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The Influence of Singlet Oxygen and Loss of Function of Fatty Acid Desaturase 7 in the Chloroplast on Aphid Resistance in Arabidopsis thaliana

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The Influence of Singlet Oxygen and Loss of Function of Fatty Acid Desaturase 7 in the Chloroplast on Aphid Resistance in *Arabidopsis thaliana*

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Abstract

Fatty Acid Desaturase 7 (FAD7) is a chloroplast-localized enzyme that alters the fatty acid content of photosynthetic membranes, and that negatively regulates plant defenses against aphids. Previous studies in the model organism Arabidopsis (*Arabidopsis thaliana*) have shown that loss-of-function mutations in FAD7 decrease population growth of the green peach aphid (GPA; *Myzus persicae* Sulzer). This study further characterized the effects of a *fad7* null mutant on aphids, and investigated the role of reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), in plant responses to aphid resistance in *fad7* as well as in wild type plants and a mutant with heightened $^1O_2$ accumulation (*flu*). Bioassays indicated that aphid resistance in *fad7* is species-specific and impacts the generalist herbivore GPA but not a specialist herbivore, the cabbage aphid (*Brevicoryne brassicae*). The effects of *fad7* on GPA appeared to be antibiotic but not antixenotic, and reduced population growth by decreasing adult fecundity and increasing juvenile development time. Compared to wild type controls, *fad7* also has increased expression of an $^1O_2$-responsive reporter gene (*AAA-ATPase:Luc*) both constitutively and in response to aphids, and aphid challenge upregulated *AAA-ATPase:Luc* in both *fad7* and in wild type plants. These results suggest that $^1O_2$ may contribute to plant defenses against aphids. A redox-sensitive green fluorescent protein (roGFP2) tagged to the chloroplast showed that GPA induce a rapid and sustained oxidative response in the chloroplast; furthermore, ROS accumulation in response to aphid feeding, as measured with luminol, was attenuated in transgenic plants with enhanced expression of an $^1O_2$ scavenger in the chloroplast (*SPS1oex*). Together, these results suggest that ROS, including $^1O_2$, are accumulating in the chloroplast in response to aphid challenge. To explore whether $^1O_2$ impacts host suitability for aphids, aphid performance was also measured on the conditional *fluorescence* (*flu*) mutant, which has normal ROS levels when grown in

continuous light but accumulates high $^1O_2$ in the chloroplast when transferred from light to dark and back to light again (ie. a light/dark/light shift). The population growth of GPA was significantly lower on *flu* exposed to a light/dark/light shift than on *flu* grown in continuous light or on wild type plants in either light treatment; thus, high $^1O_2$ conferred aphid resistance. Because $^1O_2$ generated in the chloroplast is known to impact nuclear gene expression via a retrograde signaling pathway that requires the chloroplast-localized EXECUTER1/EXECUTER2 (EX1/EX2) proteins, additional experiments were conducted to determine if EX1/EX2 were required for aphid resistance in *fad7* or *flu*. Null mutations in *EX1* and *EX2* partially reduced aphid resistance in *flu*, and eliminated resistance in the *fad7*. This indicates that $^1O_2$ accumulation and EX1/EX2 signaling contribute to aphid resistance in both genotypes. Compared to wild type plants, *fad7* had comparable chlorophyll levels and higher maximum potential quantum efficiency (Fv/Fm) of Photosystem II, which is typically the primary source of $^1O_2$ in the chloroplast. Thus, enhanced aphid resistance associated with increased $^1O_2$ accumulation did not compromise photosynthesis in *fad7*. This work demonstrates, for the first time, a role for $^1O_2$-mediated chloroplast signaling in plant defense against herbivores.

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Dedication

This dissertation is dedicated to my mother. She gave me my faith and curiosity and taught me I have more strength than I know. I would not be who I am without her.

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Chapter I

Introduction

Aphids as global pests

Aphids (Hemiptera:Aphididae) are a prolific group of phloem feeders that have coevolved with plants for millions of years to become closely associated with their host plant (Peccoud et al., 2010). Most aphids spend their entire life on their host plant, relying on it for food and shelter. There are over 4,500 extant aphid species that feed on trees, shrubs, and herbaceous plants with varying degrees of specialization (Blackman and Eastop, 2000, 2006). Aphids have evolved remarkably complicated life history strategies that can include asexual and sexual generations, often on multiple host plants of different taxa, and involve elaborate polyphenism and polymorphism (Moran, 1992). The soybean aphid (SBA; *Aphis glycine* Matsumura), for example, feeds on soybean (*Glycine max,* Fabaceae) during spring and summer months in the US and reproduces wingless (apterous) clonal female offspring parthenogenically for as many as 18 generations (Ragsdale et al., 2004). At the beginning of fall months, females will produce both winged (alate) males and females that congregate on buckthorn (*Rhamnus cathortica*, Rhamnaceae), mate, and produce eggs that overwinter. In spring, the eggs hatch as winged females and migrate back to soybean (Ragsdale et al., 2011). Generation times for aphids during the spring and summer months are often rapid, due to short periods of juvenile development before reproductive capacity (Rutledge et al., 2004).

Host selection for aphids involves a set of behavioral sequences that utilize olfactory, visual, and gustatory cues to detect a suitable host (Kring, 1967; Powell et al., 1995; Kirchner et al., 2005; Döring and Chittka, 2007). Once on the plant, aphids feed using piecing-sucking mouthparts to tap into the phloem and feed on photoassimilates with minimal detection

(Tjallingii, 2006). Specially constructed stylets maneuver between cells to reach the phloem sieve elements, tasting epidermal and mesophyll cell contents along the way. As they probe, aphids secrete two types of saliva that are crucial to successful, sustained feeding (Miles, 1999). Gelling, or sheath, saliva acts as physical support along the stylet pathway and enzymes in the saliva help break down middle lamella for sampling of cell contents (van Bel and Will, 2016). Watery saliva is secreted into cells along the stylet pathway and phloem sieve elements (Miles, 1999). Watery saliva is composed of hundreds of proteins, including pectinases, oxidases, peroxidases, and other components that help the aphid locate the phloem cells and prevent clogging in sieve elements for sustained feeding (Miles, 1999; Will et al., 2007; Harmel et al., 2008; Mutti et al., 2008). Watery saliva also contains effectors which modulate plant defense responses such that aphids remain undetected by the plant (Bos et al., 2010; Elzinga et al., 2014; Wang et al., 2015). In some interactions, plants may recognize these effectors with resistance (R) genes and mount inducible defenses (Jaouannet et al., 2014; Cui et al., 2019).

Considering the rapid generation times, advantageous life history strategies, and specialized feeding mechanisms, it is no wonder aphids have become global pests to agronomically important crops, specifically in temperate regions. For instance, SBA is able to reduce yields as much as 40% in a growing season, which results in huge economic losses (Ragsdale et al., 2007). The sugarcane aphid (*Melanaphis sacchari* Zehntner) is a devastating pest of sorghum in the US that can cost growers $23 mil in yield reductions in years with high infestations, even under conservative estimates (Zapata et al., 2018). In Europe, aphid damage was shown to result in significant loss of staple crops including 700,000 t of wheat, 850,000 t of potatoes, and 2,000,000 t of sugar beet (Wellings et al., 1989).

Aphids can damage plants and reduce yields not only from removal of photoassimilates during feeding, but also through a variety of induced responses that decrease plant fitness. Direct damage by aphid feeding can result in decreased photosynthesis, chlorosis, necrosis, and wilting or stunting of growth (Botha and Matsiliza, 2004; Botha et al., 2005a; Zhang et al., 2019). Large infestations of aphids can further reduce photosynthesis when honeydew extraction reaches levels that promote black sooty mold growth and reduce photosynthetic area of leaf tissue (Hurej and Van der Werf, 1992). Also, aphids can cause cosmetic damage to specialty crops that make products less appealing to consumers (Nebreda et al., 2005). Furthermore, aphids are an important vector of plant viruses that cause even further yield losses. The green peach aphid (GPA;*Myzus persicae* Sulzer) alone is responsible for vectoring over 100 plant viruses including *Potato leafroll virus*, *Potato virus Y*, and *Cucumber mosaic virus* (Kennedy et al., 1962; Mowry, 2005; Mondal et al., 2016; Rhee et al., 2020).

Management of aphids

Effective management of aphids in a cropping system requires multiple techniques including chemical control, biological control, and plant resistance used together in integrated pest management (IPM). Chemical control is the most widely used tactic with a broad array of insecticides that utilize varying modes of action to decrease infestations. Pesticide usage against aphids has progressed from reliance on organophosphates and carbamates, two highly toxic chemicals that affect a broad range of organisms, and now employs more efficient insecticides such as pyrethroids and neonicotinoids (Dewar and Denholm, 2017). However, chemical control still has major limiting factors. For one, aphids as a group have a propensity for developing insecticide resistance, rendering many pesticides ineffective (Kerns and Gaylor, 1992; Barber et

al., 1999; Silva et al., 2012; Zhang et al., 2017a). GPA especially shows a capacity for developing resistance, with several populations throughout the world that are resistant to multiple classes of insecticides, often with novel resistance mechanisms (Denholm et al., 1998; Bass et al., 2014). Also, while chemical control may be able to reduce populations in the field, they may not be sufficient for stopping the spread of plant viruses (Dedryver et al., 2010). For example, insecticides alone were not able to prevent the spread of the cucumber mosaic virus (CMV) transmitted by aphids in a tomato field in Alabama and decreased yields by 25% (Sikora et al., 1998). Furthermore, pesticides have several safety drawbacks for producers and consumers, are often costly, and can negatively impact the environment by causing non-target effects to beneficial organisms and groundwater contamination through runoff (Carvalho, 2017; Sharma and Singhvi, 2017; Zaller and Brühl, 2019). Therefore, although pesticides are an important tool in controlling aphids, other methods of managing aphids in the field should be utilized in conjunction with pesticides to decrease applications and mitigate harmful effects of insecticides.

Biological control (BC) of aphids in an IPM system relies on natural enemies (NE) of aphids such as entomopathogenic fungi, predators such as lady beetles or lacewings, and parasitoid wasps (Yano, 2006). There are three major strategies to BC that vary in their degree of effectiveness: inoculation, augmentation, and conservation. Current practices of biological control by individual growers likely will only utilize augmentation or conservation (Dedryver et al., 2010). Augmentation is aimed at enhancing the native population of NE through mass rearing and release, while conservation focuses on improving the agro ecosystem to be conducive for NE (Begg et al., 2017; Michaud, 2018). While BC is viewed as more beneficial for the health of the ecosystem, several environmental conditions, such as temperature and humidity,

add constraints on the establishment and success of the system (Bielza et al., 2020). Furthermore, a thorough understanding of the dynamics of the prey and predator/parasitoid biology is needed to optimize the impact of the NE. Other concerns for BC are that some NE are unable to effectively control the prey or that the prey population is not high enough to sustain the NE population, leading to an inefficient system (Kindlmann and Dixon, 1999; Rutledge et al., 2004). NE populations can also be impacted by incompatible pesticides applied to the system. There are many additional factors present in a large cropping system, such as NE dispersal and competition by other predators in the system, that decrease efficacy of the BC (Michaud, 2018). Therefore, many of the successful biological control systems to date have been in controlled systems, such as greenhouses (van Lenteren, 2012). Moreover, as BC can be costly, specialty crops that provide a higher return for fewer products are potentially better candidates for BC, which may be another reason greenhouse systems utilize BC more often (Boivin et al., 2012; Bielza et al., 2020).

In an IPM system, host plant resistance is another control tactic that can be utilized for sustainable agriculture. Plant resistance mechanisms can be generally assigned to three categorizes. Antixenosis is viewed as the plant adversely affecting insect behavior and results in herbivore selection of a new plant (Kogan and Ortman, 1978); antiobiosis is described as a plant trait that negatively impacts the insect biology and survival (Painter, 1951); and tolerance, a category much different than the other two, is considered the plant's ability to withstand and/or recover from insect damage (Painter, 1951; Strauss and Agrawal, 1999). Plant defenses can typically be considered as either constitutive or induced. Constitutive defenses can be structural and are present before herbivore attack. These include features such as trichomes, cuticular waxes, or visual appearance. For example, coloration can also impact aphid fitness in an

antixenotic fashion by making the plant less visible to the aphid during searching (Döring and Chittka, 2007). Defense compounds, such as secondary metabolites, are often employed by the plant to reduce aphid fitness. For example, in cruciferous plants, glucosinulates can be cleaved by myrosinase activity to produce toxic compounds that, when ingested, have antifeedant effects to deter aphids or decrease fecundity (Kim and Jander, 2007; Kim et al., 2008). Both GPA and the cabbage aphid (*Brevicoryne brassicae*) have shown reduced fitness on Arabidopsis (*Arabidopsis thaliana*) with increased levels of glucosinulates (Mewis et al., 2006). Plants can also accumulate phytoalexins, such as camalexin, that are also ingested and reduce aphid fitness by decreasing fecundity (Kettles et al., 2013; Louis and Shah, 2014). Perhaps the most widely used method of plant resistance to aphids is from breeding of cultivars with R genes. These genes and their homologs offer aphid resistance in several crops, such as the *Mi-1.2* gene in tomato that confers resistance to *Macrosiphum euphorbiae* (Kaloshian et al., 1995), the *Vat* gene in melons against *Aphis gossypii* (Boissot et al., 2016), and several cultivars of *Dn* wheat that are resistant to *Diuraphis noxia* (RWA; Botha et al., 2005b). Cultivars with resistance traits can be effectively utilized to control aphids in the field and decrease pesticide applications; this in turn promotes the health of the environment and slows insecticide resistance in aphid populations to prolong the life of that technology (Smith and Boyko, 2007). Furthermore, resistance traits can be crossed into high-yielding lines to further increase producer profit (Smith, 2005). The largest limiting factor to aphid-resistant cultivars is simply the lack of availability (Smith and Chuang, 2014). Therefore, it is necessary to explore the underpinnings of host-plant resistance that can be broadly related to agronomically important crops.

An emerging area of study for plant resistance to aphids involves reactive oxygen species (ROS) that accumulate as an early response to aphids. Several aphid-resistance cultivars,

including ones with resistance attributed to R genes, show increased accumulation of ROS compared with susceptible cultivars (Moloi and van der Westhuizen, 2006; Berner and Van der Westhuizen, 2010; Sytykiewicz, 2016). Also, as ROS are known to be important signaling messengers for plant defense response against abiotic and biotic stress (Foyer and Noctor, 2016), it is likely ROS are also involved in aphid resistance.

Reactive oxygen species

ROS are a consequence of the development of aerobic metabolism by both plants and animals. Consisting of singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxy radicals ($OH^\bullet$), ROS are known to be generated in various subcellular compartments under both optimal and stress conditions. ROS are highly reactive with biological components such as DNA, lipids, and proteins. While they can be generated in large amounts that can become toxic to the plant, the plant has developed antioxidant systems to scavenge ROS. Therefore, the balance between ROS production and scavenging results in the overall level of ROS accumulation.

Extracellular ROS production

Apoplast and Plasma Membrane

Both of these locations are a significant source of ROS, particularly documented when the plant is under biotic stress such as pathogen and aphid challenge (Bolwell et al., 2002). ROS can be generated at this site by NADPH oxidases, which are expressed in several forms, such as *RbohD* and *RbohF* (Suzuki et al., 2011). NAPDH oxidases transfer an electron to O_2 taken from NADPH to generate O_2^-, which is then converted into H_2O_2either spontaneously or through

dismuation catalyzed by superoxide dismutases (SODs; Kámán-Tóth et al., 2019). Activation of NADPH oxidase can happen by phosphorylation due to upstream signals to propagate ROS signaling (Kwak et al., 2003). Besides NADPH oxidases, other enzymes such as amine and polyamine oxidases and cell-wall linked peroxidases also add to the pool of apoplastic ROS (Bolwell and Wojtaszek, 1997; Kámán-Tóth et al., 2019).

Intracellular ROS production

Chloroplast

The chloroplast houses the photosynthetic machinery of the plant and is the largest source of ROS. Photosystem (PS) II continually produces $^1O_2$ during photosynthesis when excess light energy is passed from the photosystem to nearby ground state atmospheric oxygen ($^3O_2$) (Apel and Hirt, 2004). This can occur from either excited chlorophylls or energy charge separation of the PSII reaction center (Dmitrieva et al., 2020). Also, when the electron transport chain (ETC) between PSII and PSI is over-reduced and energy is again passed to $^3O_2$ (Asada, 2006). If light energy is not passed on, it can lead to irreversible damage to the reaction center of PSII (Andersson et al., 1992). Therefore, the plant has evolved a method of regulating energy by de-excitation of singlet excited chlorophyll as heat in order to protect the light harvesting complex of PSII by the thermal transfer of energy, a process termed non-photochemical quenching (NPQ; Niyogi and Truong, 2013) .

PSI is also responsible for production of O_2^- in the chloroplast. This is described in a process known as the Mehler reaction, when the reduced ferredoxin transfers an electron to $^3O_2$ instead of the principal target $NADP^+$, which would yield NAPDH for the fueling the Calvin-Benson cycle (Mehler, 1951). O_2^- production increases during periods of high light stress and/or

drought which cause photorespiration (Wingler et al., 2000), slowing the Calvin-Benson cycle and the use of NADPH which causes a lack of $NADP^+$. Therefore, the production of O_2^- protects PSI from photooxidative damage by using O_2 as an electron sink (Kangasjärvi et al., 2012). Typically, O_2^- can be rapidly converted to H_2O_2, however, heavy metal stress can cause it to be converted to $OH^\bullet$ (Winterbourn, 1995).

Aside from generation as byproducts of photosynthesis, photosensitizers, such as tetrapyrrole precursors of chlorophyll function in $^1O_2$ generation, particularly in plant-pathogen interactions (Flors and Nonell, 2006). It is also speculated that $^1O_2$ could be produced from damaged PSII repair occurring in the grana margin of the thylakoid membrane (Dogra and Kim, 2020).

Peroxisomes

Peroxisomes are single-membrane organelles with granular matrices where ROS production can occur. Peroxisomes are a major source of H_2O_2, particularly during photorespiration when there are high levels of glycolate produced in the chloroplast and sent to the peroxisome. There, glycolate oxidases form H_2O_2 from the oxidization of glycolate (del Río and López-Huertas, 2016). Furthermore, fatty acid β-oxidation and enzymatic reactions of flavin oxidases can also generate H_2O_2 (Sharma et al., 2012). O_2^- is also generated in the peroxisome in the peroxisomal matrix as a by-product when xanthine or hypoxanthine metabolized into uric acid by xanthine oxidases (Foyer and Noctor, 2003).

Mitochondria

Mitochondria are double-membrane organelles, similar to the chloroplast, with the inner membrane housing the ETC and encompassing the mitochondrial matrix. Both locations are sites for O_2^-/H_2O_2 production. In the mitochondrial ETC, there are two components where O_2 is reduced to O_2^-. Complex I, or the NADH Dehydrogenase, directly reduces O_2 to O_2^- (Møller, 2001). At Complex III the ubiquinone donates an electron to Cyt c_1 which then causes the ubiquinone to be in the unstable ubisemiquinone form which can leak electrons to O_2 to form O_2^-, similar to the way O_2^- is generated at PSII (Sweetlove and Foyer, 2004). O_2^- is then rapidly dismutated to H_2O_2 by SODs (Das and Roychoudhury, 2014). However, there is an alternative respiratory pathway in mitochondria that helps reduce ROS production by maintaining the ubiquinone in a reduced state using the alternative oxidase (AOX) (Vanlerberghe, 2013). This is known to be especially helpful in reducing ROS production during periods of drought as shown in plants with suppressed AOX which were more sensitive to drought conditions (Vishwakarma et al., 2015). They are also important in defense against phloem feeding insects with AOX silencing leading to less secondary metabolite accumulation and lower fitness (Zhang et al., 2012).

ROS Scavengers

The extent of ROS accumulation and the maintenance of a reductive/oxidative (redox) balance in cellular compartments in the plant is dependent on a network of antioxidant machinery (Foyer and Noctor, 2005). Antioxidants act to mitigate the oxidative stress that could result from the reactivity of ROS and are divided into two categories: enzymatic antioxidants which catalyze a reaction to scavenge ROS and nonezymatic antioxidants which directly react

with ROS to scavenge (Ahmad et al., 2009, 2010). The enzymatic antioxidants are distributed throughout the plant as compartment-specific isoforms and include SODs, catalases (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and guaiacol peroxidase (GPX; Davletova et al. 2005, Gill and Tuteja 2010). All of these enzymes are associated with scavenging O_2^-/H_2O_2 or regenerating substrates for the scavenging of ROS (Ahmad et al., 2010). Nonenzymatic antioxidants also have various subcellular locations and are typically positioned close to the site of ROS production. $^1O_2$ is scavenged non-enzymatically by carotenoids, flavonoids, proline, and α-tocopherol (Asada, 2006; Ramel et al., 2012b; Brunetti et al., 2018). Ascorbic acid (AsA) and reduced glutathione (GSH) are both involved in scavenging for H_2O_2 by acting as a substrate for enzymatic antioxidants such as APX (for AA) and GR and GST (for GSH) (Ahmad et al., 2009). However, AsA can be associated with $^1O_2$ scavenging as well, but this is not likely the principle scavenger *in planta* (Bodannes and Chan, 1979).

Redox Signaling in the Plant

As their name suggests, ROS are highly reactive with the biological components of the plant. Once produced, ROS can react with DNA, lipids, and proteins or be scavenged by antioxidants. The role ROS play as signaling molecules for defense is determined by ROS processing systems.

Superoxide/Hydrogen Peroxide

O_2^-/H_2O_2 production has been implicated in a number of physiological responses to abiotic and biotic stresses such as drought stress (Terzi et al., 2014), heat stress (Wang et al.,

2014), and the oxidative burst during a hypersensitive response to pathogens that can trigger programmed cell death (PCD; Levine et al. 1994). It is very important for regulating growth and development (Laloi et al., 2006). As discussed, there are several sites responsible for the production of both O_2^- and H_2O_2. However, the source depends on the stress, such as NADPH oxidase in the apoplast and cell membrane being important in the production of ROS due to herbivore challenge, yet the chloroplast and peroxisome are the primary producers during drought (Wingler et al., 2000; Chaouch et al., 2010).

Because O_2^- is rapidly dismutated to H_2O_2, it is likely that H_2O_2 is the ROS signal for physiological changes and adaptations. H_2O_2 signaling pathways can lead to physiological changes by either direct interaction, such as cross-linking of cell wall structural proteins, or by activation of transcription factors and changes in gene expression to help induce defense or tolerance to a stress (Levine et al., 1994; Mittler and Berkowitz, 2001). The interplay between ROS, specifically H_2O_2, and antioxidants is known to be diverse and complex (Foyer and Noctor, 2008; Hasanuzzaman et al., 2019). They can serve the function of redox sensing, such as with the thiol group of glutathione (Mock and Dietz, 2016). For example, H_2O_2-dependent changes in glutathione are implicated in regulation of hormonal defense pathways (Han et al., 2013a, 2013b). Because the interplay between ROS and scavengers is variable and complex, it is suggested that ROS scavenging systems should be referred to as “ROS processing systems” instead (Noctor et al., 2018).

Other mediators of H_2O_2 signal transduction are mitogen-associated protein kinases (MAPKs). These function downstream of H_2O_2 and/or Ca^{2+} to regulate various physiological responses (Waszczak et al., 2015). Chloroplast-produced H_2O_2, for example, is shown to activate a specific MAPK cascade which triggers hypersensitive response cell death (Liu et al., 2007).

However, H_2O_2 can also activate MAPKs by Ca^{2+}, which in turn activates the ser/thr kinase OXIDATIVE INDUCIBLE-SIGNALING1 (OXI1; Rentel et al. 2004). OXI1 is required in the plant for activation of specifically MAPK3/6 (Rentel et al., 2004), which cause a phosphorylation cascade that leads to expression of transcription factors (TF) or activation of NADPH oxidases. This activity continues to propagate the H_2O_2 signal and cause a similar signaling cascade in other cells.

Many of the TFs induced by MAPK3/6 are responsible for increasing gene expression of the ROS-scavenging network. *Zat12*, for instance, encodes a zinc-finger protein necessary for the upregulation of *Apx1*, which is involved in scavenging H_2O_2 in the cytosol to relieve oxidative stress (Rizhsky et al., 2004; Koussevitzky et al., 2008). Regulation of *OXI1* expression is important for resistance to herbivore stress as well, as seen in the *oxi1* Arabidopsis mutant. Shoala and colleagues (2018) demonstrated aphid resistance to GPA in the mutant because of increased callose deposition due to upregulation of the callose synthase gene *GLS5*. However, it was not shown if this response was due to MAPK3/6 activity or acted independently. This suggests that OXI1 functions to mediate stress responses by signaling for tolerance.

Singlet Oxygen

Because $^1O_2$ is the most reactive species, it has the shortest half-life of 200 ns in biological environments (Gorman and Rodgers, 1992). Therefore, responses to $^1O_2$ are likely due to interactions with biomolecules close to the site of production that initiate a chloroplast-to-nucleus retrograde signal. These may be β-carotene near PSII reaction centers, lipids of chloroplast membranes, or proteins embedded in the thylakoid membrane (Wagner et al., 2004;

Przybyla et al., 2008; Ramel et al., 2012a). In Arabidopsis there appears to be distinct pathways for $^1O_2$ retrograde signaling.

An instrumental *Arabidopsis* mutant in the study of $^1O_2$ signaling is the *flu* mutant which accumulates the potent photosensitizer protochlorophyllide (Pchlide) in the dark and upon re-illumination generates $^1O_2$ in the chloroplast (Meskauskiene et al., 2001; Camp et al., 2003). An important discovery made with the *flu* mutant was the role of EXECUTER1 (EX1) and EXECUTER2 (EX2) in signaling for programmed cell death (PCD) which is preceded by electrolyte leakage (EL; Wagner et al., 2004; Lee et al., 2007; Przybyla et al., 2008). EX1 and EX2 protein are localized to the non-appressed region of the thylakoid membrane called the grana margin (GM). EX1 oxidation by $^1O_2$ marks it for proteolysis by a thylakoid membrane-bound metalloprotease, FtsH2 (Wang et al., 2016; Dogra et al., 2017). EX2 is a putative modulator of the $^1O_2$ response in *flu* and is also believed to undergo the same process of activation, although it has not been demonstrated (Lee et al., 2007; Dogra and Kim, 2020). T-DNA mutations of *EX1* and *EX2* abrogates the response of *flu* after an LDL shift also confirming their essential role in $^1O_2$ signaling (Lee et al., 2007; Przybyla et al., 2008).

While $^1O_2$ can cause protein modification for signal transduction of PCD, the reaction of $^1O_2$ with β-carotene to produce β-cyclocitrol (β-CC) in the chloroplast signals for stress acclimation under high light stress (Ramel et al., 2012b, 2013). The reaction of $^1O_2$ with carotenoid scavengers near the reaction center of PSII yields aldehydes and endoperoxides through oxidative modification (Ramel et al., 2012a). Specifically, $^1O_2$ oxidation of the carotenoid β-carotene gives rise to β-cyclocitral (β-CC), a volatile, highly reactive electrophilic compound that can then diffuse out of the chloroplast to signal for an acclimation response to high light stress (Ramel et al., 2012b). Arabidopsis pretreated with β-CC induced changes in

expression of gene associated with oxidative stress, hormone signaling, and detoxification. Furthermore, β-CC pretreated plants were more tolerant to high light exposure in a dose-dependent manner (Ramel et al., 2012b). It is proposed that the protein METHYLENE BLUE SENSITIVITY (MBS1) is activated downstream of β-CC to transduce the signal to the nucleus for regulation of plant growth and development under high light stress (Shumbe et al., 2017). However, D'Alessandro and colleagues (2018) identified SLC14 as another downstream mediator of the $^1O_2$ signal transduced by β-CC that acts independently of MBS1. SLC14 further regulates the expression of ANAC genes, and an SLC14*oex* line was found to be resilient to high light stress, indicating SLC14 is involved in photooxidative adaptation. The authors also discovered that only 30% of gene expression change was due to β-CC in *ch1* mutants under high light stress. Therefore, it is likely that multiple pathways of $^1O_2$ signaling exist for adaptation under high light stress and defense against other abiotic and biotic stressors.

Importantly, β-CC generation occurs in the grana core (GC) of the thylakoid membrane where active PSII reside. When the D1 and D2 proteins of the PSII reaction centers are damaged, they move from the GC to the GM where EX1 and EX2 are localized (Dogra and Kim, 2020). Because of the high reactivity of $^1O_2$, it is not likely that $^1O_2$ produced in GC by active PSII would move to the GM to react with EX1 and EX2. Therefore, the pathways are not initiated by the same $^1O_2$-generating mechanism and remain relatively distinct from one another.

Reactive oxygen species and Aphids

ROS are important signaling molecules in plant stress and are likely involved in defense signaling against aphids. In plant-aphid interactions, investigations of both sides of the relationship is key to fully understand the dynamics of ROS-mediated aphid defense. Therefore,

the following sections address the questions (1) do aphids induce ROS and alter the redox status of the plant and (2) does a redox shift in the plant impact the aphid? Table I summarizes many of the relevant studies discussed in these sections.

Evidence supporting aphid-induced redox change

In the last three decades, multiple studies have addressed the question of "do aphids affect the plant redox state?" The potential role aphids play in altering the redox status of the plant was first hypothesized by Miles (1978), who suggested that oxidizing agents in aphid saliva might interact with plant hormones and pointed out the potential for plant phenolics to reduce aphid fitness. Later, Jiang and Miles (1993) discovered increased peroxidase, catechol oxidase, and SOD activity in extracts of infested leaves. Interestingly, oxidized tissue extracts were more preferred by aphids. Furthermore, in that system, Miles and Oertli (1993) demonstrated lucerne tissues infested with *Therioaphis trifolii maculate* (Buckton) were more greatly oxidized than uninfested tissue and had more soluble phenolics. The addition of ascorbate and glutathione antioxidants decreased aphid populations on the plant, again suggesting the oxidation of plant tissue benefited the aphid. These findings led Miles and his group to conclude that the aphid attempts to oxidize plant tissue while the plant works to control oxidation through a redox system.

Over the past three decades since Miles and his group first investigated the redox hypothesis, several studies have gone on to directly measure ROS accumulation in the plant and link it to aphid resistance. Argandoña et al. (2001) demonstrated H_2O_2 and peroxidase activity increased in the less susceptible cultivar of barley, Frontera, as early as 20 and 30 minutes, respectively, when infested with the greenbug (*Schizaphis graminum*). The H_2O_2 production was

theorized to be the result of NADPH oxidases activity in the apoplast. NADPH oxidases were further attributed to aphid-induced ROS production by Moloi and van der Westhuizen (2006) who showed the RWA on resistant *Dn1* wheat increased H_2O_2 accumulation and NADPH oxidase activity. When the suicide inhibitor of NADPH oxidase, diphenyleneiodonium, was applied to the plant NADPH oxidase activity and H_2O_2 accumulation decreased in the same manner. Later, in the same system, H_2O_2 accumulation was attributed to xanthine oxidases (XOs) in the peroxisome (Berner and Van der Westhuizen, 2010).

While further studies continued to identify H_2O_2 accumulation in response to aphids, O_2^- has also been identified in response to aphids. Although, it was not directly measured in aphid-infested plants until nearly a decade later. Before that, O_2^- was indirectly addressed through quantification of H_2O_2 as the result of NADPH oxidase activity. Also, Jiang and Miles (1993) indirectly observed increases in O_2^- as measured by the ability of infested leaf extracts to reduce cytochrome c. However, Mai et al. (2013) directly measured O_2^- in the leaves of pea (*Pisum sativum* L.) in response to the pea aphid (*Acyrthosiphon pisum* Harris) using the dye dihydroethidium, which fluoresces after reaction with O_2^-. This study found increased O_2^- production in response to aphids that last at least until 96 h, whereas H_2O_2 accumulation peaked 24 h after infestation before returning to normal levels. Later studies in other plant-aphid systems have observed the same trend of O_2^- production outlasting the peak of H_2O_2 (Borowiak-Sobkowiak et al., 2016; Czerniewicz et al., 2017). Furthermore, sustained O_2^- production was only observed in the resistant winter triticale cultivar, supporting the role of O_2^- in enhancing the plant defense response (Czerniewicz et al., 2017).

Factors influencing the timing of ROS in response to aphids

ROS production is an early response in plant defense against biotic stress, often characterized as an oxidative burst that triggers a hypersensitive response (Levine et al., 1994; Bolwell et al., 2002). In Arabidopsis, extracts of GPA caused a ROS burst at 2 h that faded 9 h later and required NADPH oxidases (Prince et al., 2014). This response was delayed compared with the pathogen elicitor flg22 but lasted longer and was sufficient to increase defense-related gene expression and callose deposition. A non-invasive microtest technique (NMT) that measured ROS in tobacco *in situ* found H_2O_2 accumulating in response to GPA as early as 2 hpi that peaked at 15 hpi (Ren et al., 2014). Furthermore, GPA on Arabidopsis caused a significant burst of ROS, attributed to the apoplast, as early as minutes after infestation (Xu et al., 2021).

The dynamic response of ROS is influenced by various factors, including density of the infestation and which ROS are evaluated. For example, soybean infested with the cowpea aphid (*Aphis craccicora* Koch) was infested at three different densities (10, 20, and 30 aphids) and caused both H_2O_2 and O_2^- to accumulate (Mai et al., 2017). Both H_2O_2 and O_2^- accumulation peaked earlier in tissue infested with 20 and 30 aphids compared with 10 aphids; also, O_2^- peaked earliest, at 12 h, whereas H_2O_2 peaked at 24 h. Again, in other systems such as asparagus and wheat, the moderate and heavy infestation levels appear to expedite the peak of ROS accumulation (Borowiak-Sobkowiak et al., 2016; Durak et al., 2019; Lukasik and Golawska, 2019). Also, age of the plant may influence the ROS response. For example, when one-month-old and two-month-old asparagus were infested with the asparagus aphid (*Brachycorynella asparagi* Mordvilko) H_2O_2 production was strongest in two-month-old plants, whereas O_2^- production was strongest in one-month-old plants (Borowiak-Sobkowiak et al., 2016). Furthermore, ROS accumulation has been demonstrated to peak earlier in aphid-resistant

cultivars than in susceptible cultivars (Berner and Van der Westhuizen, 2010; Czerniewicz et al., 2017; Lukasik and Golawska, 2019), indicating ROS are involved in plant defense against aphids. As a whole, the ROS response in the plant is dependent on various factors, which may allow the plant to fine-tune the defense response in relation to the stress.

In plant-pathogen interactions, ROS production is often observed as a biphasic response characterized by two distinct production phases. For example, avirulent pathogens that are detected by the plant through R genes cause a rapid, transient accumulation of ROS followed by a second phase with a greater magnitude of accumulation that is sustained. This is believed to be necessary for disease resistance. However, in virulent pathogen systems where the plant does not mount a defense response, only the first transient phase is observed (Lamb and Dixon, 1997). Furthermore, ROS accumulation for the first phase typically is due to NADPH oxidase activity. ROS accumulation in plant-aphid systems does show similarity to plant-pathogen interactions when viewed through the lens of the biphasic response. For example, H_2O_2 began accumulating in the aphid-resistant *Dn1* wheat when challenged with RWA at 3 h, peaking shortly after at 10 h, and was attributed to NADPH oxidase activity (Moloi and van der Westhuizen, 2006). A further study in that system found H_2O_2 began accumulating 8 h after attack and steadily increased until 50 h, with H_2O_2 production attributed to peroxisomal XOs (Berner and Van der Westhuizen, 2010). Viewing these studies together, this system mirrors the transient first phase and the sustained second phase of a biphasic response, along with the separate cellular locations of ROS production. This response could be involved in the aphid-resistance observed in *Dn1* wheat to RWA. Moreover, a study measuring apoplastic ROS and the redox response of the peroxisome discovered a similar trend as the *Dn1* ROS response. In Arabidopsis challenged with

GPA, a transient burst of ROS was observed in the apoplast, followed by an oxidative shift in the peroxisome, indicating ROS accumulation, that was sustained for up to 20 hpi (Xu et al., 2021).

A biphasic response could be occurring in more plant-aphid interactions, however, most studies of ROS in response to aphids do not measure early (before 24 h) and late (after 24 h) ROS accumulation or investigate specific cellular locations of ROS production. In fact, only a few studies have addressed the question of the location of ROS generation in response to aphids beyond NADPH activity in the apoplast, and often indirectly (Berner and Van der Westhuizen, 2010; Kerchev et al., 2012; Borowiak-Sobkowiak et al., 2016; Rasool et al., 2019). Therefore, studies aimed at early and late responses in the subcellular locations are needed. The chloroplast is the largest site of intracellular ROS and is important in plant-pathogen resistance through ROS-mediated chloroplast-to-nucleus retrograde signaling (Kuźniak and Kopczewski, 2020). Investigating the ROS accumulation and signaling in the chloroplast in response to aphids could provide novel mechanisms for host plant resistance.

ROS influence on aphid fitness

Observations of increased ROS in the plant after aphid-infestation does not necessarily mean the ROS is impacting the fitness of the aphid. So how does ROS impact the aphid? Potentially the ROS accumulation triggers oxidative or allelochemical compounds that are taken up by the aphid in the phloem (Krishnan et al., 2007; Zhang et al., 2017b). It has been demonstrated that aphid feeding triggers a burst of H_2O_2 in the phloem that is taken up by the aphid (Guo et al., 2020). Lei and Zhu-Salzman (2015) noted that mutants of *BOTRYTIS-INDUCED KINASE1* (*bik1*) with aphid-induced H_2O_2 accumulation had up-regulated ROS-generating and ROS-responsive genes but not ROS-metabolizing genes. In turn, GPA feeding on

bik1 had increased antioxidant activity, as well as decreased fecundity. The authors concluded the reduced fitness was due to the metabolic expense of detoxification. Moreover, aphids feeding on resistant winter triticale with higher ROS accumulation (Lukasik and Golawska, 2019) had increased ROS in their tissue, both H_2O_2 and O_2^-, within 24 h of feeding (Łukasik and Goławska, 2013). However, it is unclear in this study if the ROS is directly from the plant or the result of ingesting prooxidant compounds.

Changes in the redox status of the plant from ROS accumulation can also trigger downstream defenses. Defense responses can impact aphids either by antibiosis, such as decreasing adult fecundity and juvenile survival (Zhao et al., 2016; Sun et al., 2020), or by antixenosis, which causes the aphid to move away from the plant (Guo et al., 2019). Redox signaling in the plant can cause cell wall reorganization and cross-linking (Levine et al., 1994; Rasool et al., 2017), as well as callose deposition in the phloem (Prince et al., 2014; Durak et al., 2019), that makes feeding difficult for aphids and reduces the time spent feeding. Additionally, aphid-induced ROS triggers accumulation of defensive compounds such as phenolics that can act as feeding deterrents (Dreyer and Jones, 1981; Czerniewicz et al., 2017; Durak et al., 2019). Also, a hypersensitive response (HR) caused by an oxidative burst is followed by PCD and associated with aphid resistance (Villada et al., 2009). Furthermore, ROS can interact with phytohormones to regulate defense response such as secondary metabolite accumulation (Kuśnierczyk et al., 2008; Rasool et al., 2019).

While ROS accumulation is often connected with aphid resistance in the plant, some studies have found ROS to be only symptomatic of aphid challenge or inducing aphid susceptibility. For example, potato aphid (*Macrosiphum euphorbiae* Thomas) infestations did induce ROS on resistant *Mi-1* and susceptible *mi-1* tomato, but this was not followed by an HR

and, therefore, not attributed to R gene-mediated resistance (de Ilarduya et al., 2003). Also, subsequent infestation of *S. graminum* and *S. avenae* on plants with increased H_2O_2 from prior *S. graminum* feeding had increased reproduction, indicating H_2O_2 induced aphid susceptibility (Zhang et al., 2019).

Aphids and singlet oxygen

The previous studies reporting ROS production and accumulation in response to aphids have all dealt with H_2O_2 and/or O_2^-, neglecting $^1O_2$. This in part is due to difficulty in detecting $^1O_2$ (Koh and Fluhr, 2016), as it is highly reactive with a half-life of 200ns in biological systems (Gorman and Rodgers, 1992). Some studies have indirectly addressed the idea of $^1O_2$ being important in herbivore resistance through the generation of plant secondary metabolites such as phytoalexins (Berenbaum and Larson, 1988). Phytoalexins are induced defensive compounds that are commonly employed by plants for resistance to biotic stress, such as fungi (Flors and Nonell, 2006). Some phytoalexins, such as phenalenones or furanocoumarins, can act as photosensitizers to generate $^1O_2$ via light energy (Knox and Dodge, 1985; Flors and Nonell, 2006). Furanocoumarin is found in the plant families Umbelliferae and Rutaceae and the linear furanocoumarin, xanthotoxin, is toxic to the southern fall armyworm (SFA; *Spodoptera aridania* Cramer) (Berenbaum, 1978, 1981). When a diet of the SFA containing xanthotoxin was treated with UV light, the toxic effect on SFA was enhanced (Berenbaum, 1978). As phytoalexins can generate $^1O_2$ from UV light, this could indicate that $^1O_2$ in the media increased the toxicity to SFA. Furthermore, plants from Umbelliferae and Rutaceae were treated with UV light and generated a high flux of $^1O_2$ in the stable gas-phase on the leaf surface that were projected to be sufficient to damage herbivores on the plant (Berenbaum and Larson, 1988). While phytoalexins

in Umbelliferae and Rutaceae may serve as a source of $^1O_2$-mediated herbivore defense, it is not certain if they have potential for herbivore defense in agronomic crops.

A potential source of $^1O_2$ that is ubiquitous throughout plants is the chloroplast. Currently $^1O_2$ from the chloroplast is mainly associated with high light stress acclimation (Dogra and Kim, 2020). However, there is evidence of chloroplast-to-nucleus retrograde signaling through $^1O_2$ having overlap with abiotic and biotic stress response. In the *flu* mutant after an LDL shift, enhanced $^1O_2$ production in the chloroplast causes salicylic acid (SA) accumulation and expression of pathogenesis-related (PR) proteins *PR1* and *PR5* mediated by *ENHANCED DISEASE SUSCEPTIBILITY1* (Ochsenbein et al., 2006), which are responses that trigger plant resistance to pathogens (Rustérucci et al., 2001, 1). Key nodes in the $^1O_2$ signaling pathway of *flu* are EX1 and EX2 proteins located in the thylakoid membrane (Lee et al., 2007; Wang et al., 2016). Furthermore, resistance to a virulent strain of *Pseudomonas syringae* was activated after treatment of moderate light stress that induces $^1O_2$ accumulation (Zhang et al., 2014). Disease resistance in moderate light stressed plants was compromised by T-DNA insertion mutations of *EX1* and *EX2* that suppressed *PR1* expression; these results indicate $^1O_2$ signaling from the chloroplast via EX1/EX2 is also important in biotic stress response. Furthermore, *PR1* and SA are involved in defense against aphids (Moran and Thompson, 2001). Therefore, $^1O_2$ from the chloroplast may be a source of plant defense against aphids.

Fatty acid desaturases and plant defense

Fatty acids and desaturation

Fatty acids (FAs) are vital components of cellular function and defense signaling in the plant (Kachroo and Kachroo, 2009). Composed of carboxylic acids attached to a tail of

hydrocarbon chains, FAs vary based on the length of carbons and degree of double bonds present in the tail. Saturated FAs have no double bonds in their tail, while unsaturated FAs have one or more double bonds that are added by fatty acid desaturases (FADs) (Lee et al., 2016). In the membranes of the plant, almost all FAs are 16- and 18-carbon long (Millar et al., 2000). Furthermore, the leaf and root tissue is primarily composed of trienoic (TA) and dienoic (DA) FAs, which have three and two double bonds, respectively (Reszczyńska and Hanaka, 2020). Omega-3 fatty acid desaturases (ω-3 FADs), including ones localized to the chloroplast and endoplasmic reticulum, are essential for desaturation from DAs to TAs and influence the membrane fluidity (Teixeira et al., 2010; Hiremath et al., 2017; Li et al., 2021).

FAD7 and aphid resistance

Fatty Acid Desaturase 7 (FAD7) is a chloroplast-localized ω-3 FAD that negatively regulates aphid resistance. Loss of function of FAD7 in both Arabidopsis and tomato confers resistance to GPA and PA, respectively (Avila et al., 2012). As FAD7 is responsible for converting DAs to TAs, a loss of function results in increased levels of 16:2 and 18:2 and much lower levels of 16:3 and 18:3 (Browse et al., 1986). Notably, plants with loss of function of FAD7 have increased levels of the 18:2 FA, linoleic acid (LNA), and decreased levels of the 18:3 FA, linolenic acid (LA). The *fad7-1* mutation in Arabidopsis has between 30-40% reduced LA in foliar tissue compared to wild-type Arabidopsis (Li et al., 2021). In tomato, the mutation of *fad7*, *suppressor of prosystemin-mediated response2* (*spr2*), much more severely decreases TAs and results in up to a 90% decrease of LA in foliar tissue (Avila et al., 2012). In *spr2*, population growth of PA is decreased by over 50% compared with wild-type tomato. Comparatively, the *fad7* mutant Arabidopsis decreases GPA population growth by about 20%

compared with wild-type Arabidopsis (Avila et al., 2012; Li et al., 2021). This demonstrates the FAD7 influences aphid fitness in two different plant groups. Furthermore, the *spr2* tomato mutant has antixenotic effects on PA that signficiantly reduces host-selection. Whether the *fad7* mutation in Arabidopsis also confers antixenotic resistance is not known. The FAD7 enzyme is localized to the thylakoid membrane of the chloroplast (Andreu et al., 2007), and influences the fluidity of photosynthetic membranes. This points to the chloroplast as the source for aphid resistance in these plants and could involve ROS.

Plant defense against abiotic and biotic stress is found to be influenced by fatty acid metabolism. A novel source of aphid resistance that warrants further investigation is the loss of function of Fatty Acid Desaturase 7 (FAD7) which confers aphid resistance to tomato (*Solanum lycopersicum*, Solanaceae) and Arabidopsis (Brassicaceae) (Avila et al., 2012; Li et al., 2021). As homologs of this gene are conserved throughout several plant groups (Song et al., 2004; Andreu et al., 2007; Hiremath et al., 2017), the biochemical and physiological underpinnings of this resistance mechanism could potentially be relevant to agronomically important crops.

FADs and ROS and Aphids

FAD enzymes regulate the composition of FAs in the plant tissue and influence plant stress signaling. For example, ω-3 FADs, such as FAD7, convert LNA to LA, the main targets of enzymatic or non-enzymatic oxidation that produces oxylipins. Oxylipins are stress signaling components that contribute to plant defense against biotic stresses (Blée, 2002), including aphids (Smith et al., 2010). Moreover, the fatty acid-hydroperoxidase α-dioxygenases (α-DOX) that contributes to oxylipin synthesis has been shown to be upregulated by aphid challenge (Avila et al., 2013). Moreover, silencing α-DOX in *spr2* restored aphid susceptibility in that background

(Avila et al., 2013), indicating oxylipin synthesis altered FAs are important in aphid resistance. ROS, especially $^1O_2$, are able to react with FAs non-enzymatically, but can also influence the oxylipin synthesis through increasing enzymatic lipid peroxidation (Przybyla et al., 2008; Farmer and Mueller, 2013).

The influence of FADs on the stability of photosynthetic membranes could also potentially aid in aphid resistance by impacting the rate of $^1O_2$ generation (Hiremath et al., 2017). This hypothesis is support by preliminary data from our laboratory using the aphid-resistant Arabidopsis mutant *fad3fad7fad8.* Lipid profiling analyzing fatty acid hydroperoxides indicates enhanced accumulation of $^1O_2$ in *fad3fad7fad8* compared to wild-type plants (unpublished data by M. Mueller and F. Goggin). The profile observed by HPLC in *fad3fad7fad8* matches the characteristic fingerprint of lipid peroxidation products previously reported to occur after $^1O_2$ exposure (Farmer and Mueller, 2013). Both the *fad3/fad7/fad8* triple mutant and *fad7* single mutant have the same level of aphid resistance to GPA (unpublished data, Goggin lab). Therefore, it is possible *fad7* also has enhanced levels of $^1O_2$ that is influencing aphid resistance.

Research Objectives

The loss of function of Fatty Acid Desaturase 7 (FAD7) alters the fatty acid composition of the photosynthetic membranes of the chloroplast, as well as confers aphid resistance in Arabidopsis. This research aims to contribute to an understanding of the FAD7 aphid-resistance mechanism and explore the possibility of $^1O_2$-signaling from the chloroplast being involved in aphid resistance. To investigate the influence of the *fatty acid desaturase 7* (*fad7*) mutation of Arabidopsis on aphid resistance, we evaluated several parameters of aphid fitness on *fad7* using the generalist herbivore, the green peach aphid (GPA), *Myzus persicae* (Sulzer) and also the

specialist herbivores, the cabbage aphid, *Brevicoryne brassicae* (Chapter II). Next, we investigated the redox status of the chloroplast and the influence of aphids on $^1O_2$ accumulation in *fad7* and wild-type Arabidopsis, as well as the influence of $^1O_2$ accumulation on aphid fitness (Chapter III). Finally, we explored the role of EX1 and EX2, critical nodes in the $^1O_2$-signaling pathway, on aphid resistance in *fad7* (Chapter IV). This study is relevant to our understanding of the influence of the chloroplast in perception of environmental stress and initiation of defense responses. Moreover, it contributes to our knowledge of the *fad7* resistance mechanism and how $^1O_2$ is involved in plant defense against herbivores.

Table I. Relevant studies in ROS-Aphid interactions.

Citation **Plant System** **Aphid Species**	**Do aphids affect plant redox state?**	**Does the plant redox state affect aphids?**
Guo et al., 2020. *Nicotiana tabacum* *Myzus persicae*	**Yes.** Increased ROS after aphid infestation	**Yes. Decreased fitness.** Virulent aphid decreased elicitor protein which induces ROS burst
Sun et al., 2020. *Capsicum baccatum* *M. persicae*	**Yes.** Virulent aphid caused ROS burst compared with avirulent aphid; avirulent aphid prevented ROS burst by virulent aphid	**Yes. Decreased fitness.** Decreased ROS by avirulent aphid made *C. baccatum* susceptible to virulent aphid
Durak et al., 2019. *Thuja orientalis* *Cinara tujafilina*	**Yes.** Increased O_2^- and other free radicals (quinones)	N/A
Guo et al., 2019. *N. tabacum* *M. persicae*	N/A	**Yes**. **Decreased fitness.** H_2O_2 in the apoplast, increased by CMV, reduced aphid settling and phloem feeding, and increased short probes Silencing *RbohD* decreased H_2O_2 and restored aphid susceptibility
Shao et al., 2019. *Sorghum bicolor* *Melanaphis sacchari*	**Yes.** H_2O_2 accumulation Antioxidant activity decreased (APX, POX, GPX)	**Yes*. Decreased fitness** Aphid-resistant HN16 had more H_2O_2
Zhang et al., 2019. *Triticum aestivum* *Sitobion avenae* *Schizaphis graminum*	**Yes.** Both aphids induced H_2O_2, but *S. graminum* was significantly higher	**Yes. Increased fitness.** Both aphids had increased reproduction feeding on wheat with increased H_2O_2 from pre-infestation by *S. graminum*
Lukasik and Golawska et al, 2019. *Triticosecale* Wittm. *Ropalosiphum padi*	**Yes.** Both aphids increased H_2O_2 attributed to NADPH oxidase activity	**Yes*. Decreased fitness.** Aphid-resistant Witon accumulated more H_2O_2
Sytykiewicz, 2016. *Zea mays* *R. padi* *S. avenae*	**Yes.** Both aphid induced H_2O_2 attributed to NADPH oxidase activity	**Yes*.** Aphid-resistant cultivars had increased ROS response

N/A = not addressed

*= aphid impact evaluated by comparison between aphid-resistant and susceptible cultivars

Table I. (Continued)

Mai et al, 2013. *Glycine max* *Aphis craccivora*	**Yes.** H_2O_2 and O_2^- (first time measured) increased	N/A
Kerchev et al., 2012. *Solanum tuberosum* *M. persicae*	**Yes.** H_2O_2 accumulated in response to aphids; suppressed chloroplastic SOD expression	**Yes. Decreased fitness.** Increased antioxidant, ascorbic acid, increased aphid reproduction
Berner and Westhuizen 2010. *T. aestivum* *Diuraphis noxia*	**Yes.** H_2O_2 accumulated in response to aphid on *Dn1* Attributed to xanthine oxidase in peroxisome	**Yes*. Decreased fitness.** Aphid-resistant *Dn1* had higher ROS accumulation
Moloi 2006. *T. aestivum* *D. noxia*	**Yes.** NADPH oxidase activity and H_2O_2 increased in aphid resistant *Dn1*	**Yes*. Decreased fitness.** Aphid-resistant *Dn1* had higher ROS accumulation
Ilarduya et al. 2003. *Solanum lycopersicum* *Macrosiphum euphorbia*	**Yes.** H_2O_2 accumulated in resistant (*Mi-1)* and susceptible (*mi-1)*	**No.** Was not followed by HR Potentially only symptom development
Argandona 2001 *Hordeum vulgare* *R. padi* *S. graminum*	**Yes.** H_2O_2 accumulation and increased peroxidase activity	N/A
Jiang and Miles 1993. Lucerne *Therioaphis trifolii maculata*	**Yes.** ROS production marked as discoloration around stylet sheath and cell walls Potentially O_2^- from decreased Cytochrome C activity and increased SOD activity	N/A
Miles and Oertli 1993. Lucerne *Acyrthosiphon kondo* *Therioaphis trifolii maculata*	N/A	**Yes. Increased fitness.** Decreased oxidation of plant tissue through antioxidants decreased aphid reproduction.

N/A = not addressed

*= aphid impact evaluated by comparison between aphid-resistant and susceptible cultivars

References

Ahmad, P., Jaleel, C. A., Azoos, M. M., and Nabi, G. (2009). Generation of ROS and non-enzymatic antioxidants during abiotic stress in plants. *Botany research international* 2, 11–20.

Ahmad, P., Jaleel, C. A., Salem, M. A., Nabi, G., and Sharma, S. (2010). Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress. *Critical Reviews in Biotechnology* 30, 161–175. doi:10.3109/07388550903524243.

Andersson, B., Salter, A. H., Virgin, I., Vass, I., and Styring, S. (1992). Photodamage to photosystem II - primary and secondary events. *Journal of Photochemistry and Photobiology B: Biology* 15, 15–31. doi:10.1016/1011-1344(92)87003-R.

Andreu, V., Collados, R., Testillano, P. S., Risueño, M. del C., Picorel, R., and Alfonso, M. (2007). *In situ* molecular identification of the plastid ω3 Fatty Acid Desaturase FAD7 from soybean: Evidence of thylakoid membrane localization. *Plant Physiol* 145, 1336–1344. doi:10.1104/pp.107.109637.

Apel, K., and Hirt, H. (2004). REACTIVE OXYGEN SPECIES: Metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology* 55, 373–399. doi:10.1146/annurev.arplant.55.031903.141701.

Argandoña, V. H., Chaman, M., Cardemil, L., Muñoz, O., Zúñiga, G. E., and Corcuera, L. J. (2001). Ethylene production and peroxidase activity in aphid-infested barley. *J Chem Ecol* 27, 53–68. doi:10.1023/A:1005615932694.

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiol.* 141, 391–396. doi:10.1104/pp.106.082040.

Avila, C. A., Arévalo-Soliz, L. M., Jia, L., Navarre, D. A., Chen, Z., Howe, G. A., et al. (2012). Loss of function of FATTY ACID DESATURASE7 in tomato enhances basal aphid resistance in a salicylate-dependent manner. *Plant Physiol.* 158, 2028–2041. doi:10.1104/pp.111.191262.

Avila, C. A., Arevalo-Soliz, L. M., Lorence, A., and Goggin, F. L. (2013). Expression of α-DIOXYGENASE 1 in tomato and arabidopsis contributes to plant defenses against aphids. *MPMI* 26, 977–986. doi:10.1094/MPMI-01-13-0031-R.

Barber, M. D., Moores, G. D., Tatchell, G. M., Vice, W. E., and Denholm, I. (1999). Insecticide resistance in the currant–lettuce aphid, *Nasonovia ribisnigri* (Hemiptera: Aphididae) in the UK. *Bulletin of Entomological Research* 89, 17–23. doi:10.1017/S0007485399000036.

Bass, C., Puinean, A. M., Zimmer, C. T., Denholm, I., Field, L. M., Foster, S. P., et al. (2014). The evolution of insecticide resistance in the peach potato aphid, *Myzus persicae*. *Insect Biochemistry and Molecular Biology* 51, 41–51. doi:10.1016/j.ibmb.2014.05.003.

Begg, G. S., Cook, S. M., Dye, R., Ferrante, M., Franck, P., Lavigne, C., et al. (2017). A functional overview of conservation biological control. *Crop Protection* 97, 145–158. doi:10.1016/j.cropro.2016.11.008.

Berenbaum, M. (1978). Toxicity of a furanocoumarin to armyworms: A case of biosynthetic escape from insect herbivores. *Science* 201, 532–534. doi:10.1126/science.201.4355.532.

Berenbaum, M. (1981). Effects of linear furanocoumarins on an adapted specialist insect (*Papilio polyxenes*). *Ecological Entomology* 6, 345–351. doi:https://doi.org/10.1111/j.1365-2311.1981.tb00624.x.

Berenbaum, M. R., and Larson, R. A. (1988). Flux of singlet oxygen from leaves of phototoxic plants. *Experientia* 44, 1030–1032. doi:10.1007/BF01939914.

Berner, J. M., and Van der Westhuizen, A. J. (2010). Inhibition of xanthine oxidase activity results in the inhibition of russian wheat aphid-induced defense enzymes. *J Chem Ecol* 36, 1375–1380. doi:10.1007/s10886-010-9879-y.

Bielza, P., Balanza, V., Cifuentes, D., and Mendoza, J. E. (2020). Challenges facing arthropod biological control: Identifying traits for genetic improvement of predators in protected crops. *Pest Management Science* 76, 3517–3526. doi:https://doi.org/10.1002/ps.5857.

Blackman, R. L., and Eastop, V. F. (2000). Aphids on the world's crops: An identification and information guide. *Aphids on the world's crops: an identification and information guide.* Available at: https://www.cabdirect.org/cabdirect/abstract/20001106823 [Accessed March 2, 2021].

Blackman, R. L., and Eastop, V. F. (2006). *Aphids on the World's Herbaceous Plants and Shrubs*. New York: John Wiley & Sons.

Blée, E. (2002). Impact of phyto-oxylipins in plant defense. *Trends in Plant Science* 7, 315–322. doi:10.1016/S1360-1385(02)02290-2.

Bodannes, R. S., and Chan, P. C. (1979). Ascorbic acid as a scavenger of singlet oxygen. *FEBS Letters* 105, 195–196. doi:10.1016/0014-5793(79)80609-2.

Boissot, N., Schoeny, A., and Vanlerberghe-Masutti, F. (2016). *Vat*, an amazing gene conferring resistance to aphids and viruses they carry: From molecular structure to field effects. *Front Plant Sci* 7. doi:10.3389/fpls.2016.01420.

Boivin, G., Hance, T., and Brodeur, J. (2012). Aphid parasitoids in biological control. *cjps* 92, 1–12. doi:10.1139/CJPS2011-045.

Bolwell, G. P., Bindschedler, L. V., Blee, K. A., Butt, V. S., Davies, D. R., Gardner, S. L., et al. (2002). The apoplastic oxidative burst in response to biotic stress in plants: A three-component system. *Journal of Experimental Botany* 53, 1367–1376. doi:10.1093/jexbot/53.372.1367.

Bolwell, G. P., and Wojtaszek, P. (1997). Mechanisms for the generation of reactive oxygen species in plant defence – a broad perspective. *Physiological and Molecular Plant Pathology* 51, 347–366. doi:10.1006/pmpp.1997.0129.

Borowiak-Sobkowiak, B., Wozniak, A., Bednarski, W., Formela, M., Samardakiewicz, S., and Morkunas, I. (2016). Brachycorynella asparagi (Mordv.) Induced-oxidative stress and antioxidative defenses of *Asparagus officinalis* L. *Int. J. Mol. Sci.* 17, 1740. doi:10.3390/ijms17101740.

Bos, J. I. B., Prince, D., Pitino, M., Maffei, M. E., Win, J., and Hogenhout, S. A. (2010). A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (green peach aphid). *PLoS Genet* 6, e1001216. doi:10.1371/journal.pgen.1001216.

Botha, A.-M., Lacock, L., van Niekerk, C., Matsioloko, M. T., du Preez, F. B., Loots, S., et al. (2005a). Is photosynthetic transcriptional regulation in *Triticum aestivum L*. cv. 'TugelaDN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae)? *Plant Cell Rep* 25, 41. doi:10.1007/s00299-005-0001-9.

Botha, A.-M., Li, Y., and Lapitan, N. L. V. (2005b). Cereal host interactions with Russian wheat aphid: A review. *Journal of Plant Interactions* 1, 211–222. doi:10.1080/17429140601073035.

Botha, C. E. J., and Matsiliza, B. (2004). Reduction in transport in wheat (*Triticum aestivum*) is caused by sustained phloem feeding by the Russian wheat aphid (*Diuraphis noxia*). *South African Journal of Botany* 70, 249–254. doi:10.1016/S0254-6299(15)30242-8.

Bowler, C., Camp, W. V., Montagu, M. V., Inzé, D., and Asada, P. K. (1994). Superoxide Dismutase in Plants. *Critical Reviews in Plant Sciences* 13, 199–218. doi:10.1080/07352689409701914.

Browse, J., McCourt, P., and Somerville, C. (1986). A mutant of arabidopsis deficient in C18:3 and C16:3 Leaf Lipids 1. *Plant Physiol* 81, 859–864.

Brunetti, C., Fini, A., Sebastiani, F., Gori, A., and Tattini, M. (2018). Modulation of phytohormone signaling: A primary function of flavonoids in plant–environment interactions. *Front. Plant Sci.* 9. doi:10.3389/fpls.2018.01042.

Camp, R. G. L. op den, Przybyla, D., Ochsenbein, C., Laloi, C., Kim, C., Danon, A. (2003). Rapid induction of distinct stress responses after the release of singlet oxygen in Arabidopsis. *The Plant Cell* 15, 2320–2332. doi:10.1105/tpc.014662.

Carvalho, F. P. (2017). Pesticides, environment, and food safety. *Food and Energy Security* 6, 48–60. doi:https://doi.org/10.1002/fes3.108.

Chaouch, S., Queval, G., Vanderauwera, S., Mhamdi, A., Vandorpe, M., Langlois-Meurinne, M., et al. (2010). Peroxisomal hydrogen peroxide is coupled to biotic defense responses by

ISOCHORISMATE SYNTHASE1 in a daylength-related manner. *Plant Physiol.* 153, 1692–1705. doi:10.1104/pp.110.153957.

Cui, N., Lu, H., Wang, T., Zhang, W., Kang, L., and Cui, F. (2019). Armet, an aphid effector protein, induces pathogen resistance in plants by promoting the accumulation of salicylic acid. *Philosophical Transactions of the Royal Society B: Biological Sciences* 374, 20180314. doi:10.1098/rstb.2018.0314.

Czerniewicz, P., Sytykiewicz, H., Durak, R., Borowiak-Sobkowiak, B., and Chrzanowski, G. (2017). Role of phenolic compounds during antioxidative responses of winter triticale to aphid and beetle attack. *Plant Physiology and Biochemistry* 118, 529–540. doi:10.1016/j.plaphy.2017.07.024.

D'Alessandro, S., Ksas, B., and Havaux, M. (2018). Decoding ß-cyclocitral-mediated retrograde signaling reveals the role of a detoxification response controlled by SCL14 and ANAC102 in plant tolerance to photooxidative stress. *The Plant Cell*, tpc.00578.2018. doi:10.1105/tpc.18.00578.

Das, K., and Roychoudhury, A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Front. Environ. Sci.* 2. doi:10.3389/fenvs.2014.00053.

Davletova, S., Rizhsky, L., Liang, H., Shengqiang, Z., Oliver, D. J., Coutu, J., et al. (2005). Cytosolic Ascorbate Peroxidase 1 is a central component of the reactive oxygen gene network of Arabidopsis. *The Plant Cell* 17, 268–281. doi:10.1105/tpc.104.026971.

de Ilarduya, O. M., Xie, Q., and Kaloshian, I. (2003). Aphid-induced defense responses in *Mi-1*-mediated compatible and incompatible tomato interactions. *MPMI* 16, 699–708. doi:10.1094/MPMI.2003.16.8.699.

Dedryver, C.-A., Le Ralec, A., and Fabre, F. (2010). The conflicting relationships between aphids and men: A review of aphid damage and control strategies. *Comptes Rendus Biologies* 333, 539–553. doi:10.1016/j.crvi.2010.03.009.

del Río, L. A., and López-Huertas, E. (2016). ROS generation in peroxisomes and its role in cell signaling. *Plant and Cell Physiology* 57, 1364–1376. doi:10.1093/pcp/pcw076.

Denholm, I., Pickett, J. A., Devonshire, A. L., Devonshire, A. L., Field, L. M., Foster, S. P., et al. (1998). The evolution of insecticide resistance in the peach–potato aphid, *Myzus persicae*. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 353, 1677–1684. doi:10.1098/rstb.1998.0318.

Dewar, A. M., and Denholm, I. (2017). "Chemical Control," in *Aphids as Crop Pests, 2nd Edition* (CABI).

Dmitrieva, V. A., Tyutereva, E. V., and Voitsekhovskaja, O. V. (2020). Singlet oxygen in plants: generation, detection, and signaling roles. *International Journal of Molecular Sciences* 21, 3237. doi:10.3390/ijms21093237.

Dogra, V., Duan, J., Lee, K. P., Lv, S., Liu, R., and Kim, C. (2017). FtsH2-dependent proteolysis of EXECUTER1 is essential in mediating singlet oxygen-triggered retrograde signaling in *Arabidopsis thaliana*. *Front Plant Sci* 8. doi:10.3389/fpls.2017.01145.

Dogra, V., and Kim, C. (2020). Singlet Oxygen Metabolism: From Genesis to Signaling. *Front Plant Sci* 10. doi:10.3389/fpls.2019.01640.

Döring, T. F., and Chittka, L. (2007). Visual ecology of aphids—a critical review on the role of colours in host finding. *Arthropod-Plant Interactions* 1, 3–16. doi:10.1007/s11829-006-9000-1.

Dreyer, D. L., and Jones, K. C. (1981). Feeding deterrency of flavonoids and related phenolics towards *Schizaphis graminum* and *Myzus persicae*: Aphid feeding deterrents in wheat. *Phytochemistry* 20, 2489–2493. doi:10.1016/0031-9422(81)83078-6.

Durak, R., Bednarski, W., Formela-Luboińska, M., Woźniak, A., Borowiak-Sobkowiak, B., Durak, T., et al. (2019). Defense responses of *Thuja orientalis* to infestation of anholocyclic species aphid *Cinara tujafilina*. *Journal of Plant Physiology* 232, 160–170. doi:10.1016/j.jplph.2018.11.018.

Elzinga, D. A., De Vos, M., and Jander, G. (2014). Suppression of plant defenses by a *Myzus persicae* (green peach aphid) salivary effector protein. *Mol Plant Microbe Interact* 27, 747–756. doi:10.1094/MPMI-01-14-0018-R.

Farmer, E. E., and Mueller, M. J. (2013). ROS-Mediated Lipid Peroxidation and RES-Activated Signaling. *Annual Review of Plant Biology* 64, 429–450. doi:10.1146/annurev-arplant-050312-120132.

Flors, C., and Nonell, S. (2006). Light and singlet oxygen in plant defense against pathogens: Phototoxic phenalenone phytoalexins. *Acc. Chem. Res.* 39, 293–300. doi:10.1021/ar0402863.

Foyer, C. H., and Noctor, G. (2003). Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiologia Plantarum* 119, 355–364. doi:10.1034/j.1399-3054.2003.00223.x.

Foyer, C. H., and Noctor, G. (2005). Redox homeostasis and antioxidant signaling: A metabolic interface between stress perception and physiological responses. *The Plant Cell* 17, 1866–1875. doi:10.1105/tpc.105.033589.

Foyer, C. H., and Noctor, G. (2008). Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. *Antioxidants & Redox Signaling* 11, 861–905. doi:10.1089/ars.2008.2177.

Foyer, C. H., and Noctor, G. (2016). Stress-triggered redox signalling: what's in pROSpect? *Plant Cell Environ.* 39, 951–964. doi:10.1111/pce.12621.

Gill, S. S., and Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry* 48, 909–930. doi:10.1016/j.plaphy.2010.08.016.

Gorman, A. A., and Rodgers, M. A. (1992). Current perspectives of singlet oxygen detection in biological environments. *J Photochem Photobiol B* 14, 159–176. doi:10.1016/1011-1344(92)85095-c.

Guo, H., Gu, L., Liu, F., Chen, F., Ge, F., and Sun, Y. (2019). Aphid-borne viral spread is enhanced by virus-induced accumulation of plant reactive oxygen species. *Plant Physiology* 179, 143–155. doi:10.1104/pp.18.00437.

Guo, H., Zhang, Y., Tong, J., Ge, P., Wang, Q., Zhao, Z., et al. (2020). An aphid-secreted salivary protease activates plant defense in phloem. *Current Biology* 30, 4826-4836.e7. doi:10.1016/j.cub.2020.09.020.

Han, Y., Chaouch, S., Mhamdi, A., Queval, G., Zechmann, B., and Noctor, G. (2013a). Functional analysis of arabidopsis mutants points to novel roles for glutathione in coupling H_2O_2 to activation of salicylic acid accumulation and signaling. *Antioxid Redox Signal* 18, 2106–2121. doi:10.1089/ars.2012.5052.

Han, Y., Mhamdi, A., Chaouch, S., and Noctor, G. (2013b). Regulation of basal and oxidative stress-triggered jasmonic acid-related gene expression by glutathione. *Plant, Cell & Environment* 36, 1135–1146. doi:10.1111/pce.12048.

Harmel, N., Létocart, E., Cherqui, A., Giordanengo, P., Mazzucchelli, G., Guillonneau, F., et al. (2008). Identification of aphid salivary proteins: A proteomic investigation of *Myzus persicae*. *Insect Mol. Biol.* 17, 165–174. doi:10.1111/j.1365-2583.2008.00790.x.

Hasanuzzaman, M., Bhuyan, M. H. M. B., Anee, T. I., Parvin, K., Nahar, K., Al Mahmud, J., et al. (2019). Regulation of ascorbate-glutathione pathway in mitigating oxidative damage in plants under abiotic stress. *Antioxidants* 8, 384. doi:10.3390/antiox8090384.

Hiremath, S. S., Sajeevan, R., Nataraja, K. N., Chaturvedi, A. K., Chinnusamy, V., and Pal, M. (2017). Silencing of Fatty Acid Desaturase (FAD7) gene enhances membrane stability and photosynthetic efficiency under heat stress in tobacco (*Nicotiana benthamiana*). *INDIAN J EXP BIOL*, 10.

Hurej, M., and Van der Werf, W. (1992). The influence of black bean aphid, *Aphis fabae* Scop., and its honeydew on the photosynthesis of sugar beet. *Annals of Applied Biology* 122, 189–200.

Jaouannet, M., Rodriguez, P. A., Thorpe, P., Lenoir, C. J. G., MacLeod, R., Escudero-Martinez, C., et al. (2014). Plant immunity in plant–aphid interactions. *Front. Plant Sci.* 5. doi:10.3389/fpls.2014.00663.

Jiang, Y., and Miles, P. W. (1993). Responses of a compatible lucerne variety to attack by spotted alfalfa aphid: Changes in the redox balance in affected tissues. *Entomologia Experimentalis et Applicata* 67, 263–274. doi:10.1111/j.1570-7458.1993.tb01677.x.

Kachroo, A., and Kachroo, P. (2009). Fatty acid-derived signals in plant defense. *Annual review of phytopathology* 47, 153–176.

Kaloshian, I., Lange, W. H., and Williamson, V. M. (1995). An aphid-resistance locus is tightly linked to the nematode-resistance gene, *Mi*, in tomato. *PNAS* 92, 622–625. doi:10.1073/pnas.92.2.622.

Kámán-Tóth, E., Dankó, T., Gullner, G., Bozsó, Z., Palkovics, L., and Pogány, M. (2019). Contribution of cell wall peroxidase- and NADPH oxidase-derived reactive oxygen species to *Alternaria brassicicola*-induced oxidative burst in Arabidopsis. *Molecular Plant Pathology* 20, 485–499. doi:https://doi.org/10.1111/mpp.12769.

Kangasjärvi, S., Neukermans, J., Li, S., Aro, E.-M., and Noctor, G. (2012). Photosynthesis, photorespiration, and light signalling in defence responses. *J. Exp. Bot.*, err402. doi:10.1093/jxb/err402.

Kennedy, J. S., Day, M. F., Eastop, V. F., Commonwealth Institute of Entomology, Commonwealth Agricultural Bureaux, and Executive Council (1962). *A conspectus of aphids as vectors of plant viruses*. London: Commonwealth Institute of Entomology.

Kerchev, P. I., Fenton, B., Foyer, C. H., and Hancock, R. D. (2012). Infestation of potato (*Solanum tuberosum* L.) by the peach-potato aphid (*Myzus persicae* Sulzer) alters cellular redox status and is influenced by ascorbate. *Plant, Cell & Environment* 35, 430–440. doi:10.1111/j.1365-3040.2011.02395.x.

Kerns, D. L., and Gaylor, M. J. (1992). Insecticide resistance in field populations of the cotton aphid (Homoptera: Aphididae). *Journal of Economic Entomology* 85, 1–8. doi:10.1093/jee/85.1.1.

Kettles, G. J., Drurey, C., Schoonbeek, H., Maule, A. J., and Hogenhout, S. A. (2013). Resistance of *Arabidopsis thaliana* to the green peach aphid, *Myzus persicae*, involves camalexin and is regulated by microRNAs. *New Phytologist* 198, 1178–1190. doi:https://doi.org/10.1111/nph.12218.

Kim, J. H., and Jander, G. (2007). *Myzus persicae* (green peach aphid) feeding on Arabidopsis induces the formation of a deterrent indole glucosinolate. *The Plant Journal* 49, 1008–1019. doi:https://doi.org/10.1111/j.1365-313X.2006.03019.x.

Kim, J. H., Lee, B. W., Schroeder, F. C., and Jander, G. (2008). Identification of indole glucosinolate breakdown products with antifeedant effects on *Myzus persicae* (green peach aphid). *Plant J* 54, 1015–1026. doi:10.1111/j.1365-313X.2008.03476.x.

Kindlmann, P., and Dixon, A. F. G. (1999). Strategies of aphidophagous predators: Lessons for modelling insect predator–prey dynamics. *Journal of Applied Entomology* 123, 397–399. doi:https://doi.org/10.1046/j.1439-0418.1999.00390.x.

Kirchner, S. M., Döring, T. F., and Saucke, H. (2005). Evidence for trichromacy in the green peach aphid, *Myzus persicae* (Sulz.) (Hemiptera: Aphididae). *Journal of Insect Physiology* 51, 1255–1260. doi:10.1016/j.jinsphys.2005.07.002.

Knox, J. P., and Dodge, A. D. (1985). Singlet oxygen and plants. *Phytochemistry* 24, 889–896. doi:10.1016/S0031-9422(00)83147-7.

Kogan, M., and Ortman, E. F. (1978). Antixenosis–A new term proposed to define Painter's "Nonpreference" modality of resistance. *Bulletin of the Entomological Society of America* 24, 175–176. doi:10.1093/besa/24.2.175.

Koh, E., and Fluhr, R. (2016). Singlet oxygen detection in biological systems: Uses and limitations. *Plant Signaling & Behavior* 11, e1192742. doi:10.1080/15592324.2016.1192742.

Koussevitzky, S., Suzuki, N., Huntington, S., Armijo, L., Sha, W., Cortes, D., et al. (2008). Ascorbate Peroxidase 1 plays a key role in the response of *Arabidopsis thaliana* to stress combination. *J. Biol. Chem.* 283, 34197–34203. doi:10.1074/jbc.M806337200.

Kring, J. B. (1967). Alighting of Aphids on Colored Cards m a Flight Chamber. *Journal of Economic Entomology* 60, 1207–1210. doi:10.1093/jee/60.5.1207.

Krishnan, N., Kodrík, D., Turanli, F., and Sehnal, F. (2007). Stage-specific distribution of oxidative radicals and antioxidant enzymes in the midgut of Leptinotarsa decemlineata. *Journal of Insect Physiology* 53, 67–74. doi:10.1016/j.jinsphys.2006.10.001.

Kuśnierczyk, A., Winge, P., Jørstad, T. S., Troczyńska, J., Rossiter, J. T., and Bones, A. M. (2008). Towards global understanding of plant defence against aphids – timing and dynamics of early Arabidopsis defence responses to cabbage aphid (*Brevicoryne brassicae*) attack. *Plant, Cell & Environment* 31, 1097–1115. doi:10.1111/j.1365-3040.2008.01823.x.

Kuźniak, E., and Kopczewski, T. (2020). The chloroplast reactive oxygen species-redox system in plant immunity and disease. *Front. Plant Sci.* 11. doi:10.3389/fpls.2020.572686.

Kwak, J. M., Mori, I. C., Pei, Z.-M., Leonhardt, N., Torres, M. A., Dangl, J. L., et al. (2003). NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in Arabidopsis. *EMBO J* 22, 2623–2633. doi:10.1093/emboj/cdg277.

Laloi, C., Przybyla, D., and Apel, K. (2006). A genetic approach towards elucidating the biological activity of different reactive oxygen species in *Arabidopsis thaliana*. *J. Exp. Bot.* 57, 1719–1724. doi:10.1093/jxb/erj183.

Lamb, C., Dixon, R.A. (1997). The oxidative burst in plant disease resistance. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.* 48, 251-275. doi:10.3390/ 10.1146/annurev.arplant.48.1.251.

Lee, J. M., Lee, H., Kang, S., and Park, W. J. (2016). Fatty acid desaturases, polyunsaturated fatty acid regulation, and biotechnological advances. *Nutrients* 8, 23. doi:10.3390/nu8010023.

Lee, K. P., Kim, C., Landgraf, F., and Apel, K. (2007). EXECUTER1- and EXECUTER2-dependent transfer of stress-related signals from the plastid to the nucleus of *Arabidopsis thaliana*. *PNAS* 104, 10270–10275. doi:10.1073/pnas.0702061104.

Lei, J., and Zhu-Salzman, K. (2015). Enhanced aphid detoxification when confronted by a host with elevated ROS production. *Plant Signal Behav* 10. doi:10.1080/15592324.2015.1010936.

Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. (1994). H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79, 583–593. doi:10.1016/0092-8674(94)90544-4.

Li, J., Galla, A. L., Avila, C. A., Flattmann, K., Vaughn, K. L., and Goggin, F. (2021). Fatty Acid Desaturases in the chloroplast and endoplasmic reticulum promote susceptibility to the Green Peach Aphid, *Myzus persicae*, in *Arabidopsis thaliana*. *MPMI*. doi:10.1094/MPMI-12-20-0345-R.

Liu, Y., Ren, D., Pike, S., Pallardy, S., Gassmann, W., and Zhang, S. (2007). Chloroplast-generated reactive oxygen species are involved in hypersensitive response-like cell death mediated by a mitogen-activated protein kinase cascade. *The Plant Journal* 51, 941–954. doi:10.1111/j.1365-313X.2007.03191.x.

Louis, J., and Shah, J. (2014). Plant defence against aphids: The PAD4 signalling nexus. *J. Exp. Bot.*, eru454. doi:10.1093/jxb/eru454.

Łukasik, I., and Goławska, S. (2013). Effect of host plant on levels of reactive oxygen species and antioxidants in the cereal aphids *Sitobion avenae* and *Rhopalosiphum padi*. *Biochemical Systematics and Ecology* 51, 232–239. doi:10.1016/j.bse.2013.09.001.

Lukasik, I., and Golawska, S. (2019). Biochemical markers of oxidative stress in triticale seedlings exposed to cereal aphids. *ACTA BIOLOGICA CRACOVIENSIA* 61, 35–46. doi:10.24425/ABCSB.2019.127745.

Mai, V. C., Bednarski, W., Borowiak-Sobkowiak, B., Wilkaniec, B., Samardakiewicz, S., and Morkunas, I. (2013). Oxidative stress in pea seedling leaves in response to *Acyrthosiphon pisum* infestation. *Phytochemistry* 93, 49–62. doi:10.1016/j.phytochem.2013.02.011.

Mai, V.-C., Nguyen, B.-H., Nguyen, D.-D., and Nguyen, L.-A.-V. (2017). Nostoc calcicola extract improved the antioxidative response of soybean to cowpea aphid. *Bot Stud* 58, 55. doi:10.1186/s40529-017-0211-9.

Mehler, A. H. (1951). Studies on reactions of illuminated chloroplasts. II. Stimulation and inhibition of the reaction with molecular oxygen. *Archives of Biochemistry and Biophysics* 34, 339–351. doi:10.1016/0003-9861(51)90012-4.

Meskauskiene, R., Nater, M., Goslings, D., Kessler, F., Camp, R. op den, and Apel, K. (2001). FLU: A negative regulator of chlorophyll biosynthesis in *Arabidopsis thaliana*. *PNAS* 98, 12826–12831. doi:10.1073/pnas.221252798.

Mewis, I., Tokuhisa, J. G., Schultz, J. C., Appel, H. M., Ulrichs, C., and Gershenzon, J. (2006). Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. *Phytochemistry* 67, 2450–2462.

Michaud, J. P. (2018). Problems Inherent to augmentation of natural enemies in open agriculture. *Neotrop Entomol* 47, 161–170. doi:10.1007/s13744-018-0589-4.

Miles, P. W. (1978). Redox reactions of hemipterous saliva in plant tissues. *Entomologia Experimentalis et Applicata* 24, 534–539. doi:10.1111/j.1570-7458.1978.tb02814.x.

Miles, P. W. (1999). Aphid saliva. *Biological Reviews* 74, 41–85. doi:10.1111/j.1469-185X.1999.tb00181.x.

Miles, P. W., and Oertli, J. J. (1993). The significance of antioxidants in the aphid-plant interaction: the redox hypothesis. *Entomologia Experimentalis et Applicata* 67, 275–283. doi:10.1111/j.1570-7458.1993.tb01678.x.

Millar, A. A., Smith, M. A., and Kunst, L. (2000). All fatty acids are not equal: discrimination in plant membrane lipids. *Trends in Plant Science* 5, 95–101. doi:10.1016/S1360-1385(00)01566-1.

Mittler, R., and Berkowitz, G. (2001). Hydrogen peroxide, a messenger with too many roles? *Redox Report* 6, 69–72. doi:10.1179/135100001101536067.

Mock, H.-P., and Dietz, K.-J. (2016). Redox proteomics for the assessment of redox-related posttranslational regulation in plants. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 1864, 967–973. doi:10.1016/j.bbapap.2016.01.005.

Møller, I. M. (2001). Plant mitochondria and oxidative stress: Electron Transport, NADPH turnover, and metabolism of reactive oxygen species. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.* 52, 561–591. doi:10.1146/annurev.arplant.52.1.561.

Moloi, M. J., and van der Westhuizen, A. J. (2006). The reactive oxygen species are involved in resistance responses of wheat to the Russian wheat aphid. *Journal of Plant Physiology* 163, 1118–1125. doi:10.1016/j.jplph.2005.07.014.

Mondal, S., Wenninger, E. J., Hutchinson, P. J. S., Whitworth, J. L., Shrestha, D., Eigenbrode, S. D., et al. (2016). Comparison of transmission efficiency of various isolates of Potato

virus Y among three aphid vectors. *Entomologia Experimentalis et Applicata* 158, 258–268. doi:https://doi.org/10.1111/eea.12404.

Moran, N. A. (1992). The evolution of aphid life cycles. *Annual Review of Entomology* 37, 321–348. doi:10.1146/annurev.en.37.010192.001541.

Moran, P. J., and Thompson, G. A. (2001). Molecular responses to aphid feeding in arabidopsis in relation to plant defense pathways. *Plant Physiol* 125, 1074–1085.

Mowry, T. M. (2005). Insecticidal reduction of Potato leafroll virus transmission by *Myzus persicae*. *Annals of Applied Biology* 146, 81–88. doi:https://doi.org/10.1111/j.1744-7348.2005.03149.x.

Mutti, N. S., Louis, J., Pappan, L. K., Pappan, K., Begum, K., Chen, M.-S., et al. (2008). A protein from the salivary glands of the pea aphid, *Acyrthosiphon pisum*, is essential in feeding on a host plant. *PNAS* 105, 9965–9969. doi:10.1073/pnas.0708958105.

Nebreda, M., Michelena, J. M., and Fereres, A. (2005). Seasonal abundance of aphid species on lettuce crops in Central Spain and identification of their main parasitoids. *Journal of Plant Diseases and Protection* 112, 405–415.

Niyogi, K. K., and Truong, T. B. (2013). Evolution of flexible non-photochemical quenching mechanisms that regulate light harvesting in oxygenic photosynthesis. *Current Opinion in Plant Biology* 16, 307–314. doi:10.1016/j.pbi.2013.03.011.

Noctor, G., Reichheld, J.-P., and Foyer, C. H. (2018). ROS-related redox regulation and signaling in plants. *Seminars in Cell & Developmental Biology* 80, 3–12. doi:10.1016/j.semcdb.2017.07.013.

Ochsenbein, C., Przybyla, D., Danon, A., Landgraf, F., Göbel, C., Imboden, A., et al. (2006). The role of EDS1 (Enhanced Disease Susceptibility) during singlet oxygen-mediated stress responses of Arabidopsis. *The Plant Journal* 47, 445–456. doi:10.1111/j.1365-313X.2006.02793.x.

Painter, R. H. (1951). *Insect resistance in crop plants.* New York: Macmillan.

Peccoud, J., Simon, J.-C., von Dohlen, C., Coeur d'acier, A., Plantegenest, M., Vanlerberghe-Masutti, F., et al. (2010). Evolutionary history of aphid-plant associations and their role in aphid diversification. *Comptes Rendus Biologies* 333, 474–487. doi:10.1016/j.crvi.2010.03.004.

Powell, G., Hardie, J., and Pickett, J. A. (1995). Responses of *Myzus persicae* to the repellent polygodial in choice and no-choice video assays with young and mature leaf tissue. *Entomologia Experimentalis et Applicata* 74, 91–94. doi:10.1111/j.1570-7458.1995.tb01878.x.

Prince, D. C., Drurey, C., Zipfel, C., and Hogenhout, S. A. (2014). The Leucine-Rich Repeat Receptor-Like Kinase BRASSINOSTEROID INSENSITIVE1-ASSOCIATED

KINASE1 and the Cytochrome P450 PHYTOALEXIN DEFICIENT3 contribute to innate immunity to aphids in Arabidopsis. *Plant Physiol* 164, 2207–2219. doi:10.1104/pp.114.235598.

Przybyla, D., Göbel, C., Imboden, A., Hamberg, M., Feussner, I., and Apel, K. (2008). Enzymatic, but not non-enzymatic, 1O2-mediated peroxidation of polyunsaturated fatty acids forms part of the EXECUTER1-dependent stress response program in the flu mutant of *Arabidopsis thaliana*. *The Plant Journal* 54, 236–248. doi:10.1111/j.1365-313X.2008.03409.x.

Ragsdale, D. W., Landis, D. A., Brodeur, J., Heimpel, G. E., and Desneux, N. (2011). Ecology and management of the soybean aphid in north america. *Annual Review of Entomology* 56, 375–399. doi:10.1146/annurev-ento-120709-144755.

Ragsdale, D. W., Mccornack, B. P., Venette, R. C., Potter, B. D., Macrae, I. V., Hodgson, E. W., et al. (2007). Economic threshold for soybean aphid (Hemiptera: Aphididae). *JOURNAL OF ECONOMIC ENTOMOLOGY* 100, 10.

Ragsdale, D. W., Voegtlin, D. J., and O'neil, R. J. (2004). Soybean aphid biology in north america. *Annals of the Entomological Society of America* 97, 204–208. doi:10.1093/aesa/97.2.204.

Ramel, F., Birtic, S., Cuiné, S., Triantaphylidès, C., Ravanat, J.-L., and Havaux, M. (2012a). Chemical quenching of singlet oxygen by carotenoids in plants. *Plant Physiology* 158, 1267–1278. doi:10.1104/pp.111.182394.

Ramel, F., Birtic, S., Ginies, C., Soubigou-Taconnat, L., Triantaphylidès, C., and Havaux, M. (2012b). Carotenoid oxidation products are stress signals that mediate gene responses to singlet oxygen in plants. *Proc Natl Acad Sci U S A* 109, 5535–5540. doi:10.1073/pnas.1115982109.

Ramel, F., Ksas, B., Akkari, E., Mialoundama, A. S., Monnet, F., Krieger-Liszkay, A. (2013). Light-induced acclimation of the Arabidopsis *chlorina1* mutant to singlet oxygen. *Plant Cell* 25, 1445–1462. doi:10.1105/tpc.113.109827.

Rasool, B., Karpinska, B., Pascual, J., Kangasjärvi, S., and Foyer, C. H. (2019). Catalase, glutathione, and protein phosphatase 2A-dependent organellar redox signalling regulate aphid fecundity under moderate and high irradiance. *Plant, Cell & Environment* 43, 209–222. doi:10.1111/pce.13669.

Rasool, B., McGowan, J., Pastok, D., Marcus, S. E., Morris, J. A., Verrall, S. R., et al. (2017). Redox control of aphid resistance through altered cell wall composition and nutritional quality. *Plant Physiology* 175, 259–271. doi:10.1104/pp.17.00625.

Ren, G., Wang, X., Chen, D., Wang, X., and Liu, X. (2014). Effects of aphids *Myzus persicae* on the changes of Ca^{2+} and H_2O_2 flux and enzyme activities in tobacco. *Journal of Plant Interactions* 9, 883–888. doi:10.1080/17429145.2014.982221.

Rentel, M. C., Lecourieux, D., Ouaked, F., Usher, S. L., Petersen, L., Okamoto, H., et al. (2004). OXI1 kinase is necessary for oxidative burst-mediated signalling in Arabidopsis. *Nature* 427, 858–861. doi:10.1038/nature02353.

Reszczyńska, E., and Hanaka, A. (2020). Lipids Composition in Plant Membranes. *Cell Biochem Biophys* 78, 401–414. doi:10.1007/s12013-020-00947-w.

Rhee, S.-J., Watt, L. G., Bravo, A. C., Murphy, A. M., and Carr, J. P. (2020). Effects of the cucumber mosaic virus 2a protein on aphid-plant interactions in *Arabidopsis thaliana*. *Mol Plant Pathol* 21, 1248–1254. doi:10.1111/mpp.12975.

Rizhsky, L., Davletova, S., Liang, H., and Mittler, R. (2004). The Zinc Finger Protein Zat12 Is Required for Cytosolic Ascorbate Peroxidase 1 Expression during Oxidative Stress in Arabidopsis. *J. Biol. Chem.* 279, 11736–11743. doi:10.1074/jbc.M313350200.

Rustérucci, C., Aviv, D. H., Holt, B. F., Dangl, J. L., and Parker, J. E. (2001). The disease resistance signaling components EDS1 and PAD4 are essential regulators of the cell death pathway controlled by LSD1 in Arabidopsis. *Plant Cell* 13, 2211–2224. doi:10.1105/tpc.010085.

Rutledge, C. E., O"Neil, R. J., Fox, T. B., and Landis, D. A. (2004). Soybean aphid predators and their use in integrated pest management. *Annals of the Entomological Society of America* 97, 240–248. doi:10.1093/aesa/97.2.240.

Sharma, N., and Singhvi, R. (2017). Effects of chemical fertilizers and pesticides on human health and environment: A review. *Intern. Jour. of Agricul., Environ. and Biotech.* 10, 675. doi:10.5958/2230-732X.2017.00083.3.

Sharma, P., Jha, A. B., Dubey, R. S., and Pessarakli, M. (2012). reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany* 2012, e217037. doi:10.1155/2012/217037.

Shoala, T., Edwards, M. G., Knight, M. R., and Gatehouse, A. M. R. (2018). OXI1 kinase plays a key role in resistance of Arabidopsis towards aphids (*Myzus periscae*). *Transgenic Res*, 1–12. doi:10.1007/s11248-018-0078-x.

Shumbe, L., D'Alessandro, S., Shao, N., Chevalier, A., Ksas, B., Bock, R., et al. (2017). METHYLENE BLUE SENSITIVITY 1 (MBS1) is required for acclimation of Arabidopsis to singlet oxygen and acts downstream of β-cyclocitral. *Plant, Cell & Environment* 40, 216–226. doi:10.1111/pce.12856.

Sikora, E. J., Gudauskas, R. T., Murphy, J. F., Porch, D. W., Andrianifahanana, M., Zehnder, G. W., et al. (1998). A multivirus epidemic of tomatoes in alabama. *Plant Disease* 82, 117–120. doi:10.1094/PDIS.1998.82.1.117.

Silva, A. X., Jander, G., Samaniego, H., Ramsey, J. S., and Figueroa, C. C. (2012). Insecticide resistance mechanisms in the green peach aphid *Myzus persicae* (Hemiptera: Aphididae) I: A transcriptomic survey. *PLOS ONE* 7, e36366. doi:10.1371/journal.pone.0036366.

Smith, C. M. (2005). *Plant Resistance to Arthropods: Molecular and Conventional Approaches*. Springer Science & Business Media.

Smith, C. M., and Boyko, E. V. (2007). The molecular bases of plant resistance and defense responses to aphid feeding: Current status. *Entomologia Experimentalis et Applicata* 122, 1–16. doi:10.1111/j.1570-7458.2006.00503.x.

Smith, C. M., and Chuang, W.-P. (2014). Plant resistance to aphid feeding: Behavioral, physiological, genetic and molecular cues regulate aphid host selection and feeding. *Pest Management Science* 70, 528–540. doi:10.1002/ps.3689.

Smith, C. M., Liu, X., Wang, L. J., Liu, X., Chen, M.-S., Starkey, S., et al. (2010). Aphid feeding activates expression of a transcriptome of oxylipin-based defense signals in wheat involved in resistance to herbivory. *J Chem Ecol* 36, 260–276. doi:10.1007/s10886-010-9756-8.

Song, J., Lee, D. E., Jung, S., Kim, H., Han, O., Cho, B. H., et al. (2004). Characterization of transgenic rice plants expressing an Arabidopsis *FAD7*. *Biologia Plantarum* 48, 361–366. doi:10.1023/B:BIOP.0000041087.17353.d8.

Strauss, S. Y., and Agrawal, A. A. (1999). The ecology and evolution of plant tolerance to herbivory. *Trends in Ecology & Evolution* 14, 179–185. doi:10.1016/S0169-5347(98)01576-6.

Sun, M., Voorrips, R. E., van Kaauwen, M., Visser, R. G. F., and Vosman, B. (2020). The ability to manipulate ROS metabolism in pepper may affect aphid virulence. *Hortic. Res.-England* 7, 6. doi:10.1038/s41438-019-0231-6.

Suzuki, N., Miller, G., Morales, J., Shulaev, V., Torres, M. A., and Mittler, R. (2011). Respiratory burst oxidases: The engines of ROS signaling. *Current Opinion in Plant Biology* 14, 691–699. doi:10.1016/j.pbi.2011.07.014.

Sweetlove, L. J., and Foyer, C. H. (2004). "Roles for Reactive Oxygen Species and Antioxidants in Plant Mitochondria," in *Plant Mitochondria: From Genome to Function* Advances in Photosynthesis and Respiration., eds. D. A. Day, A. H. Millar, and J. Whelan (Dordrecht: Springer Netherlands), 307–320. doi:10.1007/978-1-4020-2400-9_14.

Sytykiewicz, H. (2016). Deciphering the role of NADPH oxidase in complex interactions between maize (Zea mays L.) genotypes and cereal aphids. *Biochemical and Biophysical Research Communications* 476, 90–95. doi:10.1016/j.bbrc.2016.05.050.

Teixeira, M. C., Carvalho, I. S., and Brodelius, M. (2010). ω-3 Fatty Acid Desaturase genes isolated from purslane (*Portulaca oleracea* L.): Expression in different tissues and response to cold and wound stress. *J. Agric. Food Chem.* 58, 1870–1877. doi:10.1021/jf902684v.

Terzi, R., Kadioglu, A., Kalaycioglu, E., and Saglam, A. (2014). Hydrogen peroxide pretreatment induces osmotic stress tolerance by influencing osmolyte and abscisic acid

levels in maize leaves. *Journal of Plant Interactions* 9, 559–565. doi:10.1080/17429145.2013.871077.

Tjallingii, W. F. (2006). Salivary secretions by aphids interacting with proteins of phloem wound responses. *J Exp Bot* 57, 739–745. doi:10.1093/jxb/erj088.

van Bel, A. J. E., and Will, T. (2016). Functional evaluation of proteins in watery and gel saliva of aphids. *Front. Plant Sci.* 7. doi:10.3389/fpls.2016.01840.

van Lenteren, J. C. (2012). The state of commercial augmentative biological control: plenty of natural enemies, but a frustrating lack of uptake. *BioControl* 57, 1–20. doi:10.1007/s10526-011-9395-1.

Vanlerberghe, G. C. (2013). Alternative oxidase: A Mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. *Int J Mol Sci* 14, 6805–6847. doi:10.3390/ijms14046805.

Villada, E. S., González, E. G., López-Sesé, A. I., Castiel, A. F., and Gómez-Guillamón, M. L. (2009). Hypersensitive response to *Aphis gossypii* Glover in melon genotypes carrying the *Vat* gene. *J Exp Bot* 60, 3269–3277. doi:10.1093/jxb/erp163.

Vishwakarma, A., Tetali, S. D., Selinski, J., Scheibe, R., and Padmasree, K. (2015). Importance of the alternative oxidase (AOX) pathway in regulating cellular redox and ROS homeostasis to optimize photosynthesis during restriction of the cytochrome oxidase pathway in *Arabidopsis thaliana*. *Ann Bot* 116, 555–569. doi:10.1093/aob/mcv122.

Wagner, D., Przybyla, D., Camp, R. op den, Kim, C., Landgraf, F., Lee, K. P., et al. (2004). The genetic basis of singlet oxygen-induced stress responses of *Arabidopsis thaliana*. *Science* 306, 1183–1185. doi:10.1126/science.1103178.

Wang, L., Kim, C., Xu, X., Piskurewicz, U., Dogra, V., Singh, S., et al. (2016). Singlet oxygen- and EXECUTER1-mediated signaling is initiated in grana margins and depends on the protease FtsH2. *PNAS* 113, E3792–E3800. doi:10.1073/pnas.1603562113.

Wang, W., Dai, H., Zhang, Y., Chandrasekar, R., Luo, L., Hiromasa, Y., et al. (2015). Armet is an effector protein mediating aphid–plant interactions. *The FASEB Journal* 29, 2032–2045. doi:10.1096/fj.14-266023.

Wang, Y., Zhang, J., Li, J.-L., and Ma, X.-R. (2014). Exogenous hydrogen peroxide enhanced the thermotolerance of *Festuca arundinacea* and *Lolium perenne* by increasing the antioxidative capacity. *Acta physiologiae plantarum*.

Waszczak, C., Akter, S., Jacques, S., Huang, J., Messens, J., and Van Breusegem, F. (2015). Oxidative post-translational modifications of cysteine residues in plant signal transduction. *J Exp Bot* 66, 2923–2934. doi:10.1093/jxb/erv084.

Wellings, P. W., Ward, S. A., Dixon, A. F. G., and Rabbinge, R. (1989). "Crop loss assessment.," in *Aphids, their biology, natural enemies and control, Vol. 2c* (Amsterdam: Elsevier Science), 49–64.

Will, T., Tjallingii, W. F., Thönnessen, A., and van Bel, A. J. E. (2007). Molecular sabotage of plant defense by aphid saliva. *Proc. Natl. Acad. Sci. U.S.A.* 104, 10536–10541. doi:10.1073/pnas.0703535104.

Wingler, A., Lea, P. J., Quick, W. P., and Leegood, R. C. (2000). Photorespiration: metabolic pathways and their role in stress protection. *Philos Trans R Soc Lond B Biol Sci* 355, 1517–1529.

Winterbourn, C. C. (1995). Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicology Letters* 82–83, 969–974. doi:10.1016/0378-4274(95)03532-X.

Xu, J., Padilla, C. S., Li, J., Wickramanayake, J., Fischer, H. D., and Goggin, F. L. (2021). Redox responses of *Arabidopsis thaliana* to the green peach aphid, *Myzus persicae*. *Molecular Plant Pathology* n/a. doi:https://doi.org/10.1111/mpp.13054.

Yano, E. (2006). Ecological considerations for biological control of aphids in protected culture. *Popul Ecol* 48, 333. doi:10.1007/s10144-006-0008-2.

Zaller, J. G., and Brühl, C. A. (2019). Editorial: Non-target Effects of Pesticides on Organisms Inhabiting Agroecosystems. *Front. Environ. Sci.* 7. doi:10.3389/fenvs.2019.00075.

Zapata, S. D., Dudensing, R., Sekula, D., Esparza-Díaz, G., and Villanueva, R. (2018). ECONOMIC IMPACT OF THE SUGARCANE APHID OUTBREAK IN SOUTH TEXAS. *Journal of Agricultural and Applied Economics* 50, 104–128. doi:10.1017/aae.2017.24.

Zhang, L., Lu, H., Guo, K., Yao, S., and Cui, F. (2017a). Insecticide resistance status and detoxification enzymes of wheat aphids Sitobion avenae and Rhopalosiphum padi. *Science China Life Sciences* 60, 1–4. doi:10.1007/s11427-017-9105-x.

Zhang, L., Oh, Y., Li, H., Baldwin, I. T., and Galis, I. (2012). Alternative Oxidase in Resistance to Biotic Stresses: Nicotiana attenuata AOX Contributes to Resistance to a Pathogen and a Piercing-Sucking Insect But Not Manduca sexta Larvae. *Plant Physiol.* 160, 1453–1467. doi:10.1104/pp.112.200865.

Zhang, S., Apel, K., and Kim, C. (2014). Singlet oxygen-mediated and EXECUTER-dependent signalling and acclimation of *Arabidopsis thaliana* exposed to light stress. *Philosophical Transactions of the Royal Society B: Biological Sciences* 369, 20130227. doi:10.1098/rstb.2013.0227.

Zhang, Y., Fan, J., Fu, Y., Francis, F., and Chen, J. (2019). Plant-Mediated Interactions between Two Cereal Aphid Species: Promotion of Aphid Performance and Attraction of More Parasitoids by Infestation of Wheat with Phytotoxic Aphid *Schizaphis graminum*. *J. Agric. Food Chem.* 67, 2763–2773. doi:10.1021/acs.jafc.8b06150.

Zhang, Y.-N., Feng, Y.-A., Li, Z., and Shao, X.-S. (2017b). Synthesis and insecticidal evaluation of phytoalexin phenalenones derivatives. *Chinese Chemical Letters* 28, 1228–1231. doi:10.1016/j.cclet.2017.04.003.

Zhao, H., Sun, X., Xue, M., Zhang, X., and Li, Q. (2016). Antioxidant Enzyme Responses Induced by Whiteflies in Tobacco Plants in Defense against Aphids: Catalase May Play a Dominant Role. *PLoS One* 11. doi:10.1371/journal.pone.0165454.

Chapter II

Impact of Loss-of-Function of *Fatty Acid Desaturase7* in *Arabidopsis* on the green peach aphid, *Myzus persicae*, and the cabbage aphid, *Brevicoryne brassicae*

Abstract

The Fatty Acid Desaturase 7 (FAD7) enzyme is widely conserved across many groups of plants and influences resistance to multiple stresses, including aphid infestation. In the model plant Arabidopsis (*Arabidopsis thaliana*), loss-of-function mutation in the *FAD7* gene decreases population growth of a generalist herbivore, the green peach aphid (GPA, *Myzus persicae*). This study used multiple bioassay designs to further characterize the effects of a *fad7* mutant on the GPA, and also to determine if *fad7* impacts a specialist herbivore, the cabbage aphid (CA, *Brevicoryne brassicae*). In choice assays, when presented with wild-type Arabidopsis (Col-0) or *fad7*, neither the GPA nor the CA showed a preference between genotypes within 48 hr. In no-choice assays the *fad7* mutation decreased the overall population growth of GPA, with significant differences in juveniles per plant occurring as early as 48 after infestation. In contrast, the CA showed comparable population growth on *fad7* and Col-0 at all time points, from 24h to 6 days. No-choice assays also demonstrated that the *fad7* mutation increased GPA development time and reduced fecundity but did not influence the mortality rates of adults or juveniles, or the weight of newly emerged adults reared on *fad7*. These results indicate that aphid resistance in the *fad7* mutant is species-specific, and that it impacts the population growth of GPA by decreasing adult fecundity and increasing juvenile development time.

Introduction

Aphids are global pests of agriculturally important crops. The green peach aphid (GPA), *Myzus persicae* Sulzer, in particular is considered one of the most damaging crop pests due to its polyphagous nature, which allows it to feed on over 400 plants in 50 different families (Eastop and Blackman, 2005; Margaritopoulos et al., 2009). Aphids cause damage directly feeding from the plant vasculature and injecting saliva that alters the physiology of the host and decrease yield (Miles, 1999; Guo et al., 2019). In addition, aphids also vector phytopathogenic viruses that can further reduce yield (Eastop, 1977; Valenzuela and Hoffmann, 2015; Aradottir and Crespo-Herrera, 2021); for example, the GPA vectors over 100 viruses including *Potato Virus Y* and *Potato Leafroll Virus* (Kennedy et al., 1962; Mowry, 2005; Hussain et al., 2016). Managing aphids in the field can be achieved through integration of chemical control, biological control, and aphid-resistant cultivars that reduce the population of aphids. However, current management practices for most crops rely heavily on chemical control due to limited availability of aphid-resistant crop varieties (Emden and Harrington, 2017). This in turn drives the development of pesticide resistance in aphid populations. GPA notably shows a capacity to develop resistance rapidly to chemical control tactics and has documented resistance to most available pesticides (Dedryver et al., 2010; Silva et al., 2012), requiring alternative methods of management. Therefore, a better understanding of mechanisms of host-plant resistance to aphids is needed to diversify integrated pest management options to combat aphids in the field.

One aspect of plant physiology that impacts levels of aphid resistance is the activity levels of Fatty Acid Desaturases (FADs) in foliage. FADs introduce double bonds into the acyl chains of fatty acids, regulating the abundance of unsaturated and polyunsaturated fatty acids (He et al., 2020). The activity levels of different FADs are dynamic and can vary in response to

different environmental conditions including insect attack. FADs influence important cellular properties such as membrane fluidity, and also regulate the availability of fatty acid precursors required for synthesis of certain plant hormones and defensive secondary metabolites (Kachroo et al., 2003; Kachroo and Kachroo, 2009). Overexpression or inhibition of certain FADs impacts levels of plant resistance to various abiotic and biotic stresses, including aphids (Upchurch, 2008). Loss-of-function of FAD7, a chloroplast-localized ω-3 FAD, resulted in resistance to the potato aphid (*Macrosiphum euphorbiae* Thomas) in tomato and GPA in Arabidopsis (*Arabidopsis thaliana* (Avila et al., 2012, Li et al., 2021).

Arabidopsis serves as a useful research tool to investigate the influence of FADs on aphid resistance. Because of its long history as a model system to study plant metabolism and its large public repositories of genetic materials, numerous lines with null mutations in FAD genes are available for study (Li et al., 2021). In addition, FAD7 and many other FADs are conserved across taxa, allowing knowledge and principles gained from studying Arabidopsis to be applied to agronomically valuable crops (Upchurch, 2008). Arabidopsis has also been used extensively to study plant-aphid interactions, and presents the opportunity to compare interactions with generalist and specialist aphids (Vos et al., 2007; Louis and Shah, 2013). GPA is a generalist aphid feeding on over 50 families of plants, while the cabbage aphid (CA, *Brevicoryne brassicae*) will only feed on members of Brassicaceae (Deloach, 1974), and both aphids readily colonize Arabidopsis. Some studies suggest that specialists would be less susceptible to plant defenses due to co-evolution with or the ability to manipulate host resistance mechanisms (Ali and Agrawal, 2012). Yet several studies evaluating generalists verses specialists in response to a range of host defenses show conflicting results, indicating the need for case-by-case analysis of each defensive trait (Mewis et al., 2006; Bidart-Bouzat and Kliebenstein, 2011).

To understand the effects of plant defenses on any aphid species and predict their effects on population dynamics, it is important to analyze how these defenses influence the various stages of the host colonization process, including host finding, host acceptance or rejection, and survival and reproduction on the host. Generally, aphids undergo a behavioral sequence that differentiates hosts from non-hosts. The initial stages of host finding are guided by attractive or repellent olfactory cues as well as visual cues such as color (Kring, 1967; Powell et al., 1995; Kirchner et al., 2005; Döring and Chittka, 2007). Once on the plant, aphid settling behavior is influenced by the morphology and chemistry of the plant surface, gustatory cues that aphids detect as they penetrate the intracellular spaces and sample plant cells, and the ease or difficulty with which they establish a feeding site in the phloem (Smith, 2005; Smith and Chuang, 2014) During the sampling of the plant, insect host acceptance may be influenced by "token stimuli," compounds that are specific to certain plant taxa and that may be toxic to generalists but attractive to specialists (Fraenkel, 1959). Depending upon all of these factors, aphids may choose to accept the host and begin prolonged feeding and reproduction, or they may reject the host and move to another plant. Plant traits that intercept any of these early stages of host finding and acceptance are considered sources of antixenotic resistance (Painter, 1951; Kogan and Ortman, 1978). Once aphids settle on the plant and begin reproduction, other host plant defenses may limit population growth by impacting aspects of the insect life cycle such as adult longevity, fecundity, juvenile survival, and development time (Coleson and Miller, 2005; Hesler and Tharp, 2005; Diaz-Montano et al., 2006). Plant traits that do not influence insect host finding or acceptance but that limit insect fitness are considered sources of antibiotic resistance (Smith, 2005). Therefore, evaluation of each stage of the selection sequence requires multiple bioassays

to fully characterize host-plant resistance and distinguish between antixenotic and antibiotic traits.

In this study, we investigated the characteristics of aphid resistance against GPA in an Arabidopsis mutant with impaired function of FAD7 (the *fad7* mutant) by performing choice and no-choice bioassays. We also tested whether aphid resistance in *fad7* impacted the specialist CA. Choice assays between wild-type and *fad7* Arabidopsis were performed to examine aphid settling during the first 48h of colonization. No-choice assays were also used to evaluate population growth within the first generation, as well as aspects of aphid fitness including adult survival and fecundity, and juvenile development and mortality. Our results suggest that *fad7* impacts GPA but not CA, and limits GPA fitness through reduction of female fecundity and increased juvenile developmental time.

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana genotypes used were the wild-type ecotype Columbia CS7000 (Col-0) and a *fad7* mutant line developed by Vaughn and coworkers (2014) that carries the *fad7-1* mutation (ABRC stock number CS71655). Standardized germination and growth of plants was achieved by germinating seeds on media prior to transplanting to soil. Surface sterilization of seeds was done by washing seeds in a 1.5 mL microcentrifuge tube with 1 mL 70% ethanol for 5 minutes. Seeds were then centrifuged for 10-15 seconds before removing the ethanol. Next, seeds were treated with 1 ml of freshly prepared bleach solution (50% bleach and 0.05% Tween 20 in autoclaved dd H_2O) for 10 minutes before another brief centrifugation and removal of the bleach solution. Finally, seeds were washed 7-8 times with autoclaved distilled H_2O before being plated

onto 1X Murashige and Skoog (MS) media (0.8% agar, 3% sucrose, pH adjusted to 5.5-5.9). Seeds were vernalized at 4 °C for 3 days and then transferred to a growth chamber (16 h L/8 h D photoperiod, 180 μmol m^{-2} sec^{-1} light intensity, 55% RH, 24 °C) to germinate. One week after germination, seedlings were transplanted in an *Arabidopsis* potting mix (4:3:2 peat moss, vermiculite, perlite) with a slow-release fertilizer (Osmocote Plus; 15-9-12, Scotts-MiracleGro Company, Marysville, OH). Two weeks after transplanting, plants were used in bioassays.

Aphid Material and Synchronization

This study utilized a generalist aphid species, the green peach aphid (GPA), *Myzus persicae* (Sulzer) and a species which feeds exclusively on Brassicaceae, the cabbage aphid (CA), *Brevicoryne brassicae* (L.). Aphid colonies were reared on cabbage plants (*Brassica rapa* cv. Joi Choi) in wood boxes with mesh on all sides at room temperature with a 16 L:8 D photoperiod, 60% RH, and 24°C. A continuous production of healthy GPA and CA morphs was maintained by replacing cabbage plants bi-weekly to keep populations low. A cohort of age-synchronized aphids was produced by placing apterous adults onto new cabbage plants to reproduce; each aphid typically produced 3-6 juveniles a day. Plants were isolated in separate cages from the rest of the colony, and adults were removed after 24 hrs. The juveniles that were produced within the 24-hr exposure period were monitored daily and collected for bioassays when they reached adulthood (typically within 6 to 8 days).

Aphid Bioassays

Choice assay. To assess host-selection of GPA and CA between Col-0 and *fad7,* we used a choice assay design modified from Louis and colleagues (2010). Each arena consisted of a 15 cm

standard round pot with one Col-0 and one *fad7* seedling planted 4 cm apart to allow the rosette leaves to almost overlap (Fig. 1). Two weeks after transplanting, I placed 10 adult female aphids were placed on a 125 mm diameter filter paper which was set equidistant between the two plants with leaves from each plant touching the filter paper. Pots were caged and the number of aphids present on each plant was counted at 4, 24, and 48 hours post infestation (hpi). Each choice arena was treated as a single replicate. Choice assays with GPA had 13 reps and those with CA had 9 reps.

No-choice assay. To assess the fitness of GPA and CA on Col-0 and *fad7*, we utilized a population growth assay that measures female fecundity similar to that done by Avila and coworkers (2012). Two weeks after transplanting to soil, Arabidopsis plants were infested with 2 adult female aphids of GPA or CA that were collected within 48h of emergence to adulthood. Plants were then caged and placed back into a growth chamber, and aphids were monitored at 24 and 48 hr, and then again at 6 days. At each timepoint, the plant was carefully examined by moving leaves gently with a paint brush. There were 19 and 20 reps of *fad7* and Col-0, respectively, for population growth assays with GPA and 16 reps per genotype for population growth assays with CA. The amount of live adults and juveniles was recorded at each timepoint and only plants on which both adult aphids were recovered were used for population growth counts.

Assays similar to those used by Pitino et al. (2011) and Li (2016) were performed to evaluate the impact of *fad7* on juvenile mortality and development. Two weeks after transplanting to soil, Arabidopsis plants were inoculated with 4 adult female GPA, which were allowed to reproduce for 24 hrs to produce a cohort of age-synchronized juveniles on the plants.

After 24 hrs, all adult aphids were removed, and age-synchronized juveniles were thinned to 10 per plant. Plants were caged during the bioassay. The number of remaining juveniles was counted every 24 hrs until either all aphids were dead or emerged as adults. Within 24 hrs of juveniles molting into adulthood, they were removed from the plants, placed into a tube and weighed using a Mettler Toledo© XS3DU Microbalance (Columbus, OH, USA). Finally, to measure the impact of the *fad7* mutation on the fecundity of aphids that were reared on this genotype, the last adult to emerge from the juvenile mortality bioassay was left on the plant and allowed to reproduce for 5 days. At 5 days, the number of juveniles produced on the plant were counted. Any plants in which the adult aphid was not alive at the end of 5 days was excluded from analysis for this fecundity assay.

Statistical Analysis

Differences in aphid fitness for choice and no-choice bioassays were analyzed using JMP®, Pro 15.2.0. (SAS Institute Inc., Cary, NC, 1989-2019.) Choice assays were analyzed using a paired t-test. The population growth assays of juvenile production, and juvenile development and survival were assessed with a Student's t-test or with Welch's t-test when data had unequal variance. Adult survival during population growth assays was evaluated with a Pearson chi-squared test for frequency of adult recovery. Significance was determined at $\alpha=0.05$.

Results

The fad7 mutation in Arabidopsis does not influence the host preference of the GPA or CA in choice assays.

When adult GPA or CA were placed in choice arenas (10 aphids/arena) between *fad7* and Col-0 plants to assess aphid host preference, aphids moved onto plants very quickly, typically within 15 minutes of being introduced into the arena. The two plant genotypes were colonized by similar numbers of aphids, and the aphids typically remained on the first plants they colonized. Both aphid species preferentially colonized the developing inflorescence of the plants compared to the rosette foliage. There was no significant difference in the numbers of GPA adults on the two genotypes at 4 hours post infestation (hpi) ($t=0.41$, $df=12$, $P=0.69$), 24 hpi ($t=0.81$, $df=12$, $P=0.44$), or 48 hpi ($t=0$, $df=12$, $P=1.00$; Figure 2A). Likewise, the numbers of CA adults on the two genotypes were comparable at 4 hpi ($t=0.115$, $df=8$, $P=0.91$), 24 hpi ($t=0.57$, $df=8$, $P=0.59$), and 48 hpi ($t=0.77$, $df=8$, $P=0.46$; Figure 2B). From initial selection to the final scoring, there were slight changes in aphid numbers between genotypes, but nothing significant in either GPA or CA. Thus, neither aphid species showed evidence of a preference between the two genotypes, and *fad7* did not appear to deter aphid settling behavior.

The fad7 mutation reduces GPA offspring numbers in no-choice assays but does not impact the CA offspring production.

No-choice assays were conducted to determine if the *fad7* mutation influences GPA or CA population growth at 24h, 48h, and 6 days after infestation (hpi or dpi, respectively). Results from the chi squared test indicated there was no significant difference between the two plant genotypes in the survival rates of the adult aphids of either species that were used to inoculate

the plants (GPA: *P*=0.16; CA: *P*=1.00). At 24 hpi, there was also no significant difference in the number of juveniles produced on *fad7* compared to Col-0 for either GPA (t=0.45, df=37, *P*=0.66; Figure 3A) or CA (t=0.077, df=29, *P*=0.94; Figure 3B). CA juvenile production was also not significantly impacted by the *fad7*mutation at 48 hr (t=0.46, df=29, *P*=0.65), or 6 days (t=0.45, df=29, *P*=0.65). Interestingly, the CA induced distorted leaf growth on both genotypes; the mature leaves curled to provide a sheltered area in which the aphids congregated and reproduced (Figure 4A) and emerging leaves were also similar affected (Figure 4B). In contrast, the GPA did not induce visible symptoms on either genotype (Figure 4C) and produced significantly fewer juveniles on *fad7* than on Col-0 at 48 hpi (t=2.08, df=37, *P*=0.045) and 6 dpi (t=4.62, df=37, *P*<0.0001). These data indicate that *fad7* reduces population growth of the generalist herbivore GPA by reducing offspring numbers, and that the effects of resistance can be detected as early as 48h after inoculation, whereas *fad7* does not appear to impact the CA, a specialist herbivore that manipulates Arabidopsis growth.

The fad7 mutation increases the development time and reduces the fecundity of the GPA. Subsequent assays focused on the effects of *fad7* on GPA to determine which components of population growth were impacted by the mutation because the previous assays did not show an effect of *fad7* on CA. To assess whether *fad7* decreases GPA population growth by decreasing juvenile survival and/or increasing development time, cohorts of newly-emerged GPA juveniles were monitored for 8 days and scored as either dead, alive, or molted to adulthood. On both genotypes, 100% of the offspring molted to adulthood by day 8, and so there was no significant difference in juvenile survival (t=0, df=18, P=1.00; Table 1). However, the juvenile development period was significantly longer on *fad7* than on Col-0, with an average of 7.6 days on *fad7*

compared to 7.3 days on Col-0 (t=2.19, df=18, P=0.042). Aphids began emerging on day 6 GPA on both Col-0 and *fad7,* but there was no significant difference between the emergence rates (t=0.54, df=18, P=0.54). On day 7, there was significantly more adult emergence on Col-0 (t=2.65, df=18, P=0.017), whereas the majority of adult emergence for aphids on *fad7* occurred on day 8 (t=2.49, df=18, P=0.023). Though there was no significant difference in the weights of the newly emerged adults (t=0.26, df=17, P=.80, Figure 5). A subset of juveniles were caged individually and retained on the plants for five days after molting to adulthood so that their fecundity could be monitored. The numbers of juveniles produced on *fad7* were significantly lower on *fad7* than on Col-0 (t=2.68, df=12, P=0.01; Figure 6).

Discussion

Choice and no-choice assays that tested host selection, acceptance, and suitability were performed to characterize GPA resistance in the *fad7* mutant. In choice assays in which aphids were free to move between genotypes, the GPA showed no preference between wild-type (Col-0) and *fad7*plants at 4 hpi or at 48 hpi; the initial choice between paired plants appeared random and aphids were not prone to move from their first host. Despite its seeming inability to discriminate between *fad7* and Col-0, by 48 hpi the GPA had significantly lower population growth on the mutant than on wild-type plants in no-choice assays designed to assess host suitability. Moreover, by 6 dpi, there were approximately 20% fewer juveniles on *fad7* than on Col-0. This reduced population growth on *fad7* was the result of reduced fecundity and increased juvenile development time, although resistance appeared to have no effect on adult or juvenile mortality or the weight of newly emerged adults. Thus, resistance appeared to be due to antibiosis rather than antixenosis, and to impact fecundity and development time more than survival.

Characterizing the impacts of any form of resistance on aphid settling behavior and life parameters is important to predict how resistance might impact aphid population growth and virus transmission in the field. No-choice bioassays indicated that *fad7* reduced GPA offspring production per live adult by 20% and increased juvenile development time by 5% compared to aphid performance on wild-type plants. GPA typically has 20 generations in a growing season and populations can double in as little as 2.5 days under optimal conditions (Deloach, 1974; Capinera, 2001); thus, even modest impacts on fecundity and generation time can compound to result in valuable reductions of GPA in the field. In an integrated pest management system, this could mean fewer pesticide sprays and less selection pressure to drive resistance, allowing the technology to be available longer. Sources of antibiosis that reduce aphid fitness without disrupting settling behavior or promoting plant-to-plant movement are also valuable from the perspective of managing virus transmission by aphids. GPA is an important vector of over 100 viruses, including many non-persistent viruses such as cucumber mosaic virus and potato virus Y that can be acquired within minutes by aphids and transmitted in an equally short period of time (Pirone and Harris, 1977; Whitfield and Rotenberg, 2016). Traits that reduce aphid population growth can delay the production of winged morphs that emerge under crowded conditions and transmit viruses long distance (Donnelly et al., 2019; Carr et al., 2020). Moreover, forms of resistance that suppress aphid fitness without being detected by the aphid would not be expected to increase plant-to-plant movement, in contrast to antixenotic traits that have been shown to promote the local spread of non-persistent viruses (Mauck et al., 2010; Rhee et al., 2020). Thus, if similar forms of aphid antibiosis were identified in agronomically important crops, they could potentially translate to reduced direct and indirect damage in the field.

The impacts of plant defense mechanisms however may vary between aphid species. This study compared the influence of *fad7* on CA versus GPA in choice and population growth assays and found that CA was unaffected by *fad7*. CA is a specialist aphid that only feeds on members of the Brassicaceae, and compared to generalist herbivores, it may be less susceptible to defenses in these plants due to coevolution than generalist feeders. For example, CA is thought to have adapted to defensive glucosinolates, using them as "token stimuli" to stimulate feeding and sequestering them to ward off predators (Cole, 1997; Goodey et al., 2015). In this study, CA was observed to congregate in larger groups than GPA and cause a leaf curling symptom not seen with GPA; potentially CA may modify the growth and physiology of the plant to increase host suitability and/or overwhelm plant defenses. Potentially, CA and GPA may also be impacted by differing defensive signaling pathways in the plant. In *spr2* tomato, aphid resistance is dependent upon salicylic acid (SA), whereas jasmonate (JA)-signaling is reduced compared to wild type plants (Li et al., 2003; Avila et al., 2012). However, CA trigger and are susceptible to JA-regulated defenses, whereas GPA appears to be less susceptible to JA-regulated defenses (Mewis et al., 2006; Kuśnierczyk et al., 2007, 2011). Potentially, CA may be insensitive to SA-dependent defenses in *fad7,* which may be compromised in JA-dependent defenses against the CA.

In addition to having varying effects on different aphids, the mode of action of resistance in plants with impaired FAD7 activity may also vary in different plant species. In contrast to our results with *fad7* in Arabidopsis, our laboratory (2012) previously demonstrated that loss-of-function of FAD7 in tomato had both antixenotic and antibiotic effects on the potato aphid (PA), *Macrosiphum euphobiae* (Thomas). In choice tests, the PA showed a strong preference for wild type plants over a *FAD7*-impaired mutant line (*spr2*) as early as 4 hpi, with almost all aphids moving from *spr2* to wild-type tomato by 24 hpi. In no-choice bioassays, the *spr2* mutation

increased the mortality of adults and juveniles in addition to decreasing fecundity; moreover, the mutant caused >50% reductions in short-term population growth of the PA, compared to the 20% reductions in GPA populations we see in Arabidopsis, and increased juvenile mortality. This resistance is obviously much stronger than in Arabidopsis. Potentially this is due to the functional redundancy of ω-3 FADs in Arabidopsis than in tomato; the *spr2* mutation typically causes up to a 90% decrease in trienoic fatty acids in tomato, whereas *fad7* in Arabidopsis typically causes 30-40% reduction (McConn et al., 1994; Li et al., 2003, 2021). However, it is worth noting that the *fad3fad7fad8* triple mutant in Arabidopsis had similar levels of aphid resistance as *fad7* despite having a near-total lack of trienoic fatty acids (Li et al., 2021). Therefore, differences in resistance characteristics between tomato and Arabidopsis may instead be due to evolutionary divergence of plant defenses modulated by fatty acid metabolism. Different plant families vary in their arsenal of induced defenses; for example, in response to the same pest, the two-spotted spider mite (*Tetranychus urticae*), Arabidopsis upregulates secondary metabolites specific to cruciferous plants such as indole glucosinolates, while tomato upregulates proteins targeting insect digestion (Martel et al., 2015). Divergence of downstream defenses regulated by homologous genes could therefore also explain differences in the characteristics and magnitude of the *fad7*-mediated resistance in tomato versus Arabidopsis. Further studies are needed to identify and compare the downstream defenses that contribute to aphid resistance in Arabidopsis and tomato plants with impaired FAD7 function.

Figure 1. Choice assay layout between wild-type Col-0 and *FAD7*-impaired (*fad7*) Arabidopsis. (**A**) plants were grown 2.5" apart and (**B**) grew close together without overlapping. Ten GPA or CA adults were placed on filter paper that was set equidistant between the two plants. Aphid position was recorded at 24 and 48 hpi.

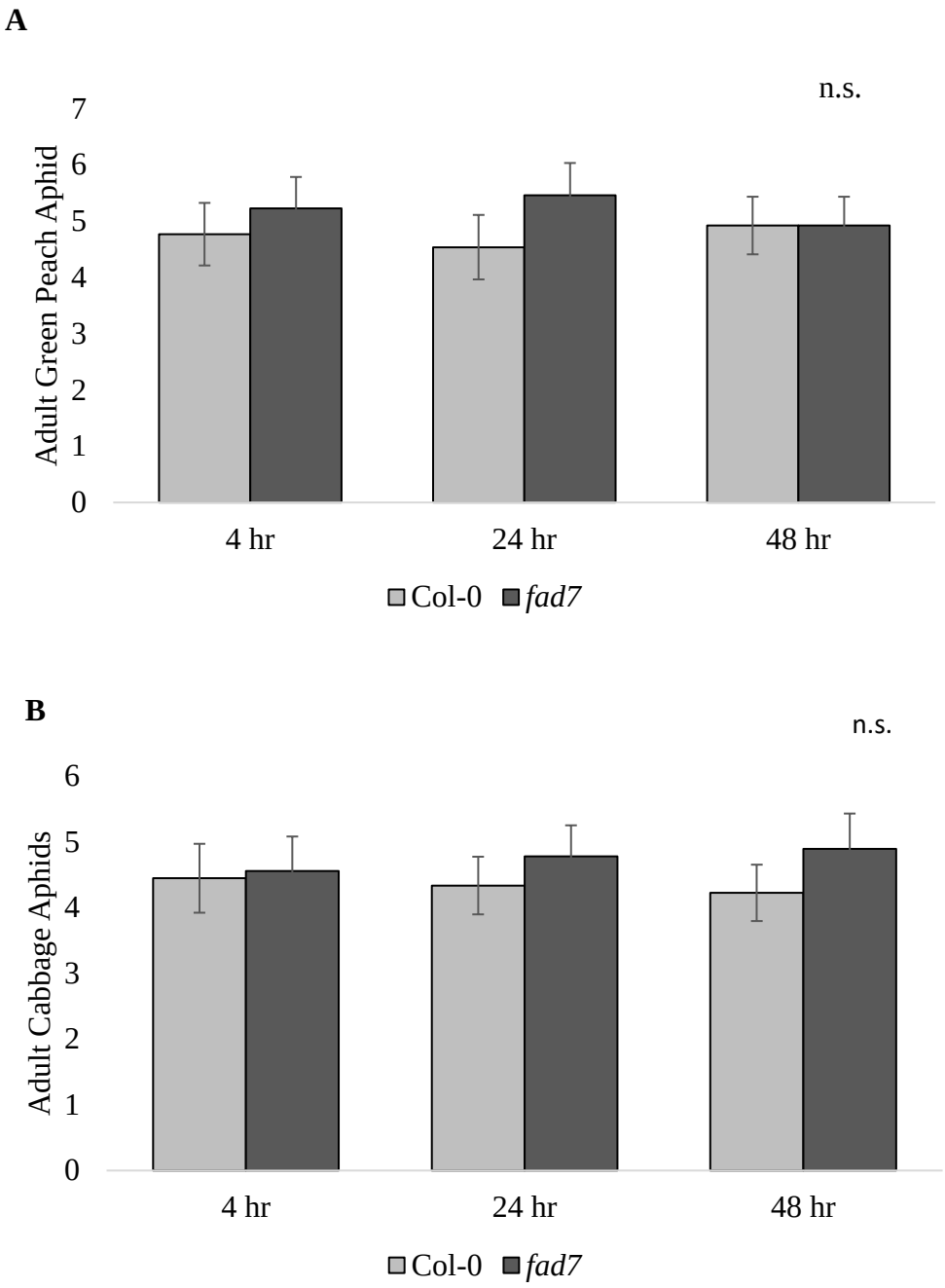

Figure 2. Choice assay on Arabidopsis with (**A**) GPA and (**B**) CA at 4, 24, and 48 hrs. Data were analyzed with a paired t-test. Error bars represent ±SE. There was no significant difference in aphid host-plant selection at any timepoint at an α=0.05, (**A**) n=13, (**B**) n=9; n.s.=not significant.

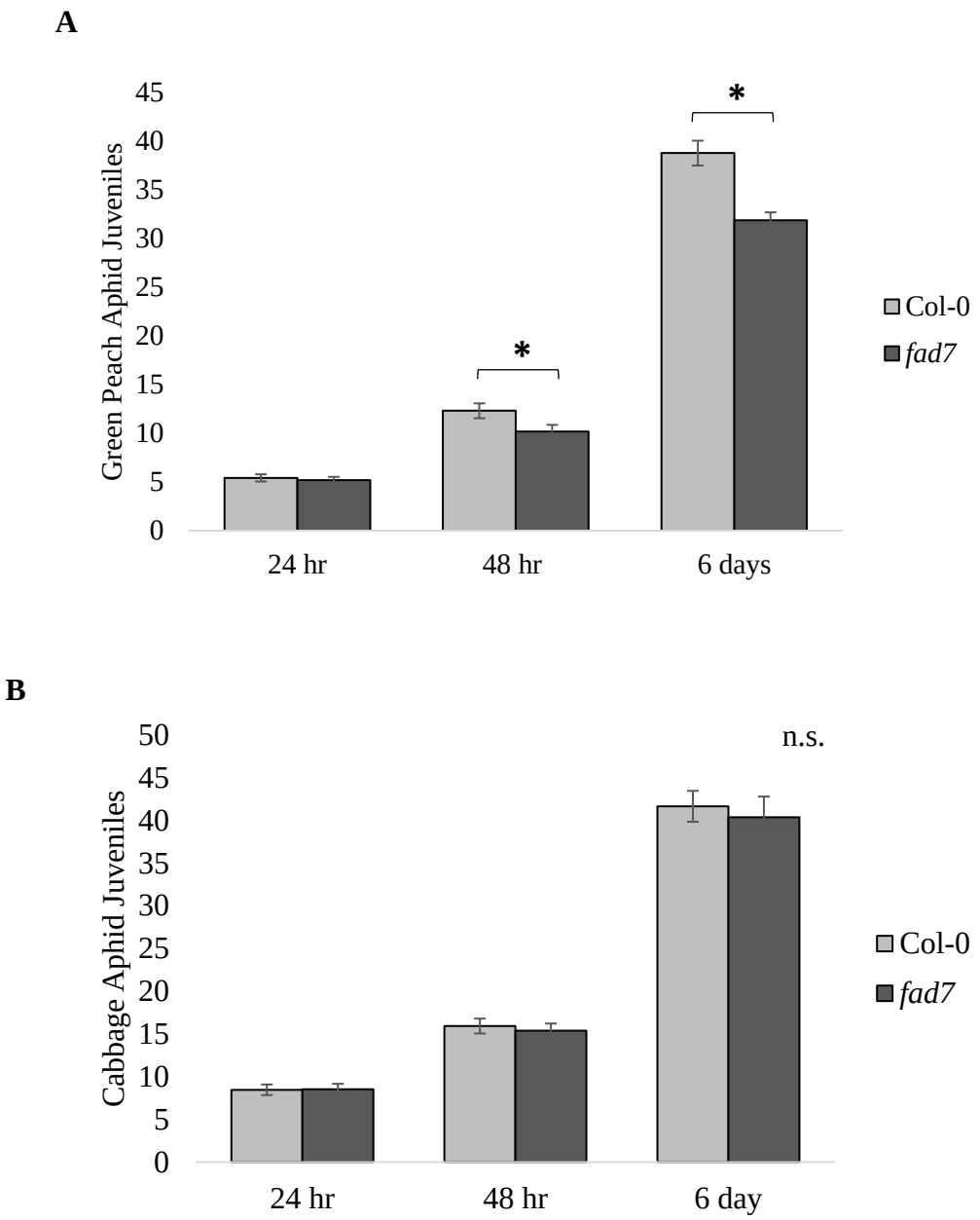

Figure 3. No-choice population growth assay of (**A**) GAP and (**B**) CA on Arabidopsis with and without *FAD7*-impaired mutations at 24 hr, 48 hr, and 6 d after infestation with two adult aphids. While GPA reproduction was not significantly different on *fad7* plants compared with wild-type Col-0 at 24 hr, there was a significant reduction in juvenile production at 48 hr and 6 d. However, the *fad7* mutation did not impact aphid fecundity of CA at any of the timepoints. Data were analyzed by student's t-test. Error bars represent ±SE and asterisks represent significant difference at α=0.05, (A) n=19 for Col-0 and 20 for *fad7*, (B) n=16; n.s.=not significant.

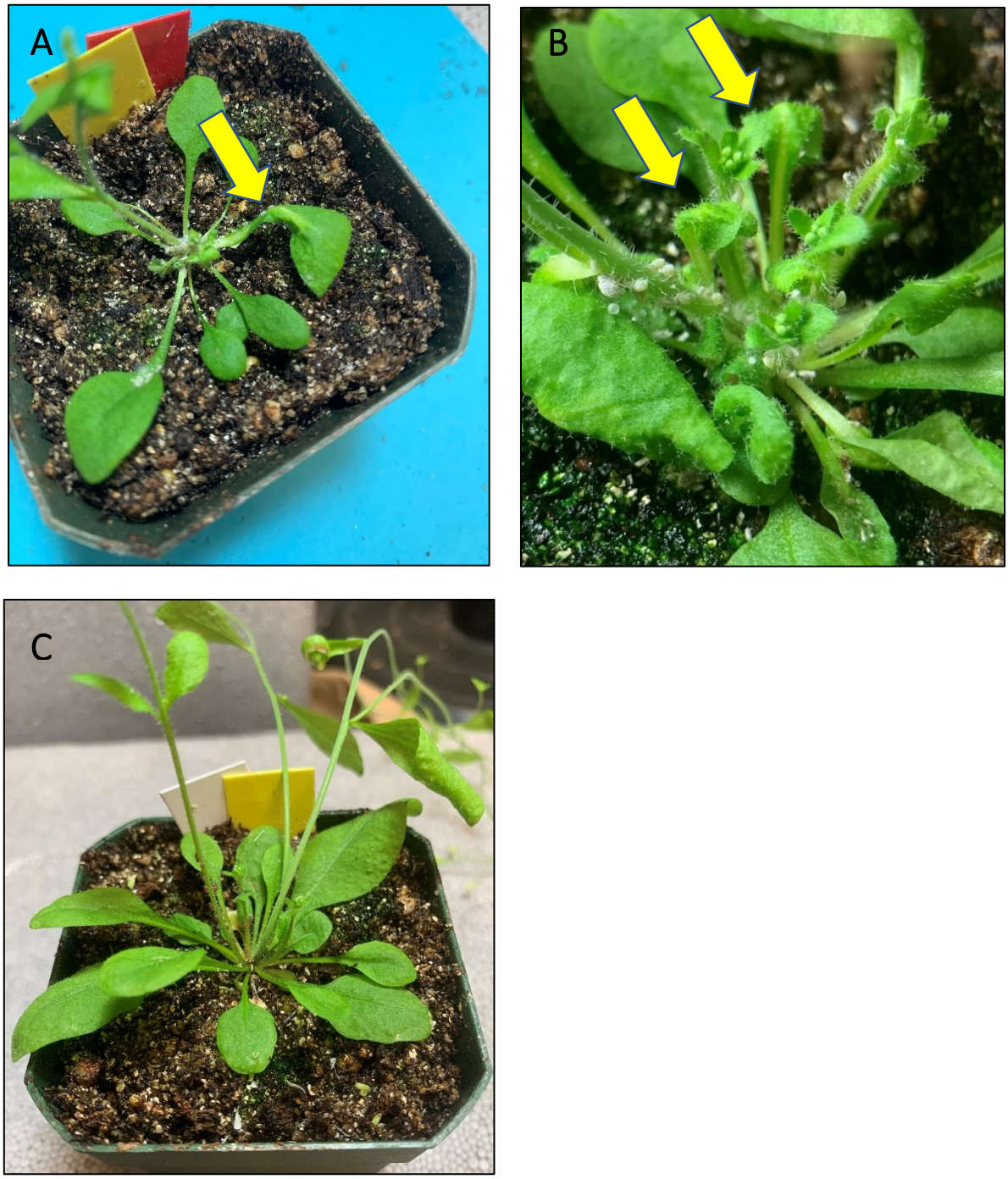

Figure 4. Impact of CA manipulation on (**A**) mature leaves and (**B**) emerging leaves and inflorescences compared with plants infested with (**C**) green peach aphid, as indicated by a yellow arrow.

Table 1. Juvenile development time and survival of green peach aphids. Data analyzed with a Student's t-test. Significant difference was determined at α=0.05 and indicated by an asterisks; n=10; n.s.=not significant.

Day		6	7	8	Total	Average Days to Emergence
% Emergence of Adults	Col-0	4.0 ± 2.5	66.8 ± 6.3	29.2 ± 7.1	100%	7.3
	fad7	1.8 ± 2.5	40.3 ± 6.3	57.9 ± 7.1	100%	7.6
P-value		0.54	0.008*	0.01*	1.0	0.042*

Note: No adult emergence before day 6.

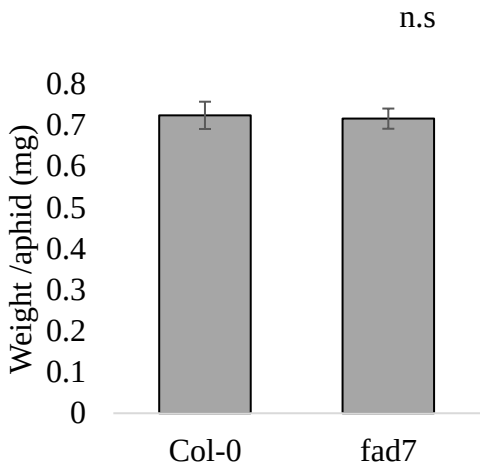

Figure 5. Weights of newly emerged adult green peach aphid on Arabidopsis. Data were analyzed with a Student's t-test. Error bars represent ±SE. Significant difference was determined at α=0.05 and indicated by an asterisks; n=10; n.s.=not significant.

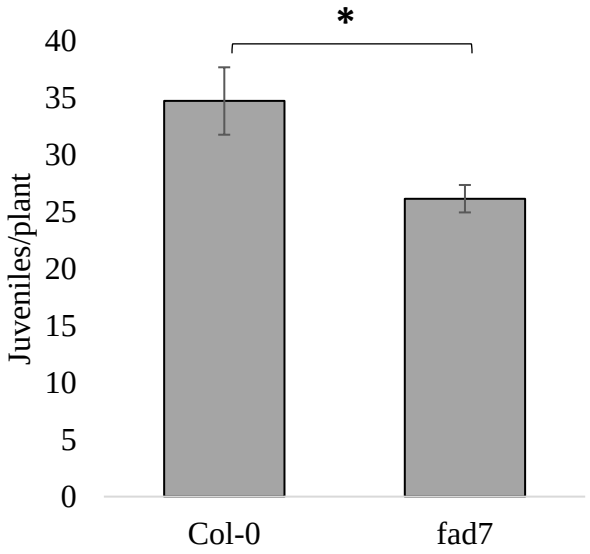

Figure 6. No-choice assay to measure GPA fecundity. Juvenile production was counted 5 days after each juvenile reared on Col-0 or *fad7* emerged to adulthood. Data were analyzed with a student's t-test. Error bars represent ±SE. There were significantly fewer juveniles on *fad7* compared with Col-0, as indicated by an asterisk, at α=0.05; n=7.

References

Ali, J. G., and Agrawal, A. A. (2012). Specialist versus generalist insect herbivores and plant defense. *Trends Plant Sci* 17, 293–302. doi:10.1016/j.tplants.2012.02.006.

Aradottir, G. I., and Crespo-Herrera, L. (2021). Host plant resistance in wheat to barley yellow dwarf viruses and their aphid vectors: a review. *Curr Opi Insect Sci* 45, 59–68. doi:10.1016/j.cois.2021.01.002.

Avila, C. A., Arévalo-Soliz, L. M., Jia, L., Navarre, D. A., Chen, Z., Howe, G. A., Meng, Q., Smith, J.E., Goggin, F.L. (2012). Loss of Function of FATTY ACID DESATURASE7 in tomato enhances basal aphid resistance in a salicylate-dependent manner. *Plant Physiol.* 158, 2028–2041.

Bidart-Bouzat, M. G., and Kliebenstein, D. (2011). An ecological genomic approach challenging the paradigm of differential plant responses to specialist versus generalist insect herbivores. *Oecologia* 167, 677. doi:10.1007/s00442-011-2015-z.

Capinera, J. L. (2001). Green Peach Aphid, *Myzus persicae* (Sulzer) (Insecta: Hemiptera: Aphididae). Available at: http://entnemdept.ufl.edu/creatures/veg/aphid/green_peach_aphid.htm

Carr, J. P., Tungadi, T., Donnelly, R., Bravo-Cazar, A., Rhee, S.-J., Watt, L. G., et al. (2020). Modelling and manipulation of aphid-mediated spread of non-persistently transmitted viruses. *Virus Res* 277, 197845. doi:10.1016/j.virusres.2019.197845.

Cole, R. A. (1997). The relative importance of glucosinolates and amino acids to the development of two aphid pests *Brevicoryne brassicae* and *Myzus persicae* on wild and cultivated brassica species. *Entomol Exp Appl* 85, 121–133. doi:https://doi.org/10.1046/j.1570-7458.1997.00242.x.

Coleson, J. L., and Miller, R. H. (2005). Antibiosis and antixenosis to *Aphis gossypii* (Homoptera: Aphididae) in *Colocasia esculenta*. *J Econ Entomol* 98, 996–1006. doi:10.1603/0022-0493-98.3.996.

Dedryver, C.-A., Le Ralec, A., and Fabre, F. (2010). The conflicting relationships between aphids and men: A review of aphid damage and control strategies. *Comptes Rendus Biol* 333, 539–553. doi:10.1016/j.crvi.2010.03.009.

Deloach, C. J. (1974). Rate of increase of populations of cabbage, green peach, and turnip aphids at constant temperatures. *Ann Entomol Soc Am* 67, 332–340. doi:10.1093/aesa/67.3.332.

Diaz-Montano, J., Reese, J. C., Schapaugh, W. T., and Campbell, L. R. (2006). Characterization of antibiosis and antixenosis to the soybean aphid (Hemiptera: Aphididae) in several soybean genotypes. *J Econ Entomol* 99, 1884–1889. doi:10.1093/jee/99.5.1884.

Donnelly, R., Cunniffe, N. J., Carr, J. P., and Gilligan, C. A. (2019). Pathogenic modification of plants enhances long-distance dispersal of nonpersistently transmitted viruses to new hosts. *Ecology* 100, e02725. doi:10.1002/ecy.2725.

Döring, T. F., and Chittka, L. (2007). Visual ecology of aphids—a critical review on the role of colours in host finding. *Arthropod-Plant Inter* 1, 3–16. doi:10.1007/s11829-006-9000-1.

Eastop, V. F. (1977). "Worldwide importance of aphids as virus vectors," in *Aphids As Virus Vectors* (Elsevier), 3–62. doi:10.1016/B978-0-12-327550-9.50006-9.

Eastop, Victor. F., and Blackman, R. L. (2005). Some new synonyms in Aphididae (Hemiptera: Sternorrhyncha). *Zootaxa* 1089, 1. doi:10.11646/zootaxa.1089.1.1.

Emden, H. F. van, and Harrington, R. (2017). *Aphids as Crop Pests, 2nd Edition*. CABI.

Fraenkel, G. S. (1959). The raison d'être of secondary plant substances. *Science* 129, 1466–1470.

Goodey, N. A., Florance, H. V., Smirnoff, N., and Hodgson, D. J. (2015). Aphids pick their poison: selective sequestration of plant chemicals affects host plant use in a specialist herbivore. *J. Chem. Ecol.* 41, 956–964. doi:10.1007/s10886-015-0634-2.

Guo, H., Gu, L., Liu, F., Chen, F., Ge, F., and Sun, Y. (2019). Aphid-borne viral spread is enhanced by virus-induced accumulation of plant reactive oxygen species. *Plant Physiology* 179, 143–155. doi:10.1104/pp.18.00437.

He, M., Qin, C.-X., Wang, X., and Ding, N.-Z. (2020). Plant unsaturated fatty acids: Biosynthesis and regulation. *Front. Plant Sci.* 11. doi:10.3389/fpls.2020.00390.

Hesler, L. S., and Tharp, C. I. (2005). Antibiosis and antixenosis to *Rhopalosiphum padi* among triticale accessions. *Euphytica* 143, 153–160. doi:10.1007/s10681-005-3060-7.

Hussain, A., Arif, M., Man, S., Hussain, B., Ali, M., and Jaffar, S. (2016). *A review on aphid-borne virus (Potato Virus Y)*. doi:10.13140/RG.2.1.1661.7844.

Kachroo, A., and Kachroo, P. (2009). Fatty acid-derived signals in plant defense. *Annu Rev Phytopath* 47, 153–176.

Kachroo, A., Lapchyk, L., Fukushige, H., Hildebrand, D., Klessig, D., and Kachroo, P. (2003). Plastidial fatty acid signaling modulates salicylic acid– and jasmonic acid–mediated defense pathways in the Arabidopsis *ssi2* mutant. *The Plant Cell* 15, 2952–2965. doi:10.1105/tpc.017301.

Kennedy, J. S., Day, M. F., Eastop, V. F., Commonwealth Institute of Entomology, Commonwealth Agricultural Bureaux, and Executive Council (1962). *A conspectus of aphids as vectors of plant viruses*. London: Commonwealth Institute of Entomology.

Kirchner, S. M., Döring, T. F., and Saucke, H. (2005). Evidence for trichromacy in the green peach aphid, *Myzus persicae* (Sulz.) (Hemiptera: Aphididae). *J Insect Physiol* 51, 1255–1260. doi:10.1016/j.jinsphys.2005.07.002.

Kogan, M., and Ortman, E. F. (1978). Antixenosis–A new term proposed to define painter's "nonpreference" modality of resistance. *Bulle Entomol Soc Amer* 24, 175–176. doi:10.1093/besa/24.2.175.

Kring, J. B. (1967). Alighting of aphids on colored cards m a flight chamber. *J Econ Entomol* 60, 1207–1210. doi:10.1093/jee/60.5.1207.

Kuśnierczyk, A., Tran, D. H., Winge, P., Jørstad, T. S., Reese, J. C., Troczyńska, J., and Bones, A.M. (2011). Testing the importance of jasmonate signalling in induction of plant defences upon cabbage aphid (*Brevicoryne brassicae*) attack. *BMC Genomics* 12, 423. doi:10.1186/1471-2164-12-423.

Kuśnierczyk, A., Winge, P., Midelfart, H., Armbruster, W. S., Rossiter, J. T., and Bones, A. M. (2007). Transcriptional responses of Arabidopsis thaliana ecotypes with different glucosinolate profiles after attack by polyphagous *Myzus persicae* and oligophagous *Brevicoryne brassicae. JXB* 58, 2537–2552. doi:10.1093/jxb/erm043.

Li, C., Liu, G., Xu, C., Lee, G. I., Bauer, P., Ling, H.Q., Ganal, M.W., and Howe, G.A. (2003). The tomato suppressor of prosystemin-mediated responses2 gene encodes a fatty acid desaturase required for the biosynthesis of jasmonic acid and the production of a systemic wound signal for defense gene expression. *The Plant Cell* 15, 1646–1661. doi:10.1105/tpc.012237.

Li, J. (2016). Influence of fatty acids and their derivatives on aphid resistance in Arabidopsis and tomato. *Theses and Dissertations* 1617, 113.

Li, J., Galla, A. L., Avila, C. A., Flattmann, K., Vaughn, K. L., and Goggin, F. (2021). Fatty acid desaturases in the chloroplast and endoplasmic reticulum promote susceptibility to the green peach aphid, *Myzus persicae*, in *Arabidopsis thaliana*. *MPMI*. doi:10.1094/MPMI-12-20-0345-R.

Louis, J., Leung, Q., V, P., J, R., and J, S. (2010). PAD4-dependent antibiosis contributes to the *ssi2*-conferred hyper-resistance to the green peach aphid. *MPMI* 23, 618–627. doi:10.1094/mpmi-23-5-0618.

Louis, J., and Shah, J. (2013). *Arabidopsis thaliana—Myzus persicae* interaction: shaping the understanding of plant defense against phloem-feeding aphids. *Front Plant Sci* 4. doi:10.3389/fpls.2013.00213.

Margaritopoulos, J. T., Kasprowicz, L., Malloch, G. L., and Fenton, B. (2009). Tracking the global dispersal of a cosmopolitan insect pest, the peach potato aphid. *BMC Ecol* 9, 13. doi:10.1186/1472-6785-9-13.

Martel, C., Zhurov, V., Navarro, M., Martinez, M., Cazaux, M., Auger, P., Migeon, A., Santamaria, M.E., Wybouw, N., Diaz, I., Van Leeuwen, T., Navajas, M., Grbic, M., Grbic, V. (2015). Tomato whole genome transcriptional response to *Tetranychus urticae* identifies divergence of spider mite-induced responses between tomato and *Arabidopsis*. *MPMI* 28, 343–361. doi:10.1094/MPMI-09-14-0291-FI.

Mauck, K. E., Moraes, C. M. D., and Mescher, M. C. (2010). Deceptive chemical signals induced by a plant virus attract insect vectors to inferior hosts. *PNAS* 107, 3600–3605. doi:10.1073/pnas.0907191107.

McConn, M., Hugly, S., Browse, J., and Somerville, C. (1994). A Mutation at the *fad8* locus of Arabidopsis identifies a second chloroplast ω-3 desaturase. *Plant Physiol.* 106, 1609–1614. doi:10.1104/pp.106.4.1609.

Mewis, I., Tokuhisa, J. G., Schultz, J. C., Appel, H. M., Ulrichs, C., and Gershenzon, J. (2006). Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. *Phytochem* 67, 2450–2462. doi:10.1016/j.phytochem.2006.09.004.

Miles, P. W. (1999). Aphid saliva. *Biol Rev* 74, 41–85. doi:10.1111/j.1469-185X.1999.tb00181.x.

Mowry, T. M. (2005). Insecticidal reduction of Potato leafroll virus transmission by *Myzus persicae*. *Anna Appl Biol* 146, 81–88. doi:https://doi.org/10.1111/j.1744-7348.2005.03149.x.

Painter, R. H. (1951). *Insect resistance in crop plants.* New York: Macmillan.

Pirone, T. P., and Harris, K. F. (1977). Nonpersistent transmission of plant viruses by aphids. *Annu Rev Phytopath* 15, 55–73. doi:10.1146/annurev.py.15.090177.000415.

Pitino, M., Coleman, A. D., Maffei, M. E., Ridout, C. J., and Hogenhout, S. A. (2011). Silencing of aphid genes by dsRNA feeding from plants. *PLOS ONE* 6, e25709. doi:10.1371/journal.pone.0025709.

Powell, G., Hardie, J., and Pickett, J. A. (1995). Responses of *Myzus persicae* to the repellent polygodial in choice and no-choice video assays with young and mature leaf tissue. *Entomol Exp Appl* 74, 91–94. doi:10.1111/j.1570-7458.1995.tb01878.x.

Rhee, S.-J., Watt, L. G., Bravo, A. C., Murphy, A. M., and Carr, J. P. (2020). Effects of the cucumber mosaic virus 2a protein on aphid-plant interactions in *Arabidopsis thaliana*. *Mol Plant Pathol* 21, 1248–1254. doi:10.1111/mpp.12975.

Silva, A. X., Jander, G., Samaniego, H., Ramsey, J. S., and Figueroa, C. C. (2012). Insecticide resistance mechanisms in the green peach aphid *Myzus persicae* (Hemiptera: Aphididae) I: A transcriptomic survey. *PLOS ONE* 7, e36366. doi:10.1371/journal.pone.0036366.

Smith, C. M. (2005). *Plant Resistance to Arthropods: Molecular and Conventional Approaches*. Springer Science & Business Media.

Smith, C. M., and Chuang, W.-P. (2014). Plant resistance to aphid feeding: behavioral, physiological, genetic and molecular cues regulate aphid host selection and feeding. *Pest Man Sci* 70, 528–540. doi:10.1002/ps.3689.

Upchurch, R. G. (2008). Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *Biotechnol Lett* 30, 967–977. doi:10.1007/s10529-008-9639-z.

Valenzuela, I., and Hoffmann, A. A. (2015). Effects of aphid feeding and associated virus injury on grain crops in Australia. *Austral Entomol* 54, 292–305. doi:https://doi.org/10.1111/aen.12122.

Vaughn, K. L., Avila, C. A., Padilla-Marcia, C. S., and Goggin, F. L. (2014). Development of *fad7-1* single mutant *Arabidopsis thaliana* plants that are resistant to aphids. *Discovery, The Student Journal of Dale Bumpers College of Agricultural, Food and Life Sciences* 15, 7.

Vos, M. de, Kim, J. H., and Jander, G. (2007). Biochemistry and molecular biology of Arabidopsis–aphid interactions. *BioEssays* 29, 871–883. doi:https://doi.org/10.1002/bies.20624.

Whitfield, A. E., and Rotenberg, D. (2016). *Disruption Of Insect Transmission Of Plant Viruses*. National Academies Press (US) Available at: https://www.ncbi.nlm.nih.gov/books/NBK390434/.

Chapter III

The chloroplast and singlet oxygen as signaling components for aphid resistance

Abstract

Previous studies suggest that aphids induce extracellular superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which could modify plant defenses. However, little is known about intracellular oxidative responses to aphids, or how other reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) influence plant responses to insects. This study used the redox-sensitive green fluorescent protein (roGFP2) to explore aphid-induced redox responses in the chloroplast, the site of greatest intracellular ROS generation in plants. This sensor revealed an oxidative shift in the chloroplast of Arabidopsis (*Arabidopsis thaliana*) in response to the green peach aphid (GPA; *Myzus persicae* Sulzer) that began within minutes of infestation and was sustained for at least 48 hours. GPA infestation of Arabidopsis also induced expression of a reporter gene (*AAA-ATPase:Luc*) that is responsive to $^1O_2$, a highly reactive molecule produced almost exclusively in the chloroplast. Furthermore, ROS accumulation in response to aphid feeding, as measured with luminol, was attenuated in transgenic plants with enhanced expression of an $^1O_2$ scavenger in the chloroplast (*SPS1oex*). These results indicate that aphid infestation induces $^1O_2$ accumulation in the chloroplast. GPA infestations were also significantly lower on a conditional mutant (*flu*) exposed to growth conditions that induce excess $^1O_2$ accumulation as compared to plants with normal $^1O_2$ levels (untreated *flu* plants, and treated and untreated wild-type plants). Furthermore, both constitutive and aphid-responsive *AAA-ATPase:Luc* expression levels were elevated in an Arabidopsis genotype with enhanced aphid resistance, the *fad7* mutant with impaired chloroplastic fatty acid desaturase activity. Together, these results suggest that production of $^1O_2$ in the chloroplast in response to aphid attack help limit aphid infestations. These findings

illustrate the importance of the chloroplast in perception and initiation of plant defenses against aphids and bring attention to the overlooked role of $^1O_2$ as a signaling component in biotic stress responses.

Introduction

Aphids are a destructive pest group of insects around the world, containing over 4300 species that are experts at exploiting plant resources (Blackman and Eastop, 2000). More than 250 species of aphids are known pests of agricultural and horticultural crops, causing damage by removing photoassimilates, altering photosynthesis, and vectoring phytopathogenic viruses to further reduce plant quality and yield (Kennedy et al., 1962; Blackman and Eastop, 2006; Botha et al., 2012). As phloem sap feeders, aphids use specialized mouthparts called stylets to pierce plant tissue and manuever between cells to reach the sieve elements of the phloem. During feeding, aphids salivate into the plant to prevent phloem occlusion and mitigate plant defenses (Harmel et al., 2008; Vos and Jander, 2009; Elzinga et al., 2014). However, there is evidence demonstrating that plant perception of aphids' saliva triggers plant defenses, including an oxidative burst of reactive oxygen species (ROS; Bos et al., 2010; Jaouannet et al., 2014).

ROS including hydrogen peroxide (H_2O_2), superoxide (O_2^-), and singlet oxygen ($^1O_2$) are important in development and environmental responses in the plant (Xia et al., 2015; Foyer and Noctor, 2016). Extracellular ROS are notably involved in biotic stress response and can accumulate in the apoplast as a result of NADPH oxidase activity at the plasma membrane which produces O_2^- that is rapidly dismutated to H_2O_2 (Bolwell et al., 2002). However, within the cell, the largest site of ROS production is the chloroplast, where ROS are unavoidable by-products of the photosynthetic electron transport chain. $^1O_2$ is continuously generated at PSII and PSI, while

PSI generates both H_2O_2 and O_2^- (Apel and Hirt, 2004). Mitochondria, peroxisomes, and the cytosol are also sources of ROS, particularly during photorespiration (Foyer and Noctor, 2000). As ROS oxidize biological components such as DNA, lipids, and proteins, uncontrolled ROS accumulation can be deleterious to the plant cell. Therefore, the plant employs several scavenging systems to regulate accumulation through various enzymatic and non-enzymatic antioxidants (Ahmad et al., 2009, 2010). The balance between ROS generation and scavenging maintains the reductive-oxidative (redox) homeostasis of subcellular compartments. The redox status is an important regulator of cell signaling and influences defense against abiotic and biotic stress (Dietz, 2003; Dietz and Scheibe, 2004).

A rapid and robust production and accumulation of ROS is one of the earliest plant responses to aphid attack (Maffei et al., 2007). Many studies have found significant accumulation almost immediately after aphid challenge that persists for hours or days (Prince et al., 2014; Ren et al., 2014, Xu et al. 2021). The importance of ROS in aphid resistance is also demonstrated with many aphid-resistant cultivars of agronomic crops having higher ROS accumulation compared with susceptible cultivars, either basally or in response to aphids (Sytykiewicz, 2016a; Czerniewicz et al., 2017; Lukasik and Golawska, 2019; Shao et al., 2019). ROS accumulation can trigger downstream defenses through gene activation, interaction with hormone signaling, and accumulation of secondary metabolites that reduce aphid fitness (Kerchev et al., 2012b; Foyer et al., 2017; Lukasik and Golawska, 2019). Guo et al. (2019) found H_2O_2 accumulation from NADPH oxidases caused the green peach aphid (GPA, *Myzus persicae* Sulzer) to have reduced feeding efficiency and would move to new plants. The *rbohD* mutant *Arabidopsis thaliana* (Arabidopsis) with impaired NADPH oxidase activity showed reduced levels of H_2O_2 and increased GPA population growth (Miller et al., 2009). To circumvent ROS

accumulation, some aphids have evolved methods of attenuating ROS production and accumulation. For example, compared to GPA populations that are not adapted to tobacco, tobacco-adapted GPA lineages downregulate expression of CathB3, a salivary cysteine protease that elicits a ROS burst in tobacco and limits aphid feeding (Guo et al., 2020). Furthermore, on an aphid-resistant accession of pepper (*Capsicum baccatum*), an avirulent GPA biotype elicited ROS accumulation lasting up to three days, whereas a virulent biotype that can overcome resistance in pepper did not induce detectable ROS. In fact, prior feeding by virulent aphids reduced the ROS burst from subsequent infestations of avirulent aphids as well as improved the fitness of the avirulent aphid (Sun et al., 2020).

The nature of the defense mechanisms triggered by ROS signaling appears to depend on a variety of factors including which ROS accumulate, where they are produced, and the timing and magnitude of accumulation (Laloi et al., 2004). Previously, plant-aphid interaction studies have focused primarily on H_2O_2, O_2^-, and their associated antioxidants, often demonstrating their importance in aphid resistance, while $^1O_2$ remains vastly understudied in plant responses to biotic stress. Also, many studies either investigate total ROS accumulation in plant tissue without specific location or focus on ROS production in the apoplast generated by NADPH oxidases (Moloi and van der Westhuizen, 2006; Miller et al., 2009; Sytykiewicz, 2016a). Investigation of the chloroplast, the site of largest intracellular ROS production, will broaden our understanding of ROS-mediated aphid resistance.

In the chloroplast, ROS generation is occuring continuously as a by-product of photosynthesis. In plant-pathogen interactions, it has been observed that certain pathogens target components of photosynthesis, thereby reducing ROS and other defense responses and increasing host-susceptibility (Zhou et al., 2015; Zeng et al., 2020). In certain plant-aphid

interactions, aphid infestation has also been reported to directly and indirectly suppress photosynthesis, which could result in an altered redox status of the chloroplast (Botha et al., 2005; Wilson et al., 2006; Hussain et al., 2015). Redox shifts in the chloroplast cause signal cascades that lead to retrograde reprogramming of the nucleus, which in some cases can enhance defense responses (Sierla et al., 2012; Kuźniak and Kopczewski, 2020). For example, chemical silencing of the chloroplast tAPX increased H_2O_2 which in turn increased expression of pathogen defense-related genes and accumulation of salicylic acid (SA), a phytohormone often involved in plant resistance to biotrophic pathogens and aphids (Morkunas et al., 2011; Maruta et al., 2012). Interestingly, in maize (*Zea mays*) cultivars resistant to the cereal aphids *Rhopalosiphum padi* and *Sitobium avenae* (F.), expression levels of the chloroplast associated *APX7* were upregulated significantly at 12 h compared with susceptible maize (Sytykiewicz, 2016b). Antioxidants are often induced in the wake of an oxidative burst to restore cellular homeostasis, but it is not possible to know from gene expression profiles alone the redox status of the chloroplast (Foyer and Noctor, 2005). Therefore, investigation of the chloroplast redox status of Arabidopsis in response to GPA could give further insight to the balance between ROS production and scavenging in response to stress.

In particular, there is a need to investigate $^1O_2$ accumulation in the chloroplast, and the potential interactions between $^1O_2$ and chloroplast membrane biology. While both H_2O_2 and O_2^- accumulate in the chloroplast and other cellular compartments, $^1O_2$ is believed to be the largest component of chloroplast ROS accumulation (Dmitrieva et al., 2020; Kuźniak and Kopczewski, 2020). Moreover, although the roles of $^1O_2$ in plant defense have not been studied as extensively as H_2O_2 and O_2^-, $^1O_2$ is the primary ROS involved in signaling for programmed cell death (PCD) and has been implicated in defense responses against pathogens such as *Pseudomonas syringae*

(Danon et al., 2006; Zoeller et al., 2012; Zhang et al., 2014). $^1O_2$-responsive defense signaling appears to be mediated by oxidation products of fatty acids (Przybyla et al., 2008). Since different unsaturated fatty acids vary in their rates of decomposition by $^1O_2$ (Watabe et al., 2007), $^1O_2$ signaling could potentially be influenced by fatty acid desaturases (FADs), which regulate the fatty acid composition of membrane lipids in the chloroplast (Upchurch, 2008; Kachroo and Kachroo, 2009). Since FADs influence the structure of photosynthetic membranes, they could potentially also impact the rate of $^1O_2$ generation (Hiremath et al., 2017). Consistent with this hypothesis, preliminary data from our laboratory suggests that the Arabidopsis mutant *fad3fad7fad8* has enhanced accumulation of $^1O_2$ compared to wild-type plants (unpublished data by M. Mueller and F. Goggin). The profile of fatty acid hydroperoxides observed by HPLC in *fad3fad7fad8* matches the characteristic fingerprint of lipid peroxidation products previously reported to occur after $^1O_2$ exposure (Farmer and Mueller, 2013). The *fad3fad7fad8* triple mutant is resistant to GPA; moreover, comparable levels of resistance are observed in the *fad7* single mutant, which is impaired in a chloroplast-localized FAD that regulates fatty acid desaturation levels in thylakoid membranes (Avila et al., 2012; Li et al., 2021). These findings suggest an possible connection interlinking aphid resistance, fatty acid desaturation, and $^1O_2$ accumulation.

To explore the potential roles of $^1O_2$ and the chloroplast in aphid resistance, it is critical to be able to measure the overall redox status of the chloroplast, to be able to detect $^1O_2$ specifically, and also to be able to manipulate $^1O_2$ accumulation artificially. Advancements in reporters for redox status and ROS as well as the availability of Arabidopsis with altered ROS production and/or accumulation have made this possible. A redox-sensitive green fluorescent protein (roGFP2) can be used to measure the overall redox status of specific cellular compartments (Stonebloom et al., 2012; Aller et al., 2013). RoGFP2 has two excitation peaks at

400 nm and 490 nm that are more responsive under oxidized and reduced states, respectively, that allows for ratiometric measurement of a cellular compartment; importantly, transgenic plants expressing this protein in chloroplasts and other plastids (i.e. the *roGFP2-plastid* transgenic line) are responsive to accumulation of H_2O_2, O_2^-, and $^1O_2$ (Brunkard et al., 2015). $^1O_2$ accumulation can be indirectly detected using a luciferase reporter under the promotor of the $^1O_2$-responsive gene *AAA-ATPase (AAA-ATPase:Luc)* that results in quantifiable luminescence as a way to measure $^1O_2$. This approach is useful because $^1O_2$ has a very short half-life (~200 ns) making direct quantification extremely difficult. Perhaps the most useful tool for studying the biological consequences of $^1O_2$ accumulation is the conditional *flu* mutant in Arabidopsis developed by Meskauskiene and coworkers (2001). When the *flu* mutant is grown in continuous light (termed a permissive photoperiod), its phenotype is comparable to wild type plants; however, if *flu* is exposed to a period of darkness, it accumulates the photosensitizer protochlorophyllide (Pchlide), which then generates high levels of $^1O_2$ in the chloroplast when plants are subsequently exposed to light (Camp et al., 2003). Thus, $^1O_2$ can be artificially induced by exposing *flu* to a non-permissive photoperiod (light/dark/light shift). Conversely, to decrease $^1O_2$ in the chloroplast, Ksas and colleagues (2015) developed a transgenic Arabidopsis line (*SPS1oex*) which overexpresses a gene for the biosynthesis of the $^1O_2$-scavenger plastoquinone-9 (PQ-9). PQ-9 that is stored outside the thylakoid membrane has been shown to decrease $^1O_2$ accumulation in high light stressed plants (Ksas et al., 2015, 2018).

Using these tools, this study explored the role of the chloroplast redox response in triggering aphid-resistance in Arabidopsis. The redox response of the chloroplast was measured in the first 48 h of GPA infestation using the *roGFP2-plastid* reporter gene. ROS accumulation was further investigated in response to aphids in wild-type Arabidopsis (Col-0), aphid-resistant

fad7, and *SPSP1oex* with increased $^1O_2$ scavenging in the chloroplast. Then, the *AAA-ATPase:Luc* reporter gene was used to assess $^1O_2$ accumulation in response to GPA, and to compare $^1O_2$ levels in wild-type (Col-0) and *fad7* genetic backgrounds. In addition, the impact of increased $^1O_2$ on aphid fitness was evaluated using the conditional *flu* mutant. Results from this study suggest aphids induce a rapid oxidative response in the chloroplast involving $^1O_2$ accumulation which then may be involved in triggering defense responses against aphids.

Materials and Methods

Plant Materials

This study used seven *Arabidopsis thaliana* genotypes: wild-type Columbia CS7000 (Col-0); the *fad7-1* mutant (ABRC stock number CS71655) developed by Vaughn and coworkers (2014); *flu/AAA-ATPase:Luc,* which was provided Dr. Klaus Apel, Boyce Thompson Institute (Baruah et al., 2009); Col-0/*AAA-ATPase:Luc* and *fad7/AAA-ATPase:Luc,* which were developed in the Goggin lab by Alnasrawi (2015) and myself, respectively; *roGFP2-plastid,* which was provided by Dr. Jake Brunkard, University of Wisconsin-Madison (Brunkard et al., 2015); and the *SPS1overexpression* (*SPS1oex*) line, which was generously provided by Dr. Michel Havaux, Bioscience and Biotechnology Institute of Aix-*Marseille* (Ksas et al., 2015). All mutants and transgenics were developed in the Col-0 ecotype of Arabidopsis.

Growth Conditions

Arabidopsis seeds were sterilized as described in Chapter II before being plated on 1X Murashige and Skoog (MS) media (0.8% agar, 3% sucrose, pH adjusted to 5.5-5.9). After three days of vernalization at 4 °C, seeds were transferred to a growth chamber to germinate.

Approximately 1 week after germination, seedlings were transferred from MS plates to an Arabidopsis potting mix (4:3:2 peat moss, vermiculite, perlite) with a slow release fertilizer (Osmocote Plus; 15-9-12, Scotts-MiracleGro Company, Marysville, OH) and placed back in the growth chamber. For experiments with the conditional *flu* mutant, all plants were germinated and grown in continuous light (100 μmol m^{-2} sec^{-1} light intensity, 55% RH, 24 °C). For experiments not including the *flu* mutation, plants were germinated and grown with a 16 h L/8 h D photoperiod, at 180 μmol m^{-2} sec^{-1} light intensity, 55% RH, 24 °C. Two weeks after transplanting, plants were used in bioassays.

Confocal imaging of roGFP2-plastid location in plant tissue

A Leica microsystems SP5 II confocal microscope with a 40X objective lens was used to image roGPF2 in the foliage tissue of the *roGFP2-plastid* Arabidopsis line and confirm its localization to the chloroplast. RoGFP2 was excited with a 488 nm laser with 12% original power, and emissions were detected at 514 nm. Chloroplasts were imaged by detecting chlorophyll autofluorescence with excitation at 488 nm and emissions measured at 633 nm with the laser at 12% original power. Images were processed by LAS AF software. In micrographs, the signals for roGFP2 and chlorophyll autofluorescence are presented as green and red, respectively.

Confirmation that the roGFP2-plastid sensor responds to chloroplastic-generated ROS

A pilot assay was conducted to confirm the responsiveness of the *roGPF2-plastid* transgenic line to oxidation in the chloroplast, and to validate a high-throughput leaf disc assay for measurement of roGFP2 redox status. The roGFP2 protein is useful for assessing the oxidative state of the cellular compartment in which it is expressed because two cysteines on the protein's surface

form a disulfide bridge under oxidizing conditions, causing a conformational change in the chromophore that influences its responsiveness to excitation at 400 and 490 nm (Stonebloom et al., 2012; Aller et al., 2013). This reversible conformational change increases roGFP2's responsiveness to excitation at 400 nm and decreases its responsiveness to excitation at 490 nm; therefore, the ratio of fluorescence emitted in response to these wavelengths can be used as a measure of the overall redox status in specific cellular compartments. To confirm that this sensor could detect ROS accumulation in the chloroplast, the ratio of fluorescence emitted in response to excitation at 490 nm compared to 400 nm (ie. the ratio of 490/400nm) was measured in leaf discs treated with water or methyl viologen (MV), a terminal oxidant of PSI that results in O_2^- and alters the redox status of the chloroplast specifically. To standardize the leaf position used for tissue collection, the 7th rosette leaf of each plant was marked with string as soon as it emerged. Three weeks after germination the 7th leaf was cut off and a leaf disc (1.1 cm^2) was collected from the apical half of the leaf using a cork borer. The leaf disc was then further cut in half perpendicular to the midrib and the halves were placed into separate wells of a 24-well Eppendorf plate with 150 µL of ddH_2O so that one half of the leaf disc could be assigned to the MV treatment group and the other half could be used as a water-treated control. Black plates were used to maximize fluorescence measurements. Plates were covered with a plastic lid and placed in the light for an hour to allow the leaf discs to recover from wounding. After the recovery period, 100 µL of water was removed from each well and replaced with 100 µL of 50 µM MV or new water for control treatments. At 4 h and 12 h after treatment, fluorescence intensities were measured at 400 and 490 nm excitation and 530 nm emission using the Cytation 3 Cell Imaging Multi-Mode Reader (Bio-Tek, Winooski, VT, USA). There were 10 reps per

treatment and data were analyzed with a Welch's t-test to account for unequal variances at both timepoints.

Evaluating potential sources of background fluorescence in the roGFP2-plastid sensor assay

An additional pilot experiment was conducted to confirm that autofluorescence from plant tissues or from 5 aphids would not skew fluorescence measurements from *roGFP2-plastid* leaf discs treated with aphids. To assess autofluorescence from foliage, fluorescence emissions at 530 nm in response to excitation at 400 and 490 nm was measured in leaf discs 30 minutes after aphid treatment from Col-0 plants that lacked the *roGFP2-plastid* transgene. Measurements were also taken from wells that contained aphids but no plant tissues to assess possible autofluorescence from the aphids themselves. In addition, the fluorescence was compared in Col-0 leaf discs with and without aphids to rule out the possibility that aphids would alter autofluorescence in the foliage. There were 6 reps per treatment group in both assays. Data comparing total fluorescence of roGFP2 reporter, Col-0, and empty wells were analyzed with a one-way ANOVA and means were separated by a Student's t-test.

Measurement of roGFP2 redox status in response to aphid infestation

A high-throughput assay (illustrated in Figure 1) was used to assess the impacts of aphid infestation on the redox status of roGFP2 localized to the chloroplast. Once the 7^{th} leaf of the rosette emerged, it was marked with string to standardize the sampled leaf. Three weeks after germination the 7^{th} leaf was cut off and a leaf disc (1.1 cm^2) was collected from the apical half of the leaf using a cork borer. The leaf disc was then further cut in half perpendicular to the midrib and the halves were placed into corresponding wells of two 24-well Eppendorf plates with 150

µL of ddH_2O. One half of the leaf disc was assigned to a treatment group and the other half could be used as an untreated control. For each experiment, two pairs of plates were prepared, for a total of four plates. Black plates were used to maximize fluorescence measurements. Plates were covered with a plastic lid and placed in the light for an hour to allow the leaf discs to recover from wounding. After the recovery period, 100 µL of the ddH_2O was removed from the wells and measurements of baseline fluorescence (the 0h time point) were taken. Fluorescence intensities were measured at 400 and 490 nm excitation and 530 nm emission using the Cytation 3 Cell Imaging Multi-Mode Reader (Bio-Tek, Winooski, VT, USA). Then, 5 adult GPA were added to one plate of each plate pair and plates were placed back in a 16 h L /8 dark photoperiod. RoGFP2 is a reversible biosensor and enabled repeated measurements to be taken of tissue. Inoculation took place at 4h into the light phase of the photoperiod, and fluorescence measurements were taken from 30 minutes to 48h after inoculation; these measurements were collected at different phases of the photoperiod, including 1 h before the dark period (12 and 36 h), 7 h into the dark period (20 and 44 h), and 2 h after the dark period (24 and 48 h). There were 48 reps per aphid treatment group. Data were analyzed as a mixed model, blocking for sample, and the means of each time point were separated with a student's t-test.

Luminol-based quantification of ROS accumulation in Col-0, fad7, and SPS1oex in response to aphids

A luminol-based ROS detection assay was used to measure ROS accumulation in response to aphids and to determine if this response differed in an aphid-resistant mutant with modified fatty acid desaturation in the chloroplast (*fad7*) or in a transgenic line with enhanced antioxidant content in the chloroplast (*SPS1oex*) compared to wild type plants (Col-0). When plants were

three weeks old, half of them were infested with aphids and enclosed in sleeve cages, while the remaining plants were left uninfested but enclosed in empty cages. Six days after infestation (dpi) total ROS accumulation in plant tissue of infested and uninfested plants was measured using a luminol assay described by Leslie and Hesse (2014). ROS was evaluated at 6 days after infestation in order to determine whether ROS accumulation persisted past the initial stages of the infestation process. Tissue from the 6^{th} or 7^{th} leaf of Arabidopsis was used to punch a leaf disc ($0.87cm^2$) near the apex of the leaf to avoid the midrib. Leaf discs were then placed in 24-well plates with 150 µL ddH_2O and set back in light for 1 h to recover from wounding. During that time, the Elicitation Solution (ES) was prepared. This was done by mixing Horseradish Peroxidase (HRP; Sigma, catalog # P6782) and Luminol (≥ 97% purity-HPLC; Sigma; catalog # A8511) in ddH_2O from 500x stock mixtures. HRP stock was prepared by dissolving 10mg/mL in autoclaved ddH_2O and luminol was prepared by dissolving 17 mg Luminol in 1 mL of 200 mM KOH. Final concentrations of HRP and Luminol were 0.2 µM. After leaf tissue recovered from wounding, the ddH_2O was removed from the wells and 200 µL of ES was added to each well using a multichannel pipet. Ten minutes after addition of the ES, luminescence was measured with the a Cytation 3 Cell Imaging Multi-Mode Reader. There were 12 reps per treatment and data were analyzed as a mixed model with genotype and infestation as main effects and plate as a random blocking factor. Mean separation was done with student's t-test.

Screening for presence of AAA-ATPase:Luc reporter gene in Col-0 and fad7

The *AAA-ATPase:Luc* transgene construct contains the hygromycin phosphotransferase (hpt) selectable marker which confers resistance to hygromycin B (Baruah et al., 2009). A hygromycin plate screen was therefore used to verify Col-0 and *fad7* backgrounds were homozygous for the

AAA-ATPase:Luc reporter gene based on methods from Harrison and colleagues (2006). Arabidopsis seeds of Col-0/*AAA-ATPase:Luc, fad7/AAA-ATPase:Luc,* and wild-type Col-0 were plated onto 1X MS agar containing 25 µg mL^{-1} hygromycin. Seeds were vernalized for 3 days at 4 °C and placed in a growth chamber to germinate for 12 h (180 µmol m^{-2} sec^{-1} light intensity, 55% RH, 24 °C). Plates were then placed in the dark for 2 d at 24 °C to produce etiolated seedlings. Hygromycin resistant seedlings had an obvious phenotype of elongated hypocotyls, while non-transformed Col-0 had short hypocotyls. Furthermore, after 3 d of a 16 h L/8 h D photoperiod, transformed seedlings all were green, whereas non-transformed Col-0 were not.

Quantification of $^1O_2$ accumulation in Col-0 in response to aphids using AAA-ATPase:Luc reporter gene and an in vitro D-luciferin multiwell plate assay

The *AAA-ATPase:Luc* reporter gene was used to evaluate the effect aphids had on $^1O_2$ accumulation in Arabidopsis. The effects of aphid infestation on reporter gene activity were measured with a high-throughput leaf disc assay similar to that used with roGFP2, with the exception that clear 24-well plates were used for luciferase activity assays. Once the 7th leaf was fully expanded (approximately 3 weeks after transplanting) leaf discs were collected, halved, and set in 150 uL of ddH_2O for an hour during wound recovery, as described for the roGFP2 assay. Then 100 uL of water was removed from each well, half of each leaf disc was infested with 5 aphids, and plates were placed back in a growth chamber with a 16 h L/8 h D photoperiod. To measure *AAA-ATPase:Luc* reporter activity at 24h after infestation, 150 uL of 150 ug/mL D-luciferin was added 30 minutes prior to measurement, the samples were incubated at room temperature in the dark for 30 min, and then luminescence was measured using the Cytation 3 Cell Imaging Multi-Mode Reader and expressed as Relative Luminescence Units (RLUs). D-

luciferin was prepared in a 200X stock solution (30mg/mL) in ddH_2O and stored at -20 °C. To make the 1X working solution, 200X was thawed and diluted 1:200 with ddH_2O to achieve a final concentration of 150 ug/mL. Care was taken to keep the working stock wrapped in foil and kept out of direct light. There were 24 reps per treatment and data were analyzed as a mixed model comparing infestation and blocking for sample. To ensure that the presence of aphids on the leaf discs did not cause any artifacts in our luminescence measurements, control measurements were also taken of leaf discs or empty wells with and without aphids in the absence of D-luciferin. Data was analyzed by one-way ANOVA and means separated with a student's t-test.

Measuring GPA fecundity on the conditional flu mutant after two photoperiod conditions

To determine the influence of $^1O_2$ accumulation in plants impacts aphids, aphid population growth, was compared on plants with and without the conditional *flu* mutation under two different photoperiod conditions, one of which induces *flu* mutants to accumulate elevated $^1O_2$ levels. The green peach aphid, *Myzus persicae* (Sulzer), was reared on cabbage plants (*Brassica rapa* cv. Joi Choi) as described in the previous chapter. Aphids used in no-choice population growth assays were synchronized as described previously and collected 48 hrs after emerging as adults. Both plant genotypes used for this assay carried the *AAA-ATPase:Luc* reporter gene to allow us to measure $^1O_2$ accumulation in parallel (see below). All plants were initially grown for two weeks in continuous light to prevent *flu* mutants from accumulating $^1O_2$ damaging levels. Then, 12h before initiation of the aphid bioassay, plants were subjected to one of two light treatments: continuous light or a light/dark/light (LDL) shift. Plants treated with the LDL shift were placed in the dark for 8 hr, causing *flu* mutants but not Col-0, to accumulate the Pchlide

photosensitizer in the chloroplast, and then moved back into the light. **A**fter re-illumination for 4 h, plants were infested with two 8-day-old adult female aphids which were allowed to feed and reproduce for 48 hr in continuous light. Juveniles were then counted before plants were harvested for luminescent quantification of the *AAA-ATPase:Luc* as described below. There were 20-22 reps per treatment and data were analyzed with a two-way ANOVA with light treatment and genotype as main effects. Means were separated with a student's t-test.

Comparison of $^1O_2$ accumulation in flu and Col-0 after photoperiod treatment using the AAA-ATPase:Luc reporter gene and the Promega© Luciferase Assay System

After assaying *flu* mutants for aphid resistance, *AAA-ATPase:Luc* reporter activity was measured in all treatment groups using the Promega© Luciferase Assay System (Madison, WI 53711) to confirm that the LDL shift induced $^1O_2$ accumulation in the *flu* background but not in the Col-0 background. The Promega© Luciferase Assay System was selected because it allowed $^1O_2$ to be measured in the entire aboveground portion of the plants used for the aphid bioassay; this was advantageous because the spatial distribution of aphids varied from plant to plant, making it impossible to collect leaf discs with uniform levels of infestation. Prior to tissue collection, aphids were removed from the plant with a soft paint brush, and then the aboveground plant material was cut, flash frozen in liquid nitrogen, and stored at -80 °C. Later, tissue was ground in liquid nitrogen and weighed out to approximately 100 mg of frozen tissue in a 2 mL Eppendorf tube. 1X lysis buffer was added and tissue was homogenized using the Genogrinder at 7500 rpm for 1 min. Samples were then centrifuged at 10,000 rpm for 5 min, and the supernatant was transferred to a new tube. 20 µL of supernatant was mixed with 100 µL of Luciferase Assay Reagent, and luminescence was measured using the GloMax® 20/20 single tube luminometer

(Promega©). The luminescence of each sample was standardized by dividing by the initial weight of the sample and displayed as Relative Luminescence Units (RLUs). There were 36 reps per treatment and data were analyzed with a two-way ANOVA with light treatment and genotype as main effects. Means were separated with a student's t-test.

Characterization of constitutive $^1O_2$ accumulation in the aphid-resistant fad7 mutant using the AAA-ATPase:Luc reporter gene and the Promega© Luciferase Assay System

To determine if loss of function of *FAD7* in Arabidopsis influences $^1O_2$ accumulation, constitutive *AAA-ATPase:Luc* reporter gene activity was measured in the *fad7* and Col-0 genetic backgrounds using the Promega© Luciferase Assay System and GloMax® 20/20 single tube luminometer. Although much lower in throughput than a plate reader-based leaf disc assay, the Promega© Luciferase Assay System appears to be more sensitive to differences in reporter gene activity levels in the plant and was ideal to measure overall constitutive $^1O_2$ levels. When plants were three weeks old, aboveground plant tissue was collected and processed with the Promega© Luciferase Assay System as described above. There were 8 and 10 reps for *fad7/ AAA-ATPase:Luc* and Col-0/*AAA-ATPase:Luc* and data were analyzed with a Student's t-test.

Examination of the influence of photoperiod on $^1O_2$ accumulation in response to aphids in fad7 and Col-0 using the AAA-ATPase:Luc reporter gene and an in vitro D-luciferin plate assay

This experiment investigated the influence of light on the persistence of aphid-induced $^1O_2$ responses by measuring AAA-ATPase:Luc reporter gene activity after 24h of aphid infestation in *fad7* and Col-0 leaf discs subjected to two different photoperiod treatments. Plants were grown for approximately three weeks after transplanting. Half of the samples (the "Light" treatment)

received a 12 h light/8 h dark/4 h light photoperiod, while an identical set of samples (the "Dark" treatment) received a 16 h light/8 h dark photoperiod; thus, while both sets of samples received the same number of hours of light and darkness, the 24h data collection time point fell during the light exposure period for one set of samples and during the dark period for the other set of samples. $^1O_2$ is very short lived (half-life ~200 ns) before it reacts with biological components or is removed through non-enzymatic antioxidants (Gorman and Rodgers, 1992; Krieger-Liszkay et al., 2008). Therefore, the 8h period of darkness preceding measurement of the "dark" samples should be sufficient for light-generated $^1O_2$ to be removed from the chloroplast prior to the reporter gene assay. Aphid infestation was initiated in the light for all samples because preliminary trials indicated that aphids did not colonize the leaf discs when introduced in the dark. Reporter gene activity was measured with the high-throughput plate assay to allow for adequate levels of replication for the eight treatment groups (*fad7* and Col-0 +/- aphids, in Light or Dark treatment groups). Plates were set up as previously described, with samples from both genotypes included on the same plates. Two plate pairs per photoperiod treatment were prepared for a total of 8 plates. After the leaf discs had recovered from wounding and half the plates had been infested with aphids (5 aphids/well), two plate pairs were placed back in a growth chamber for 24 h that were subjected to a 12 h light/8 h dark/4 h light photoperiod. Two other plate pairs were placed under an altered photoperiod in which the end of the 24 hrs finished with the 8 h dark period (16 h light/8 h dark). This gave 24 reps per treatment combination (genotype/infestation/photoperiod). *AAA-ATPase:Luc* reporter activity was measured with the luciferin *in vitro* assay. The resulting luminescence was measured 30 min after addition of D-luciferin. Data were analyzed with a mixed model comparing aphid infestation, genotype, and photoperiod treatment, blocking for sample. Means were separated with a student's t-test.

Statistical Analysis

All statistical analysis was done using JMP Pro 15.2.0. Significance was determined at α=0.05.

Results

RoGFP2 detects oxidative shifts in the chloroplast and the signal is not skewed by autofluorescence of plant tissue or aphids.

RoGFP2 is a dynamic biosensor that measures the redox potential of a compartment based on glutathione (Aller et al., 2013). The redox response of the chloroplast to aphid challenge was assessed using the roGPF2 reporter gene localized to the chloroplast in Arabidopsis. RoGFP2 is a dynamic biosensor that measures the redox potential of a compartment based on glutathione (Aller et al., 2013). The location of roGFP2 to the chloroplast was confirmed using confocal microscopy (Fig. 2). To confirm the functionality of roGFP2, its activity was induced by methyl viologen (MV). Treatment with 50 uM MV caused the redox status of the chloroplast to be significantly oxidized at 4 h ($t=4.36$, $df=9.21$, $P=0.002$) and 12 h ($t=4.34$, $df= 9.85$, $P=0.002$), as indicated by the ratio of 490/400 nm of treated tissue being up to 300% lower (Fig. 3). It was also important to test if aphid or plant autofluorescence would skew measurements of the roGFP2 signal. The amount of fluorescence from the leaf tissue of Col-0 without the reporter and empty wells was 7-fold lower than fluorescence from the reporter ($F(2,15)=79.8$, $P<0.0001$; Fig. 4A). And the addition of aphids did not influence total fluorescence of Col-0 tissue ($t=0.96$, $df=5$, $P=0.37$) or empty wells ($t=0.46$, $df=5$, $P=0.20$; Fig. 4B).

Aphids induce a rapid and sustained oxidative response in the chloroplast.

The redox response of the chloroplast to aphid challenge was assessed using the roGPF2 sensor localized to plastids in Arabidopsis. A high-throughput leaf disc assay to measure the effects of aphids over time on the redox status of *roGFP2-plastid* foliage showed that there was a significant interaction effect between aphid challenge and time (genotype x time: $F(1,88)=36.6$, $P<0.0001$; blocking; $P=0.01$; Fig. 5). Prior to infestation, the chloroplast redox status of both groups was comparable. Thirty minutes after infestation aphids caused a significant increase in oxidation of the chloroplast, as indicated by a decreased 490/400 ratio of roGFP2-plastid. Aphid-infested tissue remained significantly more oxidized compared with uninfested tissue to the end of the experiment at 48 hr ($P<0.01$ for all timepoints after 0 h). During this time, both treatment groups showed similar diurnal fluctuations in redox status during periods of light exposure; however, the two treatment groups responded differently to dark periods. As expected, the chloroplasts of uninfested tissue were in a reduced state 7 h into the dark period (20h, 44 h), during which the light reaction of photosynthesis is not active. However, the chloroplasts of infested leaf discs remained oxidized even at night. These results indicate that aphids trigger an oxidative response in the chloroplast that persists through both light and dark phases of the photoperiod.

ROS accumulation in response to aphids is attenuated in plants with increased $^1O_2$ scavenging in the chloroplasts.

Total ROS accumulation was measured, using a luminol assay, in infested and uninfested plant tissue at 6 dpi of three Arabidopsis genotypes: wild-type Col-0; aphid-resistant *fad7* with a mutation altering the fatty acid composition of the chloroplast membranes and potentially has

elevated ROS; and *SPS1oex* with a transgene which increases a $^1O_2$ scavenger outside the thylakoid membrane of the chloroplast. ROS accumulation varied by genotype and infestation (genotype x aphids: F(2,58.1)=4.61, P=0.014; blocking: P=0.37; Fig. 6). Aphids significantly induced ROS accumulation in wild-type Col-0 and aphid-resistant *fad7* by 40% and 90%, respectively. While the increase was greater in *fad7*, aphid-induced ROS accumulation was not significantly different between Col-0 and *fad7*. Interestingly, in *SPS1eox*, the transgene appeared to block this response, presumably through increased $^1O_2$ scavenging in the chloroplast. These results suggest $^1O_2$ is a major component of ROS accumulation in response to aphids at 6 dpi and further implicate chloroplastic ROS in aphid response.

AAA-ATPase:Luc reporter gene assay has minimal background luminescence from aphids and plant tissue.

Prior to the leaf disc assay with the *AAA-ATPase* reporter gene, control measurements were taken to guard against background noise of leaf tissue, aphids, and empty wells. Neither added significantly to luminescence produced by luciferase with (+) D-luciferin (F(2,15)=59.844, P<0.0001; Figure 7A). Also, aphids did not significantly alter luminescence of plant tissue in the absence of D-luciferin (t=0.85, df=5, P=0.43) or in empty wells alone (t=0.29, df=5, P=0.78; Figure 7B.).

Aphids increased $^1O_2$-responsive reporter gene AAA-ATPase:Luc after 24 h.

The $^1O_2$-responsive reporter gene *AAA-ATPase:Luc* was measured in the Col-0 background in response to aphids with an *in vitro* plate assay. Leaf discs were infested with aphids for 24 hrs before luminescence measurements. Reporter activity in response to aphids was significantly

increased by approximately 30% (F(1,46)=6.16, P=0.017; blocking: P=0.41; Fig. 8). The increased in the $^1O_2$-responsive reporter gene suggests GPA induced $^1O_2$ accumulation at 24 hpi.

$^1O_2$ accumulation triggers aphid resistance in the conditional flu mutant.

Since $^1O_2$ accumulates in response to aphids, the next step was to investigate if $^1O_2$ accumulation affects the performance of aphids. This was done using an Arabidopsis line *flu/AAA-ATPase:Luc* that expresses the $^1O_2$- responsive *AAA-ATPase:Luc* reporter gene and also carries the conditional *flu* mutation, which causes rapid accumulation of $^1O_2$ in the chloroplast upon a light/dark/light (LDL) shift. Measurement of *AAA-ATPase:Luc* reporter gene activity allowed us to confirm that after a LDL shift, *flu* plants had significantly more $^1O_2$ accumulation than *flu* plants that remained in continuous light, or Col-0 in either light treatment ($F(1, 152)=63.2$, $P<0.0001$; Fig. 9A). An aphid population growth bioassay also showed that *flu* plants exposed to a LDL shift had 20% fewer juveniles after 48 hours than other treatment groups, suggesting that $^1O_2$ produced in the chloroplast can rapidly trigger plant resistance to aphids ($F(1, 76)=6.32$, $P=0.014$; Fig. 9B).

Aphid-resistant fad7 accumulates more constitutive $^1O_2$ than wild type plants.

Previous studies have shown that loss of function of *FAD7*, which alters the fatty acid composition of thylakoid membranes in the chloroplast, confers aphid resistance and possibly also alters ROS accumulation (Li et al., 2021, unpublished data by M. Mueller and F. Goggin). To test whether *FAD7* could potentially impact $^1O_2$ accumulation, the *AAA-ATPase:Luc* reporter gene was crossed into *fad7* from Col-0 to generate a line homozygous for both *fad7* and the transgene. Constitutive reporter gene activity was 200% higher in the *fad7* background than in

Col-0 (t=3.59, df=16, P=0.0024, Fig. 10A), suggesting that *fad7* had higher $^1O_2$ accumulation than wild-type plants.

Induction of the $^1O_2$-responsive AAA-ATPase:Luc reporter gene by aphids persists through the dark phase of the photoperiod.

Next, a leaf disc assay was designed to test the accumulation of $^1O_2$ in both Col-0 and *fad7* when challenged with aphids. It also addressed the accumulation of $^1O_2$ in the plant in the dark when photosynthetic machinery is inactive. Light treatment groups were either plants under 12 h light/8 h dark/4 h light photoperiod or a 16 h light/8 h dark photoperiod, where 8 h of dark should be sufficient for $^1O_2$ produced from photosynthesis to be quenched. The main effects of genotype and aphid challenge significantly influenced $^1O_2$ accumulation, independent of photoperiod treatment (genotype: F(1,92)=6.78, P=0.01; aphids: F(1,92)=43.4, P<0.0001; light treatment: F(1,92)=1.25, P=0.27; blocking: P=0.67; no significant interaction effects; Fig. 10B). Aphid challenge increased reporter gene activity between 60 and 80% in both Col-0 and *fad7*. Overall, *fad7* had significantly higher reporter gene activity in both photoperiods. These results indicate that $^1O_2$ is accumulating in response to aphid challenge and accumulation is increased both constitutively and in response to aphids in aphid-resistant *fad7* compared with Col-0. Interestingly, however, there was no significant difference between samples collected in the light and samples collected after 8h of darkness. It is possible that expression of *AAA-ATPase:Luc* persists well after the initial $^1O_2$ stimulus is removed, or that $^1O_2$ can accumulate in the dark independent of the light reaction of photosynthesis.

Discussion

This study demonstrated that chloroplasts mediate a rapid and sustained oxidative response to aphid infestation that involves $^1O_2$. Observation of the roGFP2 reporter targeted to plastids revealed that GPA infestation on Arabidopsis caused an oxidative shift in the chloroplast that began within 30 minutes of infestation and persisted throughout the experiment, which terminated at 48h post-infestation. Furthermore, GPA challenge significantly increased ROS accumulation at 6 days post-infestation, as measured with a luminol and horseradish peroxidase system for detection of H_2O_2 and O_2^-. These results indicate that the oxidative response to aphids is highly persistent. In potato, GPA infestation has been reported to down-regulate chloroplastic superoxide dismutase, an enzymatic antioxidant that reduces the toxicity of O_2^- by converting it to H_2O_2 (Kerchev et al., 2012a). Thus, the sustained oxidative response we observed in Arabidopsis may potentially involve inhibition of antioxidant systems in the chloroplast. The results also showed that artificially enhancing the expression of an antioxidant targeted to the chloroplast (*SPS1*) attenuated ROS accumulation in response to GPA, as detected by a luminol assay. While this assay is most often used to measure extracellular ROS (Leslie and Heese, 2014), luminol is cell-membrane permeable (Shang-Guan et al., 2018), and can in some cases detect changes to ROS accumulation in intracellular compartments (Huang et al., 2013; Shang-Guan et al., 2018). In combination, results from transgenic lines expressing roGFP2 or *SPS1oex* suggest that aphids alter the redox balance of the chloroplast, and that this organelle contributes significantly to aphid-inducible ROS accumulation. This theory is further supported by the observation that GPA challenge increased expression of a $^1O_2$-responsive reporter gene (*AAA-ATPase:Luc*) in infested tissues 24 hpi. Given that $^1O_2$ is produced primarily in the chloroplast, *AAA-ATPase:Luc* induction suggests an oxidative response in this organelle. $^1O_2$ from the

chloroplast could potentially also contribute to ROS pools detected with luminol by directly oxidizing luminol (Warm and Laties, 1982; Wang et al., 2012), or, more likely, by promoting production of other ROS in the chloroplast including O_2^- and H_2O_2 (Pospíšil, 2016; Takagi et al., 2016). Since $^1O_2$ mediates retrograde signaling to reprogram the expression of defense genes in the nucleus, these observations suggest that chloroplast signaling could influence plant defenses against aphids.

Interestingly, the redox response to aphids appeared to persist during the dark phases of the photoperiod. Although the chloroplast would be expect to return to a reduced state when the light reaction of photosynthesis is deactivated by darkness, this reduced state was only observed in uninfested tissues; roGFP2 in infested leaf discs retained their oxidative status even during prolonged darkness. Similarly, induction of the $^1O_2$-responsive reporter gene *AAA-ATPase:Luc* by GPA persisted even after 8h of darkness. These responses observed in the dark phase are clearly not due to continued ROS production from photosynthesis. Previous reports indicate that $^1O_2$ production can occur in the dark in response to wounding and the pathogen elicitor flg22 in mitochondria, peroxisomes, and the nucleus, possibly as a result of lipid peroxidation (Mor et al., 2014). Thus, it is possible that $^1O_2$ generation can also occur in the dark in chloroplasts. Alternatively, the continued oxidation of the chloroplast during the dark phase may point to a drastic imbalance between antioxidants and ROS production. Several studies have discovered decreased antioxidant activity after aphid infestations that increased ROS accumulation in the plant (Lukasik et al., 2017; Kmieć et al., 2018). In either case, it appears that oxidation of the chloroplast and the $^1O_2$-signaling response can persist even in extended darkness, contributing to the prolonged nature of the oxidative response to aphid infestation.

Many prior studies have suggested that aphids induce ROS, but those have not provided a clear consensus on whether oxidative responses to aphid infestation enhance plant defenses or facilitate the infestation process. Many studies have found a correlation between ROS accumulation and aphid resistance (Miller et al., 2009; Berner and Van der Westhuizen, 2010; Kerchev et al., 2012a; Guo et al., 2019), but others have found that increased ROS or signaling components of ROS are beneficial for aphid infestations (Rasool et al., 2017; Shoala et al., 2018). In the present study, ROS accumulation at 6 dpi was increased 40% in the aphid-susceptible wild-type Col-0 and 90% in aphid-resistant *fad7* Arabidopsis, as measured with luminol. Although the difference between these genotypes was not statistically significant, the trend towards higher ROS accumulation in the aphid-resistant genotype is in line with other reports of enhanced ROS in aphid-resistant plants (Moloi and van der Westhuizen, 2006; Sytykiewicz, 2016a; Czerniewicz et al., 2017). Furthermore, compared to wild type plants, *fad7* had significantly higher expression of *AAA-ATPase:Luc* both constitutively and in response to GPA. These results suggest that *fad7* has higher $^1O_2$ levels than Col-0, possibly as a result of modified lipid peroxidation in the chloroplast. Impaired *FAD7* function in Arabidopsis results in decreases trienoic fatty acids in the chloroplast membranes (Li et al., 2021) which can act as antioxidants (Mène-Saffrané et al., 2009; Farmer and Mueller, 2013). Therefore, it is possible the reduction of TAs in *fad7* allows for increased $^1O_2$ accumulation in this genotype.

The correlation between $^1O_2$ accumulation and aphid resistance in *fad7* suggests that $^1O_2$ may contribute to plant defenses against aphids; however, to determine the true adaptive significance of $^1O_2$ accumulation in response to aphids, it is necessary to be able to manipulate $^1O_2$ levels and determine the impacts of these manipulations on aphid infestations. This study demonstrated that when $^1O_2$ accumulation in the chloroplast was artificially induced by exposing

the conditional *flu* mutant to a non-permissive light regimen, GPA population growth was reduced by 20% within the first 48 hpi. These results indicate that $^1O_2$ limits aphid infestation. Although more stable ROS such as O_2^- and H_2O_2 can cross cell membranes and interact directly with aphids during feeding (Łukasik and Goławska, 2013), $^1O_2$ is not likely to move out of the chloroplast due to its extremely short half-life (Gorman and Rodgers, 1992). Therefore, the reduced aphid fitness is likely due to $^1O_2$ signaling for defense-related nuclear gene expression changes. Characterization of the *flu* mutant has revealed that $^1O_2$ signaling plays an important role in chloroplast retrograde reprogramming of the nucleus and overlaps with disease resistance pathways (Galvez-Valdivieso and Mullineaux, 2010). For example, Ochsenbein and colleagues (2006) discovered that $^1O_2$ generated by a LDL shift in the *flu* mutant caused rapid upregulation of *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) and salicylic acid (SA) accumulation. SA further activated expression of genes encoding pathogenesis-related (PR) proteins *PR1* and *PR5*. GPA infestations on Arabidopsis have also been found to upregulate *PR* genes, including *PR1* (Moran and Thompson, 2001; Kuśnierczyk et al., 2007); furthermore, resistance to aphids resulting from loss of function of *FAD7* in tomato involves PR gene expression and requires SA accumulation, as well as expression of the SA signaling node *NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1* (*NPR1*; Avila et al., 2012). In summary, reduced aphid fitness on *flu* mutants exposed to non-permissive light conditions is likely due to defensive signaling induced by $^1O_2$, potentially including SA-dependent defenses. Development of *flu* lines with impaired SA signaling would aid in determining the defense mechanism of $^1O_2$-mediated defenses against aphids. Furthermore, additional studies are warranted to determine if $^1O_2$ is a determining factor in aphid resistance in the fad7 mutant.

Together, these data demonstrate that ROS produced in the chloroplast, especially $^1O_2$, are an important component of aphid-induced ROS accumulation, and may play a role in the perception and initiation of plant defense against aphids. This work draws attention to the need for exploring intracellular ROS in response to aphids. Furthermore, to the best of our knowledge, this is the first example of $^1O_2$ triggering aphid resistance and illustrates the importance of primary metabolism in biotic stress response, which has impacts beyond plant-aphid interactions as many defense responses have overlap between aphids and pathogens.

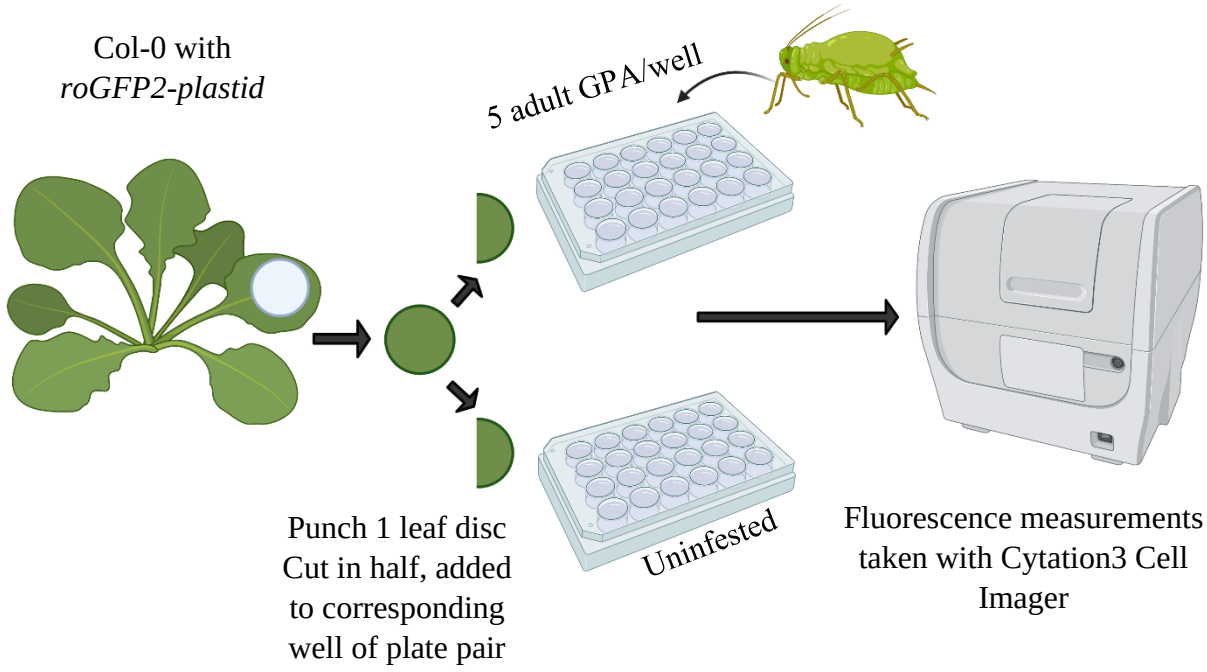

Figure 1. Diagram of high-throughput plate assay with roGFP2 and aphid infestation.

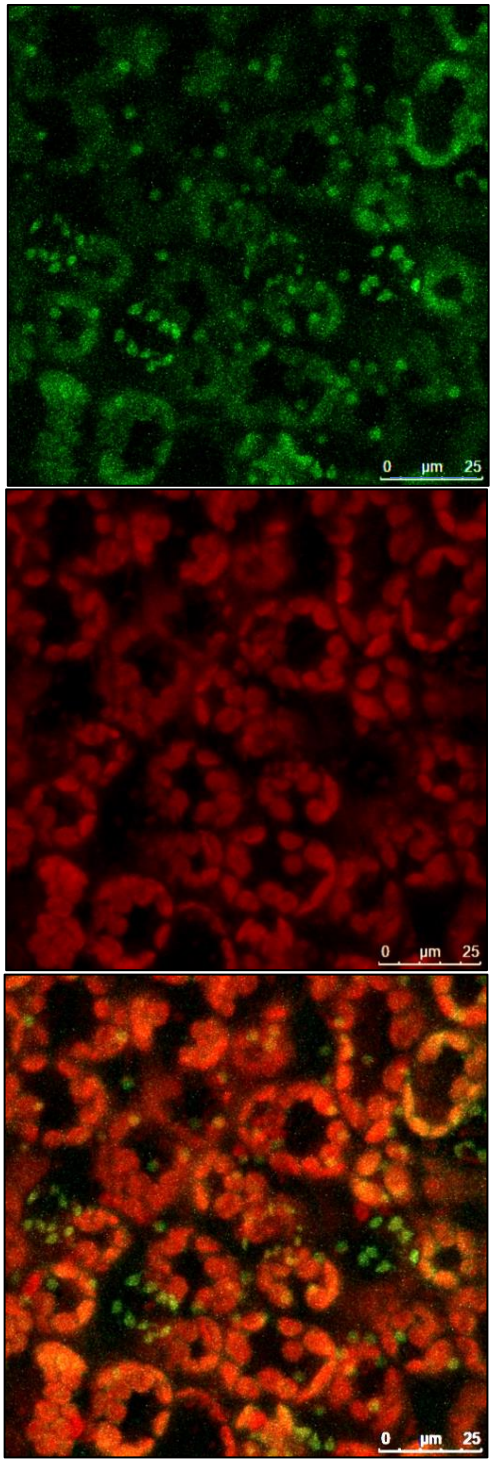

Figure 2. RoGFP2-plastid is localized to the chloroplast of Arabidopsis. Confocal microscopy detection of (**A**) fluorescence of roGFP2 (excitation: 488 nm; emission: 514 nm) with false coloring of green and (**B**) autofluorescence of chlorophyll (excitation: 488 nm; emission: 633 nm) with false coloring of red and (**C**) the overlayed image of roGFP2 and chlorophyll, with regions of overlap shown in yellow. RoGFP2 colocalized with chlorophyll in the chloroplasts of mesophyll cells, and was also evident in plastids of guard cells.

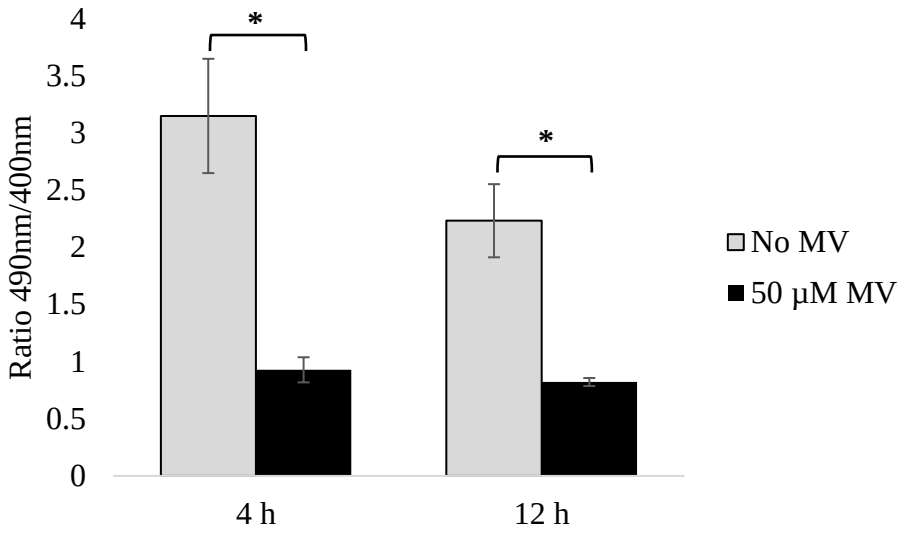

Figure 3. The *roGFP2-plastid* reporter gene is responsive to ROS. Methyl viologen (MV) is a terminal oxidant of PSI that results in superoxide in the chloroplast and was used to test the responsiveness of the *roGFP2-plastid* reporter gene to a redox change in the chloroplast. A decrease in the ratio of fluorescence emitted in response to excitation at 490 nm versus 400 nm (ie. the 490nm/400nm ratio) indicates an oxidative response. Data was analyzed with a Welch's t-test for unequal variance. Significance indicated by an asterisk and determined at α=0.05; n=10, error bars represent ±SE.

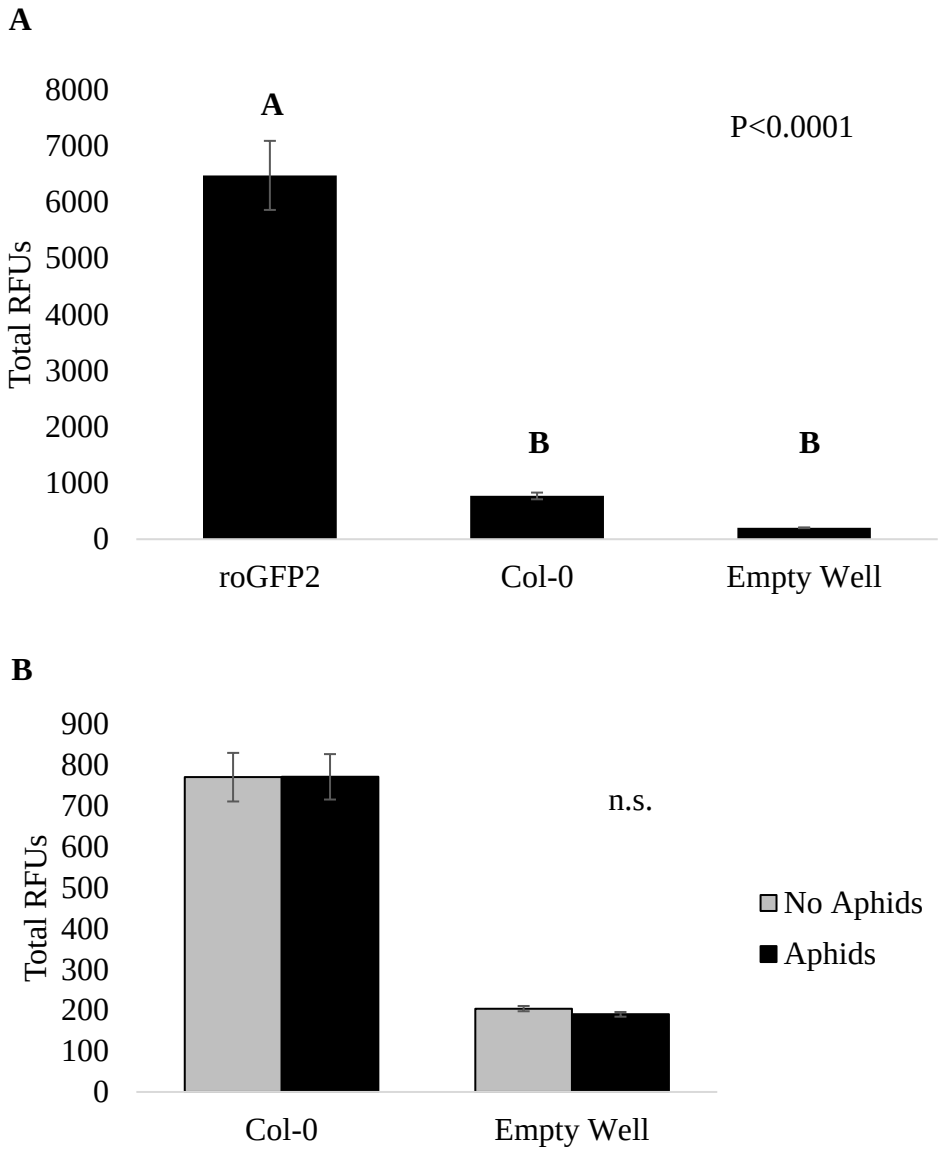

Figure 4. Autofluorescence of plant tissue and aphids are negligible. (**A**) Total fluorescence (excitation/emission: 400 nm/530 nm; 490 nm/530 nm) was measured in leaf discs of Col-0 with (roGFP2) and without (Col-0) the *roGFP2-plastid* reporter gene, and compared to empty wells of Eppendorf black well plate to confirm that the fluorescence observed in transformed plants was not due to background autofluorescnce. Data were analyzed by one-way ANOVA and means separated with a student's t-test. (**B**) Total fluorescence was also measured in untransformed Col-0 with and without aphids to confirm that aphids do not modify autofluorescence levels in plant tissue, and in wells lacking leaf discs (Empty well) with and without aphids to confirm that aphids themselves do not contribute to background autofluorescence. The autofluorescence of Col-0 without the reporter and empty wells with and without aphids were analyzed with a paired t-test. Error bars represent ±SE. Significant difference determined at α=0.05, n.s.=not significant, n=6.

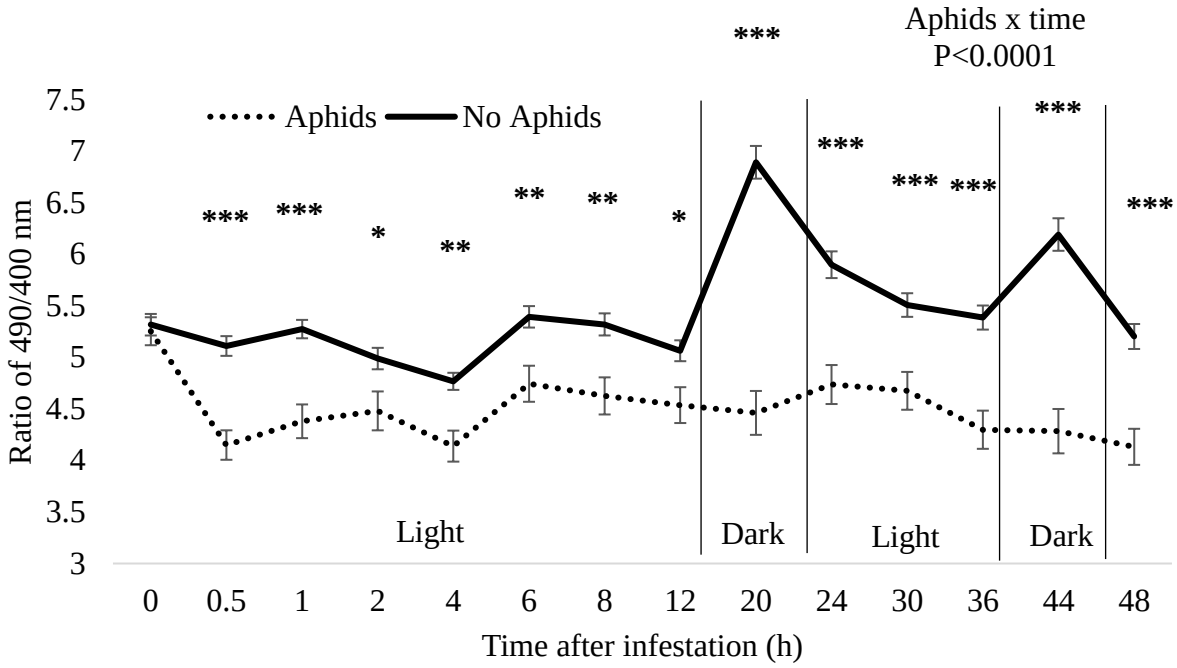

Figure 5. The roGFP2 reporter in the chloroplast shows a rapid and sustained oxidative response to aphid infestation that persists into dark periods. Data was analyzed as a mixed model, blocking for sample, and the means of each time point were separated with a student's t-test. Error bars represent ±SE. Asterisks indicate significant difference: *P<0.01, **P<0.001, ***P<0.0001, n=46.

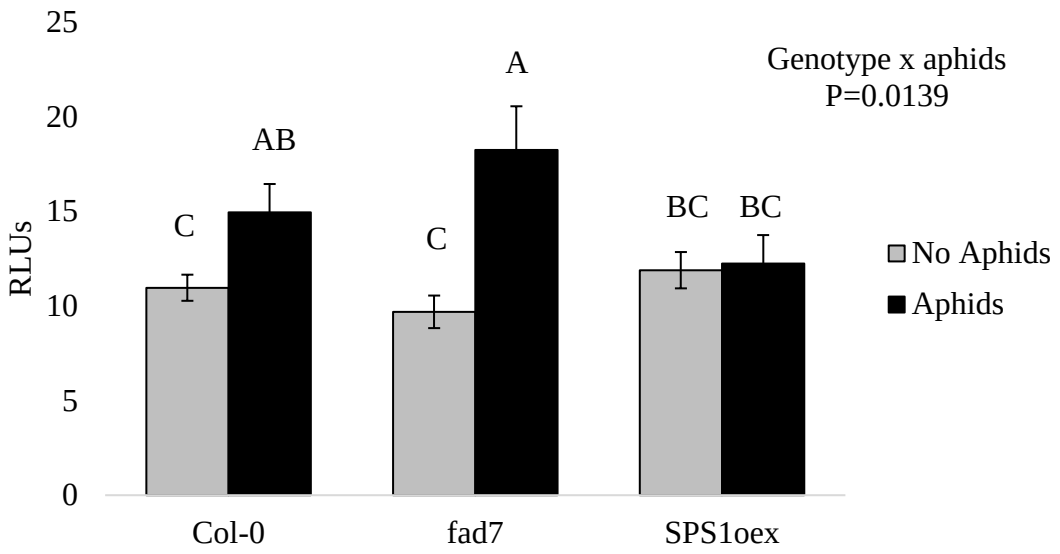

Figure 6. Overexpression of $^1O_2$ scavenger decreases total ROS accumulation in response to aphids. ROS accumulation measured by luminol in Arabidopsis was analyzed as a mixed model with genotype and infestation as main effects and plate as a random blocking factor. Error bars represent ±SE. Mean separation was done with student's t-test with significant difference at α=0.05; Means with the same letter are not significantly different; n=12. The data indicate ROS accumulates in response to aphids and may be largely from the chloroplast at 6 dpi.

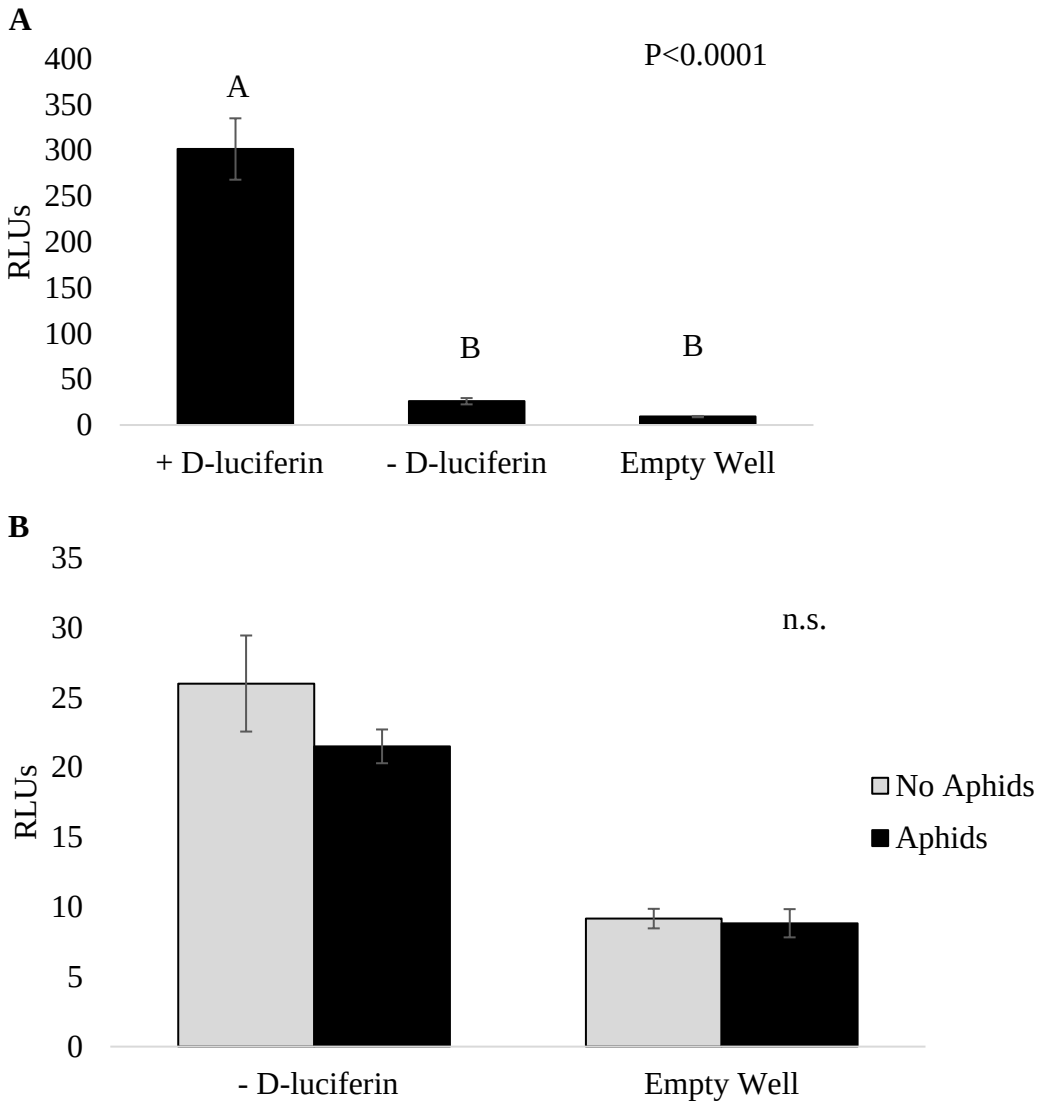

Figure 7. **The *AAA-ATPase:Luc* assay has minimal background noise from plant tissues or aphids.** (**A**) Luminescence was measured in Col-0/*AAA-ATPase:Luc* leaf discs without D-lucerferin added (-D-lucerferin) and compared to empty wells to assess the level of background noise in our luminescence measurements. Data was analyzed by one-way ANOVA and means separated with a student's t-test. (**B**) Luminescence was also measured in *Col-0/AAA-ATPase:Luc* leaf discs without D-lucerferin or in wells without leaf tissue (Empty Well) with and without aphids to confirm that aphids do not impact levels of background noise in the assay. Data was analyzed with a paired t-test. Error bars represent ±SE. Significance as indicated, ***P<0.0001, n.s.=not significant; n=6. The data indicated that background noise represented less than 10% of the luminescence readings from Col-0/*AAA-ATPase:Luc* leaf discs with D-luciferin, and that aphids did not significantly affect levels of background noise.

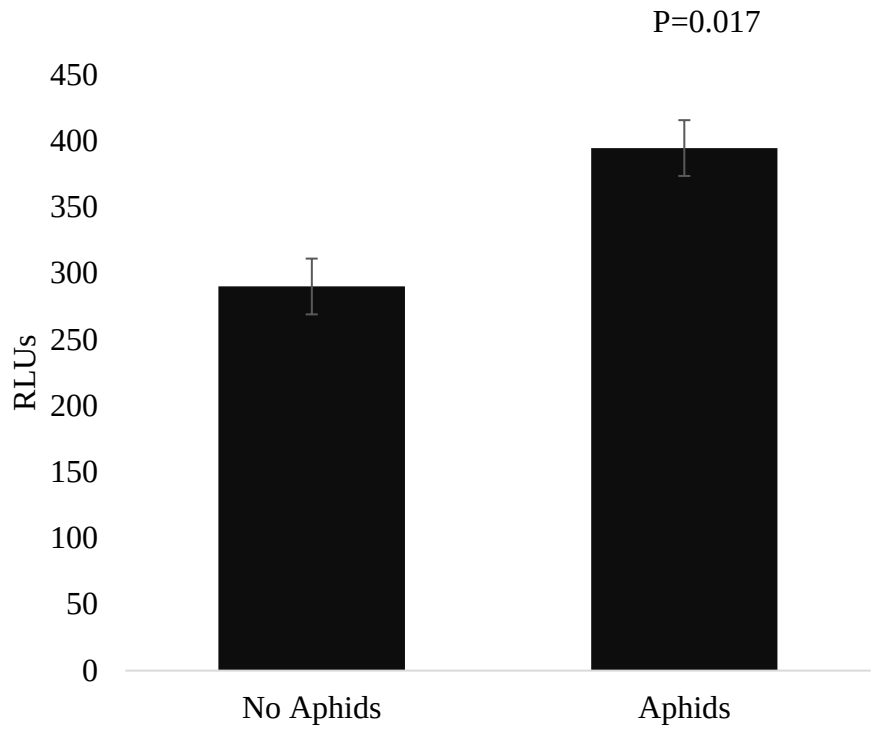

Figure 8. **Aphids increase expression of an $^1O_2$ -responsive marker gene in Arabidopsis at 24 hpi.** *AAA-ATPase:Luc* reporter activity was analyzed with a mixed model comparing leaf discs with and without aphid infestation, blocking for sample. Error bars represent ±SE. Significant difference was determined at α=0.05; n=24. The increase in reporter gene activity suggests induction of $^1O_2$ by aphid treatment.

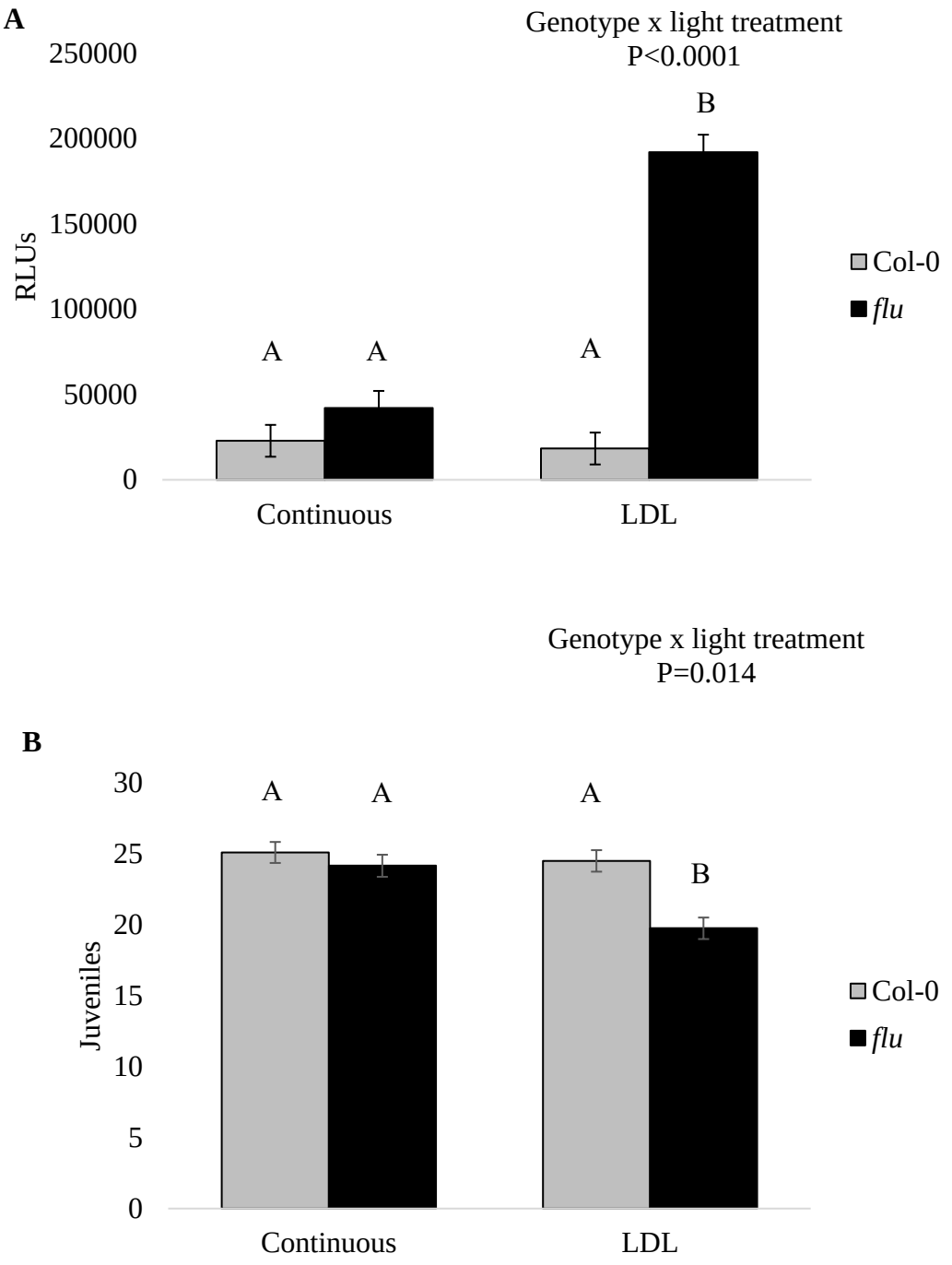

Figure 9. Induction of $^1O_2$ reduces aphid infestation. (A) *AAA-ATPase:Luc* reporter gene activity in response to continuous light or a light/dark/light (LDL) shift that allows Pchlide accumulation and excess $^1O_2$ production and (**B**) aphid juvenile production were analyzed with a two-way ANOVA. Error bars represent ±SE. Means separated by Student's t-test. Means with the same letter are not significantly different; (**A**) n=36 and (**B**) n=20-22. The data indicate increased $^1O_2$ decreased aphid population growth.

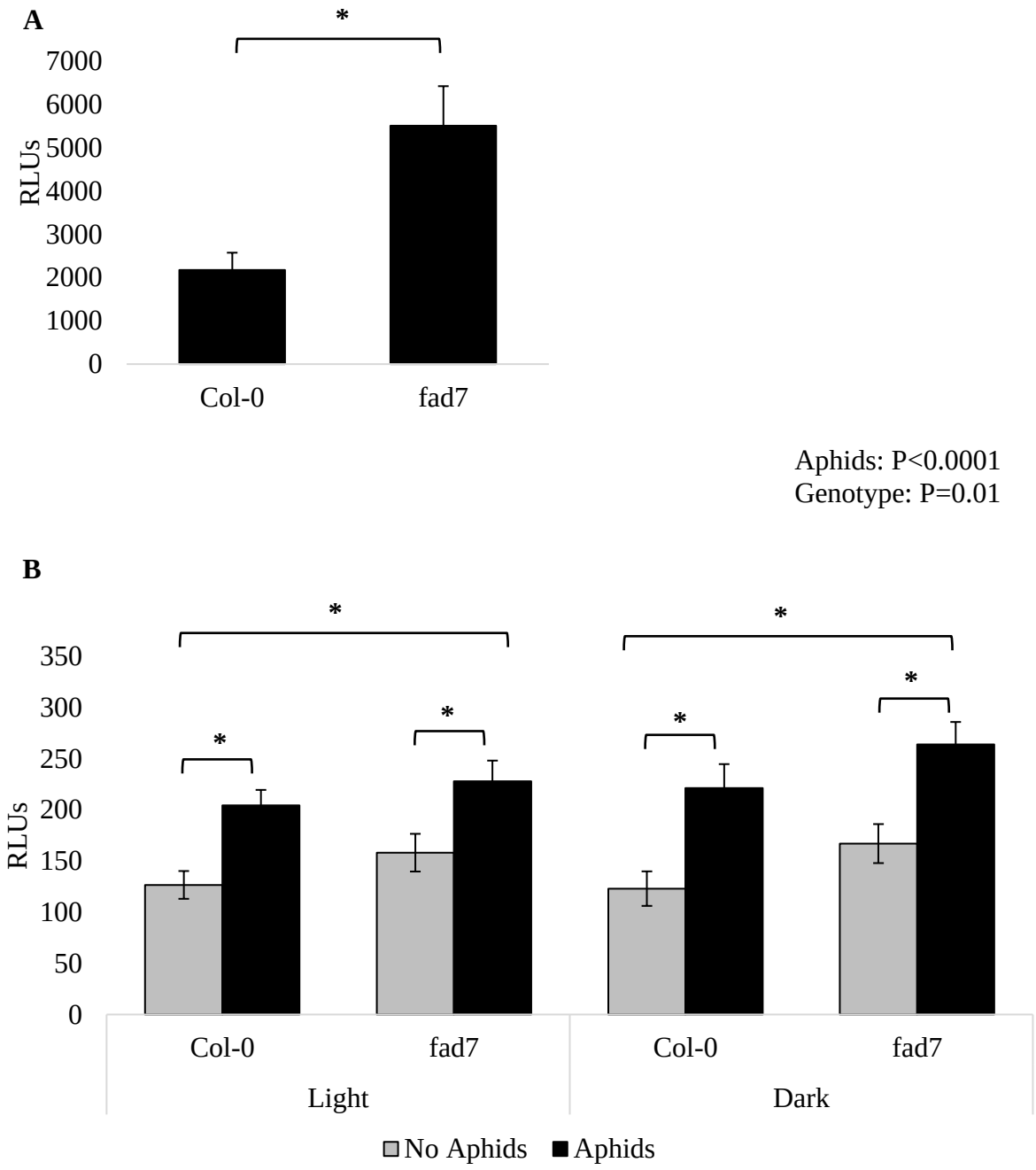

Figure 10. **Aphid-resistant *fad7* accumulates more $^1O_2$ than wild type (Col-0) plants both constitutively and in response to aphids.** (**A**) Constitutive *AAA:ATP-ase:Luc* reporter activity was analyzed with a Student's t-test and (**B**) *AAA-ATPase:Luc* reporter gene activity in response to aphids on Col-0 and *fad7* in staggered photoperiods was analyzed with a three-way ANOVA with a blocking factor for plates; altered photoperiod for light was 12 h light/8 h dark/4 h light and dark was 16 h light/ 8 h dark. Error bars represent ±SE. Means were separated with student t-test pairwise comparison. Asterisks denote significant differences between means at α=0.05; (**A**) n=8-10 and (**B**) n=24. The data indication *fad7* has increased $^1O_2$ constitutively and in response to aphids, independent of the light.

References

Ahmad, P., Jaleel, C. A., Azoos, M. M., and Nabi, G. (2009). Generation of ROS and non-enzymatic antioxidants during abiotic stress in plants. *BRI* 2, 11–20.

Ahmad, P., Jaleel, C. A., Salem, M. A., Nabi, G., and Sharma, S. (2010). Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress. *Crit. Rev. Biotechnol.* 30, 161–175.

Aller, I., Rouhier, N., and Meyer, A. J. (2013). Development of roGFP2-derived redox probes for measurement of the glutathione redox potential in the cytosol of severely glutathione-deficient rml1 seedlings. *Front. Plant. Sci.* 4.

Alnasrawi, A. M. (2015). Optimizing a luciferase-based tool for studying the effects of fatty acid desaturase 7 on singlet oxygen accumulation in *Arabidopsis thaliana*. *Theses and Dissertations* 1319, 64. doi:http://scholarworks.uark.edu/etd/1319.

Apel, K., and Hirt, H. (2004). REACTIVE OXYGEN SPECIES: Metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55, 373–399.

Avila, C. A., Arévalo-Soliz, L. M., Jia, L., Navarre, D. A., Chen, Z., Howe, G. A., Meng, Q., Smith, J.E., Goggin, F.L. (2012). Loss of Function of FATTY ACID DESATURASE7 in tomato enhances basal aphid resistance in a salicylate-dependent manner. *Plant Physiol.* 158, 2028–2041.

Baruah, A., Šimková, K., Apel, K., and Laloi, C. (2009). Arabidopsis mutants reveal multiple singlet oxygen signaling pathways involved in stress response and development. *Plant Mol Biol* 70, 547–563.

Berner, J. M., and Van der Westhuizen, A. J. (2010). Inhibition of xanthine oxidase activity results in the inhibition of Russian wheat aphid-induced defense enzymes. *J Chem Ecol* 36, 1375–1380.

Blackman, R. L., and Eastop, V. F. (2000). Aphids on the world's crops: an identification and information guide. *Aphids on the world's crops: an identification and information guide.*

Blackman, R. L., and Eastop, V. F. (2006). *Aphids on the World's Herbaceous Plants and Shrubs*. New York: John Wiley & Sons.

Bolwell, G. P., Bindschedler, L. V., Blee, K. A., Butt, V. S., Davies, D. R., Gardner, S. L., Gerrish, C., and Minibayeva, F. (2002). The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. *JXB* 53, 1367–1376.

Bos, J. I. B., Prince, D., Pitino, M., Maffei, M. E., Win, J., and Hogenhout, S. A. (2010). A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (Green Peach Aphid). *PLoS Genet* 6, e1001216.

Botha, A.-M., Eck, L. van, Jackson, C. S., Burger, N. F. V., and Schultz, T. (2012). Phloem Feeding Insect Stress and Photosynthetic Gene Expression. *Appl Photosyn*. Published by OpenTech.

Botha, A.-M., Lacock, L., van Niekerk, C., Matsioloko, M. T., du Preez, F. B., Loots, S., Venter, E., Kunert, K.J., and Cullis, C.A. (2005). Is photosynthetic transcriptional regulation in *Triticum aestivum L.* cv. 'TugelaDN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae)? *Plant Cell Rep* 25, 41.

Brunkard, J. O., Runkel, A. M., and Zambryski, P. C. (2015). Chloroplasts extend stromules independently and in response to internal redox signals. *PNAS USA* 112, 10044–10049.

Camp, R. G. L., Przybyla, D., Ochsenbein, C., Laloi, C., Kim, C., Danon, A., Wagner, D., Hideg, E., Göbel, C., Feussner, I., Nater, M., and Apel, K. (2003). Rapid induction of distinct stress responses after the release of singlet oxygen in *Arabidopsis*. *The Plant Cell* 15, 2320–2332.

Czerniewicz, P., Sytykiewicz, H., Durak, R., Borowiak-Sobkowiak, B., and Chrzanowski, G. (2017). Role of phenolic compounds during antioxidative responses of winter triticale to aphid and beetle attack. *Plant Physiol Biochem* 118, 529–540.

Danon, A., Coll, N. S., and Apel, K. (2006). Cryptochrome-1-dependent execution of programmed cell death induced by singlet oxygen in *Arabidopsis thaliana*. *PNAS* 103, 17036–17041.

Dietz, K.J. (2003). Redox control, redox signaling, and redox homeostasis in plant cells. *Int Rev Cytol* 228, 141–193.

Dietz, K.-J., and Scheibe, R. (2004). Redox regulation: an introduction. *Physiologia Plantarum* 120, 1–3.

Dmitrieva, V. A., Tyutereva, E. V., and Voitsekhovskaja, O. V. (2020). Singlet oxygen in plants: Generation, detection, and signaling roles. *Int J Mol Sci* 21, 3237.

Elzinga, D. A., De Vos, M., and Jander, G. (2014). Suppression of plant defenses by a *Myzus persicae* (green peach aphid) salivary effector protein. *MPMI* 27, 747–756.

Farmer, E. E., and Mueller, M. J. (2013). ROS-mediated lipid peroxidation and RES-activated signaling. *Annu Rev Plant Biol* 64, 429–450.

Foyer, C. H., and Noctor, G. (2000). Oxygen processing in photosynthesis: regulation and signalling. *New Phytol* 146, 359–388.

Foyer, C. H., and Noctor, G. (2005). Redox homeostasis and antioxidant signaling: A metabolic interface between stress perception and physiological responses. *The Plant Cell* 17, 1866–1875.

Foyer, C. H., and Noctor, G. (2016). Stress-triggered redox signalling: What's in pROSpect? *Plant Cell Environ.* 39, 951–964.

Foyer, C. H., Ruban, A. V., and Noctor, G. (2017). Viewing oxidative stress through the lens of oxidative signalling rather than damage. *Biochem J* 474, 877–883.

Galvez-Valdivieso, G., and Mullineaux, P. M. (2010). The role of reactive oxygen species in signalling from chloroplasts to the nucleus. *Physiol Plant* 138, 430–439.

Gorman, A. A., and Rodgers, M. A. (1992). Current perspectives of singlet oxygen detection in biological environments. *J Photochem Photobiol B* 14, 159–176.

Guo, H., Gu, L., Liu, F., Chen, F., Ge, F., and Sun, Y. (2019). Aphid-borne viral spread is enhanced by virus-induced accumulation of plant reactive oxygen species. *Plant Physiol* 179, 143–155.

Guo, H., Zhang, Y., Tong, J., Ge, P., Wang, Q., Zhao, Z., Zhu-Salzman, K. Hogenhout, S.A., Ge, F., and Sun, Y. (2020). An aphid-secreted salivary protease activates plant defense in phloem. *Current Biology* 30, 4826-4836.e7.

Harmel, N., Létocart, E., Cherqui, A., Giordanengo, P., Mazzucchelli, G., Guillonneau, F., De Pauw, E., Haubruge, E., and Francis, F. (2008). Identification of aphid salivary proteins: A proteomic investigation of *Myzus persicae*. *Insect Mol. Biol.* 17, 165–174.

Harrison, S.J., Mott, E.K., Parsley, K., Aspinall, S., Gray, J.C., Cottage. (2006). A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings following floral dip transformation. *Plant Methods.* 2, 19.

Hiremath, S. S., Sajeevan, R., Nataraja, K. N., Chaturvedi, A. K., Chinnusamy, V., and Pal, M. (2017). Silencing of fatty acid desaturase (*FAD7*) gene enhances membrane stability and photosynthetic efficiency under heat stress in tobacco (*Nicotiana benthamiana*). *Indian J Exp Biol*, 10.

Huang, Y., Chen, X., Liu, Y., Roth, C., Copeland, C., McFarlane, H. E., Huang, S., Lipka, V., Wiermer, M., and Li, X. (2013). Mitochondrial AtPAM16 is required for plant survival and the negative regulation of plant immunity. *Nat Commun* 4, 2558.

Hussain, A., Razaq, M., Zaka, S. M., Shahzad, W., and Mahmood, K. (2015). effect of aphid infestation on photosynthesis, growth and yield of *Brassica carinata* A. Braun. *Pakistan J. Zool.* 47, 1335–1340.

Jaouannet, M., Rodriguez, P. A., Thorpe, P., Lenoir, C. J. G., MacLeod, R., Escudero-Martinez, C., and Bos. J.I.B. (2014). Plant immunity in plant–aphid interactions. *Front. Plant Sci.* 5.

Kachroo, A., and Kachroo, P. (2009). Fatty acid-derived signals in plant defense. *Annu Rev Phytopathol* 47, 153–176.

Kennedy, J. S., Day, M. F., Eastop, V. F., Commonwealth Institute of Entomology, Commonwealth Agricultural Bureaux, and Executive Council (1962). *A conspectus of aphids as vectors of plant viruses*. London: Commonwealth Institute of Entomology.

Kerchev, P. I., Fenton, B., Foyer, C. H., and Hancock, R. D. (2012a). Infestation of potato (*Solanum tuberosum L.*) by the peach-potato aphid (*Myzus persicae* Sulzer) alters cellular redox status and is influenced by ascorbate. *Plant, Cell Environ* 35, 430–440.

Kerchev, P. I., Fenton, B., Foyer, C. H., and Hancock, R. D. (2012b). Plant responses to insect herbivory: interactions between photosynthesis, reactive oxygen species and hormonal signalling pathways. *Plant, Cell Environ* 35, 441–453.

Kmieć, K., Rubinowska, K., and Golan, K. (2018). *Tetraneura ulmi* (Hemiptera: Eriosomatinae) induces oxidative stress and alters antioxidant enzyme activities in elm leaves. *Environ Entomol* 47, 840–847.

Krieger-Liszkay, A., Fufezan, C., and Trebst, A. (2008). Singlet oxygen production in photosystem II and related protection mechanism. *Photosynth Res* 98, 551–564.

Ksas, B., Becuwe, N., Chevalier, A., and Havaux, M. (2015). Plant tolerance to excess light energy and photooxidative damage relies on plastoquinone biosynthesis. *Scientific Reports* 5, 10919.

Ksas, B., Légeret, B., Ferretti, U., Chevalier, A., Pospíšil, P., Alric, J., and Havaux, M. (2018). The plastoquinone pool outside the thylakoid membrane serves in plant photoprotection as a reservoir of singlet oxygen scavengers. *Plant Cell Environ.* 41, 2277–2287.

Kuśnierczyk, A., Winge, P., Midelfart, H., Armbruster, W. S., Rossiter, J. T., and Bones, A. M. (2007). Transcriptional responses of Arabidopsis thaliana ecotypes with different glucosinolate profiles after attack by polyphagous *Myzus persicae* and oligophagous *Brevicoryne brassicae*. *JXB* 58, 2537–2552.

Kuźniak, E., and Kopczewski, T. (2020). The chloroplast reactive oxygen species-redox system in plant immunity and disease. *Front. Plant Sci.* 11.

Laloi, C., Apel, K., and Danon, A. (2004). Reactive oxygen signalling: the latest news. *Curr Opin Plant Biol* 7, 323–328.

Leslie, M., and Heese, A. (2014). A Re-elicitation assay to correlate flg22-signaling competency with ligand-induced endocytic degradation of the FLS2 receptor. *Methods Mol Biol (Clifton, N.J.)* 1209, 149–62.

Li, J., Galla, A. L., Avila, C. A., Flattmann, K., Vaughn, K. L., and Goggin, F. (2021). Fatty acid desaturases in the chloroplast and endoplasmic reticulum promote susceptibility to the green peach aphid, *Myzus persicae*, in *Arabidopsis thaliana*. *MPMI*.

Łukasik, I., and Goławska, S. (2013). Effect of host plant on levels of reactive oxygen species and antioxidants in the cereal aphids *Sitobion avenae* and *Rhopalosiphum padi*. *Biochemical Systematics and Ecology* 51, 232–239.

Lukasik, I., and Golawska, S. (2019). Biochemical markers of oxidative stress in triticale seedlings exposed to cereal aphids. *Acta Biol Crac Ser Bot* 61, 35–46.

Lukasik, I., Kornacka, A., Golawska, S., Sytykiewicz, H., Sprawka, I., and Wójcicka, A. (2017). Effects of *Acyrtosiphon pisum* (Harris) infestation on the hydrogen peroxide content and activity of antioxidant enzymes in Fabaceae plants. *AJ* 40, 143–150.

Maffei, M. E., Mithöfer, A., and Boland, W. (2007). Before gene expression: Early events in plant–insect interaction. *Trends Plant Sci* 12, 310–316. doi:10.1016/j.tplants.2007.06.001.

Maruta, T., Noshi, M., Tanouchi, A., Tamoi, M., Yabuta, Y., Yoshimura, K., Yoshimura, K., and Shigeoka, S. (2012). H_2O_2-triggered retrograde signaling from chloroplasts to nucleus plays specific role in response to stress. *J Biol Chem* 287, 11717–11729.

Mène-Saffrané, L., Dubugnon, L., Chételat, A., Stolz, S., Gouhier-Darimont, C., and Farmer, E. E. (2009). Nonenzymatic oxidation of trienoic fatty acids contributes to reactive oxygen species management in *Arabidopsis. J. Biol. Chem.* 284, 1702–1708.

Meskauskiene, R., Nater, M., Goslings, D., Kessler, F., Camp, R., and Apel, K. (2001). FLU: A negative regulator of chlorophyll biosynthesis in *Arabidopsis thaliana*. *PNAS* 98, 12826–12831.

Miller, G., Schlauch, K., Tam, R., Cortes, D., Torres, M. A., Shulaev, V., Dangl, J.L., and Mittler, R. (2009). The Plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. *Sci. Signal.* 2, ra45–ra45.

Moloi, M. J., and van der Westhuizen, A. J. (2006). The reactive oxygen species are involved in resistance responses of wheat to the Russian wheat aphid. *J Plant Physiol* 163, 1118–1125.

Mor, A., Koh, E., Weiner, L., Rosenwasser, S., Sibony-Benyamini, H., and Fluhr, R. (2014). Singlet oxygen signatures are detected independent of light or chloroplasts in response to multiple stresses. *Plant Physiol.* 165, 249–261.

Moran, P. J., and Thompson, G. A. (2001). molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. *Plant Physiol* 125, 1074–1085.

Morkunas, I., Mai, V. C., and Gabryś, B. (2011). Phytohormonal signaling in plant responses to aphid feeding. *Acta Physiol Plant* 33, 2057–2073.

Ochsenbein, C., Przybyla, D., Danon, A., Landgraf, F., Göbel, C., Imboden, A., Imboden, A., Feussner, I., and Apel, K. (2006). The role of EDS1 (enhanced disease susceptibility)

during singlet oxygen-mediated stress responses of *Arabidopsis*. *The Plant Journal* 47, 445–456.

Pospíšil, P. (2016). Production of reactive oxygen species by photosystem ii as a response to light and temperature stress. *Front. Plant Sci.* 7.

Przybyla, D., Göbel, C., Imboden, A., Hamberg, M., Feussner, I., and Apel, K. (2008). Enzymatic, but not non-enzymatic, $^1O_2$-mediated peroxidation of polyunsaturated fatty acids forms part of the EXECUTER1-dependent stress response program in the *flu* mutant of *Arabidopsis thaliana*. *The Plant Journal* 54, 236–248.

Rasool, B., McGowan, J., Pastok, D., Marcus, S. E., Morris, J. A., Verrall, S. R., Hedley, P.E., Hankcock, R.D., and Foyer, C.H. (2017). Redox control of aphid resistance through altered cell wall composition and nutritional quality. *Plant Physiol* 175, 259–271.

Ren, G., Wang, X., Chen, D., Wang, X., and Liu, X. (2014). Effects of aphids *Myzus persicae* on the changes of Ca^{2+} and H_2O_2 flux and enzyme activities in tobacco. *Journal of Plant Interactions* 9, 883–888.

Shang-Guan, K., Wang, M., Htwe, N. M. P. S., Li, P., Li, Y., Qi, F., Zhang, D., Cao, M., Kim, C., Weng, H, Cen, H., Black, I.M., Azadi, P., Carlson, R.W., and Liang, Y. (2018). Lipopolysaccharides trigger two successive bursts of reactive oxygen species at distinct cellular locations. *Plant Physiol* 176, 2543–2556.

Shao, Y., Guo, M., He, X., Fan, Q., Wang, Z., Jia, J., and Guo, J. (2019). Constitutive H_2O_2 is involved in sorghum defense against aphids. *Braz. J. Bot* 42, 271–281.

Shoala, T., Edwards, M. G., Knight, M. R., and Gatehouse, A. M. R. (2018). OXI1 kinase plays a key role in resistance of *Arabidopsis* towards aphids (*Myzus persicae*). *Transgenic Res* 27, 355–366.

Sierla, M., Rahikainen, M., Salojärvi, J., Kangasjärvi, J., and Kangasjärvi, S. (2012). Apoplastic and chloroplastic redox signaling networks in plant stress responses. *ARS* 18, 2220–2239.

Stonebloom, S., Brunkard, J. O., Cheung, A. C., Jiang, K., Feldman, L., and Zambryski, P. (2012). Redox states of plastids and mitochondria differentially regulate intercellular transport via plasmodesmata. *Plant Physiol* 158, 190–199.

Sun, M., Voorrips, R. E., and Vosman, B. (2020). Aphid populations showing differential levels of virulence on *Capsicum* accessions. *Insect Sci* 27, 336–348.

Sytykiewicz, H. (2016a). Deciphering the role of NADPH oxidase in complex interactions between maize (*Zea mays L.*) genotypes and cereal aphids. *Biochem Biophys Res Commun* 476, 90–95.

Sytykiewicz, H. (2016b). Expression patterns of genes involved in ascorbate-glutathione cycle in aphid-infested maize (*Zea mays L.*) seedlings. *Int J Mol Sci* 17, 268.

Takagi, D., Takumi, S., Hashiguchi, M., Sejima, T., and Miyake, C. (2016). Superoxide and singlet oxygen produced within the thylakoid membranes both cause Photosystem I photoinhibition. *Plant Physiol* 171, 1626–1634. doi:10.1104/pp.16.00246.

Upchurch, R. G. (2008). Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *Biotechnol Lett* 30, 967–977.

Vaughn, K. L., Avila, C. A., Padilla-Marcia, C. S., and Goggin, F. L. (2014). Development of *fad7-1* single mutant *Arabidopsis thaliana* plants that are resistant to aphids. *Discovery, The Student Journal of Dale Bumpers College of Agricultural, Food and Life Sciences* 15, 7.

Vos, M. D., and Jander, G. (2009). *Myzus persicae* (green peach aphid) salivary components induce defense responses in *Arabidopsis thaliana*. *Plant Cell Environ* 32, 1548–1560.

Wang, D. M., Zhang, Y., Zheng, L. L., Yang, X. X., Wang, Y., and Huang, C. Z. (2012). Singlet oxygen involved luminol chemiluminescence catalyzed by graphene oxide. *J. Phys. Chem. C* 116, 21622–21628.

Warm, E., and Laties, G. G. (1982). Quantification of hydrogen peroxide in plant extracts by the chemiluminescence reaction with luminol. *Phytochem* 21, 827–831.

Watabe, N., Ishida, Y., Ochiai, A., Tokuoka, Y., and Kawashima, N. (2007). Oxidation decomposition of unsaturated fatty acids by singlet oxygen in phospholipid bilayer membranes. *J Oleo Sci* 56, 73–80.

Wilson, L., Heimoana, S., Constable, G., and Fletcher, R. (2006). The Effects of aphids on photosynthesis in cotton. Available at: http://www.insidecotton.com/jspui/handle/1/2546

Xia, X.-J., Zhou, Y.-H., Shi, K., Zhou, J., Foyer, C. H., and Yu, J.-Q. (2015). Interplay between reactive oxygen species and hormones in the control of plant development and stress tolerance. *JXB* 66, 2839–2856.

Xu, J., Padilla, C.S., Li, J., Wickramanayake, J., Fischer, H.D., and Goggin, F.L. (2021). Redox responses of *Arabidopsis thaliana* to the green peach aphid, *Myzus persicae. Mol Plant Pathol* .

Zeng, L., Yang, X., and Zhou, J. (2020). The xanthophyll cycle as an early pathogenic target to deregulate guard cells during *Sclerotinia sclerotiorum* infection. *Plant Signal Behav* 15, 1691704.

Zhang, S., Apel, K., and Kim, C. (2014). Singlet oxygen-mediated and EXECUTER-dependent signalling and acclimation of *Arabidopsis thaliana* exposed to light stress. *Philos Trans R Soc Lond., B, Biol Sci* 369, 20130227.

Zhou, J., Zeng, L., Liu, J., and Xing, D. (2015). Manipulation of the xanthophyll cycle increases plant susceptibility to *Sclerotinia sclerotiorum*. *PLOS Pathog* 11, e1004878.

Zoeller, M., Stingl, N., Krischke, M., Fekete, A., Waller, F., Berger, S., and Mueller, M.J. (2012). Lipid profiling of the *Arabidopsis* hypersensitive response reveals specific lipid peroxidation and fragmentation processes: Biogenesis of pimelic and azelaic acid. *Plant Physiol.* 160, 365–378. doi:10.1104/pp.112.202846.

Chapter IV

Chloroplast-generated singlet oxygen signaling through EXECUTER1/EXECUTER2 for aphid resistance

Abstract

Singlet oxygen ($^1O_2$), which is produced in the chloroplast at PSII during photosynthesis, initiates chloroplast-to-nucleus retrograde signaling via the EXECUTER1/EXECUTER2 (EX1/EX2) signaling pathway. Enhanced $^1O_2$ accumulation is correlated with aphid resistance in two different genotypes of Arabidopsis (*Arabidopsis thaliana*): the conditional *flu* mutant, which accumulates $^1O_2$ after undergoing a light/dark/light (LDL) transition, and the *fad7* mutant, which has reduced levels of fatty acid desaturation in chloroplast membranes. This study evaluated whether EX1/EX2 signaling is required for aphid resistance in these genotypes. Population growth of the green peach aphid (GPA; *Myzus persicae* Sulzer) was significantly lower on the conditional *flu* mutant after an LDL shift that increased $^1O_2$, and loss of function of EX1/EX2 in the *flu* background reduced this effect. Thus, aphid resistance in *flu* appears to be partially but not solely dependent upon EX1/EX2 signaling. A *fad7/ex1/ex2* triple mutant was then generated through crosses to observe the impact of impaired $^1O_2$-signaling on aphid resistance in *fad7*. Whereas GPA population growth was significantly lower at 48 h and 6 d after infestation on *fad7* compared with wild-type Col-0, aphid susceptibility was fully restored in the *fad7/ex1/ex2*, indicating that EX1/EX2 is required for aphid resistance in *fad7*. Because EX1/EX2 $^1O_2$-signaling often causes electrolyte leakage (EL), EL was evaluated at 6dpi in uninfested and infested tissue of *fad7*, *ex1/ex2*, *fad7/ex1/ex2*, and wild type controls. EL was significantly induced by aphid challenge but did not differ significantly among genotypes. Since EL is a hallmark of EX1/EX2-mediated programmed cell death (PCD), the lack of genotypic variation in

EL levels suggests that PCD may not be critical to aphid resistance in *fad7*. These results illustrate a connection between chloroplast retrograde signaling and plant defense against herbivores.

Introduction

Singlet oxygen ($^1O_2$) is a reactive oxygen species (ROS) predominantly produced in the chloroplast during photosynthesis at PSII by energy transfer from excited chlorophyll or charged reactions centers to molecular oxygen ($^3O_2$; Dmitrieva et al., 2020). It accumulates in response to many environmental stresses such as high light, heat, heavy metals, mechanical injury, and osmotic stress (Pospíšil and Prasad, 2014; Chen and Fluhr, 2018). As one of the most short-lived ROS, $^1O_2$ is highly unstable and quickly reacts with nearby biological molecules such as lipids, proteins, and carotenoids that can trigger chloroplast-to-nucleus retrograde signaling to influence nuclear gene expression (Triantaphylidès and Havaux, 2009; Galvez-Valdivieso and Mullineaux, 2010). At sublethal doses of $^1O_2$, retrograde signaling can contribute to adaptation to abiotic stresses by activating hormone signaling and expression of genes involved in detoxification and management of oxidative stress (Ramel et al., 2012, 2013). Furthermore, many of the responses triggered by $^1O_2$ overlap with disease resistance pathways (Ochsenbein et al., 2006; Zhang et al., 2014); for example, increased $^1O_2$ accumulation in Arabidopsis upregulated 22 transcription factors associated with plant resistance to pathogens (Zhang et al., 2014). Thus, $^1O_2$ may have multiple roles in stress-responsive signaling and could potentially contribute to biotic as well as abiotic stress responses.

In particular, $^1O_2$ may play a role in plant interactions with aphids, a large and damaging group of phloem-feeding insects. Observation of redox-sensitive and $^1O_2$-responsive sensors in

Arabidopsis (*Arabidopsis thaliana*) suggests that challenge by the green peach aphid (GPA; *Myzus persicae* Sulzer) induces an oxidative response in the chloroplast including $^1O_2$ accumulation (Chapter III). Furthermore, $^1O_2$ accumulation appears to be correlated with aphid resistance in mutant Arabidopsis genotypes that suppress aphid population growth. Compared with wild-type Col-0 plants, the *fatty acid desaturase 7* (*fad7*) mutant inhibited aphid fecundity (Chapter II) and displayed elevated $^1O_2$ levels both constitutively and in response to GPA infestation (Chapter III). The *fad7* mutation knocks out function of a chloroplast-localized ω-3 fatty acid desaturase that converts dienoic fatty acids (DAs) such as linoleic acid to trienoic fatty acids (TAs) such as linolenic acid (Li et al., 2021), and that regulates the desaturation levels and stability of photosynthetic membranes (Hiremath et al., 2017). Thus, it is likely that $^1O_2$ accumulation in *fad7* occurs in the chloroplast and results from altered lipid composition of chloroplast membranes. Artificial induction of $^1O_2$ accumulation in the chloroplast using the conditional *flu* mutant has also recently been demonstrated to enhance aphid resistance (Chapter III). In the *flu* background, plants placed in the dark accumulate a precursor of chlorophyll, protochlorophyllide (Pchlide), which generates $^1O_2$ and retrograde signaling upon re-illumination (Meskauskiene et al., 2001). Thus, *flu* plants grown in continuous light have a phenotype comparable with wild-type Arabidopsis, but upon a light/dark/light (LDL) shift, they generate enhanced levels of $^1O_2$ in the chloroplast (Camp et al., 2003). Compared to wild type controls, *flu* plants had significantly lower aphid numbers, but only after exposure to a LDL shift (Chapter III). Therefore, $^1O_2$ signaling from the chloroplast is likely involved in plant defense against aphids and may trigger retrograde signaling in response to aphids.

Because chloroplast-to-nucleus retrograde signaling of $^1O_2$ in *flu* is dependent on the plastid-localized proteins EXECUTER1 (EX1) and EXECUTER2 (EX2), EXI1/EX2 potentially

could serve as a signaling mechanism for aphid resistance. In *flu*, EX1/EX2-mediated $^1O_2$-signaling induces gene expression changes and electrolyte leakage (EL) followed by programmed cell death (PCD) and growth inhibition after an LDL shift (Wagner et al., 2004; Lee et al., 2007; Przybyla et al., 2008; Triantaphylidès et al., 2008). T-DNA insertion mutants of *EX1/EX2* (*ex1/ex2*) abolish this response in the *flu* background (Lee et al., 2007) as well as wild-type Arabidopsis after high light stress (Kim et al., 2009). Accumulation of $^1O_2$ in *flu* also causes salicylic acid (SA) accumulation and expression of pathogenesis-related (PR) proteins *PR1* and *PR5* (Ochsenbein et al., 2006); *ex1/ex2*. Increased SA and *PR1* expression overlaps with aphid resistance mediated by *fad7*. In tomato with loss of function of *FAD7* (*spr2*), aphid resistance requires SA accumulation and the SA response factor *NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1* (*NPR1*), and triggers expression of *PR1* (Avila et al., 2012). Taken together, these results identify EX1/EX2 as possible candidates for regulators of aphid-resistance. Therefore, the impact of loss of function of EX1/EX2 on aphid resistance in both *flu* and *fad7* was explored in the following work.

This study aimed to elucidate the role of EX1/EX2 in $^1O_2$-signaling for aphid resistance in both *flu* and *fad7*. I performed population growth bioassays of GPA on either *flu* or *fad7* with impaired $^1O_2$-signaling through the EX1/EX2 pathway (*ex1/ex2*). I also measured EL in the *flu* background after and LDL to determine if the typical phenotypic response of EL in *flu* was abolished by *ex1/ex2*. In the *fad7* background, I assessed EL in infested and uninfested plants to determine if EX1/EX2 signaling influenced EL in response to aphids. Finally, I measured chlorophyll content and maximum quantum efficiency of PSII in *fad7* compared with wild-type Arabidopsis to determine if inefficient photosynthesis at PSII could be a source of $^1O_2$ in the *fad7* mutant. Results from this study demonstrate that EX1/EX2 are necessary for aphid in resistance

in *fad7,* and *flu* background only partially restored aphid susceptibility and could indicate. This study is important as it further connects primary metabolism with plant defense and confirms the role of $^1O_2$ as a signaling component of aphid resistance.

Materials and Methods

Plant Materials and Growth Conditions

Six *Arabidopsis thaliana* genotypes were used in this study: wild-type Columbia CS7000 (Col-0); the *fad7-1* mutant (ABRC stock number CS71655) developed by Vaughn and coworkers (2014); *flu/AAA-ATPase:Luc,* which was provided Dr. Klaus Apel, Boyce Thompson Institute (Camp et al., 2003; Baruah et al., 2009); the $^1O_2$-signaling impaired double mutant *ex1/ex2* mutant from T-DNA insertion lines SALK002088 and SALK012127, respectively, as well as a *flu/ex1/ex2* triple mutant, which were both provided by Drs. Chanhong Kim and Keun Pyo Lee from Shanghai Center for Plant Stress Biology (Lee et al., 2007); and the *fad7/ex1/ex2* triple mutant (Appendix I). Seeds were sterilized as described in previous chapters. In brief, after sterilization with a bleach solution and 6-8 washes with autoclaved ddH_2O, seeds were plated on 1X Murashige and Skoog (MS) media (0.8% agar, 3% sucrose, pH adjusted to 5.5-5.9). Seeds were vernalized for three days at 4 °C and then placed in a growth chamber to germinate. Experimental plants were germinated and grown under a 16 h L/8 h D photoperiod, at 180 μmol m^{-2} sec^{-1} light intensity, 55% RH, and 24 °C, except in experiments including *flu* which required growth under continuous light (100 μmol m^{-2} sec^{-1} light intensity, 55% RH, 24 °C).

Aphid material, synchronization, and population growth assays

A green peach aphid (GPA; *Myzus persicae* Sulzer) colony was reared on cabbage (*B. rapae* cv. Joi Choi) as previously described in Chapter II. A cohort of age-synchronized adult GPA was used for all bioassay experiments to ensure age differences were not influencing fecundity of the aphid. Synchronization was done by placing adult GPA on new cabbage plants and letting them reproduce for 24 h. Then all adults were removed, leaving behind only the juveniles. After 6 to 8 days, the juveniles emerged as adults and were collected for bioassays. Aphid population growth assays were performed as described in Chapter 2 using two adult female GPA. Arabidopsis plants were caged for the duration of the bioassay. The number of juveniles present on the plant were scored at two time points, 48 h or 6 d after infestation. The 48 h time point allowed us to assess host suitability as early as possible, before extensive symptoms of $^1O_2$ accumulation emerged in *flu* mutants. The 6 d time point was also included for certain experiments because the impacts of *fad7* on aphid populations in some cases increase over time.

Inducing $^1O_2$ accumulation in the flu background

The method of generating $^1O_2$ in the *flu* background was carried out as described in other studies (Laloi et al., 2007; Lee et al., 2007). Wild-type Col-0 Arabidopsis, *flu*, and *flu/ex1/ex2* plants were grown initially in continuous light to prevent $^1O_2$ accumulation in the *flu* background prior to the start of the experiment. When plants were approximately two weeks old, they were placed in the dark for 8 h to generate Pchlide and then re-illuminated. In the light, plants in the *flu* background had enhanced generation of $^1O_2$. Control plants stayed in continuous light where *flu* and *flu/ex1/ex2* were phenotypically similar to Col-0. 4 h after re-illumination, plants from the LDL and control group were used for a bioassay.

Electrolyte Leakage Assay

Electrolyte leakage (EL) was measured in Arabidopsis based on modified methods of Shi and colleagues (2013). Approximately 100mg of leaf tissue was cut into 5 mm strips and incubated in 15 mL of autoclaved ddH_2O for 3 h. The initial conductivity (C_1) was then measured using an Orion Star^{TM} A212 Benchtop Conductivity Meter (Thermo Fisher Scientific, Waltham, MA USA 02451). Samples were then autoclaved for 15 min at 121 °C to induce complete electrolyte leakage. After cooling for 24 h, samples were measured again for total electrical conductivity (C_2). Values for each sample are expressed as percent electrolyte leakage using the following formula: (C_1/C_2) x 100%. In experiments comparing infested and uninfested tissue, plants from both treatments were caged to control for differences in light intensity.

Measurements of photosynthetic efficiency and chlorophyll content

Fluorescence measurements of Arabidopsis were taken using the handheld fluorescence meter MultispeQ (v1.0) (PhotosynQ LLC, East Lansing, MI). Plants were dark-adapted for 8 h before measurements. A built-in protocol of MultispeQ, "Leaf Photosynthesis MultispeQ V1.0," was used to measure minimal fluorescence in the dark-adapted state (F_o) and maximal fluorescence in the dark-adapted state (F_m), which were used to estimate the quantum efficiency of PSII (F_v/F_m). After dark-adapted measurements were taken, plants were placed back into the light for 30 min before chlorophyll fluorescence measurements were taken. Measurements were taken on the 7th leaf of a 3-week-old Arabidopsis.

Statistical Analysis

All statistical analysis was done using JMP Pro 15.2.0. Data with two main effects were analyzed with a two-way ANOVA. These included data from electrolyte leakage and the bioassay in the *flu* background. Means of significant interaction effects were separated with a student's t-test. Significant main effects were separated with student's t-test pairwise comparison. Data with one main effect were analyzed with a one-way ANOVA and means were separated with a student's t-test. This included both time points of the bioassay in the *fad7* background comparing four means. Data comparing two means were analyzed with a student's t-test, including data for F_v/F_m and chlorophyll content between Col-0 and *fad7*. All significant differences were determined at α=0.05.

Results

The ex1/ex2 mutation partially restores aphid susceptibility to the conditional flu mutant.

To determine if $^1O_2$ signaling through the EX1/EX2 pathway contributes to aphid resistance of the *flu* mutant, a bioassay was performed with wild-type Col-0, the *flu* mutant with conditionally enhanced $^1O_2$ accumulation, and the *flu/ex1/ex2* with impaired $^1O_2$ signaling. There were two treatment groups of either an LDL shift or continuous light. Population growth of GPA were influenced by both genotype and light treatment, which suggests genotypes were responding differently to the LDL shift (light treatment: $F(1,71)=18.07$, $P<0.0001$; genotype: $F(2,71)=0.42$, $P=0.66$ $F(2,71)=3.48$, $P=0.036$). In continuous light, GPA population growth was comparable between all three genotypes (Fig. 1). When treated with a LDL shift, population growth on *flu* was decreased by 17% compared with Col-0, whereas *flu/ex1/ex2* had intermediate levels of population growth between Col-0 and *flu*. This indicates impaired $^1O_2$ signaling through the

EX1/EX2 pathway is partially responsible for regulating the aphid-resistance mechanism in *flu*. Potentially other $^1O_2$-signaling responses not mediated by EX1/EX2 also play a role in aphid resistance in the *flu* background.

Loss of function of EX1/EX2 in the flu background does not decrease electrolyte leakage.

In the *flu* mutant, $^1O_2$ accumulation typically initiates electrolyte leakage (EL) which precedes programmed cell death (PCD), but is abrogated by loss of EX1/EX2 signaling (Lee et al., 2007; Przybyla et al., 2008). Thus, EL was quantified in uninfested tissue of Col-0, *flu*, and *flu/ex1/ex2* in both an LDL shift and continuous light 48 h after re-illumination to determine if this phenotype was abolished by *ex1/ex2*. EL was only significantly influenced by genotype (genotype: $F(2,12)=7.41$, $P=0.008$; light treatment: $F(1,12)= 0.04$, $P=0.85$; genotype x light treatment: $F(2,12)=0.96$, $P=0.41$; Fig. 2). Surprisingly, *flu* and *flu/ex1/ex2* were not significantly different in either light treatment group and were both significantly higher than Col-0 by 2-3%. It is possible the comparable EL between *flu* and *flu/ex1/ex2* in the LDL shift is due to missing the peak EL response by measuring EL at 48 h after re-illumination. Moreover, the higher constitutive EL in both *flu* and *flu/ex1/ex2* may indicate the *flu* mutation is "leaky", contradictory to previous studies in these mutants.

Aphid resistance in fad7 requires EX1/EX2-mediated $^1O_2$-signaling.

Previous work demonstrated a constitutive and aphid-induced increase of $^1O_2$ in the aphid-resistant *fad7* mutant compared with wild-type Col-0 Arabidopsis. To determine if $^1O_2$ signaling through the EX1/EX2 pathway contributes to the aphid resistance in *fad7*, a bioassay was done with Col-0, *fad7*, *ex1/ex2*, and *fad7/ex1/ex2*. Population growth was measured at 48 h and again

after 6 dpi. Both time points had significant difference in means of population growth between genotypes (48 hpi: $F(3,53)=3.49$, $P=0.022$; 6 dpi: $F(3,52)=3.97$, $P=0.013$; Fig. 3). At 48 hpi, population growth on *fad7* was reduced by 10% compared with Col-0, which is consistent with previous reports. However, when *fad7* had impaired $^1O_2$ signaling through the EX1/EX2 pathway (*fad7/ex1/ex2*), population growth of GPA was comparable with Col-0. Col-0 was also not significantly different than *ex1/ex2*, indicating impaired $^1O_2$ signaling through the EX1/EX2 pathway does increase aphid susceptibility on its own. This trend continued when population growth was measured again at 6 dpi, where *fad7* alone showed reduced population growth compared with Col-0. Interestingly, GPA population growth on *fad7/ex1/ex2* was actually 5% greater than even Col-0 or *ex1/ex2* at 6 dpi, although the increase was not significant. Therefore, EX1/EX2 $^1O_2$ signaling is required for aphid resistance in the *fad7* background and indicates $^1O_2$ accumulation is occuring in the chloroplast of *fad7*, where EX1 and EX2 are located.

Electrolyte leakage is increased by aphid infestation at 6 dpi.

As aphid resistance in *fad7* is dependent on EX1/EX2 signaling and EL is a common EX1/EX2-mediated response to $^1O_2$ accumulation, EL was measured in *fad7* and compared with Col-0, *ex1/ex2*, and *fad7/ex1/ex2*. EL measurements were taken from uninfested and infested tissue at 6 days after challenge with GPA. Aphids, but not genotype, had a significant influence on EL (aphids: $F(1,25)=7.33$, $P=0.012$; genotype: $F(3,25)=0.76$, $P=0.53$; aphids x genotype: $F(3,25)=0.27$, $P=0.84$; Fig. 4). If EX1/EX2 signaling were inducing EL in *fad7*, increased EL would be expected both constitutively and in response to aphids due to increased constitutive and aphid-induced $^1O_2$ accumulation. However, this was not observed, as all genotypes showed a 2-3% increase of EL in aphid-infested tissue. The EL increase in response to aphids was also not

attenuated in *ex1/ex2* or *fad7/ex1/ex2*. These results indicate EX1/EX2 is not signaling for EL in response to aphids. Possibly the EL response to aphids was triggered by the accumulation of other reactive oxygen species.

Quantum efficiency of PSII is higher in fad7, while chlorophyll content is the same.

To investigate if *fad7* causes any dysfunction of PSII that could contribute to constitutively increased $^1O_2$ in this genotype, the quantum efficiency of PSII (F_v/F_m) and chlorophyll content was measured in uninfested *fad7* and Col-0. F_v/F_m was significantly higher in *fad7* than Col-0 by 5% (t=2.69, df=17 *P*=0.015; Fig. 5), indicating more efficient electron transfer at PSII in *fad7*. There was also not a significant difference in chlorophyll content (t=0.97, df=17, *P*= 0.17; Fig. 6). These results suggest that enhanced $^1O_2$ production in *fad7* does not result from impaired PSII function.

Discussion

The role of $^1O_2$ in plant defense against aphids and other herbivores is an understudied area of host plant resistance. Three decades ago, $^1O_2$ was indirectly attributed to plant defense against herbivores through the accumulation of phytotoxins that act as photosensitizers. Namely furanocoumarin, a phytotoxin associated with the plant families Umbelliferae and Rutaceae, was shown to generate enough $^1O_2$ in a stable gas-phase on and above the leaf surface that was believed to interact with herbivores such as the southern fall armyworm, *Spodoptera eridania* (Cramer) (Berenbaum, 1978; Berenbaum and Larson, 1988). However, if or how $^1O_2$ is acting as a defense in that system was never fully measured. Furthermore, $^1O_2$ signaling for chloroplast-to-nucleus retrograde reprogramming has previously been associated with abiotic stress responses

(Kim et al., 2009; Carmody et al., 2016). The results of this study demonstrate the importance of $^1O_2$ signaling in the chloroplast of the plant for aphid resistance. The *flu* mutant had significantly decreased aphid population growth, but only after an LDL shift that caused $^1O_2$ accumulation in the chloroplast, consistent with previous work (Chapter III). Impaired $^1O_2$-signaling through the EX1/EX2 pathway reduced this effect in *flu*, partially restoring aphid susceptibility in *flu/ex1/ex2* to levels comparable with wild-type Arabidopsis.

A hallmark of the EX1/EX2-mediated $^1O_2$ response in *flu* is increased electrolyte leakage (EL) followed by programmed cell death (PCD). In this study, increased EL in the *flu* mutant after an LDL shift was not observed, indicating the peak EL response occurs in *flu* earlier than 48 h post re-illumination (hpr). Other studies investigating EL in LDL treated *flu* saw levels of EL continued to rise after re-illumination until at least 25 hpr (Laloi et al., 2007; Przybyla et al., 2008). Therefore, EL in response to $^1O_2$ in *flu* abates between 25 and 48 hpr, indicating the signaling of $^1O_2$ accumulation for EL is an ephemeral response. Interestingly, *flu* and *flu/ex1/ex2* both had elevated EL compared with wild-type Arabidopsis, irrespective of light treatment, which may point to the *flu* mutation being "leaky" as reported for other mutations (Finkelstein, 1994; Malnoë et al., 2018). However, this has not been reported in *flu*.

The EX1/EX2 $^1O_2$-signaling is initiated in the non-appressed region of the thylakoid membrane known as the grana margin. EX1 and EX2 proteins are localized specifically to the grana margin where EX1, and possibly EX2, interact with $^1O_2$ (Dogra et al., 2019). The typical site of $^1O_2$ production occurs from active PSII in the appressed thylakoid (grana core) during photosynthesis. However, the reactive nature and short half-life of $^1O_2$ (Gorman and Rodgers, 1992) severely reduce the likelihood of $^1O_2$ traveling from the grana core to the grana margin. Therefore, $^1O_2$ interacting with EX1 and EX2 is speculated to be produced by damaged PSII or

chlorophyll precursors in the grana margin (Dogra and Kim, 2020). The *fad7* mutant with altered thylakoid membrane composition has increased constitutive and aphid-induced $^1O_2$ compared with wild-type. In this study, the *ex1/ex2* mutation restored aphid susceptibility in the *fad7* background, indicating that $^1O_2$ accumulation and signaling through the EX1/EX2 pathway is necessary for *fad7* aphid resistance. Therefore, it is possible the location of enhanced $^1O_2$ production in *fad7* is the grana margin. Furthermore, compared to wild type plants, *fad7* had comparable chlorophyll levels and higher maximum potential quantum efficiency (Fv/Fm) of PS II, indicating the PSII is not dysfunctional in the *fad7* mutant. However, because increased $^1O_2$ from photosynthesis can damage active PSII, and damaged PSII is proposed to produce $^1O_2$ in the grana margin, it is still possible aspects of photosynthesis in the grana core are contributing to $^1O_2$ production in *fad7*. Therefore, other parameters of the light reaction of photosynthesis in *fad7* should be further investigated.

While EX1/EX2-mediated $^1O_2$ signaling has been documented in inducing EL, the EL response is also common in response to aphid feeding (Mai et al., 2013, 2017; Sytykiewicz et al., 2019). In Arabidopsis, GPA induced expression of a $^1O_2$-responsive reporter gene indicating increased $^1O_2$ accumulation. Therefore, an EL response to aphids, in part, could be regulated by EX1/EX2 signaling. Moreover, as *fad7* resistance is EX1/EX2-mediated, it might be expected that *fad7* EL levels would be elevated in uninfested and infested tissue. However, this study found that while aphids induced a significant increase of EL at 6 dpi, the response was not different between *fad7*, *ex1/ex2*, *fad7/ex1/ex2*, nor wild-type Arabidopsis. This indicates the aphid-induced EL response is independent of EX1/EX2 signaling. Other ROS, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) also accumulate in response to aphids and increase EL (Moloi and van der Westhuizen, 2006; Mai et al., 2013; Sun et al., 2020), which

could explain the uniform increase of EL to aphids in all genotypes regardless of functional EX1/EX2 $^1O_2$-signaling. However, as the EL response triggered by $^1O_2$ in the *flu* background was presumably diminished by 48 hpr, the evaluation of EL at 6 dpi may have missed the EX1/EX2-mediated increase of EL in *fad7*. Investigating early timepoints of EL response to aphids, therefore, may provide more insights into the EL response potentially triggered by $^1O_2$.

This work illustrates the importance the EX1/EX2 pathway in signaling for aphid resistance in *fad7*. As *FAD7* is conserved in other plant families, including agronomically important crops (Andreu et al., 2007, 7; Upchurch, 2008; Hiremath et al., 2017, 7), this work opens the door to studies of $^1O_2$-signaling for biotic stress response in multiple systems. The EX1/EX2-mediated defense in *fad7* appears to lack the characteristic response of increased EL associated with EX1/EX2 in other systems and could provide new insights into how this pathway is influenced by other physiological variables, such as altered thylakoid membrane composition. Furthermore, as *fad7* has increased photosynthetic efficiency of PSII but increased $^1O_2$ production, it offers an intriguing opportunity to elucidate other mechanisms of $^1O_2$ production in the chloroplast that have yet to be revealed.

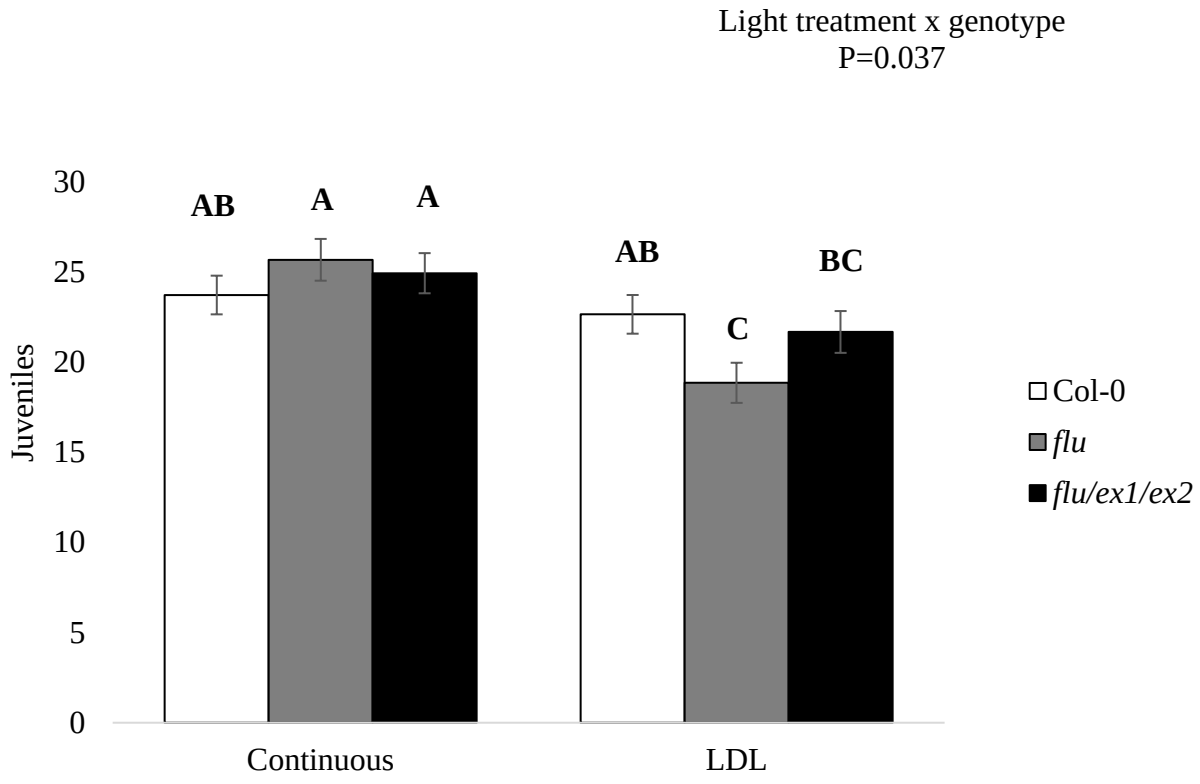

Figure 1. Impaired $^1O_2$ signaling through *ex1/ex2* mutation partially restored susceptibility to the *flu* mutant. Bioassay results of GPA juvenile production on Arabidopsis were analyzed with a two-way ANOVA at 48 hpi. In an LDL shift which increased $^1O_2$ in the *flu* background, *flu* had significantly fewer juveniles compared with Col-0, while *flu/ex1/ex2* had an intermediate level of juveniles between Col-0 and *flu*. Error bars represent ±SE. Mean were separated with student's t-test at α=0.05 and means with the same letter are not significantly different, n=13-15.

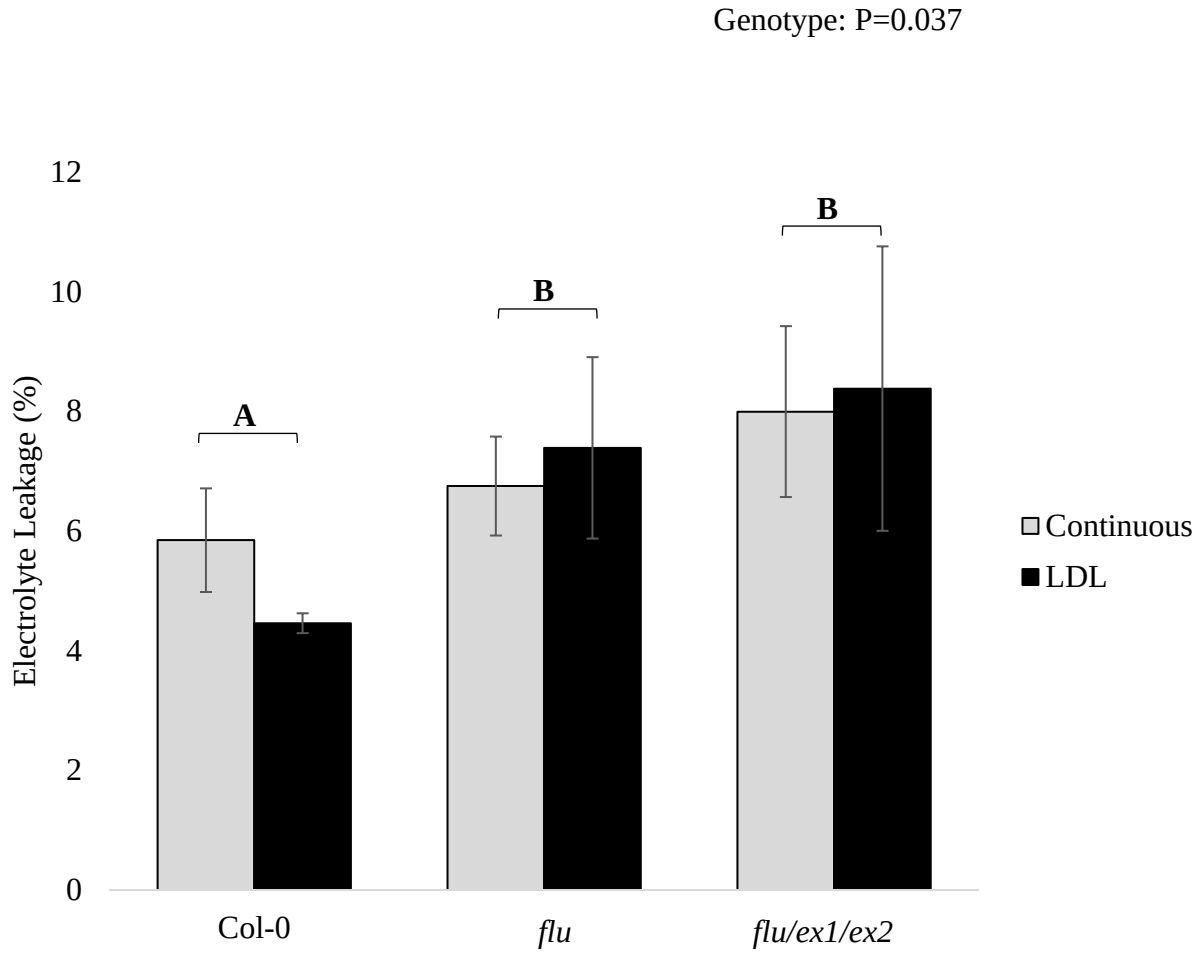

Figure 2. Electrolyte leakage of Arabidopsis is not different in continuous or light/dark/light (LDL) shift. Data of electrolyte leakage (EL) of uninfested plants 48 h after either continuous light or an LDL shift were analyzed with a two-way ANOVA. EL of *flu* and *flu/ex1/ex2* were not significantly different, but both had significantly increased EL compared to Col-0. Error bars represent ±SE. Means were separated by a student's t-test pairwise comparison with significant difference determined at α=0.05 and means with the same letters are not significantly different; n=3.

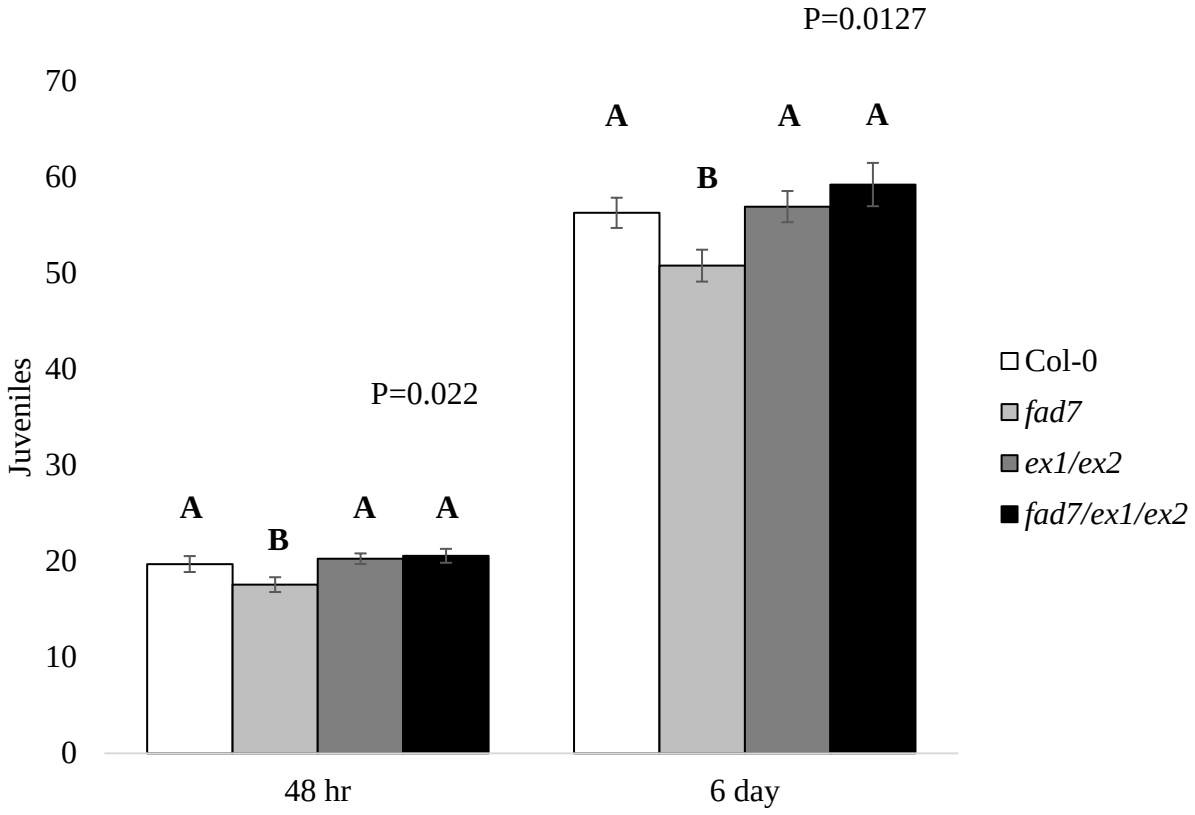

Figure 3. Aphid resistance in *fad7* requires EX1/EX2, a critical nodes in singlet oxygen signaling. Bioassay results of GPA juvenile production on Arabidopsis were analyzed with a one-way ANOVA at 48 hpi and 6 dpi. At both timepoints, *fad7* alone had significantly fewer juveniles, while the $^1O_2$-signaling impaired *fad7/ex1/ex2* was not significantly different than Col-0. Error bars represent ±SE. Mean were separated with student's t-test at α=0.05 and means with the same letter are not significantly different, n=13-15.

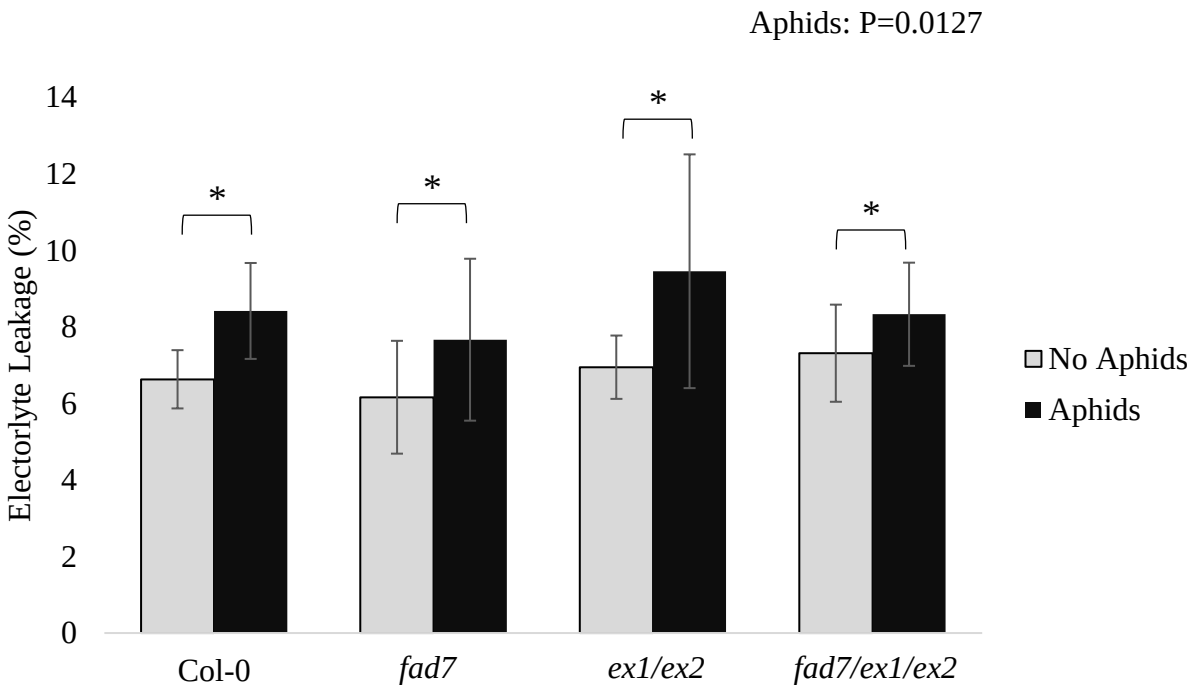

Figure 4. Aphids increase electrolyte leakage in Arabidopsis. Electrolyte leakage of Arabidopsis at 6 dpi was analyzed with a two-way ANOVA. Aphid infestation induced significantly more EL in all genotypes at 6 dpi. Means were separated with a student's t-test pairwise comparison. Error bars represent ±SE. Significance was determined at α=0.05 and indicated by asterisks; n=4.

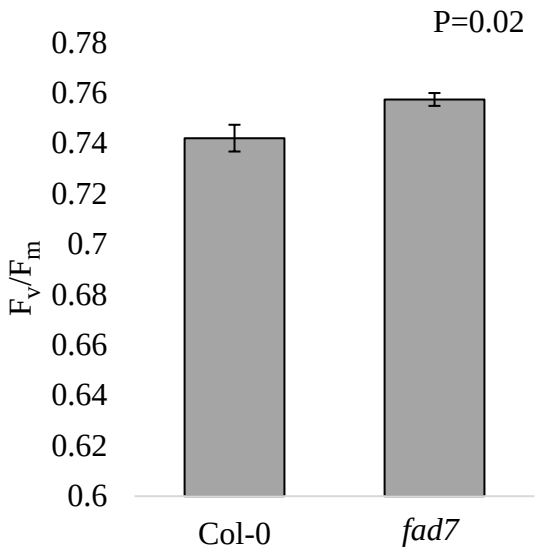

Figure 5. Quantum efficiency of photosystem II is higher in *fad7* than wild-type Arabidopsis. Quantum efficiency of PSII, estimated as F_v/F_m, of Col-0 and *fad7* was analyzed by a student's t-test. The *fad7* mutant had significantly higher maximum quantum efficiency constitutively. Error bars represent ±SE. Significant difference determined at α=0.05, n=8-10.

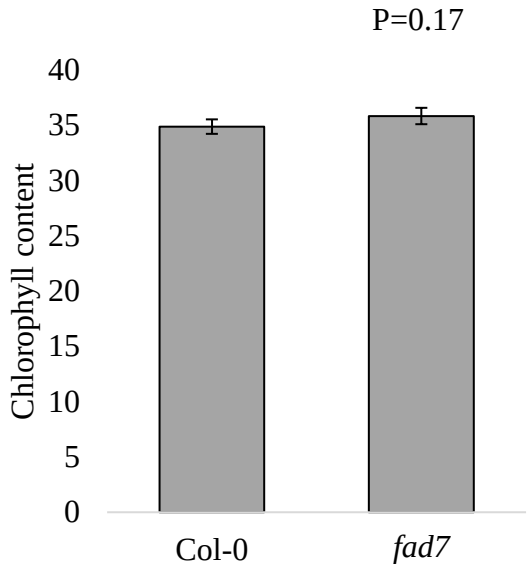

Figure 6. Chlorophyll content does differ between *fad7* and wild-type Arabidopsis. Chlorophyll content measured by SPAD 550 between Col-0 and *fad7* was analyzed by a student's t-test. There was not a significant difference in chlorophyll content of uninfested plants. Error bars represent ±SE. Significant difference determined at α=0.05, n=8-10.

References

Andreu, V., Collados, R., Testillano, P. S., Risueño, M. del C., Picorel, R., and Alfonso, M. (2007). *In situ* molecular identification of the plastid ω3 Fatty Acid Desaturase FAD7 from soybean: evidence of thylakoid membrane localization. *Plant Physiol* 145, 1336–1344. doi:10.1104/pp.107.109637.

Alnasrawi, A. M. (2015). Optimizing a luciferase-based tool for studying the effects of fatty acid desaturase 7 on singlet oxygen accumulation in *Arabidopsis thaliana*. *Theses and Dissertations* 1319, 64. doi:http://scholarworks.uark.edu/etd/1319.

Avila, C. A., Arévalo-Soliz, L. M., Jia, L., Navarre, D. A., Chen, Z., Howe, G. A., Meng, Q., Smith, J.E., Goggin, F.L. (2012). Loss of Function of FATTY ACID DESATURASE7 in tomato enhances basal aphid resistance in a salicylate-dependent manner. *Plant Physiol.* 158, 2028–2041.

Baruah, A., Šimková, K., Apel, K., and Laloi, C. (2009). Arabidopsis mutants reveal multiple singlet oxygen signaling pathways involved in stress response and development. *Plant Mol Biol* 70, 547–563. doi:10.1007/s11103-009-9491-0.

Berenbaum, M. (1978). Toxicity of a furanocoumarin to armyworms: A case of biosynthetic escape from insect herbivores. *Science* 201, 532–534. doi:10.1126/science.201.4355.532.

Berenbaum, M. R., and Larson, R. A. (1988). Flux of singlet oxygen from leaves of phototoxic plants. *Experientia* 44, 1030–1032. doi:10.1007/BF01939914.

Camp, R. G. L., Przybyla, D., Ochsenbein, C., Laloi, C., Kim, C., Danon, A., Wagner, D., Hideg, E., Göbel, C., Feussner, I., Nater, M., and Apel, K. (2003). Rapid induction of distinct stress responses after the release of singlet oxygen in *Arabidopsis*. *The Plant Cell* 15, 2320–2332.

Carmody, M., Crisp, P. A., d'Alessandro, S., Ganguly, D., Gordon, M., Havaux, M., Albrecht-Borth, V., and Pogson, B.J. (2016). Uncoupling high light responses from singlet oxygen retrograde signaling and spatial-temporal systemic acquired acclimation. *Plant Physiol.* 171, 1734–1749. doi:10.1104/pp.16.00404.

Chen, T., and Fluhr, R. (2018). Singlet oxygen plays an essential role in the root's response to osmotic stress. *Plant Physiol.* 177, 1717–1727. doi:10.1104/pp.18.00634.

Dmitrieva, V. A., Tyutereva, E. V., and Voitsekhovskaja, O. V. (2020). Singlet oxygen in plants: Generation, detection, and signaling roles. *Int J Mol Sci* 21, 3237. doi:10.3390/ijms21093237.

Dogra, V., and Kim, C. (2020). Singlet oxygen metabolism: From genesis to signaling. *Front Plant Sci* 10. doi:10.3389/fpls.2019.01640.

Dogra, V., Li, M., Singh, S., Li, M., and Kim, C. (2019). Oxidative post-translational modification of EXECUTER1 is required for singlet oxygen sensing in plastids. *Nat Commun* 10, 1–12. doi:10.1038/s41467-019-10760-6.

Finkelstein, R. R. (1994). Mutations at two new Arabidopsis ABA response loci are similar to the *abi3* mutations. *The Plant Journal* 5, 765–771. doi:https://doi.org/10.1046/j.1365-313X.1994.5060765.x.

Galvez-Valdivieso, G., and Mullineaux, P. M. (2010). The role of reactive oxygen species in signalling from chloroplasts to the nucleus. *Physiol Plant* 138, 430–439. doi:10.1111/j.1399-3054.2009.01331.x.

Gorman, A. A., and Rodgers, M. A. (1992). Current perspectives of singlet oxygen detection in biological environments. *J Photochem Photobiol B* 14, 159–176. doi:10.1016/1011-1344(92)85095-c.

Hiremath, S. S., Sajeevan, R., Nataraja, K. N., Chaturvedi, A. K., Chinnusamy, V., and Pal, M. (2017). Silencing of *fatty acid desaturase* (*FAD7*) gene enhances membrane stability and photosynthetic efficiency under heat stress in tobacco (*Nicotiana benthamiana*). *Indian J Exp Biol*, 10.

Kim, C., Lee, K. P., Baruah, A., Nater, M., Göbel, C., Feussner, I., and Apel, K. (2009). $^1O_2$-mediated retrograde signaling during late embryogenesis predetermines plastid differentiation in seedlings by recruiting abscisic acid. *PNAS* 106, 9920–9924. doi:10.1073/pnas.0901315106.

Laloi, C., Stachowiak, M., Pers-Kamczyc, E., Warzych, E., Murgia, I., and Apel, K. (2007). Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in *Arabidopsis thaliana*. *PNAS* 104, 672–677. doi:10.1073/pnas.0609063103.

Lee, K. P., Kim, C., Landgraf, F., and Apel, K. (2007). EXECUTER1- and EXECUTER2-dependent transfer of stress-related signals from the plastid to the nucleus of *Arabidopsis thaliana*. *PNAS* 104, 10270–10275. doi:10.1073/pnas.0702061104.

Li, J., Galla, A. L., Avila, C. A., Flattmann, K., Vaughn, K. L., and Goggin, F. (2021). Fatty Acid Desaturases in the chloroplast and endoplasmic reticulum promote susceptibility to the green peach aphid, *Myzus persicae*, in *Arabidopsis thaliana*. *MPMI*. doi:10.1094/MPMI-12-20-0345-R.

Mai, V. C., Bednarski, W., Borowiak-Sobkowiak, B., Wilkaniec, B., Samardakiewicz, S., and Morkunas, I. (2013). Oxidative stress in pea seedling leaves in response to *Acyrthosiphon pisum infestation*. *Phytochem* 93, 49–62. doi:10.1016/j.phytochem.2013.02.011.

Mai, V.-C., Nguyen, B.-H., Nguyen, D.-D., and Nguyen, L.-A.-V. (2017). *Nostoc calcicola* extract improved the antioxidative response of soybean to cowpea aphid. *Bot Stud* 58, 55. doi:10.1186/s40529-017-0211-9.

Malnoë, A., Schultink, A., Shahrasbi, S., Rumeau, D., Havaux, M., and Niyogi, K. K. (2018). The plastid lipocalin lcnp is required for sustained photoprotective energy dissipation in Arabidopsis. *The Plant Cell* 30, 196–208. doi:10.1105/tpc.17.00536.

Meskauskiene, R., Nater, M., Goslings, D., Kessler, F., Camp, R., and Apel, K. (2001). FLU: A negative regulator of chlorophyll biosynthesis in *Arabidopsis thaliana*. *PNAS* 98, 12826–12831. doi:10.1073/pnas.221252798.

Moloi, M. J., and van der Westhuizen, A. J. (2006). The reactive oxygen species are involved in resistance responses of wheat to the Russian wheat aphid. *J Plant Physiol* 163, 1118–1125. doi:10.1016/j.jplph.2005.07.014.

Ochsenbein, C., Przybyla, D., Danon, A., Landgraf, F., Göbel, C., Imboden, A., Imboden, A., Feussner, I., and Apel, K. (2006). The role of EDS1 (enhanced disease susceptibility) during singlet oxygen-mediated stress responses of *Arabidopsis*. *The Plant Journal* 47, 445–456.

Pospíšil, P., and Prasad, A. (2014). Formation of singlet oxygen and protection against its oxidative damage in Photosystem II under abiotic stress. *J Photochem Photobiol B* 137, 39–48. doi:10.1016/j.jphotobiol.2014.04.025.

Przybyla, D., Göbel, C., Imboden, A., Hamberg, M., Feussner, I., and Apel, K. (2008). Enzymatic, but not non-enzymatic, $^1O_2$-mediated peroxidation of polyunsaturated fatty acids forms part of the EXECUTER1-dependent stress response program in the *flu* mutant of *Arabidopsis thaliana*. *The Plant Journal* 54, 236–248. doi:10.1111/j.1365-313X.2008.03409.x.

Ramel, F., Birtic, S., Ginies, C., Soubigou-Taconnat, L., Triantaphylides, C., and Havaux, M. (2012). Carotenoid oxidation products are stress signals that mediate gene responses to singlet oxygen in plants. *PNAS* 109, 5535–5540. doi:10.1073/pnas.1115982109.

Ramel, F., Ksas, B., Akkari, E., Mialoundama, A. S., Monnet, F., Krieger-Liszkay, A., Ravanat, J., Mueller, M.J., Bouvier, F., and Havaux, M. (2013). Light-induced acclimation of the Arabidopsis *chlorina1* mutant to singlet oxygen. *The Plant Cell* 25, 1445–1462. doi:10.1105/tpc.113.109827.

Sun, M., Voorrips, R. E., van Kaauwen, M., Visser, R. G. F., and Vosman, B. (2020). The ability to manipulate ROS metabolism in pepper may affect aphid virulence. *Hortic. Res. England* 7, 6. doi:10.1038/s41438-019-0231-6.

Sytykiewicz, H., Lukasik, I., Golawska, S., and Chrzanowski, G. (2019). Aphid-triggered changes in oxidative damage markers of nucleic acids, proteins, and lipids in maize (*Zea mays L.*) seedlings. *Int. J. Mol. Sci.* 20, 3742. doi:10.3390/ijms20153742.

Triantaphylidès, C., and Havaux, M. (2009). Singlet oxygen in plants: Production, detoxification and signaling. *Trends Plant Sci* 14, 219–228. doi:10.1016/j.tplants.2009.01.008.

Triantaphylidès, C., Krischke, M., Hoeberichts, F. A., Ksas, B., Gresser, G., Havaux, M., Breusegem, F.V., and Mueller, M.J. (2008). Singlet oxygen is the major reactive oxygen species involved in photooxidative damage to plants. *Plant Physiol.* 148, 960–968. doi:10.1104/pp.108.125690.

Upchurch, R. G. (2008). Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *Biotechnol Lett* 30, 967–977. doi:10.1007/s10529-008-9639-z.

Vaughn, K. L., Avila, C. A., Padilla-Marcia, C. S., and Goggin, F. L. (2014). Development of *fad7-1* single mutant *Arabidopsis thaliana* plants that are resistant to aphids. *Discovery, The Student Journal of Dale Bumpers College of Agricultural, Food and Life Sciences* 15, 7.

Wagner, D., Przybyla, D., Camp, R. op den, Kim, C., Landgraf, F., Lee, K. P., Würsch, M., Laloi, C., Nater, M., Hideg, E., and Apel, K. (2004). The genetic basis of singlet oxygen-induced stress responses of *Arabidopsis thaliana*. *Science* 306, 1183–1185. doi:10.1126/science.1103178.

Zhang, S., Apel, K., and Kim, C. (2014). Singlet oxygen-mediated and EXECUTER-dependent signalling and acclimation of *Arabidopsis thaliana* exposed to light stress. *Philos Trans R Soc Lond., B, Biol Sci* 369, 20130227. doi:10.1098/rstb.2013.0227.

Appendix

Developing the *fad7/ex1/ex2* triple mutant

Materials and Methods

DNA Extraction and Purification

DNA from Arabidopsis was extracted using a one-step DNA extraction protocol modified from Kasajima et al (2004). This was done by first preparing a fresh dilution of 1:8 Edward's solution (ES; 200 mM Tris-HCl (pH 7.5), 25 mM NaCl, and 0.5% SDS) with TE buffer (10 mM Tris (pH 8.0) and 1 mM EDTA). 5-7 mg of Arabidopsis leaf tissue was then added to a 1.5 mL microcentrifuge tube containing 250 µL of ES and three autoclaved glass beads (3 mm). The Geno/Grinder® 2010 (SPEX SamplePrep, Metuchen, NJ) homogenized leaf tissue in ES, set at 1750 rpm for 1 min. After homogenization, 200 µL of solution was placed in a new 1.5 mL microcentrifuge tube and an equal amount of chloroform was added. After sitting for 5 min at room temperature, samples were centrifuged for 5 min at 13000 rpm and the supernatant was transferred to a new 1.5 mL microcentrifuge tube. This was washed with equal parts cold isopropyl alcohol and set at room temperature for 10 min before being spun at 13000 rpm again for 5 min. Isopropyl alcohol was removed without touching the pellet. 70% EtOH was added to wash the pellet before centrifugation for 2 min at 13000 rpm. EtOH was then removed, and the samples were dried before being diluted with 50 µL RNAse-free H_2O and heated for 10 min at 65 °C. The final DNA concentrations were diluted to 40 ng/µL to use for polymerase chain reactions. Samples were stored at -20 °C.

Screening for fad7-1/ex1/ex2 plants

The triple mutant *fad7/ex1/ex2* was developed by crossing the *ex1/ex2* double mutant as paternal material with *fad7-1* with the *glabra-1* (*gl1*) mutation as the maternal material. The *gl1* marker was used in crosses as a phenotypic marker of successful cross pollination. The GL1 protein is essential for trichome development in Arabiodpsis and any successful crosses between *ex1/ex2* and *fad7-1/gl1* would result in F1 progeny with trichomes (Marks and Feldmann, 1989). F1 progeny were grown and only plants with trichomes were kept, allowed to self-pollinate, and brought to seed. Once plants were dry, seeds were collected and replated for subsequent F2, F3, and F4 generations. Plants in the F2, F3, and F4 generations were screened using PCR for homozygous *ex1*, *ex2*, and *fad7*. PCR reactions were set up with 2.5 µL 25 mM $MgCl_2$, 5 µL 5x GoTaq Flexi buffer, 1 µL of 10 µM forward and reverse primer, 0.2 µL Taq polymerase, 12.8 µL of nuclease free water, and 2 µL of purified DNA from leaf tissue. Touchdown (TD) PCR had to be used for *Fad7* and *fad7* to increase specificity of the amplification (Korbie and Mattick, 2008). Importantly, lower concentrations of DNA were necessary for PCR genotyping of *Fad7-1* and *fad7-1* due to non-specific primer binding. PCR was run as follows for wild-type *Fad7-1* allele: 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. PCR for the mutant *fad7-1* allele used the same thermocycler program with the exception of phase I annealing temperature range from 65-56 °C and reducing to 55 °C in phase II. The forward primer for the wild-type *FAD7* was 5'-TTTCAGTGGGCTCGAAGTCC-3' and the forward primer for the *fad7* mutant was 5'-TTTCAGTGGGCTCTCGAAGACT-3'; *FAD7* and *fad7* shared a reverse primer 5'-ATCTGCGGGAAAAGATGATG-3' (Alnaswari 2015). The PCR thermocycler program for

both *EX1* and *EX2* were as follows: denaturation = 95 °C for 5 min, annealing = 52 °C for 45 sec, and extension = 72 °C for 45 sec. This was repeated for 30 cycles before a final extension at 72 °C for 5 min. The thermocycler program for *ex1* and *ex2* was the same except for an annealing temperature of 51°C. Screening for for *ex1* and *ex2* homozygous mutant lines was done with T-DNA specific primers generated by The Salk Institute Genomic Analysis Laboratory.

Results

Crosses successfully introduced the ex1/ex2 mutation into the fad7-1 background, generating a homozygous triple mutant in the F3 generation.

To study the effects that impaired $^1O_2$ signaling would have on the resistance of the *fad7-1* mutant to GPA, the *ex1* and *ex2* genes were introduced into *fad7-1*. This was done by crossing the *ex1/ex2* double mutant with *fad7-1/gl1*. For the F2 generation, 39 plants were screened, and a single plant, plant 2, was found to be homozygous for *ex1* (Fig. 2) and *fad7-1* (Fig. 3), but heterozygous for *Ex2/ex2* (Fig. 4). Next, seeds from this plant were plated and grown up to select for a homozygous *ex2* mutant. All F3 progeny had trichomes, indicating the parent line was also homozygous for *Gl1*. Twenty plants from the F3 population were screened for *Ex2* and *ex2* and 7 were *ex2* homozygous (Fig. 5). Those plants were screened again to confirm homozygosity of both *fad7* and *ex1* (data not shown).

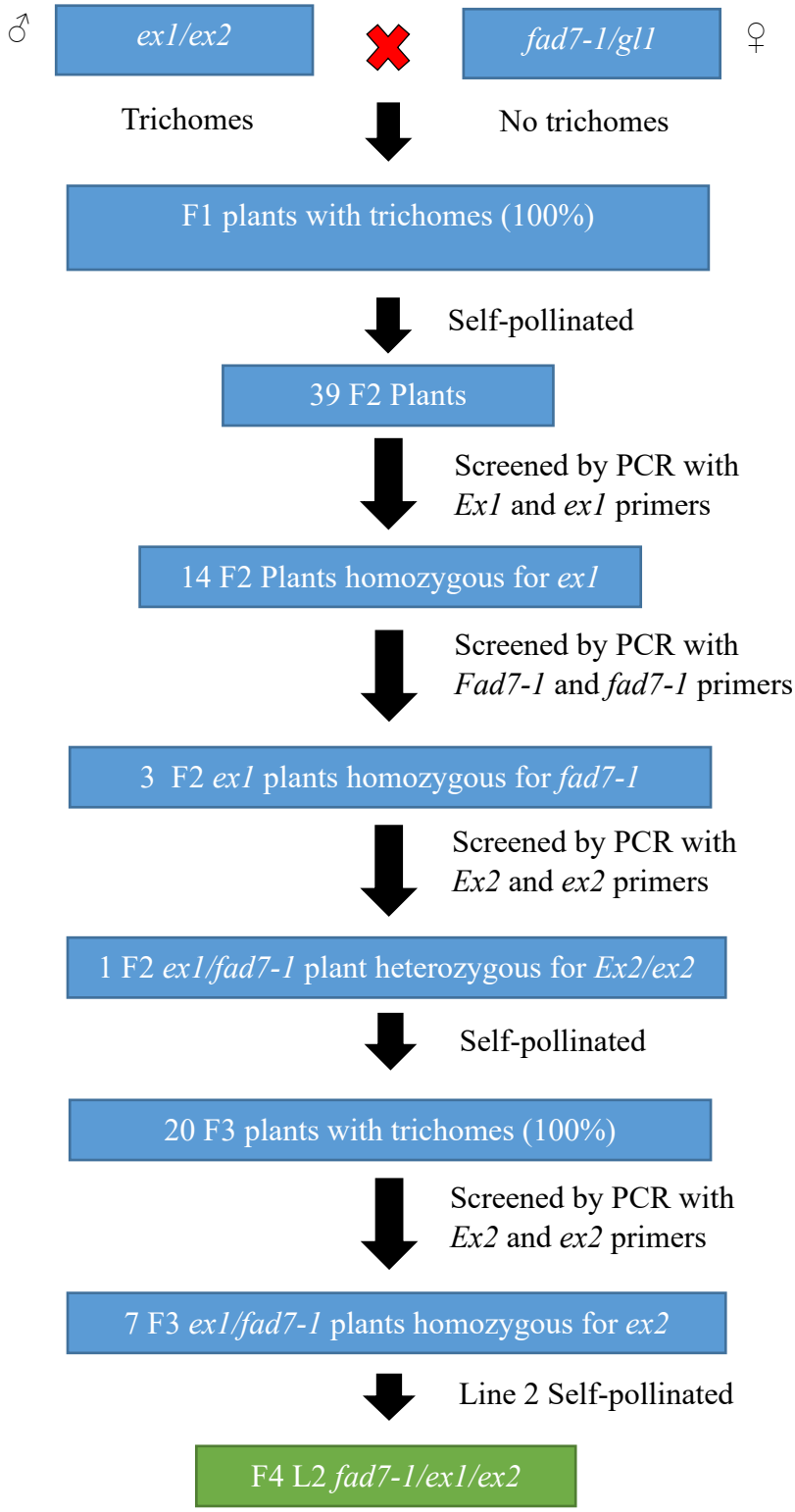

Figure 1. Schematic diagram for generating the triple mutant *fad7-1/ex1/ex2* from *ex1/ex2* and *fad7-1/gl1* Arabidopsis.

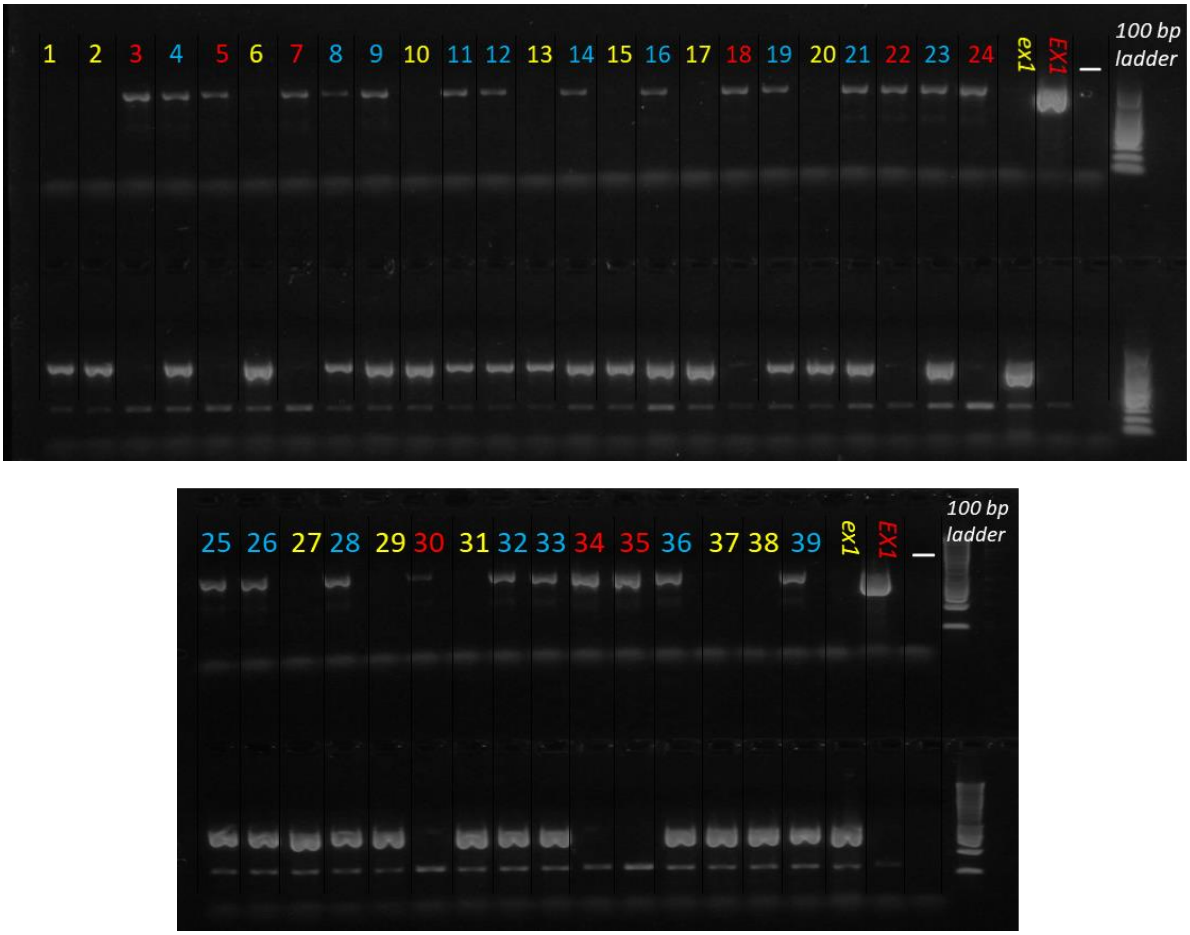

Figure 2. Screening F2 for *ex1* homozygotes. F2 Arabidopsis were screened for the presence of *EX1* (top lanes) and *ex1* (bottom lanes) alleles. Screening identified 13 plants homozygous for the *ex1* allele (yellow), 9 plants homozygous for the *EX1* allele (red), and 17 heterozygotes (blue); Minus (-) = no template control.

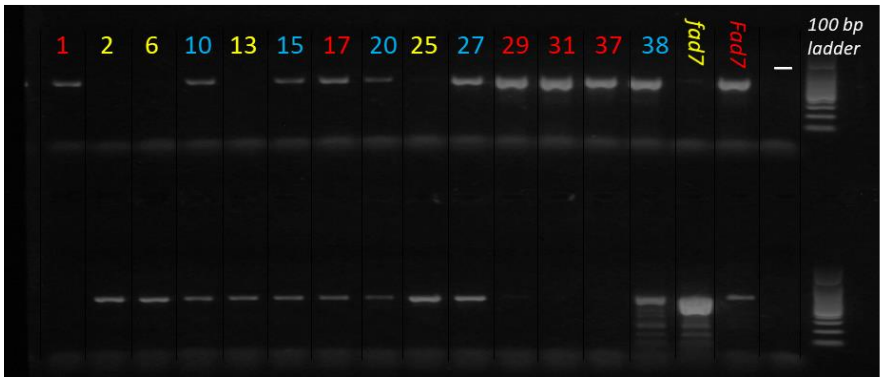

Figure 3. Screening F2 for *fad7* homozygotes. F2 Arabidopsis homozygous for *ex1* were screened for the presence of *FAD7* (top lanes) and *fad7* (bottom lanes) alleles. Screening identified 4 plants homozygous for the *fad7* allele (yellow), 5 plants homozygous for the *FAD7* allele (red), and 5 heterozygotes (blue); Minus (-) = no template control.

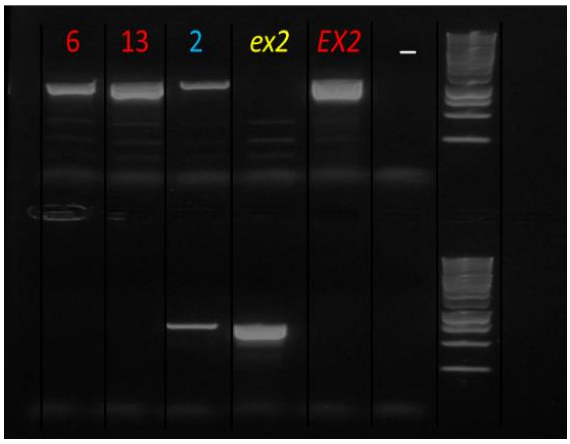

Figure 4. Screening F2 for *ex2* homozygotes. F2 Arabidopsis homozygous for *ex1* and *fad7* were screened for the presence of *EX2* (top lanes) and *ex2* (bottom lanes) alleles. Screening identified 2 plants homozygous for the *EX2* allele (red), and 1 heterozygote (blue); Minus (-) = no template control.

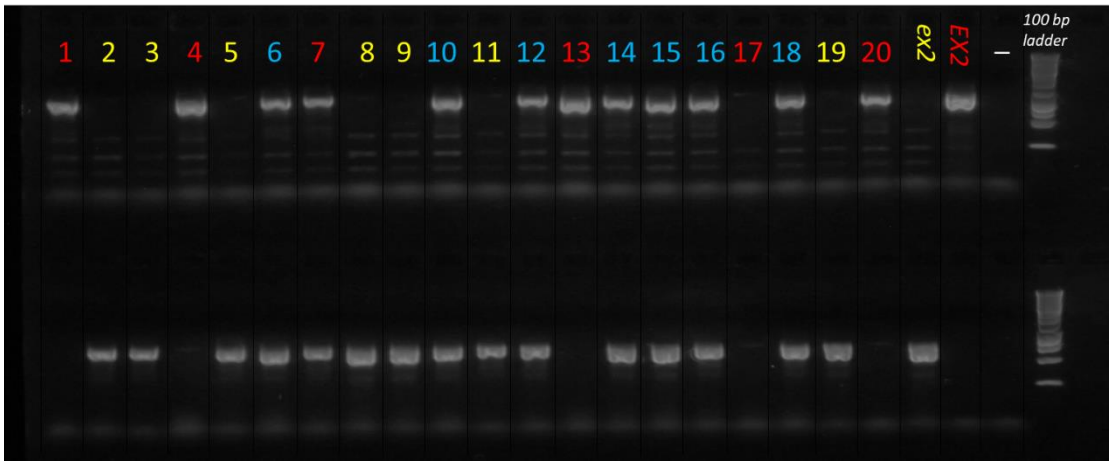

Figure 5. Screening F3 for *ex2* homozygotes. Progeny of lineage 2 of F2, which was homozygous for *ex1* and *fad7* but heterozygous for *EX2/ex2*, were screened for the presence of *EX2* (top lanes) and *ex2* (bottom lanes) alleles. Screening identified 7 plants homozygous for the *ex2* allele (yellow), 6 plants homozygous for the *EX2* allele (red), and 7 heterozygotes (blue); Minus (-) = no template control.

References

Alnasrawi, A. M. (2015). Optimizing a luciferase-based tool for studying the effects of fatty acid desaturase 7 on singlet oxygen accumulation in *Arabidopsis thaliana*. *Theses and Dissertations* 1319, 64. doi:http://scholarworks.uark.edu/etd/1319.

Korbie, D. J., and Mattick, J. S. (2008). Touchdown PCR for increased specificity and sensitivity in PCR amplification. *Nat Protoc* 3, 1452–1456. doi:10.1038/nprot.2008.133.

Marks, M. D., and Feldmann, K. A. (1989). Trichome development in *Arabidopsis thaliana*. I. T-DNA tagging of the GLABROUS1 gene. *The Plant Cell* 1, 1043–1050. doi:10.1105/tpc.1.11.1043.

Chapter V

Conclusion

Fatty Acid Desaturase 7 (FAD7) is a chloroplast-localized enzyme that alters photosynthetic membranes and influences plant defense to abiotic and biotic stresses. The loss of function of FAD7 in the model plant Arabidopsis (*Arabidopsis thaliana*) decreases population growth of the green peach aphid (GPA; *Myzus persicae* Sulzer). This suggests the chloroplast is involved in plant defense against aphids. Unavoidable by-products of photosynthesis in the chloroplast are reactive oxygen species (ROS), including $^1O_2$, that are known to regulate plant defense responses (Foyer and Noctor, 2000, 2016; Dmitrieva et al., 2020). Therefore, the chloroplast and associated ROS could be important in *fad7* aphid resistance.

In order to elucidate the *fad7* resistance mechanism and explore the connection between primary metabolism and aphid resistance, the objectives of this study were to 1) further characterize the effect of *fad7* aphid resistance on the generalist herbivore GPA compared with a specialist, the cabbage aphid (CA; *Brevicoryne brassicae*); 2) determine the effect of aphid infestation on the chloroplast redox response and $^1O_2$, and measure the impact $^1O_2$ accumulation has on aphid fitness; and 3) identify if $^1O_2$ signaling through the EXECUTER1/EXECUTER2 (EX1/EX2) pathway is involved in aphid resistance in *fad7*.

The chloroplast is a regulatory hub in the plant that connects primary metabolism and defense response, notably through ROS and associated ROS processing systems that activate defense signaling (Noctor et al., 2018; Kuźniak and Kopczewski, 2020). This study found aphid challenge caused a rapid oxidative shift in the chloroplast that was sustained even through dark periods when photosynthetic machinery was inactive; ROS accumulation was also observed in response to aphids but was attenuated in plants with increased $^1O_2$-scavenging in the chloroplast

(*SPS1oex*). These results indicate aphids are increasing ROS in the chloroplast. Whereas a growing body of evidence has demonstrated ROS accumulation in response to aphids contributes to host plant resistance (Guo et al., 2020; Sun et al., 2020), some reports have found increased ROS makes plants more susceptible (Shoala et al., 2018). Studies evaluating ROS in response to aphids have only looked at either H_2O_2 and/or O_2^-, ignoring the potential role of $^1O_2$ in signaling for aphid resistance. This study found that aphid attack does increase expression of a $^1O_2$-responsive reporter gene and that increased $^1O_2$ in the *flu* mutant after an LDL shift decreases population growth. This work demonstrated, for the first time, that $^1O_2$ is involved in plant resistance to herbivores. Furthermore, the aphid-resistant *fad7* with altered photosynthetic membranes of the chloroplast has increased constitutive and aphid-induced expression of a $^1O_2$-responsive reporter gene, suggesting aphid resistance in the mutant may be influenced by $^1O_2$. This evidence shows the chloroplast is involved in perception of plant stresses and initiating defense against aphids, a subcellular compartment that has been largely overlooked in studies of ROS-aphid interactions. Further investigations into the ROS dynamics such as timing, composition of which ROS are produced, and magnitude of ROS accumulation in the chloroplast after herbivore attack may provide clues as to how the plant senses and regulates defense responses.

As $^1O_2$ is highly unstable with a half-life of 200ns (Gorman and Rodgers, 1992), it is mainly inducing defense responses through interactions with molecules close to the production site for chloroplast-to-nucleus retrograde signaling (Galvez-Valdivieso and Mullineaux, 2010; Dogra and Kim, 2020). Oxidation of EX1 by $^1O_2$ creates a signaling cascade that alters gene expression, including increased expression for a pathogenesis-related protein (*PR1*), and induces electrolyte leakage (Lee et al., 2007; Zhang et al., 2014; Dogra et al., 2017). A null mutation of

EX1, and the close homologue *EX2*, a putative modulator of $^1O_2$ signaling, abrogates these responses. In the *fad7* background, *ex1/ex2* fully restored aphid susceptibility compared with the wild-type Arabidopsis. This response indicates both accumulation of $^1O_2$ and a reaction with the EX1/EX2 proteins is required for aphid resistance in *fad7*. Aphid resistance conferred by the *fad7* mutation in tomato (*spr2*) requires salicylic acid (SA) and the SA response factor *NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1* (*NPR1*), which triggers expression of *PR1*(Avila et al., 2012). Possibly the *fad7* mutation in Arabidopsis also requires *PR1* expression that is regulated by EX1/EX2 signaling. Further work on gene expression changes and phytohormone-regulated defense signaling in *fad7* and *fad7/ex1/ex2* will provide more insight into the defense signaling pathway of *fad7*. Also, as *fad7* is resistant to GPA but not CA, perhaps the resistance mediated by EX1/EX2 is species-specific.

The active photosystem II (PSII) of photosynthesis, housed in the appressed region of the thylakoid membranes, is typically the primary source of $^1O_2$ production in the chloroplast. This study found higher maximum potential quantum efficiency (Fv/Fm) of Photosystem II in *fad7* than wild-type Arabidopsis, in line with a previous study with *spr2* tomato (Wickramanayake et al., 2020). Also, *fad7* had comparable levels of chlorophyll with wild-type Arabidopsis, which indicates the source of $^1O_2$ is not dysfunction of the PSII. Furthermore, EX1 and EX2 are localized to the non-appressed region of the thylakoid membranes (grana margin) where damaged PSII are repaired. As it is too highly reactive, $^1O_2$ is not likely to diffuse from the grana core to the grana margin. Therefore, if $^1O_2$-signaling through EX1/EX2 is occuring, it is believed to be the result of $^1O_2$ also produced in the grana margin. Damaged PSII undergoing disassembly or reassembly are speculated to be responsible for $^1O_2$ in the grana margin, but the actual

mechanism of $^1O_2$ production in this region is still unknown (Dogra and Kim, 2020). Therefore, *fad7* could provide an opportunity to elucidate the mechanism(s) of grana-margin produced $^1O_2$.

This study significantly contributes to the field of host plant resistance by connecting primary metabolism and defense responses. Furthermore, it identifies a key signaling pathway for $^1O_2$ that has previously been assigned to plant defense against abiotic stress, also demonstrating an overlap between abiotic and biotic stress signaling. As FAD7 is widely conserved across several plant groups, including soybean and tomato (Andreu et al., 2007; Avila et al., 2012), the potential for $^1O_2$ signaling for plant defense against herbivores may reach beyond the model organism of Arabidopsis. Also, *EX1* and *EX2* homologs are also found in other plant groups (TAIR), again including soybean and tomato, providing the opportunity to evaluate *fad7* and $^1O_2$ signaling for aphid resistance in other agronomically important crops.

References

Andreu, V., Collados, R., Testillano, P. S., Risueño, M. del C., Picorel, R., and Alfonso, M. (2007). *In Situ* molecular identification of the plastid ω3 Fatty Acid Desaturase FAD7 from soybean: Evidence of thylakoid membrane localization. *Plant Physiol* 145, 1336–1344. doi:10.1104/pp.107.109637.

Avila, C. A., Arévalo-Soliz, L. M., Jia, L., Navarre, D. A., Chen, Z., Howe, G. A., Meng, Q., Smith, J.E., Goggin, F.L. (2012). Loss of Function of FATTY ACID DESATURASE7 in tomato enhances basal aphid resistance in a salicylate-dependent manner. *Plant Physiol.* 158, 2028–2041.

Dmitrieva, V. A., Tyutereva, E. V., and Voitsekhovskaja, O. V. (2020). Singlet oxygen in plants: Generation, detection, and signaling roles. *Int J Mol Sci* 21, 3237.

Dogra, V., Duan, J., Lee, K. P., Lv, S., Liu, R., and Kim, C. (2017). FtsH2-Dependent Proteolysis of EXECUTER1 Is Essential in Mediating Singlet Oxygen-Triggered Retrograde Signaling in Arabidopsis thaliana. *Front. Plant Sci.* 8, 1145. doi:10.3389/fpls.2017.01145.

Dogra, V., and Kim, C. (2020). Singlet Oxygen Metabolism: From Genesis to Signaling. *Front Plant Sci* 10. doi:10.3389/fpls.2019.01640.

Foyer, C. H., and Noctor, G. (2000). Oxygen processing in photosynthesis: regulation and signalling. *New Phytol* 146, 359–388. doi:10.1046/j.1469-8137.2000.00667.x.

Foyer, C. H., and Noctor, G. (2016). Stress-triggered redox signalling: what's in pROSpect? *Plant Cell Environ.* 39, 951–964. doi:10.1111/pce.12621.

Galvez-Valdivieso, G., and Mullineaux, P. M. (2010). The role of reactive oxygen species in signalling from chloroplasts to the nucleus. *Physiol Plant* 138, 430–439. doi:10.1111/j.1399-3054.2009.01331.x.

Gorman, A. A., and Rodgers, M. A. (1992). Current perspectives of singlet oxygen detection in biological environments. *J Photochem Photobiol B* 14, 159–176. doi:10.1016/1011-1344(92)85095-c.

Guo, H., Zhang, Y., Tong, J., Ge, P., Wang, Q., Zhao, Z., et al. (2020). An aphid-secreted salivary protease activates plant defense in phloem. *Current Biology* 30, 4826-4836.e7. doi:10.1016/j.cub.2020.09.020.

Kuźniak, E., and Kopczewski, T. (2020). The chloroplast reactive oxygen species-redox system in plant immunity and disease. *Front. Plant Sci.* 11. doi:10.3389/fpls.2020.572686.

Lee, K. P., Kim, C., Landgraf, F., and Apel, K. (2007). EXECUTER1- and EXECUTER2-dependent transfer of stress-related signals from the plastid to the nucleus of *Arabidopsis thaliana*. *PNAS* 104, 10270–10275. doi:10.1073/pnas.0702061104.

Noctor, G., Reichheld, J.-P., and Foyer, C. H. (2018). ROS-related redox regulation and signaling in plants. *Sem Cell Develop Biol* 80, 3–12. doi:10.1016/j.semcdb.2017.07.013.

Shoala, T., Edwards, M. G., Knight, M. R., and Gatehouse, A. M. R. (2018). OXI1 kinase plays a key role in resistance of Arabidopsis towards aphids (*Myzus persicae*). *Transgenic Res* 27, 355–366. doi:10.1007/s11248-018-0078-x.

Sun, M., Voorrips, R. E., van Kaauwen, M., Visser, R. G. F., and Vosman, B. (2020). The ability to manipulate ROS metabolism in pepper may affect aphid virulence. *Hortic. Res.-England* 7, 6. doi:10.1038/s41438-019-0231-6.

Wickramanayake, J. S., Goss, J. A., Zou, M., and Goggin, F. L. (2020). Loss of Function of Fatty Acid Desaturase 7 in Tomato Enhances Photosynthetic Carbon Fixation Efficiency. *Front Plant Sci* 11, 932. doi:10.3389/fpls.2020.00932.

Zhang, S., Apel, K., and Kim, C. (2014). Singlet oxygen-mediated and EXECUTER-dependent signalling and acclimation of Arabidopsis thaliana exposed to light stress. *Philos Trans R Soc Lond., B, Biol Sci* 369, 20130227. doi:10.1098/rstb.2013.0227.

The Arabidopsis Information Resource (TAIR), www.arabidopsis.org/servlets/TairObject?name=AT4G33630&type=locus, 6 April 2021.

The Arabidopsis Information Resource (TAIR), www.arabidopsis.org/servlets/TairObject?name=AT1g27510&type=locus, 6 April 2021.