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Influence of Fatty Acids and their Derivatives on Aphid Resistance in Arabidopsis and Tomato

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Abstract

Fatty acid desaturases (FADs) are enzymes that act in the chloroplast or the endoplasmic reticulum (ER) to incorporate double bonds into the acyl chains of fatty acids, and recent evidence indicates that at least one of these enzymes, FAD7, also influences plant resistance to aphids. FAD7 is an enzyme in the chloroplast that is found throughout the plant kingdom and that desaturates 16- and 18-carbon fatty acids (FAs) with two double bonds (dienoic acids) to generate FAs with three double bonds (trienoic acids). In tomato (*Solanum lycopersicum*) and the model plant *Arabidopsis thaliana*, mutants with impaired FAD7 function are more resistant to aphids than wild-type controls. Compared to wild-type plants, these mutants have increased 18-carbon FAs with one double bond (C18:1), higher 16- and 18-carbon FAs with two double bonds (C16:2 and C18:2), and lower 16- and 18-carbon FAs with three double bonds (C16:3 and C18:3). These changes in FA composition are most pronounced in galactolipids which are abundant in the chloroplast, but loss of function of FAD7 also has a modest effect on phospholipids from the endoplasmic reticulum (ER). In addition, loss of function of FAD7 also influences foliar profiles of C6 volatiles, a class of FA derivatives that have previously been reported to contribute to aphid resistance in potato and other plant species. The goal of this project was to investigate which of these changes in fatty acid metabolism contribute to aphid resistance in Arabidopsis and tomato mutants with impaired FAD7 activity. To this end, we compared aphid performance on a panel of mutant plant lines with variation in FA composition or C6 volatile production. Our results suggested that aphid resistance in plants with impaired FAD7 activity is independent of C6 volatile

synthesis, because 1) the aphid-resistant *fad7* mutant in Arabidopsis also carries a mutation in the *hydroperoxide lyase* (*HPL*) gene, which is required for C6 volatile synthesis; and 2) suppressing expression of *HPL* in a FAD7-impaired tomato line did not compromise aphid resistance in this line. Analysis of aphid resistance and FA profiles in a panel of Arabidopsis mutants with impairments in different FADs also indicated that aphid resistance was impacted by FAD activity in the ER as well as in the chloroplast. This suggested that C18 rather than C16 FAs plays a determining role in aphid resistance, since C16 FAs are exclusively synthesized in the chloroplast whereas C18 FAs are produced in both subcellular compartments. Furthermore, resistance appeared to be associated with high C18:2 levels, because mutant lines with high C18:2 displayed resistance to aphids, but other lines with low C18:2 were susceptible even if they had high C18:1 and low C18:3 levels. Potentially, C18:2 or its derivatives could contribute to defensive signaling or synthesis of defensive metabolites to combat aphid infestations. This study aids in identifying new sources of plant resistance to aphids, and advances our understanding of how FA metabolism modulates plant defenses against insects.

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Chapter I

Introduction

Introduction

Aphids as crop pests. Aphids are a group of small sap-sucking insects, which are one of the most widespread pest groups on field crops, horticultural crops and forest trees. There are more than 4,300 species of aphids, and they have a wide range of host plants (Blackman and Eastop, 1994; Lankau, 2007). For instance, the green peach aphid, *Myzus persicae*, feeds on more than 50 plant families and has hundreds of hosts including many economically important plants such as eggplant, tomato, celery, lettuce, squash, radish, and cabbage (Baker, 1994; Blackman and Eastop, 2000). The potato aphid, *Macrosiphum euphorbiae*, is also widespread across the United States. It feeds on over 200 species in 20 plant families, many of which are economically important crops (Petrovic-Obradovic, 2010; van Emden and Harrington, 2007). Its most well-known hosts include plants in the Solanaceae family such as tomatoes, and in the Asteraceae family such as lettuce (Moeller 1973; Le Guigo, et al., 2012; Legarrea, et al., 2012). Aphids feed on plant phloem sap by piercing plant tissues with their slender stylets, thereby causing little apparent physical damage to plant tissues (Walling, 2000; Blackman and Eastop, 2000). However, aphids need to extract a large amount of phloem sap to obtain enough nutrients since phloem sap contains abundant sugars but relatively low levels of amino acids which are essential for aphids (Dixon, 1998). Thus, they can cause plants to lose large amounts of photoasimilates. They are able to cause significant crop damage, including chlorosis, wilting, stunted growth, and low yields (Goggin, 2007). Moreover, aphids excrete honeydew which promotes black sooty-mold fungi, and this fungi can decrease the rate of photosynthesis and increases the rate of leaf senescence as well (Minks and Harrewijn, 1989). In addition, aphids are known as major vectors of plant viruses. The green peach aphid, *Myzus persicae*, can transmit over 100 plant viruses including cauliflower mosaic virus and turnip mosaic virus, pea leaf roll virus, potato

leaf roll virus, radish yellows virus, and cucumber mosaic virus (Kennedy et al., 1962; Kuhar et al., 2009). The potato aphid is also known as a vector of more than 60 viruses, and the most important ones include bean leaf roll virus, zucchini yellow mosaic virus, potato leaf roll virus, potato virus Y, and beet yellow net virus (Chen et al., 1991; Hanafi et al., 1989; Singh et al., 1997).

Aphid management strategies. Many management strategies have been developed to control aphids. Biological control using natural enemies such as ladybeetles, green lacewings, and parasitoid wasps is one of the common strategies to control aphids. It is a slow process for the biological agents to control the aphid population, and natural enemies are easily affected by temperature, weather, etc. Chemical control using insecticides like organophosphates, carbamates and neonicotinoids is a widespread practice (Van Toor and Teulon, 2006). However, many insecticides have a negative effect on natural enemies of aphids as well (Capinera, 2008), and many species of aphids are resistant to a variety of insecticides due to the intensive and long-term use of insecticides (Devonshire and Field, 1991; Georghiou and Lagunes-Tejada, 1991; Bass et al., 2014). Plant resistance is an important pest-management factor, and is less hazardous to the environment, humans or wildlife than chemical control. In addition, plant resistance can be compatible with other management tactics. Aphid-resistant varieties have been developed in multiple crops (Smith, 2005), and in some cases, the genes conferring resistance have been identified, including resistance genes such as the *Mi-1.2* gene in tomato and the *Vat* gene in melon (Rossi et al., 1998; Kaloshian et al., 2005; Hill et al., 2006; Dogimont et al., 2007; Stewart et al., 2009; Palliparambil et al., 2010). However, relatively little is known about the physiological or phytochemical bases of plant defense against aphids (Smith, 2005). Several lines of evidence has shown that fatty acid desaturases (FADs) are important in mediating plant responses to

abiotic and biotic stresses (Upchurch, 2008), and so here, we explore their impact on plant-aphid interactions.

Fatty Acid Desaturases and Resistance to Abiotic and Biotic Stress. A fatty acid (FA) is a carboxylic acid with a long aliphatic tail that can be either saturated (no double bonds) or unsaturated (one or more carbon-carbon double bonds) (Fig 1A). Pairs of carbon atoms connected by single bonds can be unsaturated by enzymes called fatty acid desaturases (FADs) that removing hydrogen atoms from them, converting the single bonds to double bonds (Fig 1A). In plants, most FADs are membrane proteins localized in the plastids or the endoplasmic reticulum (ER), and there are two distinct pathways-the plastid pathway and ER pathway-for the synthesis of polyunsaturated membrane FAs. There are several FADs which are responsible to synthesize C16 and C18 FAs respectively (Fig 1B).

FADs play important roles in regulating plant defenses against a variety of abiotic and biotic stresses (Upchurch, 2008; Zhang et al., 2009; Zhang et al., 2012; Chen et al, 2013; Dong et al., 2016). The unsaturated FA amounts are mainly influenced by the abundance and activity of FADs. Acclimation in plants to temperature stresses involves the levels of unsaturated FAs in membranes which modify membrane fluidity and integral protein function (Nishiuchi and Iba, 1998). The trienoic FAs (C16:3 and C18:3) in membrane lipids of plants increase low temperature survival by enhancing membrane fluidity since they have a lower melting temperature. The chloroplast membrane-localized trienoic FAs contribute to chilling tolerance and susceptibility to high temperature (Iba, 2002; Liu et al., 2006; Wang et al., 2010; Román Á et al., 2012). The high levels of C18:3 by overexpressing *FAD8* or *FAD3* can also enhance plant tolerance to salt and drought stresses (Upchurch, 2008; Zhang et al, 2005; Klinkenberg et al., 2014). The ER-localized ω-3 fatty acid desaturase FAD3 (LeFAD3) in

tomato contributes to early seedling tolerance to salinity stress (Wang et al., 2014). In addition, FADs can regulate plant responses to biotic stresses. For example, the Arabidopsis *fad7fad8* double mutant which is deficient in FAD7 and FAD8 function displays increased vulnerability to the bacterial pathogen *Pseudomonas syringae* (Yaeno et al., 2004); in contrast, suppression of the homologous *OsFAD7* and *OsFAD8* genes in rice increased resistance to the rice blast fungus *Magnaporthe grisea* (Yara et al., 2007). Defects in *suppressor of salicylic acid insensitivity2* (SSI2), a fatty acid desaturase that converts stearic acid (18:0) to oleic acid (18:1) results in increased resistance to biotrophic pathogens, but increased susceptibility to necrotrophic pathogens (Kachroo et al., 2001; Shah et al., 2001). Therefore, FADs appear to contribute to plant resistance to some stresses whereas they promote susceptibility to other stresses.

Fatty Acid Desaturases and Aphid Resistance. Reproduction of green peach aphids was lower on the Arabidopsis *ssi2* mutant deficient in SSI2 than its wild-type plant (Pegadaraju et al., 2005). FAD7 negatively regulates plant defenses against aphids in both tomato (*Solanum lycopersicum*, Solanaceae) and Arabidopsis (*Arabidopsis thaliana*, Brassicaceae) (Avila et al., 2012). The *suppressor of prosystemin-mediated responses2* (*spr2*) mutation in tomato with impaired function of FAD7 results in decreases in potato aphid settling, adult survival and fecundity compared to the wild-type control (Avila et al., 2012). The Arabidopsis *fad7* mutant with impaired FAD7 also has significantly lower numbers of green peach aphids than wild type (Avila et al., 2012). Therefore, FADs contribute plant-aphid interactions in more than one plant family. These results indicate that FADs modulate host suitability for aphids, but much further work is needed to understand how and why FAD activity and consequent regulation in FA metabolism effect on aphid infestations.

FAD7 and FA Profiles in Foliage. The *spr2* mutation in tomato, which eliminates activity of the chloroplast-localized FAD7, increases C18:2 (linoleic acid) by about 250 % and decreases C18:3 (linolenic acid) by approximately 90 % in foliage (Li et al., 2003). This is a dramatic shift in the plant's fatty acid profile, because C18:3 normally represents >50 % of the total FAs in tomato foliage. In addition, this mutation in tomato also results in a complete loss of C16:3 (hexadecatrienoic acid), and a modest increase in C16:2 (hexadecadienoic acid) and slight increase in C18:1 (oleic acid) in foliage compared to wild-type plants, but the magnitude of these change is not as great because of moving redundancy among fads in Arabidopsis. The *fad7* mutation in *Arabidopsis thaliana*, which results in impaired chloroplast-localized FAD7 function, also enhances C18:1 by 250 %, C18:2 by 230 % and C16:2 by 20-fold, but decreases C18:3 by 62 % and C16:3 by 81 % in foliage compared to controls (Browse et al., 1986) (Table 1).

Free Fatty Acids and Plant Defense. Fatty acids are usually esterified in glycerolipids or phospholipids, and they are known as free fatty acids (FFAs) when they are not esterified or not attached to other molecules. FFAs account for very small proportion of total fatty acids in healthy and intact plant cells, but the contents of some FFAs are increased in response to wounding (Conconi, et al., 1996), and insect attack (Tooker and De Moraes, 2009). A study on wounded and unwounded tomato (*Lycopersicon esculentum* var Castelmart) leaves demonstrated that levels of free C18:2 and C18:3 increased within 1 h after being mechanically wounded (Conconi, et al., 1996). An early study on castor bean (*Ricinus communis L.*) suggested that free linolenic acid (C18:2) and linoleic acids (C18:3) increase the most among the FFAs in wounded castor bean leaves, and the wound-activated phospholipase D, a phospholipid-hydrolyzing enzyme, led to an increase in diacylglycerol and free C18:3 (Ryu and Wang, 1998). Tooker and De Moraes report that the gall-inducing

caterpillar *Gnorimoschema gallaesolidaginis* greatly increased free C18:2 and C18:3 levels (Tooker and De Moraes, 2009). Another study suggests that free fatty acids C18:1 and C18:3 are released from membrane lipids, and then converted into defense signaling compounds, thereby conferring to resistant wheat plants defenses against hessian fly (Zhu et al., 2012). These studies indicate that the levels of FFAs in plants are correlated with plant defense responses. C18:3 is a precursor of jasmonic acid (JA) which is critical to induce plant defense response (Li et al., 2003), and C18:1 is a positive regulator of jasmonate signaling (Gao et al., 2011).

Mechanisms by which FA Profiles in Plants May Influence Aphids. The changes of dienoic and trienoic FAs in the *spr2* mutant are unlikely to influence the nutritional content of the plant for aphids, because phloem sap contains primarily palmitic acid (C16:0) (Madey et al., 2002). Instead, *spr2* appears to promote aphid resistance by modifying defense signaling. In particular, aphid resistance in the *spr2* mutant in tomato appears to be mediated by phytohormone salicylic acid (Avila et al., 2012). SA is a beta hydroxyl acid that is required for systemic acquired resistance (SAR) and other forms of pathogen resistance (Kachroo and Robin, 2013; Li et al., 2006), and that also participates in plant tolerance to abiotic stresses (Dong et al., 2014). Compared to wild type plants, the *spr2* mutant has enhanced accumulation of SA in response to aphid infestation; moreover, suppressing SA accumulation by introducing the *NahG* transgene results in the loss of aphid resistance in *spr2* plants (Avila et al., 2012). Virus induced gene silencing of *NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS 1* (*NPR1*), a positive-regulator of SA-dependent defenses, also makes *spr2* plants lose resistance to aphids (Avila et al., 2012).

In addition to influencing SA-dependent defense signaling, FADs also regulate the accumulation of the plant defense hormone jasmonic acid (JA). JA regulates plant responses to stresses including necrotrophic pathogens and chewing insects (De Geyter et al., 2012; Wasternack and Hause, 2013). C18:3 FAs are required for synthesis of JA, and decreased C18:3 in *spr2* with impaired LeFAD7 is assumed to inhibit synthesis of JA, thereby resulting in high susceptibility of *spr2* mutants to the tobacco hornworm larvae (Li et al., 2003). However, aphid resistance in *spr2* mutants is not dependent on JA depletion, and *spr2* mutants are still resistant to aphid when treated with methyl jasmonate (Avila et al., 2012). This evidence demonstrates that FAD7 SA-dependent defenses against aphids through NPR1-dependent salicylate signaling but independent of JA (Avila et al., 2012).

In addition to JA, FADs influence the synthesis of many other oxylipins. Oxylipins are a group of metabolites derived from oxidation of polyunsaturated FAs (Farmer and Mueller, 2013), and they contribute to stress responses in plants (Blée, 2002). Aphids are not able to synthesize oxylipins by themselves, and have to obtain them originally from phloem sap of plants (Harmel et al., 2007). The oxylipins may influence aphid-plant interactions (Harmel et al., 2007). *Cis*-12-oxo-phytodienoic acid (OPDA) plays an important role in mediating plant defenses against piercing-sucking pests including aphids (Guo et al., 2014). Green peach aphids significantly activate the synthesis of OPDA, and exogenous OPDA increases aphid resistance in radish seedlings (Guo et al., 2014). The application of exogenous OPDA strongly improves resistance to piercing-sucking insect planthopper, *Nilaparvata lugens* in rice, and the resistance is independent of JA (Guo et al., 2014). Aphids strongly induce the accumulation of 9-hydroperoxy-octadecadienoic acid (9-HPOD) derived from linoleic acid (C18:2) in potato (Gosset et al., 2009). The increased 9-HPOD or other oxylipins derived from increased levels of C18:2 in *spr2* plants may contribute to aphid

resistance (Avila et al., 2012). However, exogenous 9-HPOD increases green peach aphid infestation on Arabidopsis leaves (Nalam et al., 2012). A recent study shows that aphid feeding on *spr2* strongly upregulates transcripts encoding α-dioxygenase 1 (α-DOX1), an enzyme participated in oxylipin synthesis implicate in limiting oxidative damage; furthermore, silencing *α-DOX1* increases aphid infestation (Avila et al., 2013). These results indicate that the α-DOX1 enzyme may play a role in aphid resistance on *spr2* by regulating oxylipin synthesis.

Fatty Acid-Derived C6 Volatile Compounds in Plant Defense. Another class of oxylipin that may be affected by FAD activity are the six carbon (C6) green leaf volatiles. C6 volatiles are synthesized from the polyunsaturated octadecanoids C18:2 and C18:3 through the successive action of the enzymes lipoxygenase (LOX), hydroperoxide lyase (HPL), and alcohol dehydrogenase (ADH) (Halitschke et al., 2004). In tomato, C6 volatile compounds such as C6 aldehydes and alcohols are important constituents of flavor (Chen et al., 2004) and can act as fungicidal and antibacterial constitutes when plants encounter stress (Croft et al., 1993). The pathogenic bacteria-induced release of (E)-2-hexenal and (Z)-3-hexenol from lima bean leaves inhibits growth of the pathogenic bacterium *Pseudomonas syringae* (Croft et al., 1993). (Z)-3-hexen-1-ol is released from *N. attenuata* plants which are attacked by herbivores, and attracts predators of *Manduca sexta* larvae to decrease herbivore survival (Kessler and Baldwin, 2002). Hexenyl acetate, an acetylated C6 volatile, plays a signaling role in indirect defense against aphids by attracting their parasitoid wasp, *Aphidius colemani* (Chehab et al., 2008). The C6 volatiles were significantly increased upon infection by the gray mold pathogen, *B. cinerea*, and feeding by an herbivore, the cabbage white butterfly lavrvae, *P. rapae*, in Arabidopsis with overexpression of *HPL* compared to wild-type control (Shiojiri et al., 2006). Overexpressing HPL enhanced plant attractiveness to the parasitoid

wasp, a natural enemy of the herbivore, and promoted defenses against the fungus (Shiojiri et al., 2006). In contrast, Arabidopsis with antisense suppression of *HPL* resulted in decreased C6 volatiles, and decreased the attractiveness to the parasitoid wasp as well as increased the susceptibility to the pathogen (Shiojiri et al., 2006). The OsHPL3 enzyme in rice contributed to defenses against rice brown planthopper, but negatively impacted resistance to the rice striped stem borer by inhibiting accumulation of JA and other volatiles, and by promoting the production of C6 volatiles (Tong et al., 2012). Another study reported that antisense suppression of a *13-HPL* gene in transgenic potato plants resulted in an increase in aphid fecundity compared to wild-type control plants (Vancanneyt et al., 2001). A 13-lipoxgenase, LOXC, is essential for synthesis of C6 volatiles, tomato plants with antisense suppression of *TomloxC* have dramatically decreased C6 volatile production compared to WT plants (Shen et al., 2014). However, the altered C6 volatiles did not influence plant defenses against the bacterial pathogen, *Xanthomonas campestris* pv. *vesicatoria* (Shen et al., 2014). These studies indicate C6 aldehydes or other derivatives may play an important role in plant defenses.

The higher levels of linoleic acid (C18:2) in *spr2* mutant derives significantly more hexanal and hexanol through LOX and HPL pathway than wild-type controls; whereas the lower levels of linolenic acid (C18:3) generate less (Z)-3-hexanal and (Z)-3-hexanol via the LOX and HPL pathways than wild-type plants (Sanchez-Hernandez et al., 2006; Canoles and Beaudry, 2006). The altered C6 volatile levels in *spr2* could influence plant response to aphids.

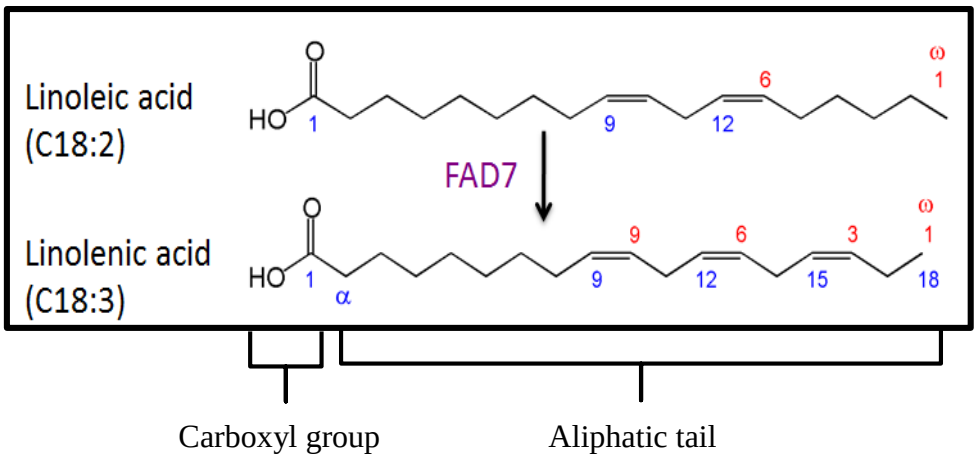
In summary, FA profiles in plants may contribute to enhanced aphid resistance by activating SA-dependent defenses and modulating C18:2- and C18:3-derived oxylipins. In

addition, SA may potentially trigger the release of free fatty acids (FFAs), because exogenous SA application to barley leaves causes increased free C18:2 and C18:3 (Weichert et al., 1999). It has been reported that accumulation of certain FFAs and their oxidation cascade is enhanced in response to stresses such as wounding (Conconi et al., 1996) and insect infestations (Zhu et al., 2012). The released FFAs from lipids may contribute to plant defenses by further promoting synthesis of oxylipins and other defense compounds (Zhu et al., 2012).

In the current study, to investigate which FA species impact aphid resistance, we investigated 1) the relative contribution of C16 and C18 FAs as well as endoplasmic reticulum (ER) and chloroplast-localized FAs in aphid resistance by comparing aphid performance and foliar FA profiles in Arabidopsis lines with loss of function of FAD3 and FAD7; 2) the relative importance of polyunsaturated FAs with single, double and triple double bounds in aphid resistance by comparing aphid performance and foliar FA profiles in Arabidopsis lines with known modifications in FA levels. We studied fatty acids first in Arabidopsis because of the availability of mutant lines for multiple FADs (chapter II). Those results suggested that 18:2 or its derivatives may contribute to resistance. One possible group of FA derivatives that are often implicated in plant defense are the C6 volatiles. In tomato, mutant plants impaired in FAD7 function are known to produce elevated levels of C6 volatiles derived from 18:2, and we chose to study their possible contribution to resistance (chapter III and IV). Tomato is a more suitable system to study C6 volatiles than Arabidopsis because the FAD mutants available in Arabidopsis happen to be generated in the Columbia ecotype, which is known to have low C6 volatile production due to defects in HPL (Duan et al., 2005; Chehab et al., 2008). The magnitude of aphid resistance in tomato plants with impaired FAD7 function is also much greater than the magnitude of aphid resistance in

Arabidopsis *fad7* mutants. The aphid fecundity on tomato mutant with impaired FAD7 was over 50 % lower than on wild-type controls; in contrast, aphid fecundity on Arabidopsis mutant with impaired FAD7 was 42 % lower than on wild-type plants. (Avila et al 2012). Therefore, we measured and compared aphid performance and C6 volatile profiles in a mutant line (*spr2*), a transgenic line (*AS-LoxC*) with antisense suppression of *TomloxC*, a transgenic line (*HPL-RNAi*) silenced for HPL, and their respective wild-type controls to investigate the potential contribution of C6 volatile to aphid resistance in tomato. To this end, we have tested whether aphid infestations on tomato increase when we suppress expression of two enzymes required for C6 synthesis, LOXC and HPL.

A



B

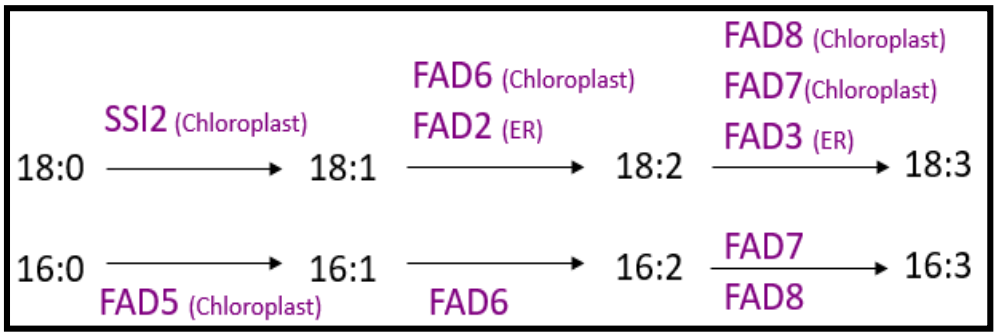


Figure 1. A) FAD7 Introduces one double bond into the acyl chain of linoleic fatty acid. B) Functions and locations of fatty acid desaturases (FADs) that contribute to synthesis of trienoic polyunsaturated FAs in Arabidopsis.

Table 1. FA Profiles in FAD Mutants.

Mutant	C16:1	C16:2	C16:3	C18:1	C18:2	C18:3
Tomato spr2 (fad7) (Li et al., 2003)	WT	↑	absent	slight ↑	2.5X ↑	90% ↓
Arabidopsis fad7 (Browse et al., 1986)	13.5X ↑	20X ↑	81% ↓	2.5X ↑	2.3X ↑	62% ↓
Arabidopsis fad3-2 (Browse et al., 1993)	normal	not detected	normal	1.3X ↑	1.5X ↑	14% ↓
Arabidopsis fad5 (Kunst et al., 1989)	WT	↓	absent	WT	WT	WT
Arabidopsis fad2-1 (Lemiuex et al., 1990)	8.3% ↓	not detected	1.2X ↑	9.1X ↑	74% ↓	22% ↓
Arabidopsis fad6 (Browse et al., 1989)	7.7X ↑	↓	not detected	6.7X ↑	34% ↑	25% ↓

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Chapter II

The Effects of Fatty Acid Desaturases on Arabidopsis Defense against Green Peach Aphids, *Myzus persicae*

Abstract

Recent evidence shows that fatty acid desaturases (FADs) can regulate levels of plant resistance to many abiotic and biotic stresses, including insect attack. These enzymes introduce double bonds into the acyl chain of fatty acids (FA). In the model plant *Arabidopsis thaliana*, there are three ω-3 FADs which convert FAs with two double bonds (dienoic acids) to FAs with three double bonds (trienoic acids): FAD3, which is localized in the endoplasmic reticulum (ER) and desaturates 18-carbon FAs, and FAD7 and FAD8, which are located in the chloroplast and act on C16 and C18 FAs. Previously we have shown that Arabidopsis mutants with impaired FAD7 function are more resistant to the green peach aphid (*Myzus persicae*) than wild-type controls. Mutants with impaired FAD7 function are enriched in 16- and 18-carbon dienoic FAs (C16:2 and C18:2) and oleic acid (C18:1), and are depleted in trienoic FAs (C16:3 and C18:3) compared to wild-type plants. This study compared aphid performance and total FA profiles in mutants with defects in FAD3, FAD7, FAD8, and other desaturases in order to determine whether 1) other ω-3 desaturases in the chloroplast or ER influence aphid resistance; 2) aphid resistance is influenced by C16 and/or C18 FAs; and 3) aphid resistance is correlated with high C18:1, high C16:2 and C18:2, or low C16:3 and C18:3. Our results showed that, compared to aphid performance on wild type plants, aphid numbers were significantly lower on mutants with impairments in the ER-localized FAD3. Levels of aphid resistance in the *fad3* mutant were comparable to levels observed in mutants with impaired function of the chloroplast-localized FAD7. This indicated that aphid resistance was impacted by FADs in the ER as well as in the chloroplast. Our results also suggested that C18 rather than C16 FAs may play a determining role in aphid resistance, since loss of function of FAD7 impacted both C16 and C18 FAs, but impaired FAD3 function only influenced C18 FAs. Moreover, elevated C18:1 or depleted C18: 3 did not appear to be

sufficient to confer aphid resistance, because the *fad2* mutant was susceptible to aphids, despite having high C18:1 and low C18:3 levels comparable to levels observed in *fad7-1*. The aphid resistant *fad7-1* line had high C18:2 compared to wild type, but *fad2* has significantly lower C18:2. These results suggested that C18:2 or its derivatives could be important in aphid resistance.

Introduction

Fatty acid desaturases (FADs) introduce double bonds into the acyl chain of fatty acids (FA), and regulate FA profiles in plants. Most FADs are membrane proteins localized in the plastids or the endoplasmic reticulum (ER). In Arabidopsis, a variety of FADs are responsible for synthesis of polyunsaturated FAs (Fig. 1). There are three ω-3 FADs which convert FAs with two double bonds (dienoic acids) to FAs with three double bonds (trienoic acids) (Arondel et al., 1992; Gibson et al., 1994): FAD3, which is localized in the endoplasmic reticulum (ER) and desaturates 18-carbon FAs, and FAD7 and FAD8, which are located in the chloroplast and act on C16 as well as C18 FAs (Browse et al., 1986 and 1993; McConn et al., 1994; Dye and Mullen, 2001; Froehlich et al., 2003). There are two FADs which convert FAs with one double bond to FAs with two double bonds: FAD2, which is localized in the ER and desaturates 18-carbon FAs (Lemiuex et al., 1990), and FAD6, which is located in the chloroplast and functions on both C16 and C18 FAs (Browse et al., 1989). Moreover, there are two chloroplast-localized FADs which convert saturated FAs to FAs with one double bond: FAD5 which desaturates 16-carbon FAs (Kunst et al., 1989), and SUPPRESSOR OF SA INSENSITIVITY2 (SSI2) which desaturates 18-carbon FAs (Kachroo et al., 2005).

FADs can modulate levels of plant defenses to many abiotic and biotic stresses, including heat and cold stress, pathogens, and insect attack (Upchurch, 2008). The relative abundance of saturated, unsaturated, and polyunsaturated FAs in the lipid pool of the plant are mainly influenced by the abundance and activity of FADs. Acclimation in plants to temperature stresses involves changes in the levels of unsaturated FAs in membranes which modify membrane fluidity and integral protein function (Nishiuchi and Iba, 1998). Trienoic FAs are the most abundant polyunsaturated FA species in plant membrane lipids, and make

up about 70 to 80 % in the chloroplast membrane lipids (Harwood, 1980; Yaeno, 2004). The levels of trienoic FA play an important role in plant adaptation to temperature stresses. Transgenic lines that have enhanced levels of chloroplast membrane-localized trienoic FAs due to overexpression of the Arabidopsis *FAD7 or FAD8* gene in tobacco leaves are more tolerant to low temperature during the early growth stage than wild type plants (Iba, 2002). Conversely, tobacco lines with decreased levels of chloroplast membrane-localized trienoic FAs due to suppressed activity of *FAD7* and *FAD8* showed enhanced resistance to high temperature (Iba, 2002). Increasing linolenic FAs (C18:3) by overexpressing of *FAD8* or *FAD3* also contribute to salt and drought tolerance (Zhang et al., 2005). Moreover, FADs influence plant defenses against a variety of biotic stresses as well. For example, suppression of the Arabidopsis *FAD7* and *FAD8* genes reduced amounts of reactive oxygen intermediates and decreased resistance to bacterial pathogen *Pseudomonas syringae* (Yaeno et al., 2004). In contrast, suppression of the homologous *OsFAD7* and *OsFAD8* genes in rice enhanced hydroperoxides and hydroxides derived from linoleic acid (C18:2) and confered resistance to the rice blast fungus *Magnaporthe grisea* (Yara et al., 2007, 2008). FADs are also involved in plant defense against herbivore feeding (Tumlinson et al., 2008). The triple mutant *fad3fad7fad8* in Arabidopsis with the loss of function of linolenic acid showed highly susceptible to insect larvae (McConn et al., 1997).

Published data from our laboratory has also demonstrated that mutants with impaired FAD7 function in tomato and Arabidopsis plants have enhanced aphid resistance; they display decreased aphid settling, survival and fecundity compared to wild type plants (Avila et al., 2012). The mutants with suppressed FAD7 are enriched in oleic acid (C18:1) and 16- and 18-carbon dienoic FAs (C16:2 and C18:2), and depleted in trienoic FAs (C16:3 and C18:3) compared to wild-type (Browse et al., 1986). The changes of FA profiles in these

mutants may be involved in aphid resistance. However, since the loss of function of FAD7 impacts the abundance of multiple FAs, we cannot be certain which of these FAs contribute to aphid resistance. The objectives for this study were to investigate whether aphid resistance is 1) affected by FA metabolism in the ER and/or only in chloroplast; 2) dependent upon change in C16 and/ or C18 FAs; 3) affected by FAs with single, double and triple double bonds.

To address objective 1 and 2, we measured aphid performance on Arabidopsis mutants *fad3-2* and *fad7-1*. FAD7 is located in chloroplast, and Arabidopsis *fad7-1* mutant has impaired FAD7 function; In contrast, FAD3 is located in the ER, and Arabidopsis *fad3-2* mutant has impaired FAD3 function. Thus, comparison of aphid performance on these two Arabidopsis mutants would determine if aphid resistance is affected by FAs in the ER or/and chloroplast. Moreover, FAD7 acts on both C16 and C18 FAs whereas FAD3 only functions on C18 FAs, since C16 FAs are synthesized exclusively in the chloroplast (Browse et al., 1993). Thus, comparing aphid performance on these two Arabidopsis mutants can also determine if aphid resistance depends on levels of C16 and/or C18 FAs. We would expect to observe reduced aphid performance on the *fad3-2* mutant relative to wild type if FA metabolism in the ER influences aphid resistance; in contrast, we expect similar numbers of aphids on *fad3* and wild type if ER-localized FADs are not important in aphid resistance.

To compare the influence of different C18 FA species on aphid resistance, we measured and compared aphid performance and FA profiles on Arabidopsis mutants *fad7-1*, *fad2-1*, and *fad2-1fad7-1*. The *fad7-1* mutant with impaired FAD7 activity has higher C18:1 FA and C18:2 FA but lower C18:3 FA levels than wild-type controls (Browse et al., 1986), whereas the *fad2-1* mutation with impaired FAD2 function results in higher C18:1 FA, and

lower C18:2 FA and C18:3 FA levels than controls (Lemiuex et al., 1990). The double mutant line *fad2-1fad7-1* is homozygous for loss of function of both FAD2 and FAD7, and was developed by crossing mutants *fad2-1* and *fad7-1/gl1* in our lab for this project. The *fad2-1fad7-1* mutant was developed in an attempt to achieve a line with higher levels of C18:1, and lower C18:2 and C18:3. Thus, measuring the aphid performance on Arabidopsis mutants *fad7-1*, *fad2-1*, *fad2-1fad7-1* and wild-type can determine the relative importance of C18 polyunsaturated FA species in aphid resistance. If high C18:1 and/or low C18:3 FAs play an important role in aphid resistance, we expect that the *fad7-1*, *fad2-1* and *fad2-1fad7-1* mutants would all be resistant to aphids. If C18:2 is more important in resistance, we expect that the *fad7-1* mutant would be resistant but *fad2-1* and *fad2-1fad7-1* mutants would be susceptible to aphids, if the double mutant *fad2-1fad7-1* has the expected FA profile.

Likewise, to compare the influence of different C16 FA species on aphid resistance, we would measure and compare aphid performance and FA profiles on the Arabidopsis mutants *fad5-1*, *fad6-1*and *fad7-1*. The *fad7-1*mutant has higher C16:1 FAs and 16:2 FAs but lower C16:3 FAs than wild-type (Browse et al., 1986); the *fad5-1* mutant with impaired FAD5 function has normal level of C16:1 FAs, a decreased C16:2 FAs and undetected C16:3 FAs (Kunst et al., 1989); and the *fad6-1* mutant with reduced FAD6 function has higher C16:1, lower C16:2 and an undetected C16:3 FA levels (Browse et al., 1989). Therefore, aphid performance on Arabidopsis mutants *fad7-1*, *fad5-1*, *fad6-1* and wild-type can be compared to assess the relative importance of C16 polyunsaturated FAs with single, double and triple bonds in aphid resistance. If C16:1 and C16:3 FAs play an important role in aphid resistance, we would expect *fad7-1* and *fad6-1* mutants to be resistant but the *fad5-1* mutant to be susceptible to aphids. If increased C16:2 is important in aphid resistance, we expect *fad7-1* to be resistant but both *fad5-1* and *fad6-1* mutant to be susceptible to aphids.

In addition to answering our three primary questions, another goal of this study was to examine whether the *glabra1* (*gl1*) mutation affects aphid resistance. Xia et al. (2010) suggested that this mutation can enhance resistance to the virulent pathogen *Pseudomonas syringae*. G*labra1* (*gl1*) is a gene that regulates the development of trichomes which are small hairs on stems, leaves, and flowers in Arabidopsis. Arabidopsis plants normally have trichomes, and *gl1* mutant causes leaves to be hairless (glabrous). The *gl1* mutant is convenient to be used as a maternal parent in genetic crosses because the presence of trichomes in the F1 plants are the product of hybridization rather than unintended self-fertilization. Perhaps for this reason, the *gl1* mutant was used as a genetic background for chemical mutagenesis studies, including the study that generated the *fad7-1/gl1* mutant (Vaughn et al., 2014). To investigate whether the *gl1* mutation contributes to the aphid resistance observed in *fad7-1/gl1*, genetic crosses were used to develop a *fad7-1* line that lacks the *gl1* mutation (Vaughn et al., 2014), and aphid performance on mutant lines *gl1*, *fad7-1/gl1*, *fad7-1* and Col-0 wild-type was compared in this study. If *gl1* mutation contributes to aphid resistance, we would expect reduced aphid performance on *gl1* and *fad7-1/gl1* as compared to Col-0 and *fad7-1*.

Materials and Methods

Plant materials

The Arabidopsis seeds *fad2-1*, *fad7-1*, *fad3-2*, *fad5-1*, *fad6-1*, *fad8* and wild-type Col-0 (CS70000) were obtained from The Arabidopsis Information Resource center (TAIR). The *gl1* and *fad7-1/gl1* mutant seeds were donated by Dr. Jyoti Shah, University of North Texas, Denton, TX. The *fad2-1fad7-1* mutant with impaired FAD2 and FAD7 functions were developed by crossing *fad2-1* and *fad7-1/gl1* mutants in our lab. Seeds were surface-sterilized

and plated on MS germination medium, then vernalized for 3 days at 4 °C. Plants were maintained in a growth chamber (23 °C; 65 % relative humidity; L13: D11 photoperiod) and grown in a peat, vermiculite, perlite (4:3:2 ratio) soil mixture supplemented with 15-9-12 Osmocote Plus fertilizer. The plants were fertilized weekly with Miracle Gro® all-purpose plant food (N:P:K=3:1:2; Scotts-MiracleGro Company, Marysville, OH), and were watered as needed with tap water. Plants were given treatments of 1 mL / 500 g Gnatrol as needed to prevent fungus gnats.

Insect materials

The green peach aphid, *Myzus persicae*, was reared on an aphid-susceptible cabbage (*Brassica oleracea* var. Joychoi) at ~20 °C and 16-hr light photoperiod. Adult aphids within 24 h of emergence to adulthood were used for the bioassays. To obtain aphids of uniform age for bioassays, 3-week-old cabbages were inoculated with wingless adult aphids, which were allowed to larviposit and then were removed after 24h. Offspring that had emerged to adulthood were collected 6 days after removing the original adults, and were transferred onto experimental Arabidopsis plants (3 apterous adults / plant, 15-20 plants per genotype). The plants were at developmental stage 5.1 (Boyes et al., 2001) at the time of infestation. After infestation, plants were covered with sleeve cages, and maintained for 7 days in a growth chamber (23 °C; 65 % relative humidity; L13: D11 photoperiod). The numbers of live and dead adults and offspring on each plant were scored 7 days after infestation (DAI).

Development an Arabidopsis *fad2-1fad7-1* double mutant line

The Arabidopsis double mutant *fad2-1fad7-1* was developed by crossing *fad7-1/gl1* (maternal parent) and *fad2-1* (pollen donor) (Flattmann, 2015). To confirm that the *fad2-1fad7-1* mutant was homozygous for both mutations, plants from the F2 generation of a *fad7-*

1/gl1 (♀) X *fad2-1* (♂) cross were screened by polymerase chain reaction (PCR) with four primer sets to detect the WT and mutant alleles of the *fad7* and *fad2* genes. 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 % SDS) by 10-fold with TE buffer (10 mM Tris-HCl (pH 8) and 1 mM EDTA) (Kasajima et al., 2004; Edwards et al., 1991). The primers for *fad7* were designed by Avila and Goggin (Fig2; Table 1) in our lab based on the mutation reported by Xia et al (2010). The primers for *fad2* were designed based on the mutation reported by Zhang et al (2012).

Touchdown polymerase chain reaction (PCR) was performed to increase amplification sensitivity and specificity using the following program: for the WT *AtFAD7* allele: initial denaturation= 95 °C for 5 min; phase I= 95 °C for 45 sec, 65-58 °C for 45 sec (reducing 1 °C per cycle), and 72 °C for 45 sec; phase II= 95 °C for 45 sec, 57 °C for 45 sec, and 72 °C for 45 (20 cycles); and final extension at 72 °C for 5 min. For the *Atfad7-1* mutation: 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.

The polymerase chain reaction (PCR) was performed using the following program: for the WT *Atfad2-1*: initial denaturation= 95 °C for 5 min; phase I= 95 °C for 45 sec, 64-57 °C for 45 sec (reducing 1 °C per cycle), and 72 °C for 45 sec; phase II= 95 °C for 45 sec, 56 °C for 45 sec, and 72 °C for 45 (20 cycles); and final extension at 72 °C for 5 min. For the mutation *Atfad2-1*: initial denaturation= 95 °C for 5 min; phase I= 95 °C for 45 sec, 60-53 °C for 45 sec (reducing 1 °C per cycle), and 72 °C for 45 sec; phase II= 95 °C for 45 sec, 52 °C for 45 sec, and 72 °C for 45 (20 cycles); and final extension at 72 °C for 5 min.

PCR products were visualized by electrophoresis. PCR products were run on a 1 % agarose gel at 250 V for 16 min in a Scooter gel electrophoresis box (Biokey American Instruments Inc.). 20 µl of reaction PCR product was loaded into gel wells, 5 µl of 100 bp DNA ladder (Invitrogen) was loaded to measure the size of PCR product. The ®UVP Biodoc was used to visualize the gel.

Tissue collection and fatty acid profile analysis

Leaf tissue was collected from plants infested with aphids as described above, and also from plants mock-infested with empty cages. At 8 days after inoculation, leaf tissue was collected from the infested plants by removing aphids with a soft brush, detaching the leaflets, and weighing followed by flash freezing in liquid nitrogen (3 replicates/genotype). The same collected method was used for the control plants. The collected leaf samples were stored at -80 °C until extraction.

To confirm and compare the C16- and C18-total fatty acid profiles in Arabidopsis mutants *fad2-1*, *fad7-1*, *fad2-1fad7-1*, *fad3-2*, fad5-1, *fad6-1* and wild-type Col-0, lipids were extracted using the Bligh and Dyer method (1959) followed by preparation of fatty acid methyl esters (FAMEs) (Shipley et al., 1993). In brief, frozen leaf tissue was ground in liquid nitrogen and transferred into a 15 ml glass vial. 2 ml of chloroform and 2 ml of methanol as well as 100 µg of internal standards (pentadecanoic acid, C15:0, NU-CHEK PREP, Inc.) were added to the samples. After incubating the samples on ice for 1 min, 1 ml of H_2O was added. The mixture was centrifuged at 4,000 rpm for 5 min at room temperature. Then the organic phase was transferred into a 7 ml Kimbal vial (VWR), and dried down under nitrogen flow. The extraction was repeated twice by adding 1ml chloroform into the remaining sample residue, and transferring organic phase into Kimbal vials followed by drying down.

Subsequently, 1ml 5 % KOH in methanol was added to the dried organic phase for saponification at 65 °C for 90 min. 1 ml of 14 % boron trifluoride in methanol was added for methylation at 65 °C for 30 min. Then the samples were dehydrated using a $MgSO_4$ column and purified using a silicic acid column. The silicic acid column was prewashed by hexane. Finally, 5 % ethyl ether was used for washing the methylated fatty acids (FAMEs) off the column. The FAMEs were dried down under nitrogen flow and dissolved into100 µl of hexane and then transferred into inserts of gas chromatography autosampler (GC, HP 6890; Agilent) vials. Then 1 µl of each sample was injected in the split mode with an autosampler (Agilent Technologies) for GC-MS (gas chromatography-mass spectrometry) analysis. The split ratio of sample was 3:1. The GC-MS system used a FAMEWAX column (30 m x 0.25 mm with a 0.25 µm film thickness, Restek, Bellefonte, PA). The initial temperature of GC oven was 130 °C, and 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 the external standard (nuchek mixture reference, NU-CHEK PREP, Inc.), and quantified based on an internal standard (pentadecanoic acid, C15:0, NU-CHEK PREP, Inc.) that does not naturally occur in Arabidopsis.

Statistical analysis

All the statistical analysis was done with JMP ® v 11 (SAS Institute Inc.). The data were analyzed by one-way ANOVA, and means were separated using Tukey-Kramer HSD for multiple testing and student's *t* test for analyses that involved comparisons of only 2 treatment groups (eg. uninfested & infested plants).

Results

Foliar C16 and C18 FA abundance in Arabidopsis mutant lines and wild-type Col-0

To identify which FAs profile are correlated with aphid performance in Arabidopsis FAD mutants, FA levels were measured by GC-MS (Fig 3). In uninfested plants, the data showed that the *fad7-1* mutant has significantly higher C16:2 and C18:2 but lower C16:3 levels than wild-type Col-0. The *fad2-1* mutant has significantly higher C18:1 than wild-type. The *fad5-1* mutant has significantly higher C16:0 but lower C16:3 than wild-type. The *fad6-1* mutant has significantly higher C18:1, lower C16:3 and C18:3 levels than wild-type. Compared to the *fad2-1* mutant, the *fad2-1fad7-1* mutant had significantly lower C18:1 and C16:3, and significantly higher C16:2 and C18:2. Compared to the *fad7-1*, the double mutant had similar levels of all FAs.

To determine whether the aphid infestation influences foliar C16 and C18 FA abundance in Arabidopsis, total FA profiles were compared in leaf tissue collected from the infested plants bioassay and uninfested control (Fig 4). All but two of the Arabidopsis genotypes (Col-0 and *fad5-1*) showed significant changes in FA profiles in response to aphids. C16:3 and C18:3 were significantly increased in response to aphids in *fad2-1* mutants than uninfested ones. C16:2 and C18:1 were also significantly higher in aphid infested double mutants *fad2-1fad7-1* than control. The aphid infested *fad6-1* mutant plants had markedly higher C18:3 levels than uninfested plants. The C16:2 and C18:2 were significantly higher in aphid infested *fad7-1* mutants than control.

The role of fatty acid metabolism in the ER and in chloroplast in plant defenses against green peach aphids.

Green peach aphid numbers were significantly lower on *fad7-1/gl1* (p=0.01), *fad3-2* (p=0.028), and the triple mutant *fad3fad7fad8* (p=0.047) than their wild-type controls respectively (Col-0 is the background for *fad3-2 or fad3fad7fad8*; *gl1* is a control for *fad7-1/gl1*) (Fig 5A). To understand whether FAD8, the other chloroplast-localized FAD which has similar function to FAD7, also negatively regulates plant defenses against aphids, the green peach aphid performance was measured on Arabidopsis mutant *fad8*. However, aphid numbers on *fad8* (p=0.71) were not significantly different from the wild-type Col-0 (Fig 5A).

The impact of the *gl1* mutation in *fad7-1/gl1* mutant on Arabidopsis defenses against green peach aphids.

To understand whether the *gl1* mutation in the aphid resistant mutant *fad7-1/gl1* affects Arabidopsis defenses against aphids, green peach aphid performance was compared on *fad7-1*, *fad7-1/gl1*, *gl1* and Col-0 at 7-DAI. Numbers of green peach aphids were significantly lower on both *fad7-1/gl1* (p=0.01) and *fad7-1* mutants (p=0.0011) than their wild-type controls respectively (Col-0 is the background for *fad7-1*; *gl1* is a control for *fad7-1/gl1*) (Fig 5B). However, aphid numbers on *gl1* (p=0.4390) were not significantly different from the wild-type Col-0. These data showed that both *fad7-1* and *fad7-1/gl1* mutants were resistant to green peach aphids compared to wild-type, and the *gl1* did not significantly influence aphid numbers.

The importance of C16 fatty acids in plant defenses against green peach aphids.

To assess the relative importance of C16 polyunsaturated fatty acids with single, double and triple bonds in aphid resistance, aphid performance on Arabidopsis mutants with higher C16:1 and 16:2 and lower C16:3 (*fad7-1* mutant), normal level of C16:1, lower C16:2

and undetected C16:3 (*fad5-1* mutant), and higher C16:1, lower C16:2 and undetected C16:3 (*fad6-1* mutant) and wild-type Col-0 was measured at 7-DAI. The green peach aphid numbers were significantly lower on *fad7-1* mutants (p=0.0057) than the wild-type control plants (Fig 6). In contrast, aphid numbers on *fad5-1* (p=0.8073) and *fad6-1* (p=0.9965) mutants were not significantly different from the wild-type at α=0.05. In some assays, the *fad6-1* mutant was notably smaller than the other genotypes, and/or appeared to have a fungal infection. Because of this variability in the size and health of the *fad6-1* mutant from assay to assay, this mutant was tested a total of 7 times (table 2). In some assays, aphid numbers were lower on *fad6-1* than wild type, but this tended to occur when *fad6-1* plants were smaller or affected by fungus. In 3/7 assays (including the assay presented in Fig 6), aphid numbers on *fad6-1* were comparable to wild type. Therefore, the fad6 mutation did not appear to have a strong or consistent effect on aphids. From our data on *fad5-1* and *fad6-1*, it appears that mutations that influenced C16 without affecting C18 do not confer aphid resistance.

The importance of C18 fatty acids in plant defenses against green peach aphids.

To determine the relative importance of C18 polyunsaturated fatty acid species with single, double, and triple bonds in aphid resistance, green peach aphid numbers were measured on Arabidopsis mutants *fad2-1, fad7-1, fad2-1fad7-1* and wild-type controls Col-0 at 7-DAI. Green peach aphid numbers were significantly lower on *fad7-1* (p=0.0005) and *fad2-1fad7-1* mutants (p=0.0011) than the wild-type control plants (Fig 7). In contrast, aphid numbers on *fad2-1* (p=0.4390) were not significantly different from the wild-type. These data showed mutants lines with high C18:2 and low C18:3 mutants were resistant to aphids, but the *fad2-1* mutant, which has low C18:2 and C18:3 levels, was susceptible.

Development of an Arabidopsis *fad2-1fad7-1* double mutant line.

The F2 generation was screened by PCR using primer sets specific to the WT and mutant alleles of *fad7* and *fad2*. The PCR product for WT *Atfad7-1* and mutant *Atfad7-1* was 582 base pairs (Fig 8A-B); the PCR product for WT *Atfad2-1* and mutant *Atfad2-1* was 549 base pairs (Fig 8C-D). The *fad2-1fad7-1* double mutant line was generated by crossing *fad2-1* and *fad7-1/gl1* mutants (Flattman et al., 2015), and PCR identified that the *fad2-1fad7-1* mutant line was homozygous *fad7* mutation (lane 11 in Fig 8A-B) and *fad2* mutation (lane 11 and 12 in Fig 8C-D).

Discussion

Many studies have indicated that FAs play important roles in regulating plant defensive signaling and impact plant defenses against pests, including aphids (Avila et al., 2012; Zhu et al., 2011; Kachroo and Kahroo, 2009; Upchurch, 2008). We hypothesized that the effects of FAD7 on aphids are mediated through variation in the host plant's FA profiles. The C16 and C18 FA profiles were changed in Arabidopsis mutant *fad7-1* deficient in FAD7. FAD5, FAD6 and FAD7 participate in regulating C16 profiles. Our results showed that the *fad5-1* and *fad6-1* mutants had significantly decreased C16:3, and normal levels of C16:2 compared to wild-type control Col-0, but the *fad7-1* mutant had markedly higher C16:2 and lower C16:3 than wild-type control Col-0 (Fig 3). The green peach aphid numbers were significantly lower on *fad7-1* mutants than the wild-type control plants, but aphid numbers on *fad5-1* and *fad6-1* mutants did not significantly differ from the wild-type (Fig 6). Thus, it is unlikely C16 play an important role in aphid resistance because the *fad7-1* mutant was resistant but *fad5-1* and *fad6-1* mutants were susceptible to aphids. The current study also suggested that both FAD7 and FAD3 in Arabidopsis act as negative regulators of aphid

resistance, since resistance was observed in the *fad3-2* and *fad7-1* mutant (Fig 5A). FAD7 functions on both 16- and 18-carbon FAs whereas FAD3 only regulates 18-carbon FAs (Browse et al., 1993). Thus, the level of 18-carbon FAs may be more important than 16-carbon FAs in aphid resistance. FAD7 is located in chloroplast, and FAD3 is located in the ER. Our results indicated that aphid resistance is influenced by FADs in the ER as well as chloroplast.

Furthermore, the data indicated that both *fad7-1* and *fad2-1fad7-1* mutants were resistant to aphids, but the *fad2-1* mutant was susceptible to aphids (Fig 7). Compared to wild-type control Col-0, the *fad7-1* mutant had significantly higher C18:2 and normal level of C18:1 and C18:3, the *fad2-1fad7-1* mutant had significantly higher C18:2 but lower C18:3, and normal C18:1, the *fad2-1* mutant had significantly higher C18:1, and normal levels of C18:2 and C18:3 (Fig 3). This suggested that neither C18:1 nor C18:3 was likely important in aphid resistance, but C18:2 may play a role in regulating plant defenses against aphids. The C18 FA levels in *fad2-1fad7-1* mutant were not significantly different from those in *fad7-1* mutant. This result suggested that the *fad2-1fad7-1* mutant may only contained the *fad7-1* single mutation. However, the results from *fad2-1* and *fad7-1* mutants can still indicated that C18:2 may play a role in aphid resistance, even though if the results of *fad2-1fad7-1* mutant were excluded.

The results from comparison of aphid performance on *fad8*, *fad3fad7fad8*, *fad3-2*, and *fad7-1/gl1* and wild-type plant indicated that the *fad8* mutation did not contribute to aphid resistance in the triple mutant *fad3fad7fad8*. This is consistent with previous reports showing that FAD8 expression is very low above 20 °C (Li et al., 2003), and that it does not contribute significantly to FAD activity at 23 °C, the temperature at which this bioassay was performed. In addition, we used *fad7-1/gl1* mutant with *fad7-1* and *gl1* mutations and *fad7-1* mutant with

single *fad7-1* mutation in our aphid bioassay, but the *gl1* mutation which influences the development of trichomes did not affect the aphid resistance; both *fad7-1/gl1* and *fad7-1* mutants were resistant to green peach aphids as compared to their controls (*gl1* for *fad7-1/gl1*; Col-0 for *fad7-1*) (Fig 5B).

The results of the comparisons in C16 and C18 FA abundance between aphid infested and uninfested Arabidopsis lines showed that the abundance of some FA species was impacted by aphid infestation in some mutant lines (*fad2-1*, *fad2-1fad7-1*, *fad6-1*, *fad7-1*), but was not markedly altered by aphid infestation in wild-type plants or the *fad5-1* mutant (Fig 4). These results indicated that aphids can modify foliar FA profiles, at least in genotypes with defects in fatty acid desaturation.

FAD7 and other FAs are unlikely to influence the nutritional content of the plant for aphids because phloem sap contains primarily palmitic acid (C16:0) (Madey et al., 2002). However, the changed FA substrate in resistant mutants may indirectly influence plant defensive signaling. Plant defense against insects is a complex process, involving a variety of plant biochemical factors and pathways. Previous work in our laboratory has shown that loss of function of FAD7 in tomato enhances aphid-inducible accumulation of salicylic acid (SA) that is required for aphid resistance in *spr2* (Avila et al., 2012). The mechanisms through which FAD 7 influence SA signaling are as yet unknown. In addition to the impact of FADs on SA signaling, other pathways through these desaturases could impact plant defenses against aphids. In Arabidopsis, petiole exudates from the aphid resistant mutant *ssi2*, which has suppressed levels of C18:1, have antibiotic effects on aphids (Pegadaraju et al., 2005; Louis et al., 2010). In tomato and Arabidopsis, α-dioxygenase 1 (α-DOX1) which is involved in the synthesis of oxylipins derived from C18:2 and C18:3, contributes to plant defenses against aphids (Avila et al., 2013). Oxylipins generated from 18:2 by α-DOX1 may contribute

to aphid resistance in *spr2*. Our study would help to understand how FAD7 modulates plant defensive signaling and identifying the network of signal transduction.

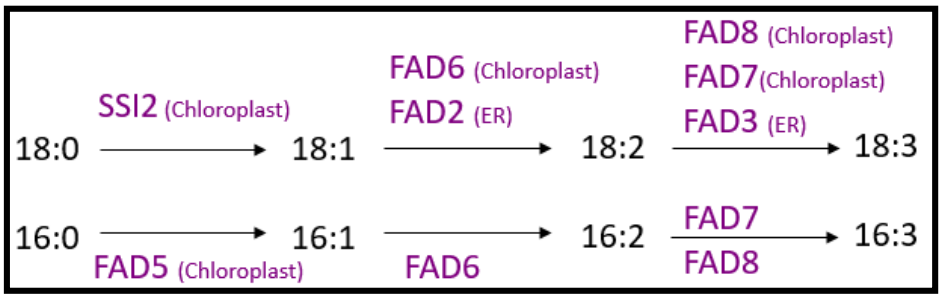


Figure 1. Functions and locations of fatty acid desaturases (FADs) that contribute to synthesis of trienoic polyunsaturated FAs in Arabidopsis.

Figure 2. The primer design for *FAD7* gene and *FAD2* gene. Red fonts represent the mutation sights. The *fad7* mutation was reported by Xia et al (2010), and the *fad2* mutation was reported by Zhang et al (2012).

Table 1. PCR primers

GENE	PRIMARY SOURCE (TAIR)	PRIMERS	SEQUENCE	AMPLICON LENGTH	PCR program
Fatty acid desaturases 7 (*FAD7*)	AT3G11170	WT*AtFAD7*(F) WT*AtFAD7*(R) Mu*AtFAD7*(F) Mu*AtFAD7*(R)	5'-TTTCAGTGGGCTCGAAGTCC-3' 5'-ATCTGCGGGAAAAGATGATG-3' 5'-TTTCAGTGGGCTCGAAGACT-3' 5'- ATCTGCGGGAAAAGATGATG-3'	582 bp (bases 1447 to 2029)	Touchdown PCR Annealing temp 57°C Touchdown PCR Annealing temp 55°C
Fatty acid desaturases 2 (*FAD2*)	AT3G12120	WT*AtFAD2*(F) WT*AtFAD2* (R) Mu*AtFAD2*(F) Mu*AtFAD2*(R)	5'-CCTAACTGGTATCTGGGTCACAG-3' 5'-AACGAGGGATGAGTGTGCTG-3' 5'-CCTAACTGGTATCTGGGTCACAA-3' 5'-AACGAGGGATGAGTGTGCTG-3'	549 bp (bases 1595 to 2143)	Touchdown PCR Annealing temp 56°C Touchdown PCR Annealing temp 55°C

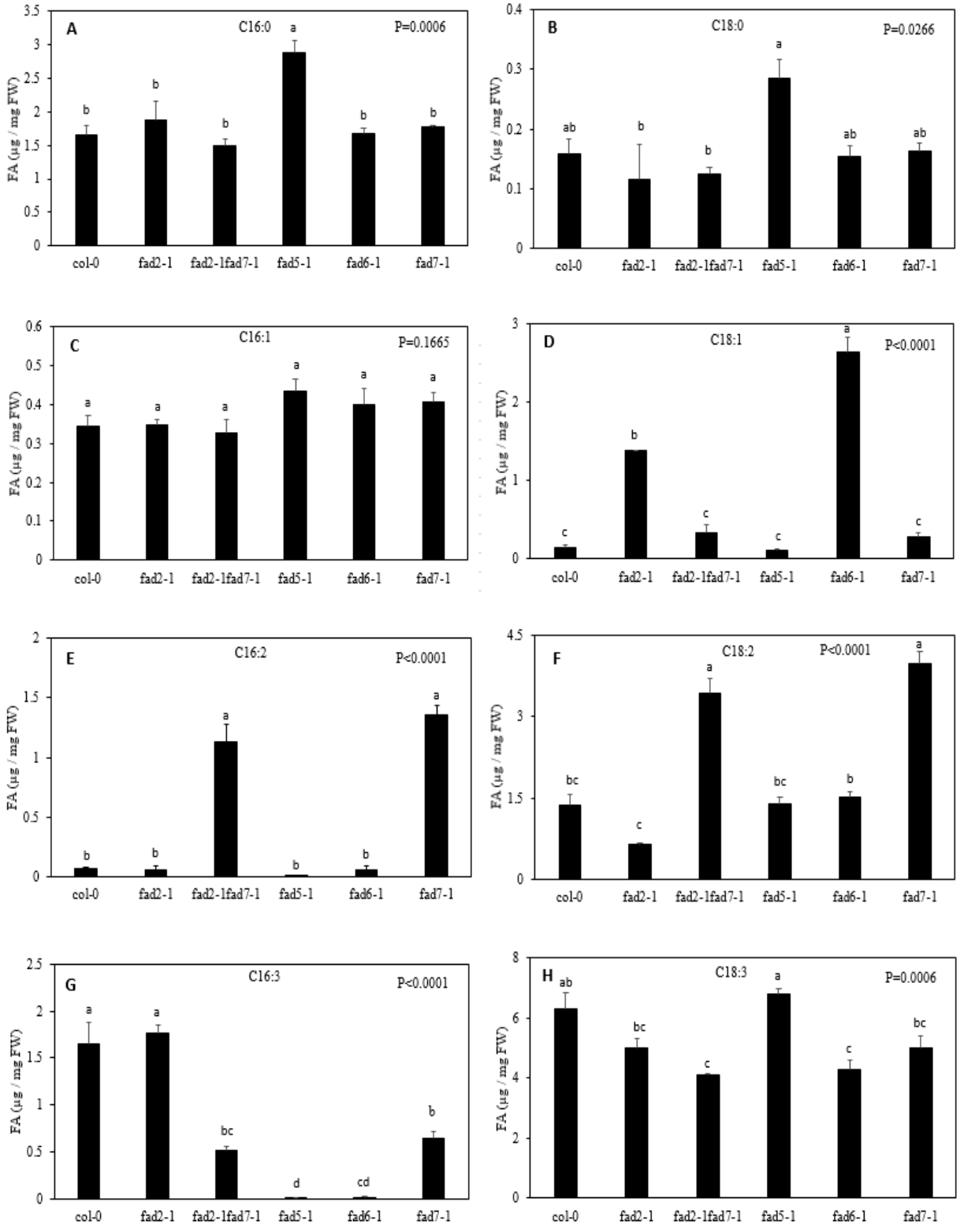


Figure 3. Fatty acid levels in Arabidopsis lines. Bars having the same letter are not significantly different among Arabidopsis lines at α=0.05 according to Tukey-Kramer HSD test, and error bars represent SEM (n=3). FW, Fresh weight.

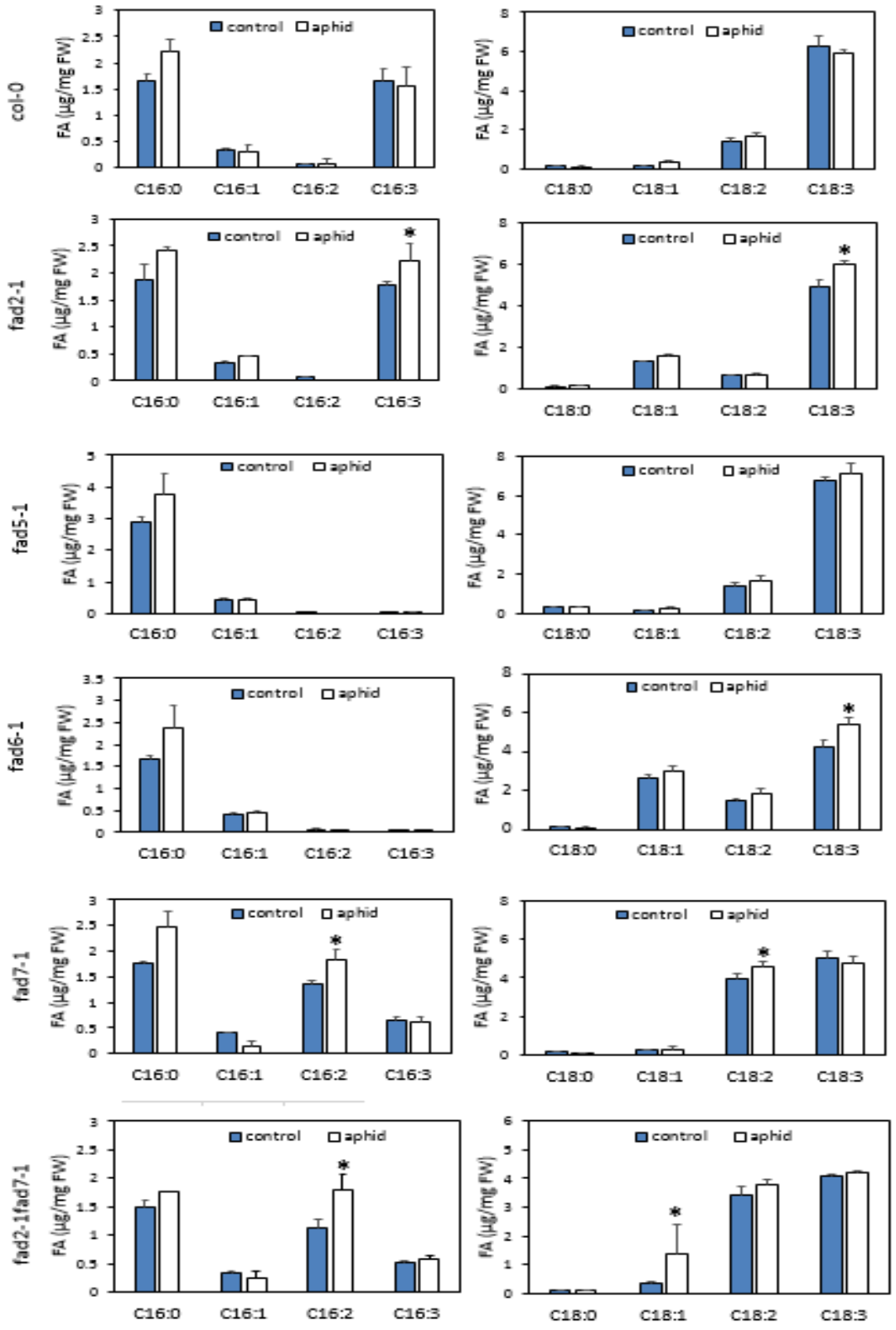
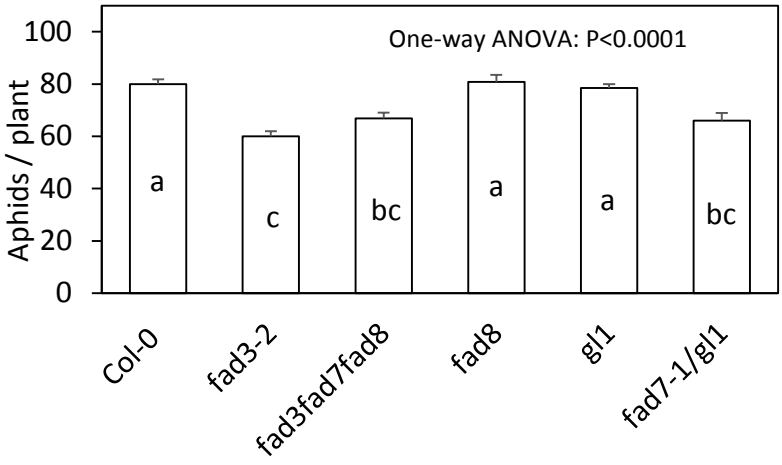


Figure 4. C16 and C18 fatty acid level comparisons between aphid infested and uninfested Arabidopsis plants. The error bars represent SEM (n=3). Asterisks denote a significant difference between aphid-infested and control plants according to student's *t* test ($p<0.05$). FW, Fresh weight.

A)



B)

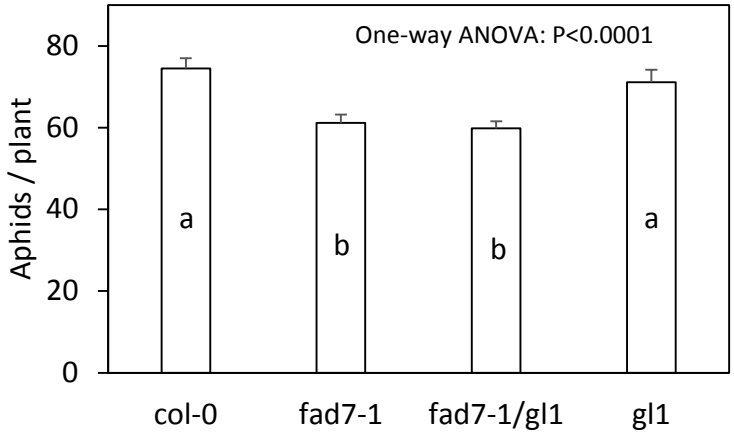


Figure 5. Aphid performance on Arabidopsis lines impaired in conversion of dienoic to trienoic FAs. Bars having the same letter are not significantly different at α=0.05 according to Tukey-Kramer HSD test, and error bars represent SEM (n=20). A) Aphid numbers were compared on mutant lines for FADs localized in the ER (FAD3) or the chloroplast (FAD7, FAD8). B) Aphid numbers were also compared on plants that carried the *fad7-1* mutation with and without the *glabra* (*gl1*) mutation. These experiments were repeated at least twice with similar results (data not shown).

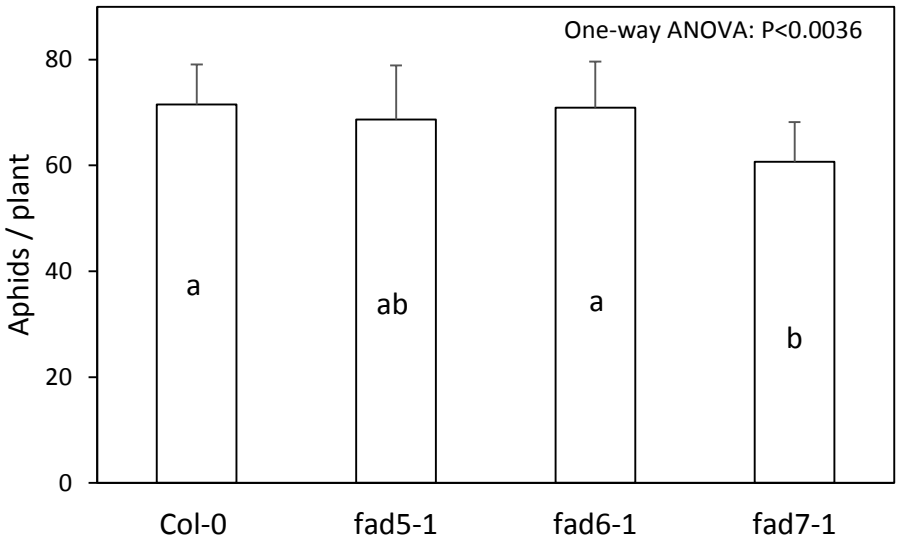


Figure 6. Aphid performance on Arabidopsis lines with impairments in synthesis of monoenoic or dienoic FAs. n=15. Bars having the same letter are not significantly different at α=0.05 according to Tukey-Kramer HSD test, and error bars represent SEM. This experiment was repeated at least 2 times with similar results (data are shown in table 2).

Table 2. Summary of Assays on *fad6-1* mutant

Assay Number	*fad6-1* Size	Fungi Apparent	Live Offspring on *fad6-1*	Live Offspring on WT
1	Smaller	No	39.00±2.89B	53.73±2.89A
2	Smaller, unhealthy	No	28.75±4.69B	41.08±3.45A
3	Smaller, unhealthy	Yes	23.45±2.04B	33.35±2.04A
4	Smaller	No	70.13±3.06B	81.13±3.06A
5	Same Size	No	61.50±2.81A	64.35±2.81A
6	Same Size	No	62.65±2.35A	64.90±2.35A
7*	Same Size	No	70.87±2.53A	71.53±2.53A

* Number 7 was included in Fig 6.

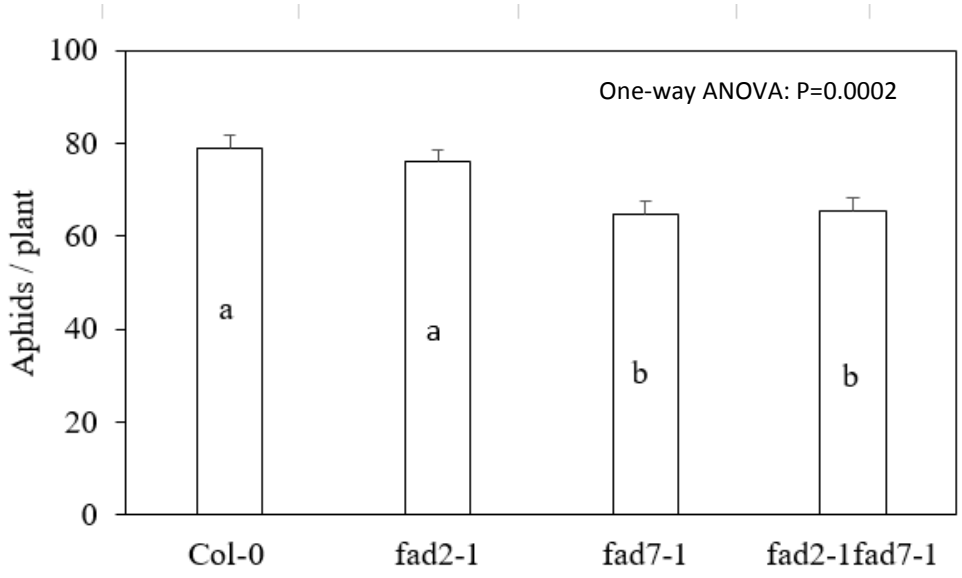


Figure 7. Aphid performance on Arabidopsis lines with different profiles of C18 FAs. n=15. Bars having the same letter are not significantly different at α=0.05 according to Tukey-Kramer HSD test, and error bars represent SEM. This experiment was repeated twice with similar results (data not shown).

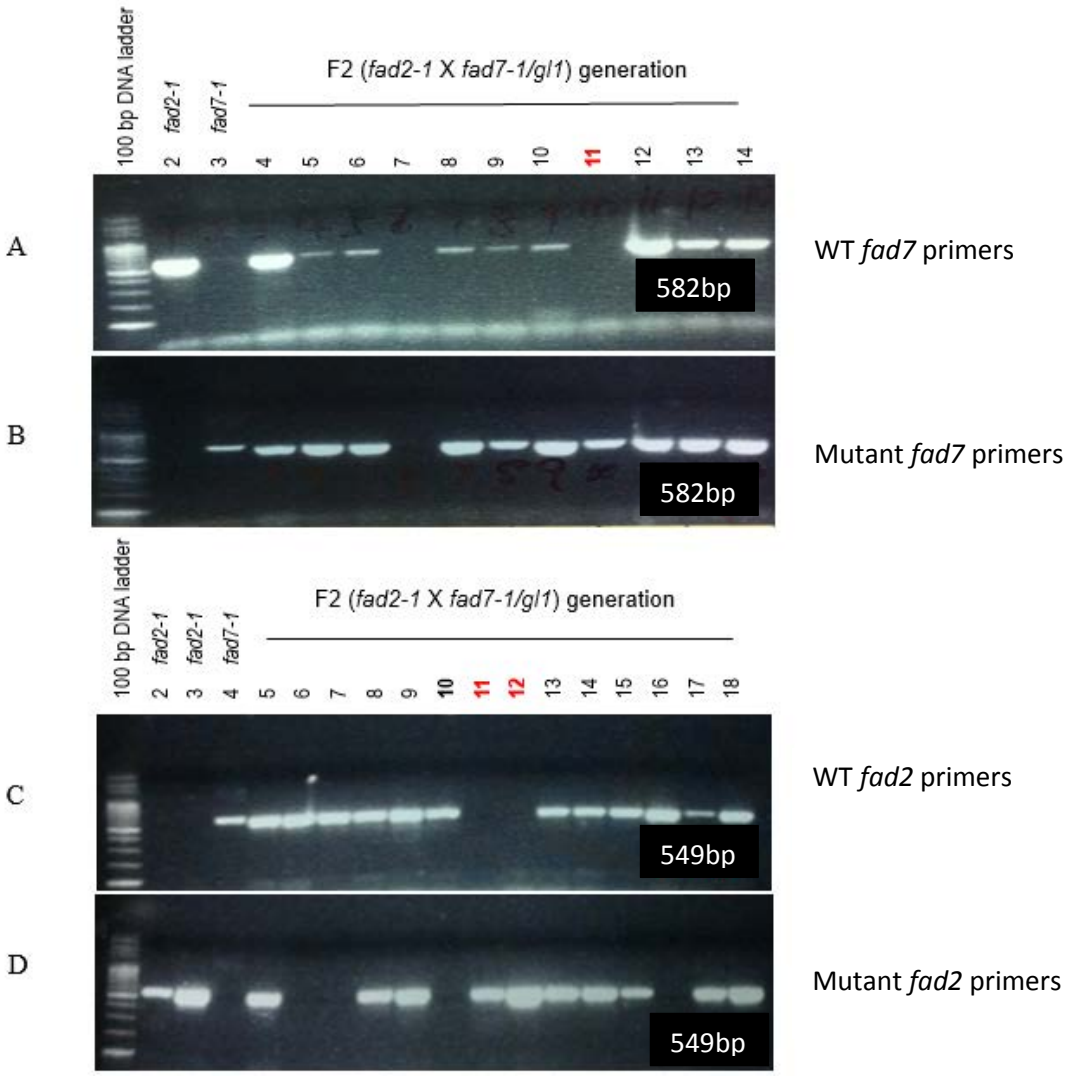


Figure 8. PCR confirmation of *fad2-1fad7-1* double mutants. A) The PCR product for WT *fad7-1* by WT *fad7* primers; B) The PCR product for mutant *fad7-1* by mutant *fad7* primers. C) The PCR product for WT *fad2-1* by WT *fad2* primers; D) The PCR product for mutant *fad2-1* by mutant *fad2* primers.

References

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Chapter III

The Effects of Biosynthetic Pathways Leading to C6 Foliar Volatiles on Plant-Aphid Interactions

Abstract

In tomato, enhanced aphid resistance has been reported in *suppressor of prosystemin-mediated responses2* (*spr2*), a mutant line with impaired function of fatty acid desaturase 7 (FAD7) and altered profiles of fatty acid-derived C6 volatiles. C6 volatiles such as (Z)-3-hexenal and hexanal are important components of the green leaf aroma of plants, and they have been implicated in aphid resistance in potato and other plant species. A goal of this study was to investigate the potential contribution of C6 volatiles to aphid resistance in tomato. To this end, we have suppressed expression of two enzymes required for C6 volatile synthesis, lipoxygenase C (LOXC) and hydroperoxide lyase (HPL), and we have compared volatile profiles, aphid host preference, survival and fecundity in the silenced lines (*AS-LoxC* and *HPL-RNAi*) with wild-type controls and *spr2* mutants. Suppression of HPL expression increased aphid host preference and growth in 5-week-old plants but did not significantly alter aphid numbers in 3-week-old seedlings, possibly because C6 volatile production in wild-type plants was much lower at 3 weeks than at 5 weeks. In contrast, the *spr2* mutation diminished aphid performance at both developmental stages, and antisense suppression of LOXC expression had no significant effects on aphids at either stage. At 5 weeks, the *spr2* mutant and the lines silenced for HPL or LOXC all displayed diminished levels of (E)-2-hexenal and (Z)-3-hexen-1-ol compared to wild-type plants, but unlike the silenced lines, *spr2* had elevated levels of hexanal. To determine if hexanal contributes to aphid resistance in *spr2*, we introduced the silencing construct for HPL into *spr2* through genetic crossing. Although silencing HPL in the *spr2* mutant decreased hexanal accumulation, aphid resistance in these *spr2HPL-RNAi* plants was comparable to aphid resistance in the *spr2* parent. These results suggest that resistance in the *spr2* mutant is independent of C6 volatiles, although these volatiles may contribute to resistance in some other tomato genotypes.

Introduction

The potato aphid, *Macrosiphum euphorbiae*, is one of the most widespread aphid species in the United States. It feeds on a wide range of economically important plants such as potatoes, tomatoes, rose, kale, lettuce and spinach. Potato aphids are able to induce a variety of effects on their hosts, causing chlorosis, decreased growth rates, wilting, stunted growth, low yields and death (Blackman and Eastop, 2000; Goggin, 2007). Moreover, they excrete honeydew which may decrease the rate of photosynthesis and increases rate of leaf senescence as well as attracts black sooty-mold fungi (Minks and Harrewijn, 1989). In addition, the potato aphid is able to transmit over 60 viruses as a vector such as the zucchini yellow mosaic virus, potato leaf roll virus and potato virus Y (Chen et al., 1991). A large amount of research is focused on the biological and chemical control of aphids (Minks and Harrewijn, 1989; Bass et al., 2014) and resistance gene mediated plant defenses (Rossi *et al.*, 1998; Kaloshian *et al*., 2005; Hill et al., 2006; Dogimont et al., 2007; Pegadaraju et al., 2007; Palliparambil et al., 2010). Plant defense is an important aphid-management factor, and is less hazardous to the environment, humans, or wildlife. However, relatively little is known about the physiological or phytochemical bases of plant defense against aphids (Smith, 2005). A number of studies have indicated that fatty acid desaturases (FADs) appear to play an important role in mediating plant defenses against abiotic and biotic stresses.

A fatty acid (FA) is a carboxylic acid with a long aliphatic tail that is unsaturated (consists of carbon-carbon double bonds) or saturated (no carbon-carbon double bonds). Pairs of carbon atoms connected by single bond can be unsaturated by FADs which remove hydrogen atoms from the carbon atoms, converting the single bonds to double bonds. In plants, most FADs are membrane proteins localized in the plastids or the endoplasmic

reticulum (ER), and are responsible for the synthesis of polyunsaturated membrane FAs. FADs in plants influence susceptibility to a variety of abiotic and biotic stresses (Upchurch, 2008; Chen et al, 2013; Dong et al., 2016). The trienoic FAs (C16:3 and C18:3) in membrane lipids of plants contribute low temperature survival by enhancing membrane fluidity. Artificially increasing levels of 16:3 and 18:3 in chloroplast membranes by overexpressing *FAD7 or FAD8* gene in tobacco leaves enhances chilling tolerance in the early growth stages (Iba, 2002). Conversely, decreasing of 16:3 and 18:3 in chloroplast membranes by suppressing the function of FAD7 and FAD8 enhances tobacco resistance to high temperature (Iba, 2002). Increasing 18:3 levels by overexpressing of chloroplast-localized ω-3 fatty acid desaturase FAD8 or ER-localized ω-3 fatty acid desaturase FAD3 also enhance tolerance to salt and drought stress (Upchurch, 2008; Zhang et al, 2005; Klinkenberg et al., 2014). Moreover, FADs influence plant defenses against a variety of biotic stresses as well. For example, suppressing the *OsFAD7* and *OsFAD8* genes in rice increased resistance to the rice blast fungus *Magnaporthe grisea* (Yara et al., 2007). Defects in suppressor of salicylic acid insensitivity2 (SSI2), a FAD that converts stearic acid (C18:0) to oleic acid (C18:1), result in increased resistance to biotrophic pathogens whereas increased susceptibility to necrotrophic pathogens (Kachroo et al., 2001; Shah et al., 2001).

Recently, it was also discovered that a ω-3 FAD negatively regulates plant defenses against aphids in tomato (*Solanum lycopersicum*, Solanaceae) and Arabidopsis (*Arabidopsis thaliana*, Brassicaceae) (Avila et al., 2012). LeFAD7 is a chloroplast-localized ω-3 FAD, and desaturates 16- and 18-carbon fatty acids with two double bonds (dienoic acids; C16:2 and C18:2) to FAs with three double bonds (trienoic acids; C16:3 and C18:3) (Li et al., 2003). The *suppressor of prosystemin-mediated responses2* (*spr2*) mutant in tomato (*Solanum*

lycopersicum, Solanaceae), which has a point mutation that results in the loss of function of LeFAD7, has enhanced resistance to potato aphids (*Macrosiphum euphorbiae*) compared to normal or wild-type plants (Avila et al., 2012). Moreover, the Arabidopsis *fad7-2* mutant with impaired FAD7 function, is also significantly resistant to green peach aphids as compared to wild type. Therefore, FADs directly or indirectly influence plant-aphid interactions in more than one plant family. Previous experiments have demonstrated that the *spr2* tomato mutant with loss of function of FAD7 results in decreases in aphid settling, survival and fecundity (Avila et al., 2012). The aphid mortality rate was over 50 percent higher on *spr2* than on wild-type controls (Avila et al., 2012). These results indicate that FADs or their derivatives modulate host suitability for aphids.

One way that FADs could possibly influence plant defenses against aphids is by affecting C6 volatile synthesis. Linoleic acid (C18:2) and linolenic acid (C18:3) are precursors for a variety of compounds including C6 volatiles. C6 volatile compounds such as C6 aldehydes and alcohols are not only important constituents of plant flavor (Chen et al., 2004), but also can act as fungicidal and antibacterial constitutes when plants encounter stresses (Croft et al., 1993). The release of C6 volatiles can be increased by pathogens and herbivore infestations (Heiden et al., 2003; Shiojiri et al., 2006; Turlings et al., 1995; Chehab et al., 2007). The pathogenic bacteria-induced release of (E)-2-hexenal and (Z)-3-hexenol from lima bean leaves inhibits growth of the pathogenic bacterium *Pseudomonas syringae* (Croft *et al*., 1993). In addition to acting as direct defenses, C6 volatiles can also promote indirect defenses; for example, the C6 volatile hexenyl acetate attracts the parasitoid wasp *Aphidius colemani*, a natural enemy of aphids (Chehab et al., 2007). (Z)-3-hexen-1-ol is released from tobacco plants (*Nicotiana attenuata*) which are attacked by *Manduca sexta*

caterpillar, and attracts predators of *Manduca sexta* larvae to decrease herbivore survival (Kessler and Baldwin, 2002). In tomato, C6 volatiles are synthesized from the polyunsaturated C18:2 and C18:3 through the successive action of the enzymes lipoxygenase (LOX) and hydroperoxide lyase (HPL) (Halitschke et al., 2004; Canoles and Beaudry, 2006) (Fig.1). A family of metabolites derived from oxidation of polyunsaturated FAs are generated through 13-LOX and 9-LOX catalyzed pathways. C6 volatiles are formed by 13-LOX pathway, and in tomato, there are six genes (*TomloxA-F*) encoding different isoforms of LOX (LOXA-F) (Chen et al., 2004). LOXA, LOXB, LOXE are enzymes in 9-LOX pathway, and not involved in C6 volatile synthesis; LOXC, LOXD and LOXF are enzymes in 13-LOX pathway, and LOXC is essential for synthesis of C6 volatiles (Griffiths et al., 1999; Chen et al., 2004; Shen et al., 2014). 13-HPL (hereafter described as HPL), cleaves hydroperoxides formed by 13-LOX into C6 volatiles. Similar to LOX, in addition to 13-HPL there are two other subfamilies of HPLs: 9-HPL which cleaves 9-hydroperoxides, and 9/13-HPL which cleaves 9-and 13-hydroperoxides (Scala et al., 2013; Froehlich et al., 2001; Fauconnier et al., 1997). It is reported that antisense expression of a *13-HPL* gene in potato resulted in increased aphid susceptibility (Vancanneyt et al., 2001). HPL catalyzes synthesis of hexanal and hexanol, which have antibiotic activity against aphids when tested in vitro (Hildebrand et al., 1993). Another report indicated that overexpression of a tea HPL gene (*CsiHPL1*) in tomato (*Solanum lycopersicum*) affected resistance to a herbivore *Prodenia litura* and a fungus *Alternaria alternata* f. sp. *lycopersici* (AAL) by regulating jasmonic acid gene expression and C6 volatile emission (Xin et al., 2014). These studies suggest that C6 volatiles play an important role in plant interactions with aphids and other pests.

In the aphid-resistant tomato mutant *spr2*, impaired FAD7 functions results in

increased C18:2 and decreased C18:3, which in turn results in significantly more hexanal and hexanol, and less (Z)-3-hexanal and (Z)-3-hexanol than observed in wild-type plants (Canoles and Beaudry, 2006; Sanchez-Henandez et al., 2006). The altered C6 volatile levels in *spr2* could influence plant responses to aphids. The goal of this study was to investigate the potential contribution of C6 volatiles to plant defenses against aphids in tomato. We tested whether aphid infestations on tomato increase when we suppress expression of two enzymes required for C6 volatile synthesis, LOXC and HPL. We compared potato aphid performance and C6 volatile profiles in a mutant tomato line with impaired function of FAD7 (*spr2*), transgenic lines silenced for LOXC or HPL, and their respective wild-type controls; we also developed a line *spr2HPL-RNAi* that is deficient in both FAD7 and HPL expression.

Materials and methods

Plant and insect materials

Seven tomato (*Solanum lycopersicum L.*) genotypes were used in this study: Castlemart (CM), *suppressor of prosystemin-mediated responses 2* (*spr2*) with impaired FAD7 activity, M82, a transgenic line (*AS-LoxC*) with antisense suppression of *TomloxC*, Flora-Dade, a transgenic line (*HPL-RNAi*) silenced for HPL, and double mutant line (*spr2HPL-RNAi*) deficient in both FAD7 and HPL expression. The *spr2* mutant carries a point mutation that prevents the expression of a functional LeFAD7 protein (Li et al., 2003), and CM is the genetic background for *spr2*. The antisense transgenic line *AS-LoxC* inhibits the expression of the lipoxygenase C (LOXC) protein by expressing an antisense construct and thus silencing the gene post-transcriptionally (Tieman et al., 2012). M82 is the wild-type control for *AS-LoxC*. The transgenic line *HPL-RNAi* inhibits the expression of the hydroperoxide lyase (HPL) protein by introducing an RNA interference (RNAi) construct for

HPL into Flora-Dade (Shen et al., 2014), and Flora-Dade is the wild-type control for the *HPL-RNAi*. Moreover, a double mutant line *spr2HPL-RNAi* was produced by crossing *spr2* and *HPL-RNAi* (described below). Seeds of CM and *spr2* have been provided by Gregg Howe at Michigan State University; M82, *AS-LoxC*, Flora-Dade and *HPL-RNAi* have been provided by Harry Klee and Denise Tieman at the University of Florida.

All the tomato control and mutant plants were grown in LC1 Sunshine potting mix (Sungro Horticulture, Bellevue, WA) with 15-9-12 Osmocote slow-release fertilizer (Scotts-MiracleGro Company, Marysville, OH), and kept in growth chambers (Controlled Environments, Inc., Winnipeg, Canada) at 23 °C and L16:D8 photoperiod, watered with a dilute nutrient solution containing 1000 ppm $CaNO_3$ (Hydro Agri North America, Tampa, FL), 500ppm $MgSO_4$ (Giles Chemical Corp, Waynesville, NC), and 500ppm 4-18-38 Gromore fertilizer (Gromore, Gardena, CA). This study used the potato aphid (*Macrosiphum euphorbiae*, isolate WU11) which was reared on an aphid-susceptible tomato cultivar (UC82), potato (*Solanum tuberosum* Linnaeus), and jimson weed (*Datura stramonium* Linnaeus) plants in Conviron growth chambers (Controlled Environments, Inc., Winnipeg, Canada) at 20 °C and 16-hr light photoperiod.

Development and characterization of tomato *spr2HPL-RNAi* double mutant line

The double mutant line *spr2HPL-RNAi* was produced by crossing *spr2* and *HPL-RNAi*. The *spr2* was the maternal parent and *HPL-RNAi* was the pollen donor. The (*spr2* x *HPL-RNAi*) F_1 hybrid generation was self-pollinated to obtain the (*spr2* x *HPL-RNAi*) F_2 generation. The (*spr2* x *HPL-RNAi*) F_2 plants were screened by PCR for presence or absence of the *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'; DNA was isolated using the DNAeasy® plant mini kit (Qiagen, Maryland) and touchdown PCR was performed to increase amplification sensitivity and specificity using the following program: 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. Then plants that were homozygous for the *spr2* mutation were screened for the presence of *HPL* transgene using the NPTII (Neomycin phosphotransferase II) primers: forward 5'-GCAATATCACGGGTAGCCAA-3'; reverse 5'-GCCGTGTTCCGGCTGTCA-3'. NPTII is resistant to kanamycin, and frequently used for selecting transgenic plants (Suratman et al., 2013). PCR was performed using the following program: 95 °C for 5 min; 95 °C for 45 sec, 50 °C for 45 sec, and 72 °C for 45 sec (30 cycles); and final extension at 72 °C for 5 min. PCR products were separated by electrophoresis on 1 % agarose gels.

Identification of the *HPL* gene in the Arabidopsis with *fad7-1 mutant*

Whether the plants carry a mutant or wild-type allele for the *HPL* gene was investigated by PCR using the HPL primers: forward 5'-GGACCGTTTAGATTACTTCTGGTT-3'; reverse 5'- CGGAAGTCTCCGATGAGAAC-3'. The primers for *HPL* were designed based on genomic DNA (AT4G15440.1) by adding the 10 bp deleted sequence by Avila in our lab. The primers can only amplify Arabidopsis lines having a complete *HPL* genomic sequence (222 bp amplicon). The PCR amplification conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 45 s; 55 °C for 45 s, and 72 °C for 45 sec, and a final extension at 72 °C for 5 min. For the separation of

PCR products, 1 % agarose gel electrophoresis was conducted.

Aphid performance bioassays

Aphid host preference

Settling behavior was measured by placing adult aphids between paired genotypes and allowing them to colonize the plants or move back and forth between them. Wingless adult potato aphids within 24 h of emergence to adulthood were released in the middle of the styrofoam choice arenas (15 cm diameter) between leaflets with uniform node position on intact tomato plants. Offspring production by adults can indicate host acceptance, so the location of adults and offspring production were recorded at different time points (1h, 6h, 24h and 48h) after release. There were 10-15 replicate pairs for each combination of genotypes. Five-week-old tomato plants were used in this study; younger plants were too fragile to allow placement of the choice arenas.

Aphid survival and fecundity

Aphid survival and fecundity were measured by confining adult aphids to individual leaflets using lightweight clip cages and allowing to count living and dead aphids (adults and offspring) in each cage of the plants. Wingless adult potato aphids within 24 h of emergence to adulthood were caged to leaflets with uniform node position on three-week-old and five-week-old tomato plants. Total living and dead aphids including adults and offspring were recorded at six days after inoculation (6-DAI). There were at least 10 replicate plants each genotype, 4 adults per cage, and 2 cages each plant. All plants inoculated with aphids were maintained in Conviron growth chambers (Controlled Environments, Inc., Winnipeg, Canada) at 23 °C and 16L: 8D photoperiod.

Tissue collection and volatile analysis

The third completely expanded leaf tissue was collected from three-week-old and five-week-old tomato plants Castlemart, *spr2*, M82, *AS-LoxC*, Flora-Dade, and *HPL-RNAi*. The other set of foliar tissue was collected from five-week-old tomato plants Castlemart, *spr2*, Flora-Dade, *HPL-RNAi* and *spr2HPL-RNAi*. The leaflets were quickly detached, weighed, and placed in 20-mL glass vials. Then the leaflets were flash frozen by adding liquid nitrogen, and the internal standard d32-pentadecane were added into the vials. The leaflets were quickly grinded followed by adding 1 mL of 50 % $CaCl_2$ solution, and the vial was immediately capped then stored at -20 °C. The samples were analyzed by Dr. Denise Tieman (Horticultural Sciences, University of Florida). Prior to the collection of volatiles from the headspace by a solid-phase microextraction (SPME) fiber (65 µm PDMS-DVB; Supelco), the samples were thawed at room temperature, and then were heated at 40 °C for 5 min with intermittent shaking in the autosampler's heating block. Then the SPME fiber was inserted into the vials in the heating block, and volatiles were collected on the fiber for 15min. The fiber was injected into the GC sample inlet, and desorbed for 1 min at 250 °C. Gas chromatography (HP 6890; Agilent) analysis used a column (30 m x 0.25 mm with a 1 µm film thickness; Agilent J&W DB-5ms). The initial temperature of the GC oven was 35 °C for 1 min, and was ramped to 150 °C at a rate of 5 °C /min then ramped to 250 °C at a rate of 10 °C /min. Volatiles were quantified based on an internal standard (d32-pentadecane), and identified by comparing retention times and mass spectra to known standards (SigmaAldrich, St. Louis, MO). C6 volatile values were normalized to the internal standard as described below. The peak areas of the internal standard (d32-pentadecane) from all the samples were averaged, and the average value was used as the expected peak area for the internal standard. The recovery rate of the internal standard was calculated by dividing the observed peak areas

for the standard by the expected peak area average. The samples that had less than 10 % were excluded as outliers. To the end, the normalized C6 volatile values were obtained by multiplying the estimated amount of C6 volatiles by the observed peak areas for the internal standard, and then divided by the expected areas for the internal standard.

Statistical analysis

The statistical analysis was done with JMP ® v 11 (SAS Institute Inc.). The mean differences between aphid performances in WT versus mutant plants in the host preference assays were analyzed by matched pairs one sided *t*-tests within each time point. Aphid survival and fecundity were analyzed by one-way ANOVA and Tukey-Kramer HSD, $P < 0.05$ indicated statistical significance. Foliar C6 volatile levels from five-week-old tomato plants Castlemart, *spr2*, Flora-Dade, *HPL-RNAi* and *spr2HPL-RNAi* were analyzed using Wilcoxon test.

Results

Aphid Host Preference

Pair-wise choice assays were performed to determine if aphid settling behavior differed on five-week-old tomato plants with modified C6 volatile production (*spr2, AS-LoxC, HPL-RNAi*) compared to their respective wild-type control lines (cultivars Castlemart, Flora-Dade, and M82, respectively). The number of adults that settled on each genotype was tracked over the first 48 h of exposure, and offspring numbers were also recorded because reproduction is a well-established marker of host plant acceptance (Powell et al., 2006). When *spr2* was compared to Castlemart (Fig. 2A), the number of adults on the two genotypes was comparable 1h after introducing aphids into the choice arena (p=0.19), but the proportion

of aphids on *spr2* decreased steadily over time and was significantly lower compared to Castlemart at 6 h ($p=0.0051$), 24 h ($p<0.0001$), and 48 h ($p<0.0001$) after inoculation. Offspring (Fig 2B) were first observed at 6h, and were significantly lower on *spr2* compared to Castlemart at 24 h ($p<0.0001$), and 48 h ($p<0.0001$). These results indicate that aphids strongly preferred the wild-type control to the *spr2* mutant, and suggest that aphid host preference manifested itself after the aphids had the opportunity to sample the plants. In contrast, the number of adult aphids (Fig. 2C) and offspring (Fig. 2D) on *AS-LoxC* and its wild-type control M82 did not differ significantly at any time point ($P > 0.05$). When *HPL-RNAi* was compared to its wild-type control Flora-Dade, the numbers of adult aphids were significantly higher on *HPL-RNAi* at 6 h ($p=0.0375$), 24 h ($p=0.0088$) and 48 h ($p=0.0376$) (Fig. 2E); and the numbers of offspring were significantly higher on *HPL-RNAi* at 24 h ($p=0.0437$) and 48 h ($p=0.0301$) (Fig. 2F). These data suggest that aphid host preference is enhanced on the *HPL-RNAi* line compared to wild-type plants, whereas host preference is unaffected in the *AS-LoxC* line and is reduced on the *spr2* mutant compared to their respective control lines.

Aphid survival and fecundity

No-choice assays were used to assess whether adult aphid survival and fecundity as well as the offspring survival differed among tomato lines with variation in C6 volatiles. Adult aphids were confined to individual leaflets on three-week-old and five-week-old tomato plants using lightweight clip cages. The live and dead aphids including adults and offspring were recorded at six days after inoculation (6-DAI). Adult survival on three-week-old *spr2* significantly differed from aphid performance on the wild-type control Castlemart (Fig. 3A). However, on five-week-old plants, 100 % of adults on *spr2* died before the end of

the assay, and the number of live adults was significantly higher than wild-type control (Fig. 4A). The total numbers of offspring (live and dead), which is a measure of adult fecundity, were over 50 % lower on *spr2* than on Castlemart on three-week-old plants (p=0.0017) (Fig. 3D) and five-week-old plants (p<0.0001) (Fig. 4D). The offspring survival on three-week-old *spr2* decreases over 9-fold as compared to wild-type control Castlemart (p<0.0001) (Fig. 3G). At five weeks after germination, *spr2* had significantly lower offspring survival (P<0.0001) as well (Fig. 4G). In contrast, there were no significant differences in adult survival, fecundity, or offspring survival on either three-week-old or five-week-old *AS-LoxC* as compared to its control M82 (Fig. 3B, E, and H; 4B, E, and H). Adult survival on three- and five-week-old *HPL-RNAi* were not significantly different from survival on the control Flora-Dade (Fig. 3C and 4C). The total numbers of offspring (live and dead) on *HPL-RNAi* were not significantly different from its control Flora-Dade at three weeks (Fig.3F), but were significantly higher than its control Flora-Dade at five weeks (p=0.0355) (Fig. 4F). The offspring survival did not show significant difference between three-week-old *HPL-RNAi* and its control Flora-Dade (Fig. 3I). The offspring survival was significantly higher on five-week-old *HPL-RNAi* (p=0.0276) (Fig. 4I). These data indicated that *spr2* mutant decreased aphid survival and fecundity, *HPL-RNAi* mutant did not affect adult survival but increased adult fecundity and offspring survival. Moreover, when compared the two assays with different aged plants, the five-week-old *spr2* mutant more significantly affects adult aphid survival than three-week-old. The five-week-old *HPL-RNAi* mutant more significantly affect adult fecundity and offspring survival than three-week-old. Therefore, non-choice assays to assess aphid performance in the double mutant *spr2HPL-RNAi* were done at five weeks to focus on a timepoint when both *spr2* and *HPL-RNAi* impact aphid resistance. The purpose of assays on the double mutant *spr2HPL-RNAi* was to investigate whether aphid resistance would be

compromised on *spr2* by introducing the silencing construct for *HPL* into *spr2*.

Adult survival on five-week-old *spr2HPL-RNAi* was significant lower than on the wide-type controls Castlemart and Flora-Dade, but was similar to *spr2* (Fig.5A). All of the original confined adults on five-week-old *spr2HPL-RNAi* and *spr2* were dead (Fig.5A). The total numbers of offspring (live plus dead) on five-week-old *spr2HPL-RNAi* were significantly lower than its controls ($p<0.0001$) (Fig. 5B), but were similar to *spr2*. The five-week-old *spr2HPL-RNAi* had significantly lower live offspring survival than wide-type controls Castlemart and Flora-Dade ($P<0.0001$), but did not statistically differ from *spr2* (Fig. 5C). These data indicated that levels of aphid resistance in the *spr2HPL-RNAi* double mutant were comparable to levels of resistance in *spr2*.

Foliar Volatiles

C6 volatile profiles were compared at three- and five weeks among the tomato genotypes with differing levels of aphid resistance. The foliar volatiles were collected from the headspace by a solid-phase microextraction (SPME) fiber, and quantified on GC-MS to determine if any specific volatiles were correlated with aphid resistance. The foliar C6 volatiles in five-week-old wild-type plants were higher than in three-week old plant controls except (Z)-3-hexen-1-ol in Castlemart (Table 1). However, the variation among samples was large. For instance, within the five-week-old Castlemart treatment group, one sample showed ~170 times higher amount of (E)-2-hexenal than the other sample. Because we did not add an internal standard when the foliar tissue was collected, we could not determine if this variation was due to biological variation or to errors to sample collection, shipping or analysis. For this reason, we used this data for exploratory purposes only, and are not presenting a statistical analysis of this data.

However, we added an internal standard when we collected the other set of foliar tissue from five-week-old tomato plants. The foliar C6 volatile measurement in those five-week-old tomato plants showed that the *spr2* mutation resulted in significantly higher levels of hexanal (P= 0.014, Fig 6A), lower levels of (Z)-3-hexen-1-ol (P= 0.014, Fig 6D), and normal levels of (Z)-3-hexenal and (E)-2-hexenal than the wild-type control Castlemart (Fig 6B-C). The abundance of foliar C6 volatiles in five-week-old *HPL-RNAi* did not significantly differ from the wild-type control Flora-Dade (Fig 6). When we measured the C6 volatiles in the double mutant *spr2HPL-RNAi*, we found that levels of hexanal were lower in this line than in *spr2* (Fig 6A). In addition, levels of (Z)-3-hexen-1-ol were significantly lower in *spr2HPL-RNAi* than in wild-type Castlemart and Flora-Dade plants, but were comparable to *spr2* and *HPL-RNAi* (Fig 6D).

Identification of the *HPL* gene in the Arabidopsis *fad7-1* mutant

To investigate whether Arabidopsis *fad7-1* mutant carries a mutant or wild-type allele for the *HPL* gene, the PCR was conducted. The PCR product for wild-type allele of *HPL* gene in Nossen was 222 base pairs (Supplemental Fig lane 5 and 6) which can be amplified by the wild-type HPL primers; in contrast, Col-0 (Supplemental Figure lane 7 and 8), *gl1* (Supplemental Figure lane 3 and 4), and *fad7-1/gl1* (Supplemental Figure lane 1 and 2) carry a mutant allele for the *HPL* gene which can not be amplified by the wild-type HPL primers.

Discussion

Several investigations have shown that a number of C6 volatile organic compounds in plants play a role in plant responses to biotic factors (Dicke and Baldwin, 2010; Christensen and Kolomiets, 2011). C6 volatiles are mainly derived from the C18 polyunsaturated FA

substrates C18:2 and C18:3, and the relative abundance of these two compounds is regulated by FAD7, which converts C18:2 to C18:3. The tomato mutant *spr2* with impaired FAD7 function showed resistance to potato aphids; thus this study sought to investigate whether C6 volatiles contribute the aphid resistance. In all three wild-type lines (Castlemart, M82, and Flora-Dade), the five-week-old plants released higher levels of C6 volatiles than three-week-old ones. The variation of C6 volatiles between two plant growth stages indicated that the levels of foliar C6 volatiles was influenced by plant development. Compared to wild-type Flora-Dade plants, the tomato line *HPL-RNAi* with suppression of HPL expression showed increased aphid host preference and growth in five-week-old plants but did not significantly alter aphid numbers in three-week-old seedlings; this difference in how *HPL-RNAi* affects aphids in three- versus five-week-old plants may be due to the fact that C6 volatile synthesis in wild-type plants increases dramatically between three and five weeks. In contrast, the *spr2* mutation enhanced aphid resistance at both three- and five-week old plants. The fact that aphid resistance in *spr2* appeared to be independent of plant age even though volatiles increased with age suggests that resistance in this genotype is not heavily dependent on volatiles. Antisense suppression of LOXC expression also had no significant effects on aphid infestations at either plant development stage.

This study confirmed that C6 volatile formation in the aphid-resistant mutant *spr2* with impaired FAD7 function differs from wild-type control. The *spr2* mutation that impaired FAD7 desaturase activity resulted in increased formation of hexanal and decreased (Z)-3-hexen-1-ol in five-week-old *spr2* plants (Fig 6A & D). To determine if hexanal contributes to aphid resistance in *spr2*, we introduced the silencing construct for HPL into *spr2* through genetic crossing, although silencing HPL in the *spr2* mutant suppressed hexanal

accumulation, aphid resistance in these *spr2HPL-RNAi* plants was comparable to aphid resistance in the *spr2* parent. In addition, the levels of (Z)-3-hexen-1-ol in five-week-old *spr2*, *HPL-RNAi* and *spr2HPL-RNAi* plants were comparable (Fig 6D), but *spr2* and *spr2HPL-RNAi* plants were resistant to aphids whereas *HPL-RNAi* plants were more susceptible to aphids. These results indicate that resistance in the *spr2* mutant is independent of C6 volatiles, although these volatiles may contribute to resistance in some other tomato genotypes. In addition, the aphid-resistant *fad7* mutant in Arabidopsis also carries a mutation in the *HPL* gene, which is required for C6 volatile synthesis. Thus, aphid resistance in *spr2* appears to be due to other consequences of altered fatty acid profiles.

HPL is an important enzyme for synthesis of foliar C6 volatiles in tomato (Chen et al., 2004; Shen et al. 2014). However, foliar levels of the C6 volatiles in five-week-old transgenic line *HPL-RNAi* did not show significantly difference from wild-type control Flora-Dade (Fig 6). The five-week-old *HPL-RNAi* plant was significantly more susceptible to aphids than its wild-type control Flora-Dade. These results indicated that aphid susceptibility in *HPL-RNAi* may not be related to C6 volatiles. The HPL and allene oxide synthase (AOS) pathways are respectively involved in the synthesis of C6 volatiles and JA, and exhibit crosstalk each other to function in stress responses (Liu et al., 2012; Halitschke et al., 2004; Howe et al., 2002; Creelman et al., 2002). The HPL-depleted mutant rice had increased levels of JA and reduced C6 aldehyde, and JA-signaling defenses were activated against rice bacterial blight pathogen *Xanthomonasoryzaepvoryzae* (*Xoo*) (Liu et al., 2012). The aphid susceptibility in HPL-RNAi may be related to oxylipin metabolites such as JA in AOS pathway.

In conclusion, the aphid-resistant mutant *spr2* has modified profiles of FA-derived C6 volatiles synthesized through the HPL pathway. Suppression of HPL expression causes a

modest increase in aphid fecundity on five-week-old plants, but HPL is not essential to aphid resistance in *spr2* mutants. Volatiles synthesize through the HPL pathway appear to contribute to basal defenses in tomato, but are not critical to *spr2*-dependent resistance.

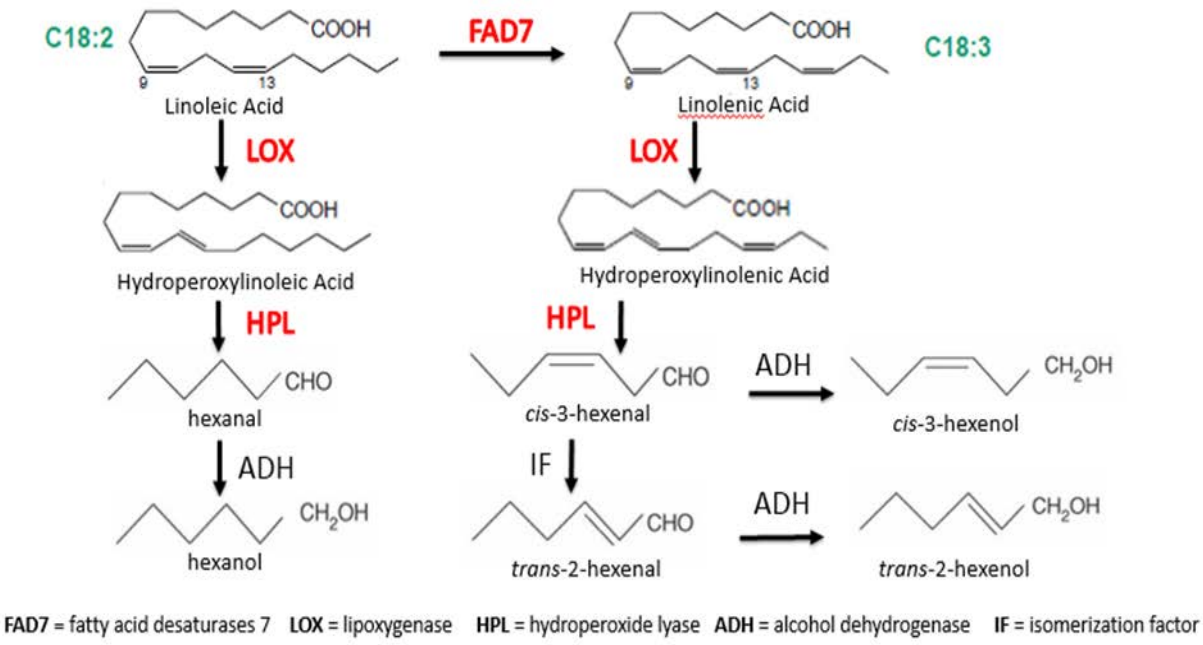


Figure 1. Biochemical pathway for synthesis of C6 volatiles in tomato. In tomato, C6 volatiles are synthesized from the polyunsaturated fatty acids linoleic acid (C18:2) and linolenic acid (C18:3) through the successive action of the enzymes lipoxygenase (LOX), hydroperoxide lyase (HPL), and alcohol dehydrogenase (ADH). Fatty acid desaturase 7 (FAD7) is an omega-3 FAD that desaturates linoleic acid to generate linolenic acid.

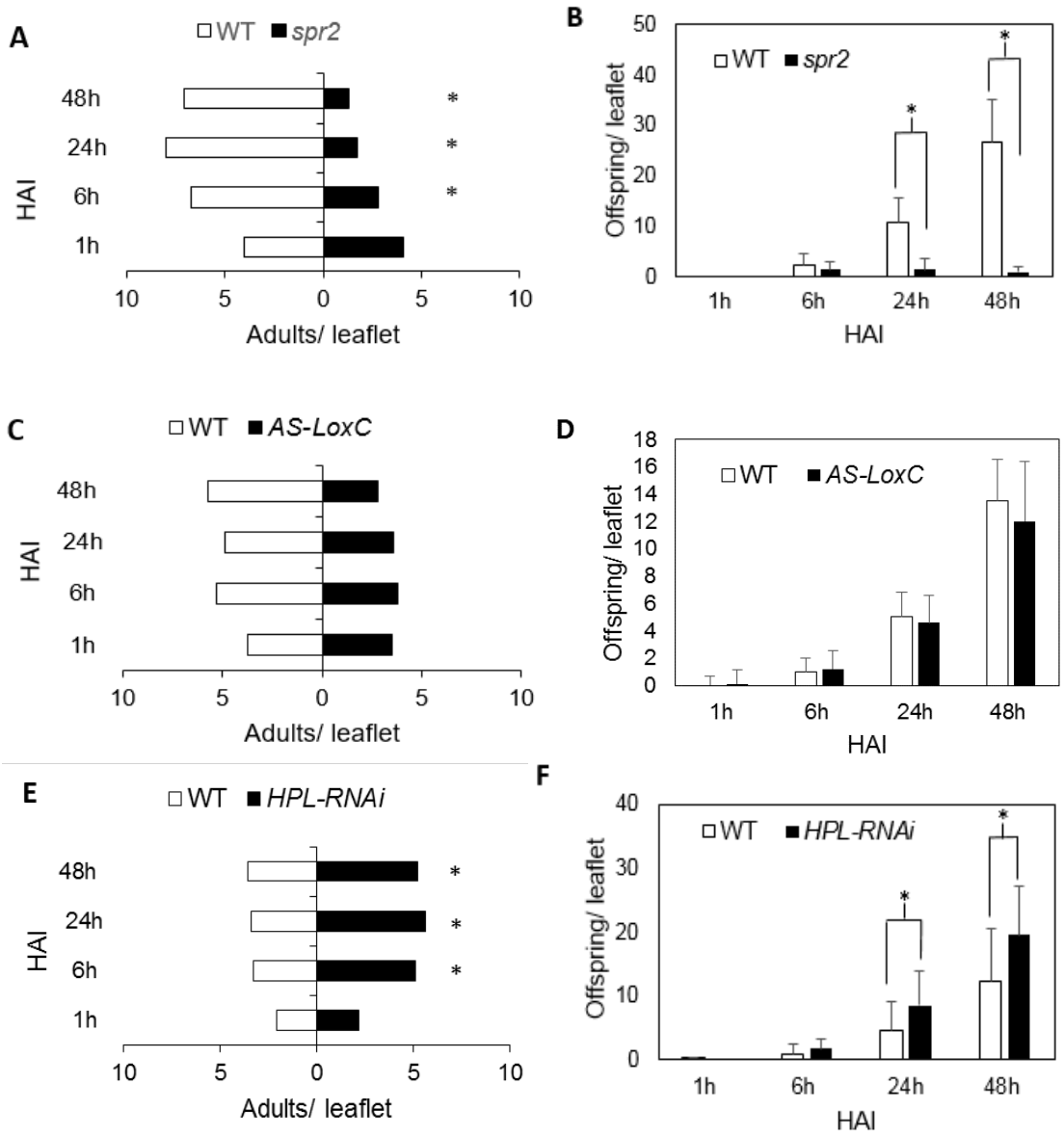


Figure 2. Aphid host preference on tomato lines with modified volatile synthesis pathways at five weeks. Choice assays were performed to compare aphid settling on *spr2*, *HPL-RNAi*, *AS-LoxC* with settling behavior on the respective wild-type (WT) controls from each line: Castlemart (CM), M82, and Flora-Dade (FD). Adult potato aphids were offered a choice of two plants from different genotypes (14 aphids per pair of plants; 10 pairs of plants for panels A-D, and 15 pairs of plants for panels E-F). Aphid settling behavior was assessed by recording on which plant the adults were located, and how many offspring they produced at 1h-, 6h-, 24h- and 48hrs- after inoculation (HAI). The data for each comparison was analyzed by Matched pairs one-sided *t*-tests within each time point in JMP® v 11 (SAS Institute, NC). Asterisks (*) indicates statistically significant differences at α=0.05, and error bars represent SEM.

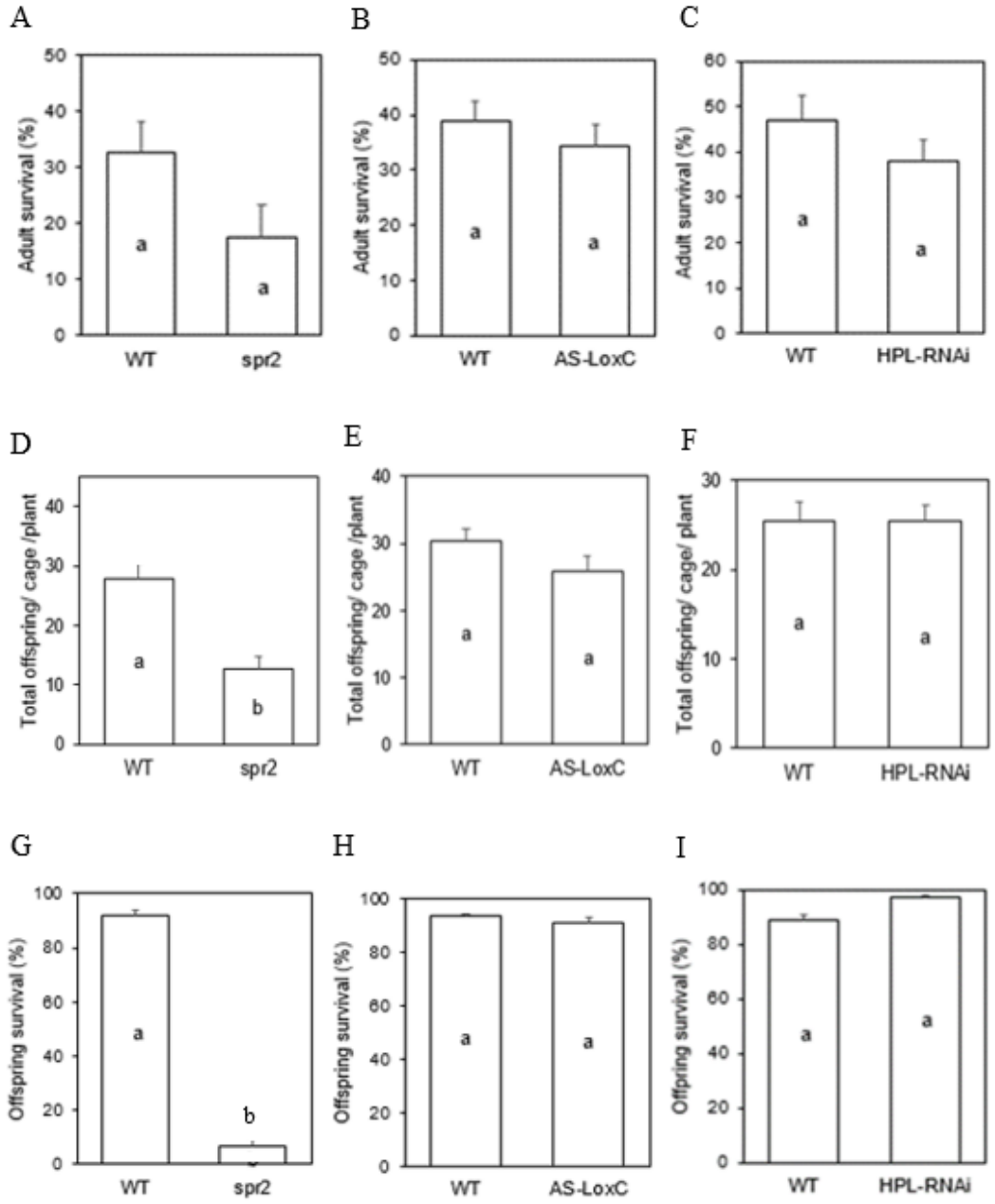


Figure 3. **Aphid survival and reproduction on tomato lines with modified volatile synthesis pathways at three weeks.** The average number of adults and offspring each cage each plant was analyzed among different genotypes by one-way ANOVA and student's *t*-test with JMP ® v 11 (SAS Institute, NC). Bars having the same letter are not significantly different at α=0.05, and error bars represent SEM.

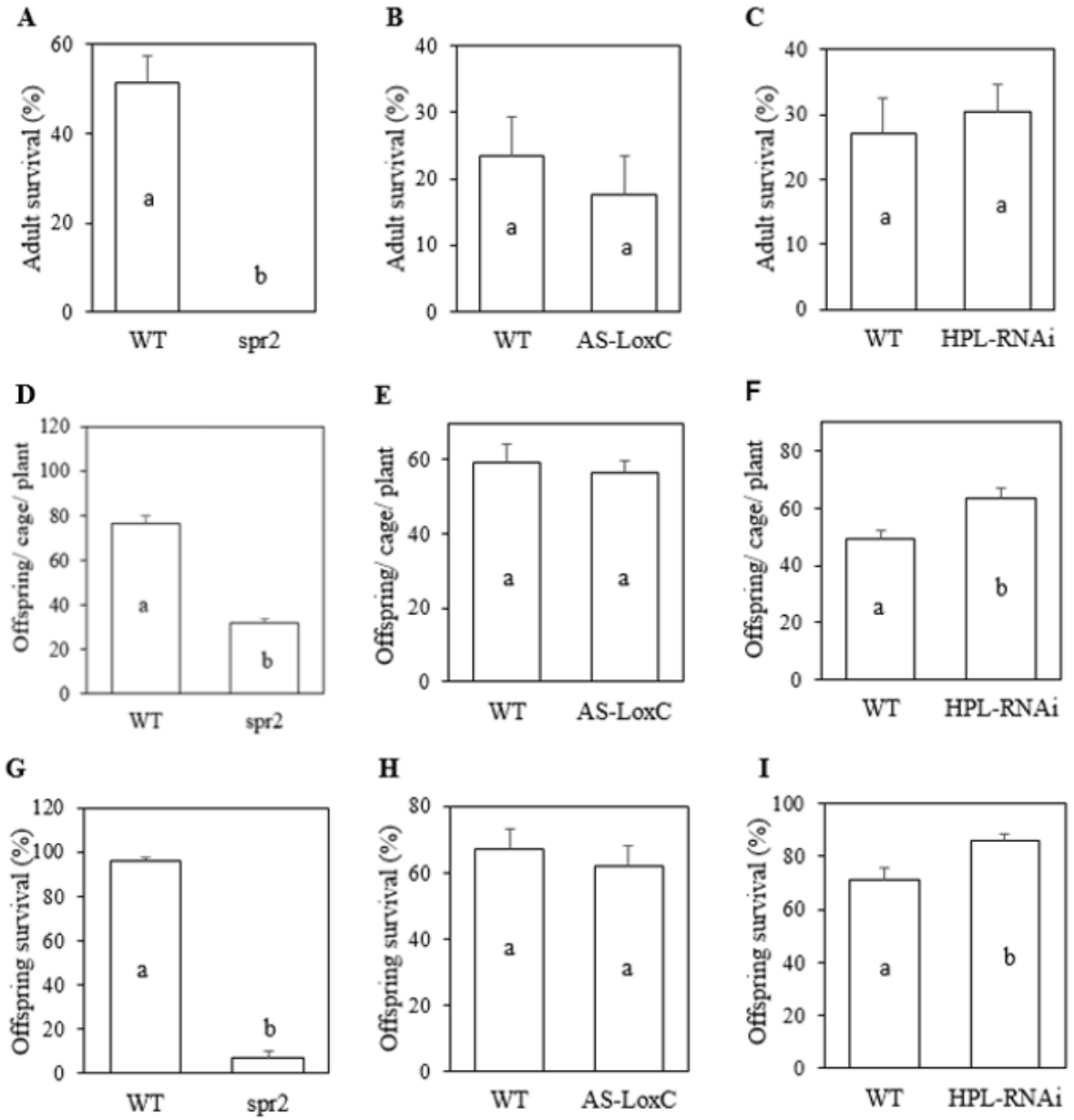


Figure 4. **Aphid survival and reproduction on tomato lines with modified volatile synthesis pathways at five weeks.** The average number of adults and offspring each cage each plant was analyzed among different genotypes by one-way ANOVA and student's *t*-test with JMP ® v 11 (SAS Institute, NC). Bars having the same letter are not significantly different at α=0.05, and error bars represent SEM. Bars having the same letter are not significantly different at α=0.05, and error bars represent SEM.

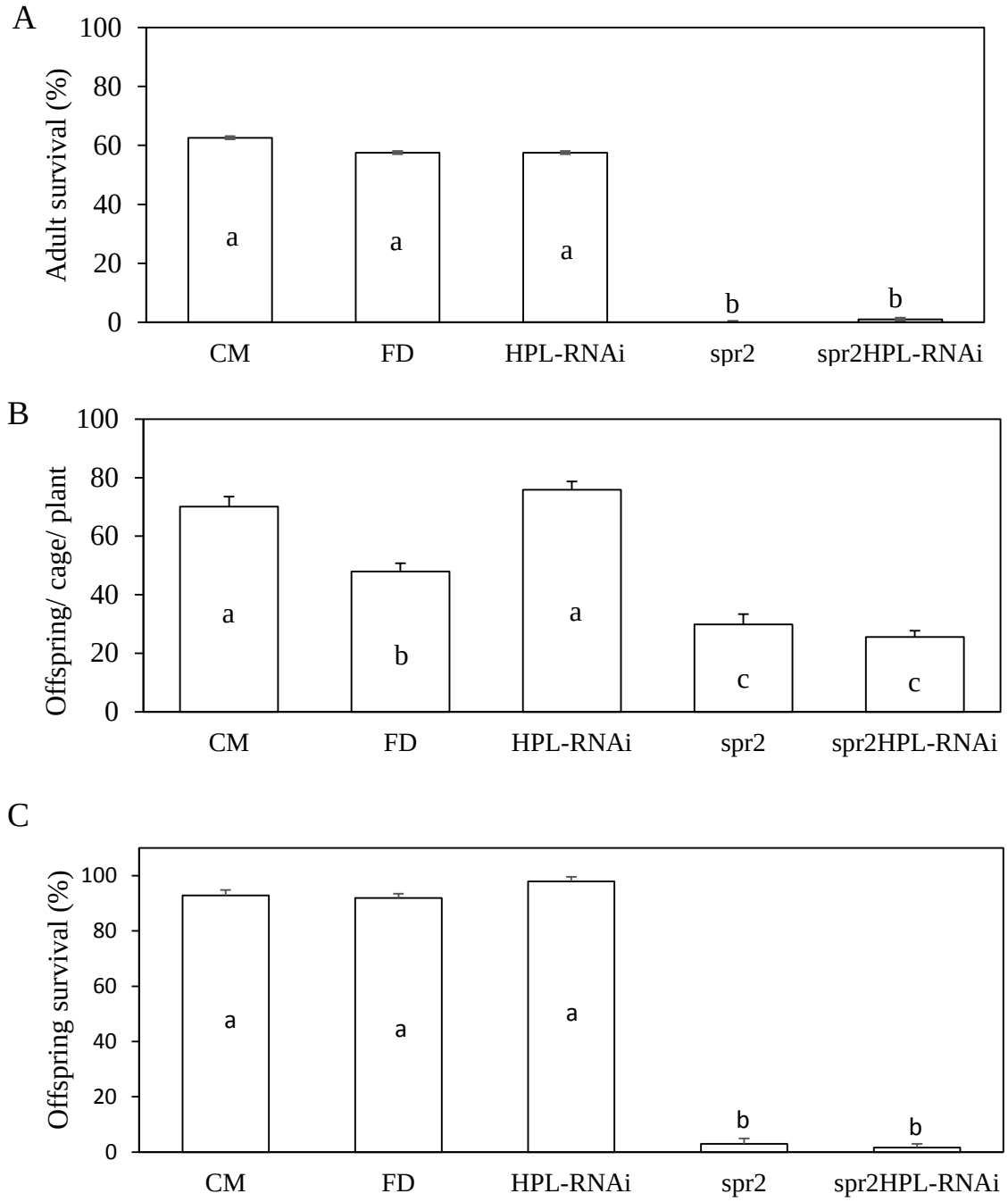


Figure 5. **Comparison of aphid survival and reproduction on plants with modifications in both fatty acid desaturation and hydroperoxide lyase expression.** A non-choice assay on five-week-old plants was used to compare aphid performance on a line with impairments in both fatty acid desaturation and the hydroperoxide lyase pathway (*spr2HPL-RNAi*) to aphid performance on single mutants (*spr2* and *HPL-RNAi*) and their respective wild-type controls (CM and FD). The average number of adults and offspring each cage each plant was analyzed among different genotypes by one-way ANOVA and Tukey-Kramer HSD with JMP ® v 11 (SAS Institute, NC). Bars having the same letter are not significantly different at α=0.05, and error bars represent SEM. Bars having the same letter are not significantly different at α=0.05, and error bars represent SEM.

Table 1. Comparison of C6 volatiles from foliage of three- and five-week-old tomato plants with modifications in volatile biosynthesis pathways. Headspace volatiles were collected from crushed foliage of *spr2*, *AS-LoxC*, *HPL-RNAi*, and their respective wild type controls (CM, M82, and FD) by a solid-phase microextraction and were analyzed by GC-MS (n≥4).

Genotype	Plant age (wk)	(Z)-3-hexenal ±SD (ng/gfw)	Hexanal±SD (ng/gfw)	(E)-2-hexenal±SD (ng/gfw)	(Z)-3-hexen-1-ol±SD (ng/gfw)
CM	3	36370±61343	315±514	1928±3227	2482±4571
spr2	3	44±82	1860±2558	23±41	13±31
M82	3	723±685	17±14	249±180	190±144
AS-LoxC	3	0±0	0±0	0±0	0±0
FD	3	342±249	5±4	172±76	88±71
HPL-RNAi	3	585±985	22±25	47±38	82±100
CM	5	73338±143196	469±837	2767±4299	2237±3872
spr2	5	6499±15182	9054±13494	287±531	544±1102
M82	5	5396±7607	177±101	1274±618	921±484
AS-LoxC	5	0±0	6±14	6±13	4±10
FD	5	3579±3748	92±111	676±581	560±634
HPL-RNAi	5	288±550	17±25	121±169	97±153

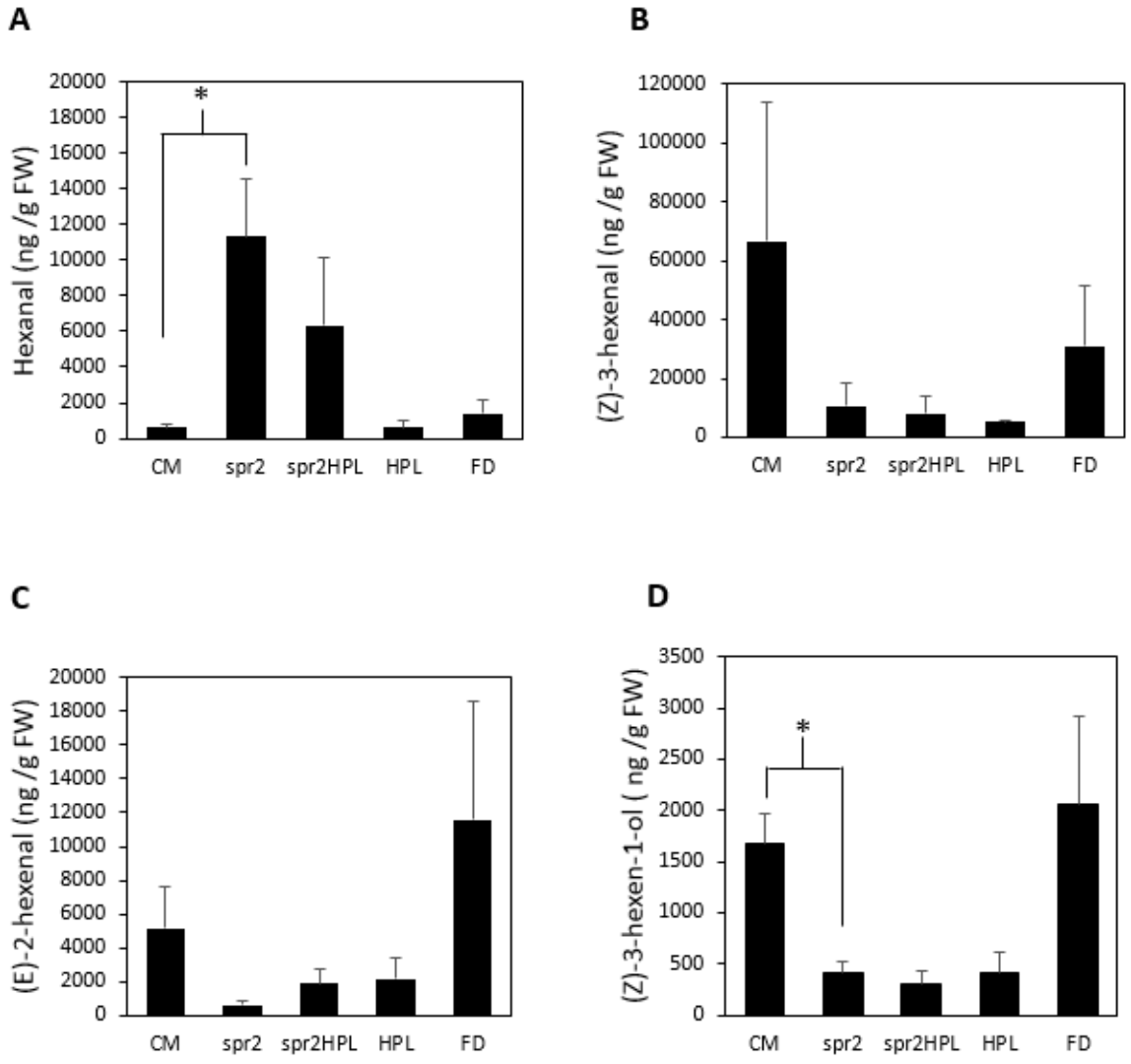
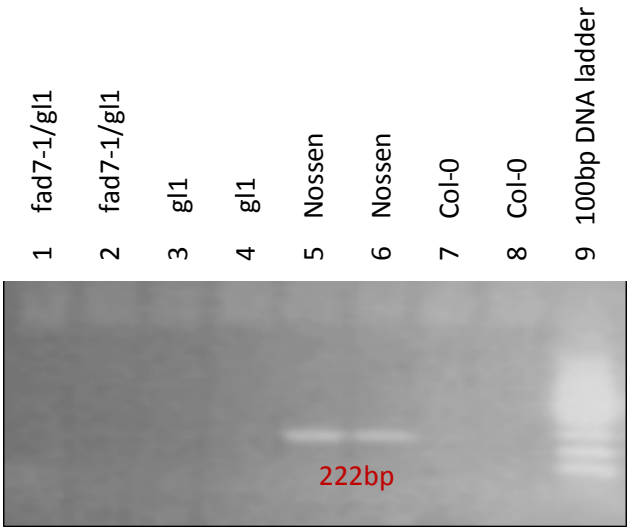


Figure 6. Comparison of C6 volatile levels in five-week-old plants with modifications in both fatty acid desaturation and hydroperoxide lyase expression. C6 volatile levels were compared on a line with impairments in both fatty acid desaturation and the hydroperoxide lyase pathway (*spr2HPL-RNAi*) to aphid performance on single mutants (*spr2* and *HPL-RNAi*) and their respective wild-type controls (CM and FD). C6 volatile levels were analyzed by Wilcoxon test with JMP ® v 11 (SAS Institute, NC). Asterisks (*) indicates statistically significant differences at α=0.05 between transgenic lines and their controls. Error bars represent SEM ($n \geq 3$).



Supplemental figure. PCR identification of the *HPL* gene in Arabidopsis lines.

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Chapter IV

The Impact of C6 volatiles on Plant Defenses at the Seedling Stage

Abstract

The potato aphid, *Macrosiphum euphorbiae*, has four nymphal instars, and each instar lasts 1.5 to 3 days. An unfertilized female can give birth to more than 60 offspring on potato. The short life cycle and high reproductivity make aphids hard to control. Host resistance can defend against aphids by influencing aphid development and decreasing aphid reproductivity. C6 volatiles play a role in some plant defenses against aphids. To investigate whether C6 volatiles contribute to plant defenses at the seedling stage, we compared the daily adult survival and fecundity, and daily development of juveniles on 3-week-old tomato lines with modified C6 profiles and their respective wild-type controls. We did not detect any significant defferences in aphid performance on wild type plants compared with *HPL-RNAi*, a transgenic line in which C6 volatiles were reduced by suppression expression of the biosyntheic enzyme hydroperoxide lyase (HPL). These results suggest that C6 volatiles generated by HPL do not contribute significantly to resistance in WT tomato seedlings, at least in the genetic background used to generate *HPL-RNAi*. In contrast, the *spr2* mutation caused significant decreases in adult survival and fecundity, juvenile survival and growth on 3-week-old plants. This mutation influences C6 volatile profiles by modifying the availabily of fatty acid precursors used for C6 volatile synthesis. Previous experiments have shown that 5-week-old *spr2* is depleted in (Z)-3-hexen-1-ol, but elevated in hexanal levels, and that artificially suppressing hexanal production in *spr2* does not alter aphid resistance. Together, these findings suggest that the antibiosis observed *spr2* plants is due to factors other than C6 volatiles.

Introduction

The potato aphid, *Macrosiphum euphorbiae*, grows through four nymphal instars, and the time for each instar ranges from 1.5 to 3 days, depending on temperature (De Conti et al., 2011; Macgillivray and Anderson, 1964). It takes about 6 to 12 days for potato aphids to develop from birth (the first instar) to reproductive maturity (adult), and adults usually live for about 10 days to a month (De Conti et al., 2011; Lamb et al., 2009; Raboudi, et al., 2011; Kaloshian et al., 1997). Each female can give birth to about 67 offspring on potato at 21.8 °C (MacGillivray and Anderson, 1958). The short life cycle and high reproductivity allow them to quickly increase population on their host plants. Potato aphids can be a major pest on some economic crops such as lettuce, potato and tomato (Tomescu and Negru, 2003).

A variety of insecticides were used to control this species (Steene et al., 2003). However, resistance to some insecticides has been reported in the potato aphid (Foster et al, 2002), and emphasizing the need for aphid-resistant varieties. Several sources of resistance to potato aphids have been reported in tomatoes or their wild relatives (Musetti and Neal, 1997; Kohler and St Clair, 2005; Rossi et al., 1998). Plant defend themselves against insects through multiple pathways which are generally classified into constitutive and inducible defenses (Chen, 2008; Wang et al., 2004; Jin et al., 2011; Kim and Jander, 2007; Levy et al., 2005). Constitutive defenses such as surface waxes exist in the plant even in the absence of the pest (Jenks et al., 1994); in contrast, inducible defenses are activated in response to stresses including insect attack (Chen, 2008). For instance, C6 volatiles can be released upon insect infestation (De Vos and Jander, 2010), and play the important roles in plant defenses.

As chapter III described, the aphid-resistant mutant line, *spr2* with impaired function of FAD7 has not significantly altered profiles of fatty acid-derived C6 volatiles relative to wild-type control in 3-old-week plants. However, 5-week-old *spr2* has altered profiles of fatty acid-derived C6 volatiles with enhanced levels of hexanal, and reduced levels of (Z)-3-hexenal, (E)-2-hexenal and (Z)-3-hexen-1-ol relative to wild-type control. To investigate whether the C6 volatile hexanal contributed defenses against aphids in *spr2*, we have compared volatiles, aphid survival and fecundity after 6 days inoculation, and aphid host preference on the silenced line *HPL-RNAi* with wild-type control and *spr2* mutant. However, the day-by-day adult survival and fecundity, and daily development of juveniles on theses tomato lines have not been studied yet. This chapter is to complete these objectives.

Materials and methods

Plant and insect materials

Four tomato (*Solanum lycopersicum L.*) genotypes were used in our bioassay: Castlemart (CM), *suppressor of prosystemin-mediated responses 2* (*spr2*), Flora-Dade and a transgenic line silenced for HPL (*HPL-RNAi*) (See chapter III for a description of these lines). All plants were grown in LC1 Sunshine potting mix (Sungro Horticulture, Bellevue, WA) with 15-9-12 Osmocote slow-release fertilizer (Scotts-MiracleGro Company, Marysville, OH), and kept in growth chambers (Controlled Environments, Inc., Winnipeg, Canada) at 23 °C and 16L: 8D photoperiod, watered with a dilute nutrient solution containing 1000 ppm $CaNO_3$ (Hydro Agri North America, Tampa, FL), 500ppm $MgSO_4$ (Giles Chemical Corp, Waynesville, NC), and 500ppm 4-18-38 Gromore fertilizer (Gromore, Gardena, CA). Newly emerged within 24 h

wingless potato aphid (*Macrosiphum euphorbiae*, isolate WU11) was used for the bioassay. Aphids were reared on an aphid-susceptible tomato cultivar (UC82), potato (*Solanum tuberosum* Linnaeus), and jimson weed (*Datura stramonium* Linnaeus) plants in Conviron growth chambers (Controlled Environments, Inc., Winnipeg, Canada) at 20 °C and a 16L: 8D photoperiod.

Aphid performance bioassays

Adult longevity and fecundity

Adult longevity and fecundity were measured by recording the living days of each adult and its offspring numbers on each leaflet of the plants. Wingless adult aphids (collected within 24 h of emergence to adulthood) were individually confined to single leaflets with uniform node position of 3 week old tomato plants (CM, *spr2*, Flora-Dade, and *HPL-RNAi*) using lightweight clip cages (2 cages/plant; 10-15 replicate plants/genotype). Plants were maintained in growth chambers at 23 °C and 16L: 8D photoperiod. The status of each adult (alive or dead) and the number of offspring it produced were recorded daily, and juveniles were removed from the cages daily after counting to prevent overcrowding. The assay was terminated at 12 days after inoculation.

Adult longevity was analyzed using the Reliability and Survival method and the χ^2 test as well as regression analysis. The adults that did not die within the observation period were considered censored values. The average lifetime fecundity per adult and average daily fecundity per adult was compared among different tomato lines using analyzed by one-way ANOVA and Tukey-Kramer HSD.

Juvenile mortality growth and development

In order to produce age-synchronized first instar juveniles, wingless adult aphids were confined to individual leaflets using clip cages (5 adults/cage), and were removed 24 h later, after producing at least 5 juveniles in each cage. 5 juveniles were kept in each cage, 2 cages were set up on each plant, and 10-14 replicate plants for each genotype. Three-week-old tomato plants were used for aphid inoculation, and maintained at 23 °C and 16L: 8D photoperiod. Juvenile mortality and development was measured by monitoring the status of each juvenile (alive, dead, or molted to adulthood) was recorded daily until all the juveniles were dead or molted to adulthood. Newly emerged adults were removed from cages within 24h of emergence, and put into an empty tube for weighing. Net weight of adults was calculated by gross weight (adult plus tube) minus the weight of empty tubes. The average mass per adult per plant among different tomato lines was analyzed by one-way ANOVA and student's *t*-test.

Results

Adult longevity and fecundity

Aphid bioassays were used to assess whether adult aphid longevity and fecundity were different among tomato lines with variation in C6 volatiles. The results showed that the adult survival rate was more than 50 % lower on *spr2* plants than on the wild-type control Castlemart (Fig. 1A), which was consistent with the report of Avila et al (2012). The adults that did not die within the observation period (12 days) were considered censored values, and the average number of days that adults survived on *spr2* mutant line (4 ± 0.2 days) was significantly lower ($p < 0.0001$) than on the wild-type control Castlemart (8 ± 0.5 days). Adult survival on *HPL-RNAi*

did not differ significantly from that on the wild-type control Flora-Dade (Fig. 1B), and the average number of days that adults survived on *HPL-RNAi* line (9 ± 0.4 days) was not different from wild-type control plants (8 ± 0.4 days).

Adult fecundity per day was calculated by the number of offspring produced that day divided by the number of surviving adult females. The average daily fecundity each adult female on *spr2* and *HPL-RNAi* plants did not significantly differ from daily fecundity on respective wild-type control plants (Fig. 2A). However, the average lifetime fecundity of each adult female was significantly lower on *spr2* than on the wild-type control Castlemart ($p < 0.0001$), which was consistent with the report of Avila et al (2012). In contrast, the average lifetime fecundity of each female did not show any significant difference on *HPL-RNAi* and its wild-type control Flora-Dade (Fig. 2B).

Juvenile mortality and development

In a separate assay, survival and development of juvenile was also monitored on the same four tomato lines. The results showed that juvenile mortality was almost 50 % higher on *spr2* plants than on the wild-type control Castlemart (Fig. 3A), but juvenile mortality on *HPL-RNAi* did not differ significantly from that on the wild-type control Flora-Dade (Fig. 3B). Juveniles started molting to adulthood on day 7 on Castlemart; in contrast, on *spr2*, no juveniles had molted to adulthood even on last experimental day (Fig. 4A). Juveniles started emerging to adulthood on day 6 on *HPL-RNAi* plants and on day 7 on its wild-type control Flora-Dade (Fig. 4B). These results showed that juvenile development was significantly faster on wild-type control Castlemart than on *spr2* plants; but was not influenced by silencing HPL. The average

mass of adults on *spr2* was also significant lower than its wild-type control Castlemart (Fig. 5A); whereas no significantly difference in average mass of adults is shown between *HPL-RNAi* and its wild-type control Flora-Dade plants (Fig. 5B).

Discussion

Aphid performance on host plants is usually assessed by measuring aphid host preference, aphid survival, fecundity and development. This study measured adult survival, and daily as well as lifetime fecundity on two tomato lines with variation in C6 volatiles and their wild-type controls respectively. The results indicated that the *spr2* mutant line with impaired FAD7 function was more resistant to aphid adults than its wild-type control due to the significantly lower survival and lifetime fecundity on *spr2* as compared to the wild-type control. In contrast, the RNAi suppression of HPL did not impact aphid adults because neither adult survival nor fecundity significantly differed on the *HPL-RNAi* line and on the wild-type control Flora-Dade. This is consistent with previous from no-choice bioassays (chapter III). These results indicate that plant age may influence the effects of HPL on aphids.

The significantly higher juvenile mortality rate on *spr2* compared to its wild-type control indicated that loss function of FAD7 increased plant defenses against aphid juveniles. There was no difference in total numbers of live juveniles and adults that emerged from juveniles on the *HPL-RNAi* line as compared to its wild-type control Flora-Dade on any of the days in this study. This indicated that suppression of HPL did not impact juvenile development. Loss function of FAD7 remarkably decreased the aphid emergence as result of higher juvenile mortality. In comparison, suppression of HPL did not influence juvenile development.

The mass of individual aphid is also one of parameters for estimating aphid performance on host plants. The current study indicated that impaired FAD7 function also significantly decreased the weight of the adults that developed on this line; in contrast, suppression of HPL did not influence aphid masses.

Previous work on the influence of C6 volatiles in tomato on aphids showed that silencing HPL resulted in increased aphid numbers on 5-week-old plants, but had no significant effect on aphid infestations on 3-week-old plants (CHIII). This difference between developmental stages could be due to difference in the abundance of C6 volatiles, which increase with plant age. Potentially, the volatile levels in 3-week-old wild type plants are not high enough to deter aphids, alternatively, the short term aphid performance assays used in CHIII were not sensitive enough to detect subtle differences in aphid performance between 3-week-old Flora-Dade and *HPL-RNAi*. To address this question, in this study we undertook a detailed analysis of aphid survival, development, a fecundity on 3-week-old Flora-Dade and *HPL-RNAi*. Since the FAD7 impaired mutant *spr2* is known to be resistant to aphids at 3 weeks after planting, it was included for comparison. Previous experiments demonstrated that the *spr2* mutation results in decreases in aphid settling, adult survival and fecundity (Avila et al., 2012). Adult mortality was over 50 percent higher on *spr2* than on wild-type controls, aphid fecundity significantly decreased over time on *spr2* than on wild-type plants, and lifetime offspring was over 50 percent lower than wild-type plants (Avila et al., 2012). The results of current experiment were consistent with the data of Avila et al (2012). In addition, this study investigated aphid juvenile mortality, development, and mass of emerged adults. This work allowed us to expand upon what was previously known about the effects of *spr2* on aphid biology.

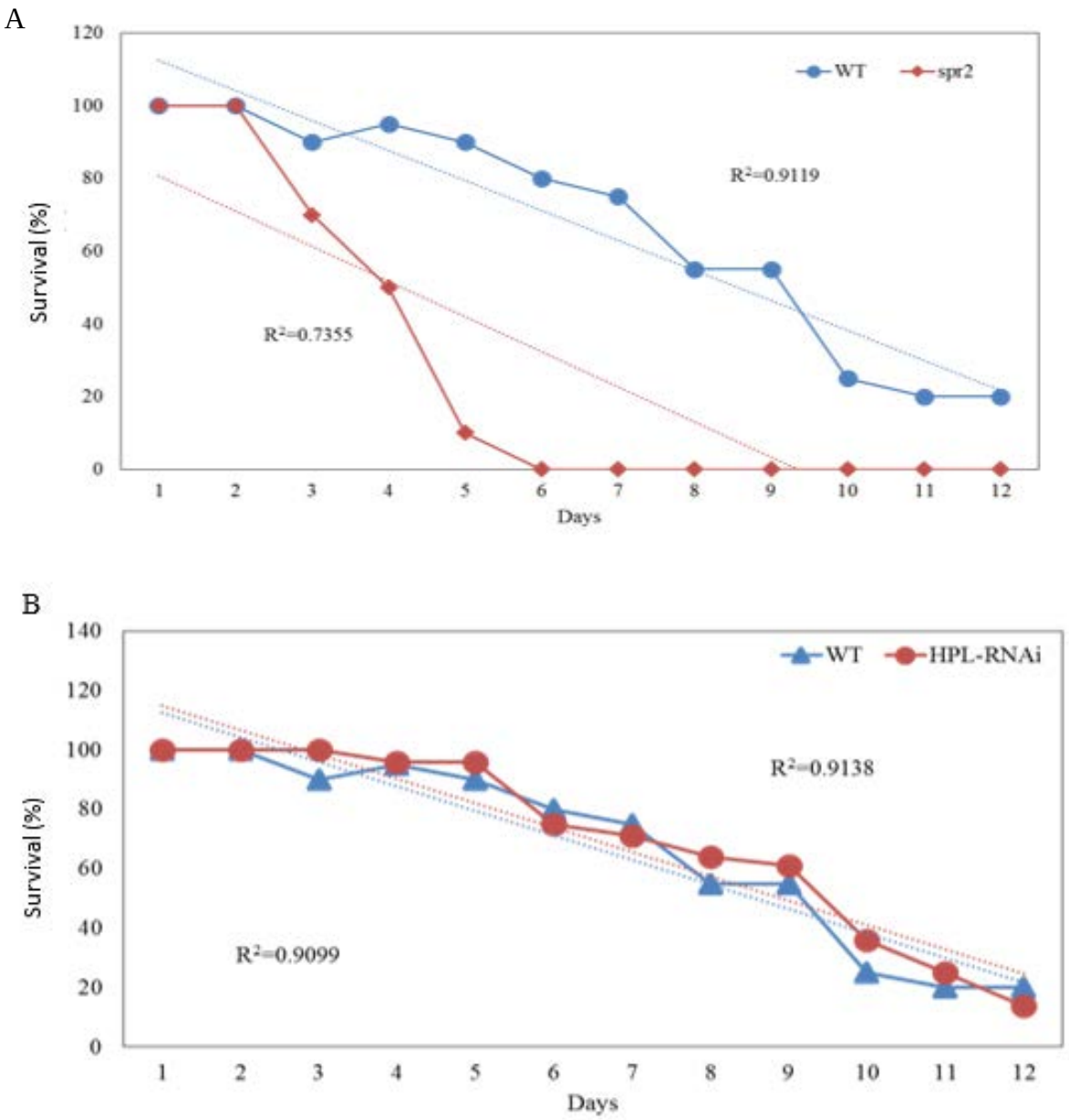


Figure 1. Adult survival on 3-week-old tomato lines with modified volatile profiles. The newly emerged adult females within 24h were singly caged on *spr2*, *HPL-RNAi* and their respective wild-type controls. The adults were monitored daily to track the survival until 12 days after inoculation. Regression analyses were used to estimate aphid survival.

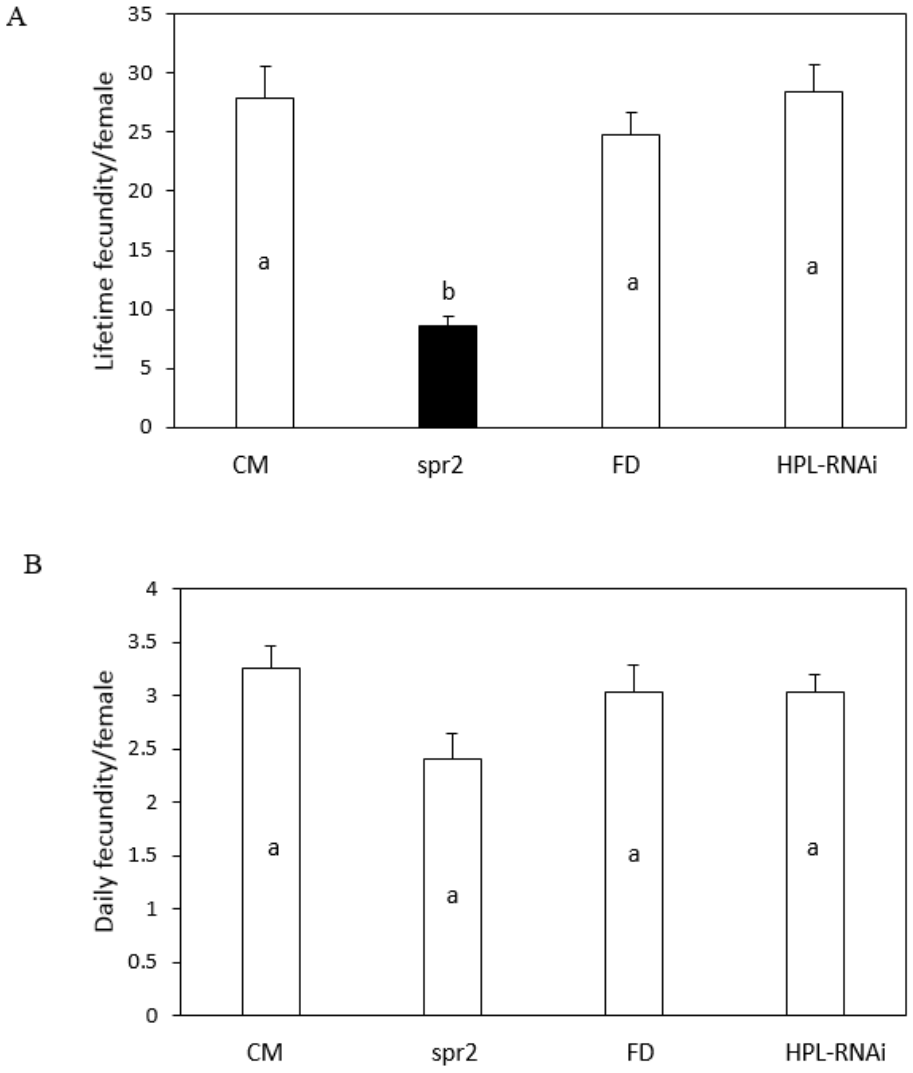


Figure 2. Adult daily fecundity and lifetime fecundity on 3-week-old tomato lines with modified volatile profiles. The newly emerged adult females within 24h were singly caged on *spr2*, *HPL-RNAi* and their respective wild-type controls. The adults were monitored daily to track their offspring. One-way ANOVA and Tukey-Kramer HSD were used to analyze. Bars having the same letter are not significantly different at α=0.05, and error bars represent SEM.

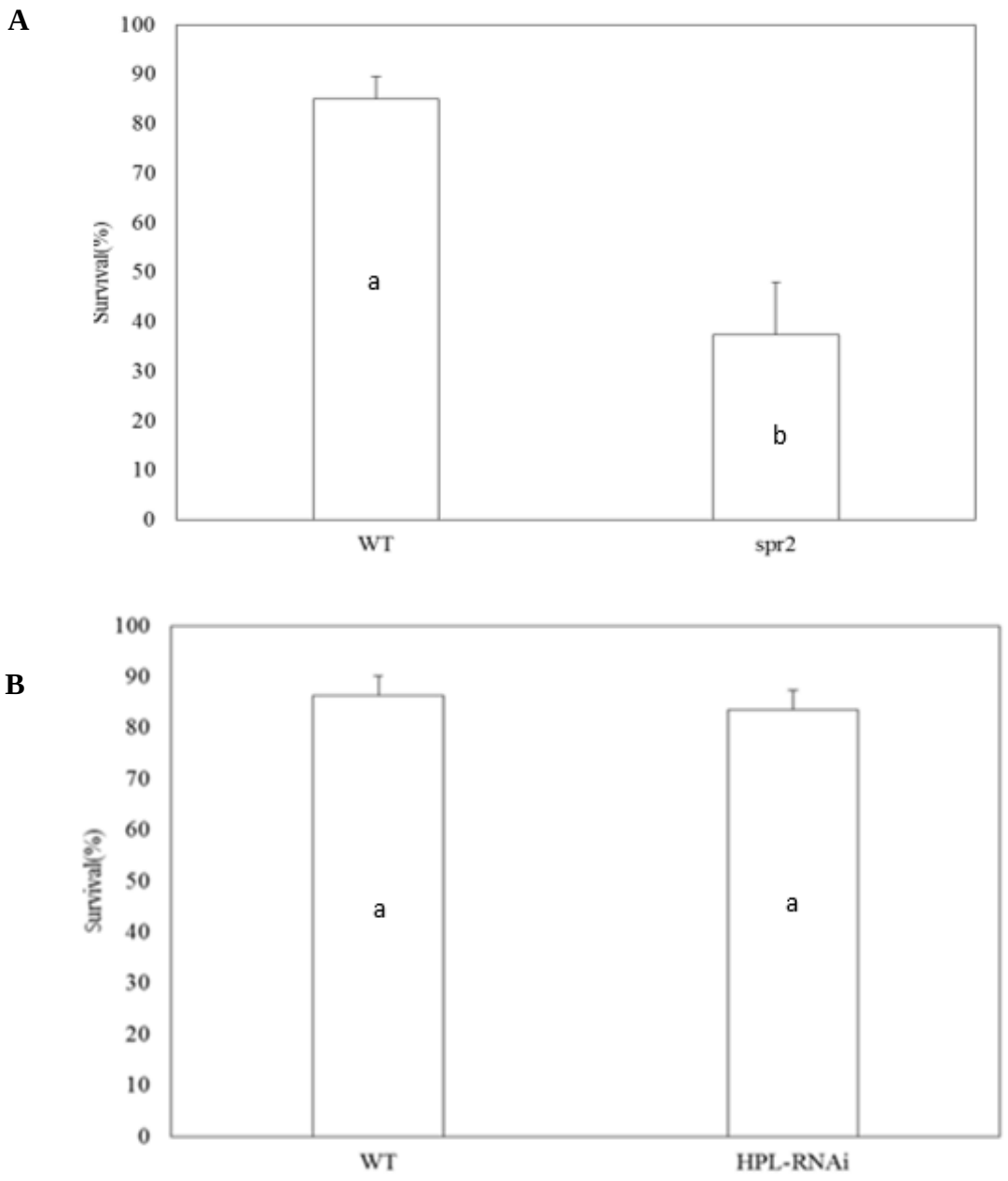
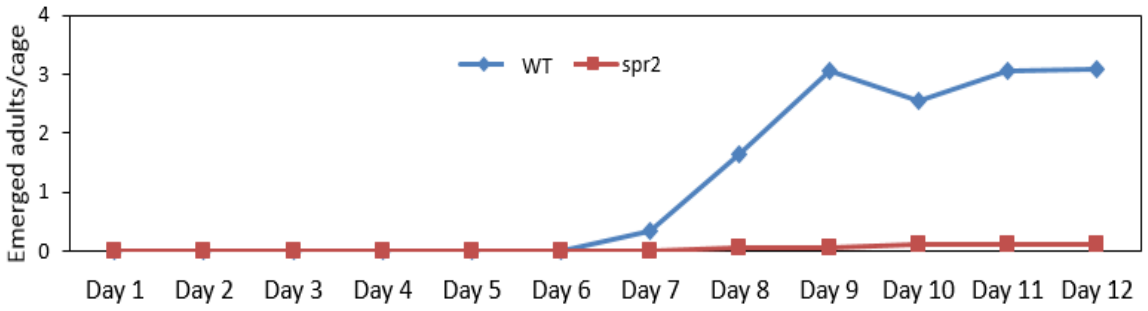


Figure 3. Juvenile survival on 3-week-old tomato lines with modified volatile profiles. Juveniles were caged on *spr2*, *HPL-RNAi* and their respective wild-type controls. The juvenile status (alive or dead, or molted to adulthood) was monitored daily until all the juveniles were dead or molted to adulthood. One-way ANOVA and student's *t*-test were used to analyze. Bars having the same letter are not significantly different at α=0.05, and error bars represent SEM.

A



B

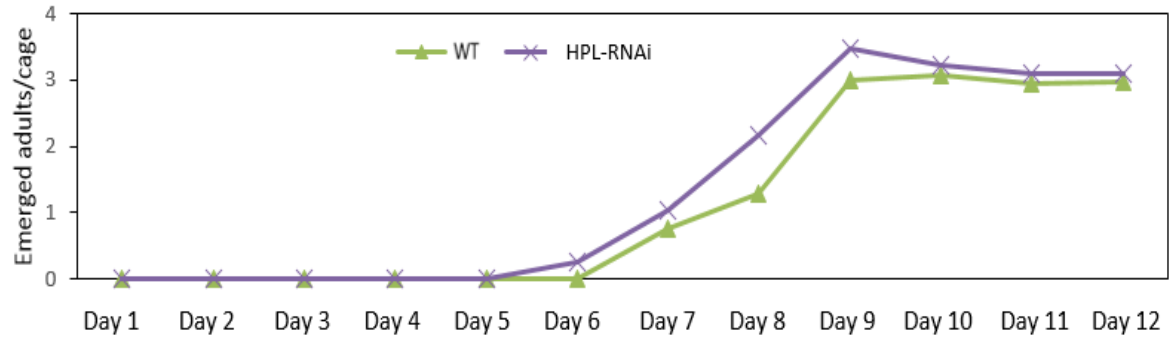


Figure 4. **Juvenile development on 3-week-old tomato lines with modified volatile profiles**. Juveniles were caged on *spr2*, *HPL-RNAi* and their respective wild-type controls. The juvenile development stage was monitored daily until all the juveniles were dead or molted to adulthood.

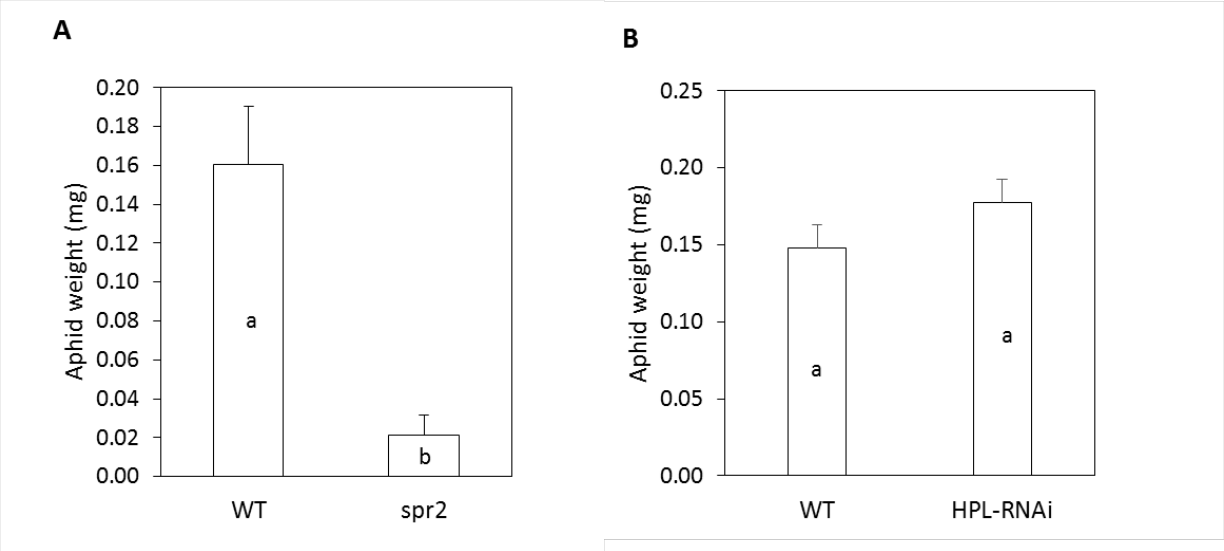


Figure 5. Size of adults developed on 3-week-old *spr2, HPL-RNAi* plants and their respective wild-type controls. Newly emerged adults were removed from cages within 24h of emergence and weighed. The average mass per adult per plant among different tomato lines was analyzed by one-way ANOVA and student's *t*-test.

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Chapter V

Conclusions

The mutant lines in Arabidopsis and tomato, which are deficient in a fatty acid desaturase, FAD7, have enhanced aphid resistance. Loss of function of FAD7 impacts the abundance of multiple C16 and C18 in the endoplasmic reticulum (ER) and chloroplast. The alteration of FA profiles may be involved in plant defenses against aphids directly or indirectly. In trying to identify which of these FAs and their derivatives impact aphid resistance, we studied 1) the relative contribution of C16 and C18 as well as ER- and chloroplast- localized FAs in aphid resistance; 2) the relative importance of C16 and C18 with single, double and triple double bounds in aphid resistance. Our results indicated that aphid resistance was impacted by FADs in the ER as well as in the chloroplast, and C18 may play a more vital role rather than C16 FAs in aphid resistance. Moreover, C18:2 or its derivatives may contribute to plant defenses against aphids. One possible group of FA derivatives that are often implicated in plant defense are the C6 volatiles. Aphid-resistant mutant *spr2* with impaired FAD7 activity has modified profiles of FA-derived C6 volatiles synthesized through the HPL pathway. Thus, we tested aphid performance on tomato lines with suppressing expression of two enzymes required for C6 synthesis, lipoxygenase C (LOXC) and hydroperoxide lyase (HPL). Suppression of HPL expression causes a modest increase in aphid fecundity on five-week-old plants, but HPL is not essential to aphid resistance in *spr2* mutants. The HPL pathway appears to contribute to basal defenses in tomato, but is not critical to *spr2*-dependent resistance. Instead, aphid resistance in *spr2* may be due to other consequences of altered fatty acid profiles. In tomato and Arabidopsis, α-dioxygenase 1 (α-DOX1) which is involved in the synthesis of oxylipins derived from C18:2 and C18:3, contributes to plant defenses against aphids. Oxylipins generated from 18:2 by α-DOX1 may

contribute to aphid resistance in *spr2*. Our study contributes to our understanding of how FAD7 modulates plant resistance to biotic stresses.