasmonate treatment (Figure 2C), antisense suppression of FAD7 (Figure 3A), the separate and combined effects of spr2 and NahG (Figure 7A and B), and VIGS silencing of NPR1 (Figure 8). A fixed number of young adult aphids were confined to a single leaflet using a clip cage (4-5/cage and 3-4 cages/plant; exact numbers for each assay are reported in the figure legends), and aphid reproduction and survival was assessed 6 days after inoculation. Values for the individual cages (sub-replicates) 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® v8.0. A third assay type allowed precise measurement of the longevity and daily offspring production of individual adult aphids on WT and spr2 (Figure 5). Newly-emerged wingless adults (<24 hr within emergence to adulthood) were confined to clip cages (1 aphid/cage; 2 sub-replicate cages/plant) and every 24 hrs adult survival was monitored and offspring were counted and removed from the cages. Plants were maintained in a greenhouse (21-27°C; 16:8 L:D) 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 lifespans (days lived), total offspring production, and daily fecundity (total offspring production/days lived) were analyzed by One-way ANOVA. Additionally, regression analyses were performed to estimate the aphid daily survival rate and fecundity with JMP® v8.0. A fourth assay type was utilized to compare aphid host preference between WT and spr2 (Figure 4). Choice tests were performed by placing 10 newly-emerged wingless adult aphids on a Styrofoam choice arena (15 cm diameter) between paired leaflets (6th or 7th node position) on intact WT 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, Victoria, Canada) around the base of the leaflet petiole (Figure 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 would 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 hrs after release, and analyzed by paired t-tests with JMP® v8.0. 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 (2 aphids/plant; 18 plants per genotype) at growth stage 5.10 (Boyes et al., 2001). Seven days after infestation, the total number of aphids were counted and analyzed by One-way ANOVA. Means were separated using Student’s t-test with JMP® v8.0.

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greenhouse and growth chamber maintenance. We would also like to thank our anonymous
reviewers for helpful feedback.
Figure Legends

Figure 1. Jasmonate synthesis and perception in plants. Mutations in tomato that block JA synthesis or perception are represented in bold. Abbreviations are as follows: AOC = Allene oxide cyclase; AOS = Allene oxide synthase; COI1 = Coronatine insensitive 1; EOT = Epoxy-9,11,15-octadecatrienoic acid; FAD = Fatty acid desaturase; JA = jasmonic 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; and OPDA = 2-oxo-phytodienoic acid; OPR = 12-oxo-phytodienoic acid reductase. Closed arrows represent biosynthetic steps, whereas the open arrow represents recognition of JA-Ile by the COI1/JAZ co-receptor (Sheard et al., 2010). This figure was modified from Schaller (Schaller, 2001).

Figure 2. Aphid infestations are reduced on spr2 but are unaffected by methyl jasmonate treatment or by other mutations that impair JA signaling. A-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 five days after inoculation and analyzed by One-Way ANOVA. Mean separations were performed using Student’s t-tests. Values (±SEM) labeled with different letters differ significantly at α=0.05. C. WT and spr2 plants were treated with methyl jasmonate (MeJA, 75 μM), and inoculated with aphids 24 h after treatment (5 aphids/ cage; 3 clip cages/ plant; 10 plants/ treatment group). Live offspring were counted six days after inoculation, and the average numbers of offspring per cage per plant were analyzed by Two-Way ANOVA. **Denotes a significant main effect of genotype at P<0.0001. D. Expression of jasmonic acid-responsive PROTEINASE INHIBITOR II gene (PI-II) was monitored by RT-PCR 24 h after MeJA treatment. Expression of constitutive RIBOSOMAL PROTEIN L2 (RPL2) gene is presented as a loading control. (N=10 for A, N=8 for B, and N=10 for C).

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 (5 aphids/cage; 3 cages/plant; 12 plants/genotype), and the total number of aphids was recorded after 6 days. Cultivar L402 was used as the untransformed WT control.
B. Adult green peach aphids were confined on individual Arabidopsis plants using sleeve cages (2 aphids/plant; 18 plants/genotype), and the total number of aphids per plant was recorded after 7 days. Aphid numbers were analyzed by One-Way ANOVA, and mean separations were performed using Student’s t-tests. Values (±SEM) labeled with different letters differ significantly at $\alpha=0.05$.

Figure 4. Loss of function of FAD7 reduces aphid host acceptance. Wingless adult aphids (10 adults/arena) were placed on choice arenas (A) between paired 6-week-old plants of spr2 and the 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. 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 in the arenas. Marked pair-wise comparisons denote significant differences according to paired t-tests at $\alpha=0.05$ (*) or $\alpha=0.001$ (**). Error bars indicate ±SEM (N=10 pairs).

Figure 5. Loss of function of FAD7 decreases aphid survival and fecundity. Newly-emerged adult female aphids were caged on spr2 or WT (cv. Castlemart) plants (1 aphid/cage; 2 cages per plant; 14 plants/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 bars (±SEM) having different letters are significantly different at $\alpha=0.05$.

Figure 6. Loss of function of FAD7 enhances expression of the SA-responsive gene Pathogenesis-Related Protein 4 (P4) and local salicylic acid (SA) accumulation in response to aphid feeding, but suppresses expression of the JA-responsive gene Proteinase inhibitor II (PI-II). A-B. 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 WT mock-inoculated control, normalized using the $RPL2$ gene, and analyzed by two-way ANOVA. Error bars represent ±SEM (N=4). **= indicates P<0.001.

C-D. WT (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; 5 plants/genotype/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-tests. Bars of the same color (±SEM) that have different lowercase letters are significantly different at $\alpha=0.05$. Bars with different capital letters show significant differences in total SA content (free + bound).

**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 was self-pollinated, and the F2 generation was screened by PCR for the presence or absence of the NahG transgene, the 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 WT LeFAD7 allele and lacking the NahG transgene; 2) WT/NahG* = plants carrying the WT LeFAD7 allele and NahG; 3) spr2/NahG = plants that are homozygous for the spr2 mutation in LeFAD7 but that lack NahG, and 4) spr2/NahG* = double mutant plants that are homozygous for the spr2 mutation and carry NahG. All four bulks were inoculated with potato aphids (5 aphids/cage; 3 cages/plant; 14-17 plants/bulk), and six days 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 salicylic acid content was measured in six randomly-selected samples/bulk (C). The average number of total, dead and living aphids/cage/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 color (±SEM) with different letters differ significantly at $\alpha=0.05$. Capital letters above the bars in panel C denote significant differences in total (free + bound) SA content.

**Figure 8. NPR1 is upregulated by aphid feeding and contributes to aphid resistance in the spr2 mutant.** A. Expression of the NPR1 gene was measured 48 hrs after inoculation in spr2 and WT (cv. Castlemart) plants infested with aphids by by RT-qPCR using RPL2 as reference gene. B-D. Virus induced gene silencing (VIGS) using the Tobacco rattle virus (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 reference gene (B). Plants were challenged with the potato aphid (4 aphids/cage; 4 cages/plant; 8 plants/treatment group), and the total number of live adults and offspring was recorded 6-days after inoculation (C).
Local total, free, and bound SA was measured one day after aphid count (D). Values were analyzed by ANOVA, and mean separations were performed using Student’s t-tests. Bars (±SEM) having different letters are statistically different at α=0.05. Capital letters above bars in panel C denote significant differences in total (free + bound) SA content. (N= 7, 8, 6, and 8 respectively).

Figure S1. Loss of jasmonic acid sensitivity in jai1-1 mutant does not enhance expression of SA-responsive gene P4 (A), but suppresses expression of the JA-responsive gene PI-II (B). WT (cv. Castlemart) and jai1-1 tomato plants were challenged with the potato aphid or mock-inoculated with empty cages, and P4 and PI-II transcript abundance was analyzed 48 hrs after inoculation. Expression values were calculated by RT-qPCR relative to the WT mock-inoculated control, normalized using the RPL2 gene, and analyzed by two-way ANOVA. Error bars represent ±SEM (N=4). **= indicates P<0.0001, and n.s.= no significant difference at α=0.05.

Figure S2. Effect of catechol on aphid performance. One-month-old spr2 tomato mutant plants were infiltrated with catechol (1mM) or water as no-treatment control using a needless syringe. Seven plants per treatment were inoculated with 4 adult aphids/cage (2 cages/plant). After 6 days the offspring/plant was counted. Bars (± SEM) having different letter are statistically different at α=0.05.

Figure S3. Impact of spr2 and NahG on Foliar Fatty acid (FA) profiles. FAs were measured by GC-MS in spr2, NahG, the corresponding WT tomato cultivars Castlemart (CM) and Moneymaker (MM), and a spr2XNahG cross (5 plants/treatment group). Plants homozygous for the spr2 mutation were selected by PCR for the presence [(spr2xNahG+)F4] or absence [(spr2xNahG-)F4] of the NahG transgene. Total lipids were extracted according to the Bligh and Dyer method (Bligh and Dyer, 1959), and fatty acid methyl esters (FAMES) were prepared and analyzed following the methodology of Shipley et al. (Shipley et al., 1993) and Chen et al (Chen et al., 2005). FAMEs were quantified based on the internal standard (C15:0) in each sample. FA content was analyzed by One-Way ANOVA, and p-values were adjusted for multiple testing using Bonferroni method to control the family-wise error rate. Mean separations were performed using Student’s t-tests. Bars (±SEM) having different letters are statistically different at α=0.05.


Farmer EE, Johnson RR, Ryan CA (1992) Regulation of expression of proteinase-inhibitor genes by methyl jasmonate and jasmonic acid

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Wu C, Jia L, Goggin FL (2011) The reliability of TRV-based VIGS experiments in tomato is influenced by the size of the vector control. Mol. Plant Pathol. 12: 299-305


Figure 1. Jasmonate synthesis and perception in plants. Mutations in tomato that block JA synthesis or perception are represented in bold. Abbreviations are as follows: AOC= Allene oxide cyclase; AOS= Allene oxide synthase; COI1= Coronatine insensitive 1; EOT= Epoxy-9,11,15-octadecatrienoic acid; FAD= Fatty acid desaturase; JA= jasmonic 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; and OPDA= 2-oxo-phytodienoic acid; OPR= 12-oxo-phytodienoic acid reductase. Closed arrows represent biosynthetic steps, whereas the open arrow represents recognition of JA-Ile by the COI1/JAZ co-receptor (Sheard et al., 2010). This figure was modified from Schaller (Schaller, 2001).
Figure 2. Aphid infestations are reduced on spr2 but are unaffected by methyl jasmonate treatment or by other mutations that impair JA signaling. A-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 five days after inoculation and analyzed by One-Way ANOVA. Mean separations were performed using Student's t-tests. Values (±SEM) labeled with different letters differ significantly at α=0.05. C. WT and spr2 plants were treated with methyl jasmonate (MeJA, 75 μM), and inoculated with aphids 24 h after treatment (5 aphids/ cage; 3 clip cages/ plant; 10 plants/ treatment group). Live offspring were counted six days after inoculation, and the average numbers of offspring per cage per plant were analyzed by Two-Way ANOVA. **Denotes a significant main effect of genotype at P<0.0001. D. Expression of jasmonic acid-responsive PROTEINASE INHIBITOR II gene (PI-II) was monitored by RT-PCR 24 h after MeJA treatment. Expression of constitutive RIBOSOMAL PROTEIN L2 (RPL2) gene is presented as a loading control. (N=10 for A, N=8 for B, and N=10 for C).
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 (5 aphids/cage; 3 cages/plant; 12 plants/genotype), and the total number of aphids was recorded after 6 days. Cultivar L402 was used as the untransformed WT control. B. Adult green peach aphids were confined on individual Arabidopsis plants using sleeve cages (2 aphids/plant; 18 plants/genotype), and the total number of aphids per plant was recorded after 7 days. Aphid numbers were analyzed by One-Way ANOVA, and mean separations were performed using Student’s t-tests. Values (±SEM) labeled with different letters differ significantly at α=0.05.
Figure 4. Loss of function of FAD7 reduces aphid host acceptance. Wingless adult aphids (10 adults/arena) were placed on choice arenas (A) between paired 6-week-old plants of spr2 and the 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. 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 in the arenas. Marked pair-wise comparisons denote significant differences according to paired t-tests at $\alpha=0.05$ (*) or $\alpha=0.001$ (**). Error bars indicate ±SEM (N=10 pairs).
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C-D. WT (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; 5 plants/genotype/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-tests. Bars of the same color (±SEM) that have different lowercase letters are significantly different at α=0.05. Bars with different capital letters show significant differences in total SA content (free + bound).
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Figure 8. *NPR1* is upregulated by aphid feeding and contributes to aphid resistance in the *spr2* mutant. A. Expression of the *NPR1* gene was measured 48 hrs after inoculation in *spr2* and WT (cv. Castlemart) plants infested with aphids by RT-qPCR using *RPL2* as reference gene. B-D. Virus induced gene silencing (VIGS) using the Tobacco rattle virus (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 reference gene (B). Plants were challenged with the potato aphid (4 aphids/cage; 4 cages/plant; 8 plants/treatment group), and the total number of live adults and offspring was recorded 6-days after inoculation (C). Local total, free, and bound SA was measured one day after aphid count (D). Values were analyzed by ANOVA, and mean separations were performed using Student’s t-tests. Bars (±SEM) having different letters are statistically different at $\alpha=0.05$. Capital letters above bars in panel C denote significant differences in total (free + bound) SA content. (N= 7, 8, 6, and 8 respectively).