Fatty Acid Desaturases in the Chloroplast and Endoplasmic Reticulum Promote Susceptibility to the Green Peach Aphid *Myzus persicae* in *Arabidopsis thaliana*

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Fatty acid desaturases (FADs) in plants influence levels of susceptibility to multiple stresses, including insect infestations. In this study, populations of the green peach aphid (*Myzus persicae*) on *Arabidopsis thaliana* were reduced by mutations in three desaturases: *AtFAB2/SSI2*, which encodes a chloroplastic stearyl-[acyl-carrier-protein] 9-desaturase, and *AtFAD7* or *AtFAD3*, which encode ω -3 FADs in the chloroplast and endoplasmic reticulum (ER), respectively. These data indicate that certain FADs promote susceptibility to aphids and that aphids are impacted by desaturases in both the chloroplast and ER. Aphid resistance in *ssi2*, *fad3*, and *fad7*, singly or in combination, might involve altered signaling between these subcellular compartments. C18:1 levels are depleted in *ssi2*, whereas C18:2 accumulation is enhanced in *fad3* and *fad7*. In contrast, *fad8* has higher than normal C18:2 levels but also high C18:1 and low C18:0 and does not impact aphid numbers. Potentially, aphids may be influenced by the balance of multiple fatty acids (FAs) rather than by a single species, with C18:2 promoting aphid resistance and C18:1 promoting susceptibility. Although the *fad7* mutant also accumulates higher-than-normal levels of C16:2, this FA does not contribute to aphid resistance because a triple

mutant line that lacks detectable levels of C16:2 (*fad2fad6fad7*) retains comparable levels of aphid resistance as *fad7*. In addition, aphid numbers are unaffected by the *fad5* mutation that inhibits C16:1 synthesis. Together, these results demonstrate that certain FADs are important susceptibility factors in plant-aphid interactions and that aphid resistance is more strongly associated with differences in C18 abundance than C16 abundance.

Keywords: FAD2, FAD3, FAD6, FAD7, SSI2, FAB2, fatty acid desaturase, GLABRA1, green peach aphid, stearyl-acyl carrier protein desaturase

Aphids, a large group of piercing-sucking insects specialized to feed on phloem sap, are among the world's most common and damaging insect pests. There are more than 4,300 aphid species, and nearly all field crops, horticultural plants, and forest trees are impacted by one or more species (Blackman and Eastop 1994, 2000). Aphid attack can cause significant direct damage to their hosts, including chlorosis, wilting, and stunted growth (Goggin et al. 2017). Furthermore, aphids can also cause extensive indirect damage by transmitting viruses and promoting the growth of sooty mold (Minks and Harrewijn 1989; Stevens and Lacomme 2017). One of the most cosmopolitan aphid pests, the green peach aphid (*Myzus persicae*), has a worldwide distribution and feeds on more than 50 plant families, including a diversity of crops, such as potato, tobacco, sugarbeet, cabbage, peach, and spinach (Baker 1994; Blackman and Eastop 2000). This damaging species can transmit over 100 plant viruses, including potato leaf roll virus, cauliflower mosaic virus, turnip mosaic virus, pea leaf roll virus, radish yellows virus, and cucumber mosaic virus (Kennedy et al. 1962; Kuhar et al. 2009). Further complicating their management, the green peach aphid and certain other aphids have also developed resistance to multiple insecticides (Bass et al. 2014; Foster et al. 2017). The interaction of the green peach aphid with the model plant *Arabidopsis thaliana* represents a useful experimental system in which to explore plant-aphid interactions (Louis and Shah 2013).

Because of the importance of aphids as crop pests and the prevalence of pesticide resistance among these pests, host plant resistance is a particularly important component of aphid management (Van Emden 2017). However, our ability to select for aphid resistance in crops is impeded by our limited understanding of the host factors that influence aphids. These so-called “stealthy” herbivores can evade many of the plant defenses normally triggered by insect damage, although they induce a variety of reactions that overlap with plant defenses against pathogens (Kaloshian and Walling 2005; Walling 2008). In addition to constitutive and

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induced defenses, the success of invaders such as pathogens or aphids is also influenced by so-called susceptibility factors or host traits that can promote the infestation process (Eckardt 2002; Li et al. 2020; van Schie and Takken 2014). Because of their key role in modulating immunity, susceptibility factors are common targets for genome editing and other approaches to engineer enhanced disease resistance in crops (Zaidi et al. 2018).

Many susceptibility factors are negative regulators of plant immunity that most likely function to manage the balance between growth and defense in the plant. Without proper regulation by other genes, receptors for pathogen detection or immune signaling and defense genes often cause excessive cell death and stunted growth in plants (Ning et al. 2017). In the absence of infection, negative regulators limit these costs to plant growth by suppressing expression of immune genes or mediating degradation of their protein products; however, in the presence of a virulent pathogen that is not effectively recognized by the host plant, these negative regulators can also function as susceptibility factors, impeding appropriate immune responses (Li et al. 2020; van Schie and Takken 2014). For example, NRL1 in potato suppresses cell death by promoting proteolysis of a positive regulator of immunity (SWAP70), and it promotes infection by virulent strains of the late blight pathogen *Phytophthora infestans*

that express the Pi02860 effector protein, because Pi02860 causes NRL1 to degrade SWAP70 even in the presence of the pathogen (He et al. 2018). Another example of a susceptibility factor is the SS12 fatty acid desaturase (FAD) in rice that promotes infection by the causal agents of leaf blight and blast (Jiang et al. 2009; Li et al. 2020). Interestingly, loss of function of SS12 in *Arabidopsis* reduces susceptibility to the green peach aphid *Myzus persicae* (Louis et al. 2010a; Pegadaraju et al. 2007), and FATTY ACID DESATURASE 7 (FAD7) also promotes susceptibility to aphids in *Arabidopsis* and tomato (Avila et al. 2012). Thus, FADs may represent important susceptibility factors for aphid infestation and further research is warranted to understand their impacts on plant-aphid interactions.

Fatty acid desaturases (FADs) located in the plastids or the endoplasmic reticulum (ER) introduce double bonds into the acyl chain of fatty acids (FA), regulating the level of desaturation of FAs incorporated into membrane- or storage lipids (Fig. 1). The majority of plant FAs have acyl chains of 18 or 16 carbons (C18 or C16); they are synthesized in plastids and either incorporated into galactoglycerolipids or exported to the ER for phospholipid synthesis. FA desaturation is initiated in the chloroplast by FAD5, which acts on C16:0, or by S-ACP-DES7 (stearoyl-[acyl-carrier-protein] 9-desaturase 7), which

Fig. 1. Fatty acid metabolism in the chloroplast and endoplasmic reticulum (ER). Mutations that inhibit FA desaturation are represented in red. C18:0 bound to an acyl carrier protein (18:0 ACP) is desaturated in the chloroplast stroma by a stearoyl-acyl carrier protein desaturase encoded by FAB2/SSI2. All other desaturases shown here are membrane-associated proteins. Esterified C16:0, C16:1, and C16:2 are desaturated exclusively in the chloroplast, whereas esterified C18:1 and C18:2 can be desaturated in the chloroplast or ER. Extensive metabolic interconnections exist between these two subcellular compartments. FAs synthesized in the chloroplast are either incorporated into galactolipids via the prokaryotic pathway (green arrows) or are exported in the form of free FAs to the ER, in which they are used for phospholipid synthesis. Phospholipids synthesized in the ER are also a source of diacylglycerol for production of chloroplast galactolipids via the eukaryotic pathway (blue arrow). It is currently uncertain which phospholipid or phospholipid derivative is transported from the ER to the chloroplast for this purpose (yellow arrow). Lipids can also be exchanged from the ER to the chloroplast via the trigalactosyldiacylglycerol transport complex (solid purple arrows) or from the chloroplast to the ER via as-yet-uncharacterized mechanisms (dashed purple arrows). C = cytosol, IM = inner membrane of chloroplast, OM = outer membrane of chloroplast. This figure was created with BioRender and represents a synthesis of models presented by Karki et al. (2019), Mehrshahi et al. (2014), and Somerville and Browse (1996).

acts on C18:0 and is encoded by the *FATTY ACID BIOSYNTHESIS 2* gene (*FAB2*, also known as *SSI2*) (Kachroo et al. 2005; Kunst et al. 1989). The resulting monoenoic FAs can be further desaturated by chloroplast-localized FAD6, which acts on both C18:1 and C16:1, or by ER-localized FAD2, which desaturates C18:1 (Browse et al. 1989; Lemieux et al. 1990). In addition, there are three ω -3 FADs that convert dienoic FAs to trienoic FAs (Arondel et al. 1992; Gibson et al. 1994): FAD3, which is localized in the ER and synthesizes C18:3, and FAD7 and FAD8, which are located in the chloroplast and generate both C18:3 and C16:3 (Browse et al. 1986, 1993; Dyer and Mullen 2001; Froehlich et al. 2003; McConn et al. 1994). Trienoic FAs are the most abundant polyunsaturated FA species in plant membrane lipids and make up about 70 to 80% of the FAs in the chloroplast membrane lipids (Harwood 1980; Yaeno et al. 2004).

FADs can modulate levels of plant defenses to many stresses, including pathogens and insects (Upchurch 2008). Polyunsaturated FAs generated by FADs are precursors for multiple oxylipins that contribute to plant defense. C18:3 is required for synthesis of 12-oxo-phytodienoic acid, jasmonic acid, and other jasmonates, and so ω -3 FADs are critical to jasmonate-dependent defenses against many pests; as a result, the *fad3fad7fad8* triple mutant in *Arabidopsis* is highly susceptible to fungus gnat larvae as well as oomycete infection (McConn et al. 1997; Vijayan et al. 1998). In addition, dienoic and trienoic FAs give rise to a multitude of other oxylipins via lipoxygenases, hydroperoxide lyase, and other enzymatic and nonenzymatic pathways, and many of these oxylipins function as phytoalexins or defense signals (Blée 2002). For example, green leaf volatiles generated by the hydroperoxide lyase pathway attract natural enemies, prime defense signaling in neighboring foliage, and can be directly toxic to insects and pathogens (Scala et al. 2013). In addition to providing substrates for oxylipin synthesis, FADs also have indirect effects on defense signaling; for example, suppression of the *Arabidopsis* *FAD7* and *FAD8* genes reduces the accumulation of reactive oxygen intermediates and decreases resistance to bacterial pathogen *Pseudomonas syringae* (Yaeno et al. 2004).

While FADs contribute to plant defenses against certain pests, they appear to increase susceptibility to other stressors. Suppression of *OsFAD7* and *OsFAD8* expression in rice confers resistance to the rice blast fungus *Magnaporthe grisea* (Yara et al. 2007, 2008), and loss of function of *FAD7* in tomato and *Arabidopsis* also confers aphid resistance (Avila et al. 2012). Settling, survival, and fecundity of the potato aphid (*Macrosiphum euphorbiae*) is significantly lower on tomato mutants with impaired *FAD7* function compared with wild-type (WT) controls (Avila et al. 2012). Furthermore, the *ssi2* mutation in *FAB2*, which impairs function of S-ACP-DES7, decreases population growth of the green peach aphid on *Arabidopsis* (Pegadaraju et al. 2007). Thus, FADs may act as susceptibility factors in certain biotic interactions, including aphid infestation. The goals of this study were to characterize the effects of the major FADs in *Arabidopsis* on susceptibility to the green peach aphid and to correlate these effects with foliar FA profiles.

RESULTS

Influence of *FATTY ACID BIOSYNTHESIS 2* (*FAB2*) and *FAD5* on aphid resistance and FA profiles.

To assess the effects of desaturases that synthesize monoenoic FAs on aphids, we assayed FA profiles and aphid population growth on the *Arabidopsis* *ssi2* and *fad5* mutants, which inhibit the synthesis of C18:1 and C16:1, respectively. The *ssi2* mutation in the *FAB2* gene causes a 10–20 fold decrease in the $\Delta 9$ desaturase activity of S-ACP-DES7, which converts C18:0 to C18:1 in the chloroplast stroma (Fig. 1) (Kachroo et al. 2001). The *ssi2* mutant

was stunted (Supplementary Fig. S4) and supported significantly fewer aphids than the WT background for this mutant, the Nossen ecotype (Fig. 2). The lower aphid numbers on *ssi2* appeared to be due to reduced adult fecundity rather than to increased mortality, because dead aphids were absent in most cages. The reduced aphid fecundity observed on *ssi2* is consistent with previous reports that this mutant has antibiotic effects on the green peach aphid (Louis et al. 2010a and b; Pegadaraju et al. 2005). Compared with Nossen, *ssi2* had more than 16-fold higher levels of C18:0 accumulation and significantly lower levels of C18:1, C18:2, and C18:3 but did not promote C16:0 accumulation or deplete C16:1 levels (Fig. 3). This is consistent with a previous report that both the mutant and WT isomers of S-ACP-DES7 have a very strong substrate preference (88:1) for C18:0 rather than C16:0 (Kachroo et al. 2001). The stunted growth of *ssi2* (Supplementary Fig. S4) could potentially reduce host suitability for aphids; however, prior studies have shown that petiole exudates from *ssi2* inhibit aphid growth on artificial diets, indicating that aphid resistance in *ssi2* is due, at least in part, to altered plant chemistry rather than to altered plant morphology (Louis et al. 2010a). In contrast to *ssi2*, the *fad5* mutation did not significantly impact aphid population growth (Fig. 2). This mutation impairs a chloroplastic palmitoyl-monogalactosyldiacylglycerol $\Delta 7$ -desaturase that converts C16:0 to C16:1 (Fig. 1) (Heilmann et al. 2004). Compared with WT plants, the *fad5* mutant had similar C18 FA levels, significantly higher C16:0 levels, and significantly lower levels of C16:3 accumulation (Fig. 3). The differences in aphid susceptibility and FA profiles between *ssi2* and *fad5* suggest that C18 FAs have more influence on aphid numbers than C16 FAs.

Influence of *FAD2* and *FAD6* on aphid resistance and FA profiles.

To assess the effects of desaturases that synthesize dienoic FAs on aphids, aphid population growth and foliar FA profiles were also measured in mutants with impairments in Δ -12 FADs in the ER (*fad2*) or chloroplast (*fad6*). Aphid population growth

Fig. 2. Effects of desaturases that synthesize monoenoic FAs on aphid numbers. Plants were inoculated with three adults per plant, and the total number of live aphids was counted 7 days after inoculation on *ssi2*, *fad5*, and their respective wild-type controls (Nossen and Col-0, respectively) ($n = 15$). The data for each comparison was analyzed by Student's *t* tests in JMP (SAS Institute). An asterisk (*) indicates statistically significant differences at $\alpha = 0.05$; ns indicates no statistically significant differences at $\alpha = 0.05$. In the box plot, the bottom and top boundaries of the box indicate the 25th and 75th percentiles and a line within the box marks the median. Whiskers above and below the box indicate the 10th and 90th percentiles.

was not significantly influenced by the *fad2* mutation, which decreases C18:2 synthesis in the ER (Fig. 4). Compared with WT Col-0 controls, this mutant had approximately 51% lower C18:2 levels than WT plants, 10-fold higher C18:1 levels, and 19% lower C18:3 levels (Fig. 5). C16 levels were unaffected by *fad2*, consistent with the fact that C16 FAs are not desaturated in the ER. The *fad6* mutation also did not appear to have a strong impact on aphid numbers, because in assays in which the *fad6* mutant showed normal growth and development, aphid numbers were comparable on *fad6* and Col-0 (Fig. 4; Supplementary Fig. S7). Interestingly though, the *fad6* mutant had much more variable growth than any other genotype in this study. In certain assays, *fad6* formed smaller rosettes than the WT control Col-0 (Supplementary Fig. S4), and, in these assays, aphid numbers were lower on the mutant than on Col-0 (Supplementary Fig. S7). No significant differences in FA profiles were observed between small *fad6* plants and *fad6* plants that were comparable in size to Col-0 (Supplementary Fig. S5). This suggests that the reduced aphid numbers on small *fad6* plants might be due to reduced plant health or surface area for colonization rather

than to differences in FA profiles. Regardless of size, *fad6* plants accumulated significantly more C18:1 and significantly less C18:3 and C16:3 than WT controls (Supplementary Fig. S5), which is consistent with the ability of the chloroplast-localized FAD6 enzyme to act on both C16 and C18 substrates (Browse et al. 1989). C18:2 levels in *fad6* were comparable to levels in WT plants and significantly higher than levels in *fad2* plants, indicating that FAD2 has a stronger impact on C18:2 accumulation than FAD6; however, neither mutation completely blocked dienoic FA accumulation (Fig. 5). These results indicate that partial loss of Δ -12 FAD activity in the ER or chloroplast does not dramatically impact aphid population growth.

Influence of FAD7 and Glabra1 on aphid resistance.

To confirm the effects of an ω -3 FAD involved in trienoic FA synthesis on aphids, we also assessed whether aphids are impacted by the *fad7-1* mutation, which impairs FAD7 function in the chloroplast. This mutation was generated in a Col-0 accession that also carried an additional mutation (*gll*) at the *GLABRA1* locus, and Xia et al. (2010) reported that defects in systemic acquired resistance (SAR) that had previously been attributed to *fad7* were, in fact, due to impairment of cuticle development by *gll*. To discriminate between the potential effects of *gll* versus *fad7* on aphids, aphid numbers on the original *fad7-1* mutant line (referred to here as *fad7gll*) were compared with numbers on *gll* as well as to the WT genetic background Col-0. In addition, by crossing *fad7gll* with Col-0, selfing the F1 generation, and screening subsequent

Fig. 3. Effects of *ssi2* and *fad5* on fatty acid (FA) profiles. Total FAs were extracted from foliage and were methylated, and fatty acid methyl esters were analyzed by gas chromatography-mass spectrometry. The data for each comparison was analyzed by Student's *t* test in JMP (SAS Institute) ($n = 3$). Asterisks (*) indicate statistically significant differences at $\alpha = 0.05$; ns indicates no statistically significant differences at $\alpha = 0.05$. In the box plot, the bottom and top boundaries of the box indicate the 25th and 75th percentiles and a line within the box marks the median.

Fig. 4. Influence of Δ -12 fatty acid desaturases (FADs) in the endoplasmic reticulum (*fad2*) or chloroplast (*fad6*) on aphid numbers. Plants were inoculated with three adults per plant, and the total number of live aphids was counted 7 days after inoculation on *fad2*, *fad6*, and their wild-type control (Col-0) ($n = 15$). The *P* value reported is from a one-way analysis of variance (ANOVA) to determine if any differences exist among genotypes. Boxes having the same letter are not significantly different at $\alpha = 0.05$, according to one-way ANOVA. In the box plot, the bottom and top boundaries of the box indicate the 25th and 75th percentiles and a line within the box marks the median. Whiskers above and below the box indicate the 10th and 90th percentiles.

generations for both mutations, we also generated a line (referred to here as *fad7*) that lacks the *gll* mutation and is homozygous for the *fad7-1* mutation (Vaughn et al. 2014). Aphid numbers on *fad7gll* and *fad7* were significantly lower than numbers on their respective controls *gll* and Col-0 (Fig. 6A), confirming prior findings that loss of function of *FAD7* confers aphid resistance (Avila et al. 2012). Furthermore, there were no significant differences in population growth between *fad7gll* and *fad7* or between *gll* and Col-0 (Fig. 6A). The comparable aphid numbers observed on *gll* and Col-0 are also consistent with recent findings that the *gll* mutation does not strongly influence the incidence of naturally occurring aphid infestations on field-grown *Arabidopsis* (Sato et al. 2019). Thus, although GLABRA1-dependent cuticle formation may regulate SAR against bacteria (Xia et al. 2010), it does not impact local defenses against aphids in WT *Arabidopsis* or *fad7* mutants. These data show that the *fad7gll* and *fad7* mutants

are equally resistant to green peach aphids and that the *gll* mutation did not significantly influence aphid numbers or contribute to aphid resistance in *fad7gll*. Loss of function of *FAD7* in *Arabidopsis* appeared to affect population growth primarily by affecting adult fecundity rather than aphid survival; dead adults or juveniles were absent in most cages.

Influence of other ω -3 FADs in the chloroplast and ER on aphid resistance.

Because loss of function of *FAD7* results in aphid resistance, assays were conducted to determine whether other ω -3 FADs that synthesize trienoic FAs in the chloroplast (*FAD8*) or the ER (*FAD3*) impacted aphids and to quantify their impacts on FA profiles. Aphid numbers on the *fad3* mutant were significantly lower than numbers on Col-0 and were comparable to numbers on both genotypes that carried the *fad7* mutation (Fig. 6B). Similar to *fad7*, *fad3* appeared to reduce offspring production but not increase mortality; only 15% of replicates had any mortality, and even these plants had only one or two dead aphids. These results demonstrate that loss of function of the ER-localized *FAD3* decreases aphid fecundity and confers a comparable level of aphid resistance as loss of function of the chloroplast-localized *FAD7*. Whereas *fad7* influenced the accumulation of both C16 and C18 FAs, *fad3* affected only the C18 profile (Fig. 7). In fact, the only statistically significant difference in FA profiles between *fad3* and the WT control Col-0 was an increase in C18:2 in *fad3* (Fig. 7G). This data supports the hypothesis that

Fig. 5. Influence of *fad2* and *fad6* on fatty acid (FA) profiles. Total FAs were extracted from foliage and were methylated, and fatty acid methyl esters were analyzed by gas chromatography-mass spectrometry. The *P* values reported are from one-way analyses of variance (ANOVA) to determine if any differences exist among treatments. Bars having the same letter are not significantly different at $\alpha = 0.05$, according to **A, B, C, and E**, one-way ANOVA or **D, F, G, and H**, the Tukey-Kramer honest significant difference test ($n = 3$). In the box plot, the bottom and top boundaries of the box indicate the 25th and 75th percentiles and a line within the box marks the median.

Fig. 6. Influence of the *gll* mutation and ω -3 fatty acid desaturases (FADs) in the endoplasmic reticulum (*fad3*) or chloroplast (*fad7*, *fad8*) and on aphid numbers. Plants were inoculated with three adults per plant, and the total number of live aphids was counted 7 days after inoculation. The *P* values reported are from one-way analyses of variance to determine if any differences exist among treatments. Boxes having the same letter are not significantly different at $\alpha = 0.05$, according to the Tukey-Kramer honest significant difference test ($n = 20$). In the box plot, the bottom and top boundaries of the box indicate the 25th and 75th percentiles and a line within the box marks the median. Whiskers above and below the box indicate the 10th and 90th percentiles.

Fig. 7. Influence of ω -3 fatty acid desaturases on fatty acid (FA) profiles. Total FAs were extracted from foliage and were methylated, and fatty acid methyl esters were analyzed by gas chromatography-mass spectrometry. The P values reported are from one-way analyses of variance (ANOVA) to determine if any differences exist among treatments. Bars having the same letter are not significantly different at $\alpha = 0.05$, according to one-way ANOVA (C16:1 panel) and the Tukey-Kramer honest significant difference test (all other panels) ($n = 4$). In the box plot, the bottom and top boundaries of the box indicate the 25th and 75th percentiles and a line within the box marks the median. Whiskers above and below the box indicate the 10th and 90th percentiles.

aphid population growth is influenced by C18 rather than C16 FAs. It also suggests that C18:2 may play a role in aphid resistance in *fad3* and that resistance does not require significant depletion of trienoic FAs.

Compared with WT plants (Col-0), the *fad8* mutation did not significantly impact aphids, whereas aphid numbers on both genotypes carrying the *fad7* mutation were significantly lower than numbers on Col-0 (Fig. 6B). Although the *fad7* and *fad8* mutants differed in their effects on aphids, their FA profiles were similar; the primary differences between these two mutants was that *fad8* had significantly lower levels of C18:0 than *fad7* as well as significantly higher levels of C16:2 and C18:2 (Fig. 7C, G, and E). C18:1 levels were also more than 60% higher in *fad8* than in *fad7*, but whether this difference was statistically significant varied depending upon whether the data were analyzed with a Student's *t* test ($P = 0.0378$) or the more conservative Tukey's ($P = 0.2055$). The differences in aphid numbers between *fad7* and *fad8* suggest that aphid resistance in *fad7* may be influenced by the abundance of more than one FA rather than by a single FA species.

Aphid populations on the *fad3fad7fad8* triple mutant were significantly smaller than populations on Col-0 and were comparable to levels on the *fad3* and *fad7gl1* lines, indicating that the resistance associated with the *fad3* and *fad7* mutations do not have additive effects on aphid populations (Fig. 6B). However, the *fad3fad7fad8* triple mutant had significantly higher levels of C16:2 and C18:2 and significantly lower C18:3 than all other genotypes, demonstrating that the individual mutations had an additive effect on FA profiles (Fig. 7). Compared with *fad7*, the triple mutant also had significantly lower C18:0 and significantly higher C18:1 (Fig. 7). Together, these results indicated that aphids are influenced by ω -3 FADs in both the chloroplast and ER and may be affected by more than one C18 FA.

Aphid numbers and FA profiles in double and triple mutants with impairments in ω -3 FAD and Δ -12 FAD activity.

To assess whether dienoic FA accumulation is required for aphid resistance in the *fad7* mutant, a *fad2fad7* mutant was

developed through genetic crosses and CRISPR-Cas9 was employed to mutagenize *FAD6* in a *fad2fad7* genetic background, generating a *fad2fad6fad7* mutant. The triple mutant line selected for this study had lost *Cas9* by segregation and carried a 38-bp deletion in *FAD6* in the same region as the *fad6-1* mutation (Supplementary Fig. S2), at the putative active site of the FAD6 enzyme (Browse et al. 1989). Aphid numbers on *fad2fad7* and *fad2fad6fad7* were comparable to numbers on *fad7* and were significantly lower than numbers on Col-0 (Fig. 8), indicating that *fad7*-mediated aphid resistance was intact in the double and triple mutants. C16:2 and C16:3 were undetectable in *fad2fad6fad7*, demonstrating that the mutation introduced into *FAD6* blocked the function of the FAD6 enzyme (Supplementary Fig. S6). C18:1 levels in the triple mutant were comparable to levels observed in the *fad6* mutant and significantly higher than all other genotypes. The ratio of C18:2 to C18:1 in *fad2fad6fad7* was also significantly lower than in *fad7* or WT plants and was comparable to levels observed in *fad2* and *fad6* single mutants, while the ratio of C18:2 to C18:1 in the *fad2fad7* mutant was intermediate between *fad7* and *fad2* (Supplementary Fig. S6). These observations reveal that the *fad2* mutant and CRISPR Cas9 mutagenesis of FAD6 had additive inhibitory effects on the conversion of C18:1 to C18:2. Despite differences in the ratio of C18:2 to C18:1, however, total C18:2 abundance in the triple mutant remained elevated relative to the WT control Col-0 (Supplementary Fig. S6). These data suggest that the *fad2* mutation does not fully block FAD2 function and indicate that the impairments in Δ -12 desaturase activity in *fad2fad6fad7* were not sufficient to counteract the strong effects of *fad7* on C18:2 accumulation. Given that *fad2fad6fad7* was resistant to aphids but lacked detectable levels of C16:2, these results also indicate that the heightened C16:2 accumulation observed in *fad7* is not required for aphid resistance.

DISCUSSION

Population growth of a generalist herbivore, the green peach aphid, are reduced on *Arabidopsis* lines with impairments in *FAD7*, *FAD3*, and *SSI2*. This indicates that certain FADs promote

Fig. 8. Combined influence of an ω -3 fatty acid desaturase (FAD) (*fad7*) and Δ -12 FADs (*fad2* and *fad6*) on aphid numbers. Aphid population growth on *Arabidopsis* lines impaired in conversion of dienoic to trienoic fatty acids. The *P* value reported is from a one-way analysis of variance to determine if any differences exist among treatments. Boxes having the same letter are not significantly different at $\alpha = 0.05$, according to the Tukey-Kramer honest significant difference test ($n = 20$). In the box plot, the bottom and top boundaries of the box indicate the 25th and 75th percentiles and a line within the box marks the median. Whiskers above and below the box indicate the 10th and 90th percentiles.

susceptibility to aphids. Several lines of evidence suggest that the effects of these desaturases on aphids are mediated through desaturation of C18 rather than C16 FAs. Although the *fad7* mutant accumulates higher-than-normal levels of C16:2 as well as C18:2, a triple mutant line that lacks detectable levels of C16:2 (*fad2fad6fad7*) nonetheless retains levels of aphid resistance comparable to those of *fad7*. Aphid numbers are also unaffected by the *fad5* mutation that inhibits C16:1 synthesis; in contrast, aphid population growth is diminished by the *ssi2* mutation, which inhibits C18:1 synthesis. Furthermore, the *fad3* mutant had levels of resistance comparable to those of the *fad7* mutant even though *fad3* only impacts C18 levels. Thus, aphid resistance is more strongly associated with differences in C18 abundance than C16 abundance.

A comparison of the multiple desaturase mutants examined in this study suggests that aphids are impacted by more than one C18 FA (a summary of all mutants is provided in Supplementary Fig. S8). A previous study on *ssi2* hypothesized that aphid resistance in this mutant is due to decreased levels of C18:1 (Louis et al. 2010a). Except for *ssi2*, all other genotypes that displayed aphid resistance in the current study had higher than normal levels of C18:2; in fact, this was the only FA that differed significantly between *fad3* and WT controls. Therefore, C18:2 may potentially contribute to aphid resistance in certain genotypes. However, elevated C18:2 levels alone were not sufficient to confer resistance to *fad8*. Notably, this mutant had nearly twice as much C18:1 and half as much C18:0 than WT plants; possibly the effects of heightened C18:2 accumulation in this genotype were countered by elevated C18:1 levels or other changes in the FA profile. Together, these data suggest that aphids are impacted by more than one C18 FA and that C18:2 may promote aphid resistance whereas C18:1 promotes susceptibility.

The observation that the *fad3* mutation reduces aphid numbers also demonstrates that aphids are impacted by desaturases in the ER as well as by the chloroplastic desaturases disrupted by the *ssi2* and *fad7* mutations. This may be due to the extensive metabolic exchange between the ER and chloroplast. The ER forms numerous points of physical contact with the outer membranes of plastids, and these membrane contact sites are thought to facilitate nonvesicular lipid transfer (Michaud and Jouhet 2019; Wang and Benning 2012). Hemifusion between the membranes of the ER and chloroplast could, theoretically, also enable enzymes from one organelle to act on substrates in another organelle (Mehrshahi et al. 2014). While the mechanisms of interorganelle exchange are not fully understood, it is well-established that the pathways for lipid biosynthesis in the chloroplast and the ER are intertwined (Fig. 1) (Karki et al. 2019; Mehrshahi et al. 2014; Somerville and Browse 1996). Chloroplast membranes are composed primarily of galactolipids, and the diacylglycerol required for their synthesis in the chloroplast is derived entirely (in C18 plants) or in part (in C16 plants) from phosphatidylcholine (PC) or PC-derived intermediates imported from the ER (Michaud and Jouhet 2019). This PC pool may be desaturated by FAD3 prior to export (Karki et al. 2019); thus, FAD3 activity in the ER may impact the FA pool in the chloroplast. Phospholipid synthesis in the ER also requires FAs synthesized in chloroplasts, including C18:1, desaturated by the *FAB2/SSI2* gene product (Michaud and Jouhet 2019). Moreover, chloroplast-localized ω -3 desaturases have been reported to contribute to C18:3 accumulation in lipids synthesized in the ER in olive and birch (Hernández et al. 2016; Martz et al. 2006) and may also partially compensate for null mutations in FAD3 in *Arabidopsis* (Browse et al. 1993). Thus, the influence of *FAB2/SSI2*, *FAD7*, and *FAD3* on plant-aphid interactions could be mediated through their impacts on lipid metabolism in the chloroplast, the ER, or both cellular compartments. Furthermore, an emerging body of evidence suggests that lipid signaling between

the ER and chloroplast is important to plant stress responses (Liu and Li 2019; Michaud and Jouhet 2019). ER-chloroplast membrane contact sites have been identified as a location for several lipid transfer proteins involved in SAR to pathogens, including AZELAIC ACID INDUCED 1 (*AZI1*), EARLY *ARABIDOPSIS* ALUMINUM INDUCED 1 (*EARL1*), and DEFECTIVE IN INDUCED RESISTANCE 1 (*DIR1*) (Cecchini et al. 2015). Moreover, the *ssi2* mutation, which constitutively activates multiple components of SAR, also constitutively activates the expression of a marker (*BIP3*) of the unfolded protein response, a stress-responsive intracellular signaling pathway initiated in the ER (Iwata et al. 2018). Potentially, altered signaling between the chloroplast and ER may contribute to aphid resistance in one or more of the *ssi2*, *fad3*, or *fad7* mutants.

The effects of FADs on aphids indeed appear to be due to their influence on defensive signaling rather than to direct effects of FAs on aphids. It is unlikely that decreased desaturase activity would decrease the nutritional quality of plants for aphids because phloem sap is reported to contain primarily unsaturated FAs (Madey et al. 2002). Instead, the *fad7* mutation likely promotes defense signaling and possibly also secondary metabolite production against aphids. Similar to *fad7* in *Arabidopsis*, the *spr2* mutation in *SIFAD7* in tomato confers strong resistance to the potato aphid *Macrosiphum euphorbiae* and this resistance requires salicylic acid (SA) signaling; resistance is diminished by the *NahG* transgene, which suppresses SA accumulation, and by transient silencing of *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1* (*NPR1*), a key regulator of many SA-dependent defenses (Avila et al. 2012). Aphid resistance in *spr2* is also diminished by suppressing expression of LIPOXYGENASE1 (*LOX1*), which can act on C18:2 or other FAs to generate multiple oxylipins (Avila et al. 2013). Potentially the altered FA content of *FAD7*-impaired plants may result in a shift in the oxylipin profile generated by *LOX1*, favoring compounds with activity against aphids. Similarly, resistance to rice blast in *OsFAD7*- and *OsFAD8*-silenced rice lines is associated with enhanced hydroperoxides and hydroxides derived from C18:2, indicating that C18:2-derived oxylipins may be important to resistance (Yara et al. 2007, 2008). Further work is needed to characterize the mechanisms of aphid resistance in plants with impaired *FAD7* activity, and to determine whether *FAD3* influences plant-aphid interactions through similar or divergent pathways.

Similar to loss of function of *FAD7*, the *ssi2* mutation also impacts aphids by modifying plant immunity. Although petiole exudates from *ssi2* inhibit aphid growth on artificial diets, this inhibitory effect is not due to direct effects of FAs because the addition of C18:0 or C18:1 to an artificial diet has no effect on aphid population growth (Louis et al. 2010a). Instead, aphid resistance in *ssi2* depends upon altered defense signaling, although it has different signaling requirements than *fad7*-mediated aphid resistance (Louis et al. 2010a and b; Pegadaraju et al. 2005). In contrast to aphid resistance in *FAD7*-impaired tomato, aphid resistance in *ssi2* is not impaired by the *NahG* transgene or a null mutation in *NPR1* (Pegadaraju et al. 2005). Aphid resistance in *ssi2* does, however, require an aphid-inducible lipase (*MPL1*) and *PHYTOALEXIN DEFICIENT4*, suggesting that this resistance is due to altered lipid signaling (Louis et al. 2010a and b). Furthermore, analysis of other *ssi2*-dependent phenotypes indicate that this mutation promotes defense signaling by releasing nitric oxide (NO) production from negative regulation by C18:1. The *ssi2* mutation causes increased constitutive SA accumulation and enhanced resistance to multiple pathogens, including the oomycete *Peronospora parasitica* and the bacterial pathogen *Pseudomonas syringae* (Kachroo et al. 2001; Shah et al. 2001). Second-site mutations that restore C18:1 accumulation in *ssi2* inhibit constitutive SA accumulation and restore WT levels of susceptibility to *Peronospora parasitica* (Kachroo et al. 2003,

2004). C18:1 acts as a negative regulator of NO signaling by binding to NITRIC OXIDE ASSOCIATED1 (NOA1) and promoting its degradation; conversely, C18:1 depletion in *ssi2* promotes NO production and consequent defense signaling (Mandal et al. 2012). Furthermore, a null mutation in *NOA1* compromises the enhanced resistance of the *ssi2* mutant to *Pseudomonas syringae* (Mandal et al. 2012). Based on these observations of pathogen resistance in *ssi2*, aphid resistance in this mutant may also depend upon altered defensive signaling mediated by C18:1 depletion. Notably, NO has been implicated in Russian wheat aphid resistance responses in wheat, and exogenous NO reduces aphid feeding on pea (Moloi and van der Westhuizen 2014; Woźniak et al. 2017). Therefore, further work is warranted to determine if NO signaling contributes the effects of desaturases on aphid numbers.

In summary, plant desaturases in both the chloroplast and ER promote susceptibility to aphids. FAD7 appears to predispose plants to aphid attack by inhibiting SA-dependent defenses, while the stearoyl-acyl carrier protein desaturase encoded by *FAB2/SSI2* may facilitate aphid population growth by suppressing NO signaling; in both cases, these effects are likely mediated through the influence of desaturases on C18 FA profiles and lipid signaling. The potential role of desaturases in stress-responsive signaling between the ER and chloroplast also merits further study. These results shed light on the influence of plant primary metabolism on plant immunity against aphids, an important group of insect pests whose mode of parasitism bears many similarities to plant pathogens (Kaloshian and Walling 2005).

MATERIALS AND METHODS

Plant materials.

Seeds for *Arabidopsis thaliana* lines carrying the *fad2-1*, *fad3-2*, *fad5-1*, and *fad6-1* mutations as well as the WT Columbia ecotype (Col-0, accession CS70000) were obtained from the *Arabidopsis* Biological Resource Center. Seeds for *gll* (*GLABRA1*), Nossen, and *ssi2* (*SUPPRESSOR OF SALICYLIC ACID INSENSITIVITY2*) were donated by J. Shah, University of North Texas, Denton, TX, U.S.A., who also provided seeds for the *fad7-1* mutant, which also carries the *gll* mutation (Xia et al. 2010); for clarity, *fad7-1* is referred to here as *fad7gll*. The triple mutant line *fad3fad7fad8* was obtained from J. Browse, Washington State University, Pullman, WA, U.S.A. A mutant line (referred to here as *fad7*) that carries only the *fad7-1* mutation without the *gll* mutation was developed by Vaughn et al. (2014). The *fad2fad7* mutant was developed by crossing *fad2-1* and *fad7-1gll* (described below). Seeds were surface-sterilized and plated on Murashige and Skoog germination medium (PhytoTechnology Laboratories, Shawnee Mission, KS, U.S.A.), then, were vernalized for 3 days at 4°C. After transfer to soil (a 4:3:2 mixture of peat, vermiculite, and perlite) supplemented with 15-9-12 Osmocote Plus fertilizer (Scotts-MiracleGro Company, Marysville, OH, U.S.A.), plants were maintained in a Conviron growth chamber (Controlled Environments Inc., Pembina, ND, U.S.A.) at 23°C, with a L13: D11 photoperiod and around 65% relative humidity. The plants were fertilized weekly with Miracle Gro all-purpose plant food (Scotts-MiracleGro Company) and were watered as needed with tap water.

Development of an *Arabidopsis fad2fad7* double mutant line.

The *Arabidopsis* double mutant *fad2fad7* was developed by crossing *fad7gll* (maternal parent) and *fad2* (pollen donor) and screening the F2 generation for plants homozygous for both mutations, using allele-specific PCR primers (Flattmann 2015). To select against the *gll* mutation, only progeny with trichomes were saved, because plants homozygous for *gll* lack trichomes (Oppenheimer et al. 1991). DNA was extracted using an extraction buffer

that was made by diluting Edwards solution (200 mM Tris-HCl [pH 7.5], 250 mM NaCl, 25 mM EDTA, and 0.5% sodium dodecyl sulfate) 10-fold with TE buffer (10 mM Tris-HCl [pH 8] and 1 mM EDTA) (Edwards et al. 1991; Kasajima et al. 2004). PCR primers were designed to distinguish between the WT *AtFAD7* allele and the *fad7-1* mutation reported by Xia et al. (2010) and between the WT *AtFAD2* allele and the *fad2-1* mutant sequence reported by Zhang et al. (2012) (Supplementary Fig. S1). The primers are as follows: forward primer for the WT *AtFAD7* allele, 5'-TTTCAGTGGGCTCGAAGTCC-3' and reverse primer, 5'-ATCTGCGGAAAAGATGATG-3'; forward primer for the *fad7-1* mutant allele, 5'-TTTCAGTGGGCTCGAAGACT-3' and reverse primer, 5'-ATCTGCGGAAAAGATGATG-3'; forward primer for the WT *Atfad2-1* allele, 5'-CCTAACTGGTATCTGGGTCA TAG-3' and reverse primer, 5'-AACGAGGGATGAGTGTGCTG-3'; forward primer for the *fad2-1* mutant allele, 5'-CCTAAC TGGTATCTGGGTACACAA-3' and reverse primer, 5'-AACGAGGGATGAGTGTGCTG-3'. Touchdown PCR was performed to increase amplification sensitivity and specificity. For the WT *AtFAD7* allele, initial denaturation = 95°C for 5 min; phase I = 95°C for 45 s, 65 to 58°C for 45 s (reducing 1°C per cycle), and 72°C for 45 s; phase II = 95°C for 45 s, 57°C for 45 s, and 72°C for 45 s (20 cycles); and a final extension at 72°C for 5 min. For the *Atfad7-1* mutation, the same program was used but with annealing temperatures of 65 to 56°C in phase I and 55°C in phase II. For the WT *AtFAD2* allele, the annealing temperatures were adjusted to 64 to 57°C in phase I and 56°C in phase II. For the *fad2-1* mutant allele, annealing temperatures of 60 to 53°C in phase I and 52°C in phase II were used. PCR products were visualized by agarose gel electrophoresis.

CRISPR-Cas9 mutagenesis of FAD6.

A CRISPR construct encoding four single guide RNA (sgRNA) to knock out *FAD6* in *Arabidopsis* was generated according to the protocols developed by Ma et al. (2015). Each sgRNA contained the *Arabidopsis* U6-29 promoter, a 20-bp target sequence from *AtFAD6*, a guide RNA sequence, and the Pol III terminator (Supplementary Fig. S2A). The four target sequences within *AtFAD6* were selected using the CRISPR-PLANT online web tool and were tested to minimize off target hybridization (CRISPR/Cas9 target online predictor) and optimize GC content (40 to 60%) and secondary structure formation (Zuker 2003). Double-stranded sgRNA adaptors were generated by combining 1.0 µM of forward primer with an AATG overhang on the 5' end and 1.0 µM reverse primer with an AAAC overhang in 0.5× TE solution, incubating in a thermal cycler at 90°C for 10 min, and returning to room temperature with a decrease of 0.2°C per second. To generate each of the sgRNA clones, double-stranded sgRNA adaptors (Supplementary Table S1) were ligated into the pYLSgRNA-AtU6-29 vector (Addgene plasmid 66203) at Bsa I restriction sites downstream of the *Arabidopsis* U6-29 promoter. Two PCR reactions were performed to amplify i) the U6-29 promoter and the sgRNA sequence, and ii) the sgRNA sequence and the Pol III terminator sequence. Overlap PCR was performed with primers containing BSA I and the four unique nucleotide sequences to assemble each of the four sgRNA cassettes. Finally, all four sgRNA cassettes were inserted together into the plant expression vector pYLCRISPR/Cas9Pubi-H by the Golden Gate method (Engler et al. 2008). The design of the construct containing all four sgRNAs is represented in Supplementary Figure S2B. The pYLCRISPR/Cas9Pubi-H vector (Addgene plasmid 66187) also carries Cas9 driven by a maize ubiquitin promoter and a hygromycin selection marker driven by the double cauliflower mosaic virus 35S promoter. The resulting final vector pYLCRISPR/Cas9Pubi-H-AtFAD6T₁T₂T₃T₄ was transformed into *Escherichia coli* and the plasmid was checked for the presence

of the insert by PCR and sequencing. The plasmid with the correct orientation of sgRNAs was transformed in *Agrobacterium tumefaciens* GV3101. This construct was introduced into the *Arabidopsis fad2fad7* double mutant by *Agrobacterium*-mediated transformation according to the methods of Bechtold and Bouchez (1995). To screen for transformants, surface-sterilized T0 seeds were plated on 1× Murashige and Skoog plates containing a final concentration of 25 µg/ml of hygromycin and hygromycin-resistant seedlings were selected according to Harrison et al. (2006). DNA from hygromycin-resistant plants was extracted with Edwards solution (Kasajima et al. 2004), and PCR was performed to confirm the presence of the *Cas9* gene. Lines positive for *Cas9* were then screened to identify mutations using two primer sets, one to amplify the region flanking targets 1 and 2 and the other to amplify a region flanking targets 3 and 4 (Supplementary Table S1). Deletions were identified by gel electrophoresis, and amplicons were eluted from the gel and were sequenced to confirm mutations. Lines that showed heterozygous deletions were propagated and screened in subsequent generations to select for lines that were homozygous for a deletion in *FAD6* and that had lost the *Cas9* gene by segregation. The line utilized for this study (*fad2fad6fad7*) was confirmed to carry a 38-bp deletion between targets 3 and 4 (Supplementary Fig. 2C and D), which includes the site mutagenized in the *fad6-1* line (Zhang et al. 2009).

Aphid bioassays.

The green peach aphid *Myzus persicae* was reared on cabbage (*Brassica oleracea* var. *Joychoi*) at about 24°C and with a 16-h light photoperiod. To produce cohorts of age-synchronized aphids for bioassays, cabbage plants were inoculated with wingless adult aphids that were allowed to larviposit on plants for 24 h. Adult aphids were removed after 24 h, and the age-synchronized nymphs were monitored daily to track development. Apterous adult aphids for bioassays were collected within 24 h of emergence to adulthood. *Arabidopsis* plants at developmental stage 5.1 (Boyce et al. 2001) were challenged with three apterous adults per plant, using sleeve cages that covered the entire plant (15 to 20 replicate plants per genotype). After infestation, plants were maintained in a Conviron growth chamber (23°C, 65% relative humidity, 13-h light and 11-h dark photoperiod), and the numbers of live and dead adults and offspring on each plant were scored 7 days after infestation.

FA profile analysis.

To assess the FA profiles of the mutant genotypes used in aphid bioassays, uninfested plants from each genotype were grown in empty sleeve cages and leaf tissue for FA analysis was collected from these plants 8 days after the beginning of the bioassay, when the plants were at developmental stage 6.0 (Boyce et al. 2001). Uninfested tissues were selected for analysis because preliminary measurements of FAs in WT plants (Col-0) indicated that the levels of aphid infestation observed in our bioassays did not significantly impact FA abundance in foliage (Supplementary Fig. S3). Leaf tissue was weighed and flash-frozen in liquid nitrogen (three to four replicates per genotype). Total FAs were extracted and methylated using an acid-catalyzed transmethylation protocol (Li-Beisson et al. 2013) combined with a purification method by Shipley et al. (1993). Fatty acid methyl esters (FAMES) were dried down under nitrogen flow and were dissolved into hexane, then, were transferred into inserts of gas chromatography (GC) autosampler vials (HP 6890; Agilent). One microliter of each sample was injected in the split mode with an autosampler (Agilent Technologies) for GC-mass spectrometry (MS) analysis. The GC-MS system used a FAMEWAX column (30 m × 0.25 mm with a 0.25 µm film thickness; Restek). The initial temperature of the GC oven was 130°C, which was

ramped to 225°C at a rate of 7°C/min, held for 12 min. FAMES were identified by comparing retention times and mass spectra to external standards (Nuchek mixture reference; Nu-Chek Prep, Inc.) and were quantified based on an internal standard (pentadecanoic acid, C15:0; Nu-Chek Prep, Inc.) that does not naturally occur in *Arabidopsis*. The abundance of each FA was expressed relative to the total moles of FA in each sample (i.e., in mol %); this compensates for any sample-to-sample differences in FA extraction efficiency.

Statistical analysis.

All the statistical analysis was done with JMP (SAS Institute Inc.). The data were analyzed by one- or two-way analysis of variance, and, when appropriate, means were separated using the Tukey-Kramer honest significant difference (for comparisons of multiple treatment groups) or Student's *t* test (for comparisons of paired genotypes).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Addgene: <https://www.addgene.org/66203> and <https://www.addgene.org/66187>
 CRISPR-PLANT online web tool: <https://www.genome.arizona.edu/crispr>
 CRISPR/Cas9 target online predictor: <https://cctop.cos.uni-heidelberg.de>

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