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Identification and Characterization of Cowpea Aphid Salivary Proteins and the Cowpea Resistance Mechanism to the Cowpea Aphid

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DEDICATIONS
To My Family and Friends

ABSTRACT OF THE DISSERTATION

Identification and Characterization of Cowpea Aphid Salivary Proteins and the Cowpea Resistance Mechanism to the Cowpea Aphid

by

Jacob Robert MacWilliams

Doctor of Philosophy, Graduate Program in Biochemistry and Molecular Biology
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Dr. Isgouhi Kaloshian, Chairperson

Cowpea (*Vigna unguiculata*) is a vital crop for semiarid regions of the world. Cowpea is able to withstand harsh abiotic stresses prevalent in these regions but is susceptible to the cowpea aphid (*Aphis craccivora*). Unlike most aphids, cowpea aphids are phytotoxic and damage cowpea even at low populations. Aphids feed on plant phloem sap and while feeding deposit saliva which contain proteinaceous effectors to disrupt plant defenses and alter plant physiology. The composition of the cowpea aphid saliva has not been well studied and how cowpea aphids manipulate their hosts remain unresolved. While resistance to cowpea aphids has been identified in an African cowpea line, and the genetic determinants of the resistance mapped to two QTLs, the underlying resistance mechanisms remain unknown. In Chapter One, liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to identify the proteome of the

cowpea aphid saliva. One of the proteins identified, diacetyl/L-xylulose reductase (DCXR), was functionally characterized using Agrobacterium-mediated transient expression and *in vitro* biochemical analyses. DCXR is a member of short-chain dehydrogenases/reductases invovled in both carbohydrate and dicarbonyl metabolism. I showed that cowpea aphid infestation transiently induces the cytotoxic dicarbonyl, methylglyoxal, in cowpea. Recombinant cowpea aphid DCXR (AcDCXR) was able to detoxify methylglyoxal *in vitro* and to oxidize the carbohydrate xylitol to xylulose and expression of AcDCXR in pea (*Pisum sativum*) improved aphid fecundity. Using resistant and susceptible cowpeas, and various aphid infestation approaches and behavioral assays, I determined the nature of the resistance in Chapters Two and Three. In Chapter Two, the susceptible interaction is elaborated and the resistance mechanism revealed to be localized in the phloem and to involve both antibiosis and antixenosis. In Chapter Three, aphid dispersal assays indicate aphids prefer susceptible cowpea to resistant. In addition, using timecourse infestation of resistant and susceptible cowpeas and RNASeq, I determined the genes regulated in both susceptible and resistant responses. The transcriptome analyses identified major differences in susceptible and resistant cowpea including involvement of multiple plant hormones and defense related genes. A subset of these genes are candidates for further exploration as the source of resistance.

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General Introduction

One of the important crops in the subtropical and tropical regions of the world is cowpea (*Vigna unguiculata*). Cowpea is a highly nutritional legume that is consumed in several ways. Multiple parts of the plant are edible including the leaves, pods, and grain (Hall et al., 1997; Singh, 2014). The cowpea grain, with over 30% protein content, is an essential and inexpensive component of the human diet in Western and Central African countries (Singh et al., 2002). Over 7 million tonnes of cowpea grain were produced in 2018 worldwide. Though the majority of cowpea production is in Africa, cowpea is grown across the world in areas with warm climates. In Africa, Nigeria and Niger are the highest producers with 2.6 million and 2.3 million tonnes, respectively (FAO, 2020). While the grain is the part used for human consumption, the fodder is also used as animal feed (IITA, 2009). Cowpea has a multitude of varieties that have different growth physiology, maturity time, and seed phenotype (Singh, 2014).

Cowpea growth is optimal with daytime temperatures between 21-36°C and night temperatures from 16-31°C (Singh, 2014). Cowpea is able to thrive in the warmer areas of the world not only because it can withstand heat stress, but it can also withstand drought stress (Hall et al., 2002; Hall, 2004). Cowpea does not need much water compared to other crops, only requiring 200-350 mm of water (Singh, 2014). Other crops like soybean (*Glycine max*) or maize (*Zea mays*) need substantially more water with 450-750 mm and 500-800 mm, respectively (Brouwer and Heibloem, 1986). However, elevated temperatures

above 40°C or low temperatures below 15°C results in poor plant growth and loss of flower and grain set (Singh, 2014). In addition to withstanding heat and drought conditions, cowpea can also endure other harsh negative plant growth conditions such as low soil fertility and can grow in a multitude of soil pH ranges (Singh, 2014). Cowpea can grow in soils that are up to 85% sand and low in organic matter content. This success growing in poor soil quality is because of its ability to fix sufficient atmospheric nitrogen through its nodules and is able to extract soil-bound phosphorus when phosphorus is limited (AATF, 2012; Singh, 2014).

While cowpea is able to withstand harsh abiotic conditions, it faces a number of production constraints. One the most important of these constraints is a devastating pest, the cowpea aphid (*Aphis craccivora*) (Fig 0.1). Cowpea aphids are especially deadly to the early stages of cowpea plants (Ofuya, 1995; Obeng-Ofori, 2007; Singh, 2014). Cowpea aphids feed on the plant phloem sap, primarily on young cowpea seedlings but they can also infest flower buds, flowers, and pods on older plants (Jackai and Daoust, 1986; Singh et al., 1996). When feeding, the cowpea aphid can generate damage in multiple forms including chlorosis and necrosis, pseudogalling (leaf curling), stunted growth, and delay in flower initiation (Fig 0.2) (Jackai and Daoust, 1986; Goggin et al., 2017; Omoigui et al., 2017). The damage can build up leading to plant death and up to 50% loss of yield (Ofuya, 1995; Obopile and Ositile, 2010).

Not only are cowpea aphids' toxic to their host plants, a lineage of cowpea aphids has been discovered in Kentucky that are toxic to one of their natural predators. The Asian ladybeetle (*Harmonia axyridis*) is a natural predator of aphids but when the larvae fed on one biotype of cowpea aphids, they all died within seven days (White et al., 2017). The mechanism of toxicity to the Asian ladybeetle is not known, but it is species-specific. Other ladybeetle species like the seven-spotted ladybeetle (*Coccinella septempunctata*) and spotted ladybeetle (*Coleomegilla maculate*) were able to feed on and tolerate the toxic cowpea aphid biotype (Jackson et al., 2017; Lenhart et al., 2018).

Similar to a number of aphids, cowpea aphids generate direct damage through feeding as well as cause indirect damage by acting as vectors for a number of plant viruses (Chan et al., 1991; Singh, 2014). Indirect damage is also caused by the honeydew, or waste product, excreted by aphids. At high infestation levels, the excreted honeydew can build up on plant tissues and lead to the growth of black sooty molds reducing the photosynthetic ability of the plant (Reynolds, 1999).

To control aphid infestation, farmers rely on spraying chemical pesticides. These pesticides often are toxic to beneficial insects, cause harm to humans and pollute the environment (Souleymane et al., 2013). The focus of this study is to better understand the relationship between cowpea and the cowpea aphids to develop alternate means to control the aphid. Additionally, to characterize

phenotypically and molecularly a known source of resistance to cowpea aphid in a resistant cowpea line.

Aphid Background

One of the most devastating pests to agriculture are aphids. Aphids belong to the Hemipteran order of insects with over 5000 known species, that have a range of colors, morphological structures, and host plants (McGavin, 1993; Remaudiere and Remaudiere, 1997; Sorenson, 2009). Of these 5000 species, only about 450 species infest plants that are grown as crops (Blackman and Eastop, 2007; Sorenson, 2009). Of these 450 species, only 14 species are considered to be serious pests to agriculture. Cowpea aphid is among these 14 devastating species (Sorenson, 2009).

Based on the damage they inflict on their host plants; aphids can be categorized as phytotoxic or non-phytotoxic. Phytotoxic aphids cause extensive direct damage on the plant while non-phytotoxic aphids lead to indirect damage (Nicholson et al., 2012). Phytotoxic aphids, like the Russian wheat aphid (*Diuraphis noxia*) and greenbug (*Schizaphis graminum*), generate damage at low population levels, most likely due to their salivary protein contents deposited into the plant while feeding (Nicholson et al., 2012; Nicholson and Puterka, 2014). Most aphids can be classified as non-phytotoxic and generally lead to no damage symptoms induced by their feeding (Nicholson and Puterka, 2014; Chaudhary et al., 2015). Similar to these phytotoxic aphids, cowpea aphids also

cause direct damage to cowpea manifested as chlorosis, necrosis, and pseudogllaing of leaves as well as stunted growth (Fig 0.2) (Jackai and Daoust, 1986; Goggin et al., 2017; Omoigui et al., 2017). These phytotoxic damage symptoms are not limited to cowpea; similar damage phenotypes have been reported on Amaranth (*Amaranths hybridus* L) infested with cowpea aphids (Loudit et al., 2018).

Aphid Lifecycle

Aphids have complex life cycles. The majority of aphids undergo a holocyclic life cycle (Fig 0.3). The holocyclic life cycle consists of two parts, the summer life cycle and the winter life cycle. In the summer life cycle, the aphids reproduce without mating (parthenogenetically) and give live birth (viviparous), while in the winter life cycle, the aphids reproduce through mating and laying eggs for overwintering (Blackman and Eastop, 2000; Sorenson, 2009). Holocyclic aphids can be further categorized as monecious or dioecious (Sorenson, 2009; Moran 1992; Ogawa and Miura, 2014). The monecious cycle occurs on a single primary host plant (Fig 0.3A). In contrast, in the dioecious cycle, the asexual and sexual parts of the life cycle occur on different hosts (Fig 0.3B). Dioecious aphids alternate between a woody plant host for overwintering and on herbaceous plants during the summer season. At the end of summer, aphids develop into winged sexual males and into winged sexual females (gynoparae) to migrate

back to the woody host to lay eggs for overwintering (Dixon and Dewar, 1974; Sorenson, 2009).

The start of the summer life cycle begins in the spring, when a fundatrix emerges from an egg that was laid for overwintering. The fundatrix gives live birth parthenogenetically to a large number of parthenogenetic females that continue through the summer lifecycle (Moran, 1992; Ogawa and Miura, 2014). Each of these parthenogenetic females begin their development in their mothers ovarioles and are born as first instar nymphs. The mothers not only contain their daughters in an embryonic state, they also contain their granddaughters called telescoping generations (Kindlmann and Dixon, 1989). The first instar nymphs undergo four molts to reach adulthood when they give birth to their daughters. Because of this mode of reproduction, aphid populations are able to grow at high rates. To deal with overcrowding, the production of winged (alatae) aphids is triggered to allow dispersal and spread of the colony from their wingless (apterae) counterparts (Johnson, 1965).

The second part of the holocyclic life cycle is cued from the environment by changes in, day length, temperature, and food availability. Once cued the aphids enter the winter lifecycle, where sexual males and females are generated (Blackman and Eastop, 1989; Hutchinson and Bale, 1994). The generation of males occurs through the absence of one of the two X chromosomes. The XX/XO sex determination in aphids allows for only female progeny with XX chromosomes to be generated after sexual reproduction (Hales et al., 2002;

Jaquiery et al., 2012). The sexual males and females will then produce fertilized cold-resistant eggs for over-wintering. In the spring, the eggs will hatch with a single fundatrix to begin the cycle over again (Moran, 1992).

The other aphid life cycle that can occur is known as the anholocyclic life cycle. The anholocyclic life cycle differs from the holocyclic life cycle by consisting of only the summer life cycle (Fig 0.3). The majority of anholocyclic aphids are located in warmer climates (Moran, 1992). Similar to dioecious aphids, anholocyclic aphids can alternate hosts depending on host availability (Moran, 1992; Sorenson 2009). Cowpea aphids are almost always anholocyclic due their distribution in warmer regions of the world (CABI, 2019).

Aphid Feeding

Aphids feed on their host plant phloem sap by inserting their flexible needle like stylets into plant tissues. Phloem sap has poor nutritional quality; it is rich in sugars and carbohydrates but is lacking in essential amino acids (Douglas, 1993; Dixon, 2012). To make up for this nutritional deficiency, aphids house a primary endosymbiont, *Buchnera aphidicola* (Douglas, 1998; Shigenobu et al., 2000). Together, *B. aphidicola* and the aphid cooperate to synthesize the full spectrum of required amino acids, both essential and nonessential (Hansen and Moran, 2011; Feng et al., 2019).

Unlike other herbivore pests, aphids navigate their stylets to the phloem in ways that generate minimal mechanical damage, (Tjallingii and Esch, 1993;

Tjallingii, 2006). To minimize damage and maximize feeding, aphids deposit saliva throughout the path to the phloem and once in the phloem. Saliva contains proteinaceous compounds that likely assist in manipulating plant defenses and metabolic processes for aphid's benefit (Miles, 1999; Will et al., 2007; Kaloshian and Walling, 2016). Aphid saliva is composed of two types, a gelling saliva and a watery saliva (Miles, 1999). As the name indicates, the gelling saliva is continuously secreted during the pathway phase when the stylet navigates to the phloem and forms a sheath around the aphid stylets. It is thought that this sheath protects the aphid stylets from apoplastic defenses and acts as a lubrication for the stylets in the plant tissues (Miles, 1999; Tjallingii, 2006; Walling, 2008). The main proteinaceous component of the gelling saliva is a cysteine rich protein SHP, and the gelling of the sheath is likely caused by the oxidation of the cysteine sulfhydryl groups producing disulfide bonds (Tjallingii, 2006; Carolan et al., 2009; Will et al., 2012). This utilization of a sheath as protection is not aphid specific as it is a common feature conserved across other phytophagous hemipterans (Morgan et al., 2013).

The proteinaceous composition of both saliva types has been investigated using liquid chromatography and tandem mass spectrometry (LC-MS/MS) with a focus on the content of watery saliva. The watery saliva proteinaceous profile has been identified with LC-MS/MS for the following aphid species: cowpea aphid, potato aphid (*Macrosiphum euphorbiae*), pea aphid (*Acyrthosiphon pisum*), Russian wheat aphid, wheat aphid (*Sitobion avenae*), rose-grain aphid

(*Metopolophium dirhodum*), green peach aphid (*Myzus persicae*), greenbug, and Chinese gall aphid (*Schlechtendalia chinensis)* (Harmel et al., 2008; Carolan et al., 2009; Cooper et al., 2010; Carolan et al., 2011; Cooper et al., 2011; Rao et al., 2013; Vandermoten et al., 2014; Chaudhary et al., 2015; Thorpe et al., 2016; Boulain et al., 2018; Loudit et al., 2018; Yang et al., 2018). LC-MS/MS was employed to identify the watery saliva proteinaceous profile of a cowpea aphid population native to Riverside, California, and is described in Chapter One. Previously, a study of cowpea aphid salivary proteins had been performed with a cowpea aphid population native to Gabon, Africa (Loudit et al., 2018).

Improved saliva collection techniques and technological advances in LC-MS have improved the ability to detect proteins in aphid saliva. The initial proteomic studies identified only a handful of proteins from aphid saliva (Harmel et al., 2008; Carolan et al., 2009). In recent years, over a hundred proteins have been identified from the saliva of different aphid species (Chaudhary et al., 2015). Besides the advancement in technology and saliva collection, another factor for the increase in the discovery of salivary proteins is the increase in the availability of aphid genome and transcriptome sequences and bioinformatics tools. The first aphid genome was for the pea aphid and was published in 2010 (IAGC, 2010). Since then, five additional aphid genomes have been publicly available as well as several aphid transcriptomes (Agunbiade et al., 2013; Nicholson et al., 2015; Mathers et al., 2017; Wenger et al., 2017; Teixeira et al., 2018; Thorpe et al., 2018).

These watery saliva proteomic studies have identified numerous proteins including a set that has been conserved among the different aphid species. These conserved proteins are described as a core set of aphid effectors that maybe used across different aphid species as a way to manipulate the plant (Thorpe et al., 2016). This manipulation could be through the disruption of plant defense signaling or by modifying the plants' metabolic processes by inducing a physiological sink to use the plants resources as the aphids own (Burd, 2002; Kaplan et al., 2011; Thorpe et al., 2016). Outside of this core set of aphid proteins, there are unique proteins that have only been identified in a single aphid species or a biotype of a species that seem to be necessary for a specific aphid-host interaction (Thorpe et al., 2016; Boulain et al., 2019). One of these unique species-specific proteins is diacetyl/L-xylulose reductase (DCXR) that was identified in the cowpea aphid saliva. Identification and characterization of DCXR is described in Chapter One.

Plant Defense – Pattern Triggered Immunity

Plants lack an adaptive immune system and must rely on their innate immune system to defend themselves (Jones and Dangl, 2006; Andolfo and Ercolano, 2015). Plants innate immune system employs a tiered response to pathogens. This tiered immunity is referred to as the zig-zag model (Jones and Dangl, 2006). This model is believed to expand to more than just pathogens and encompass plant interactions with pests such as aphids (Jaouannet et al., 2014). In the zig-

zag model, plants utilize a large number of pattern recognition receptors (PRRs) located on the plant cell surface for surveillance (Zipfel et al., 2006). These PRRs recognize conserved molecules that originate from the pathogen or pest called herbivore-associated molecular patterns (HAMPs). The recognition of the HAMP by the PRR leads to the initiation of pattern triggered immunity (PTI) by the plant to protect itself (Jones and Dangl, 2006; Boller and Felix, 2009; Andolfo and Ercolano, 2015). Activation of PTI can result in chemical defenses like production of reactive oxygen species (ROS), structural defenses like callose deposition, and downstream signaling like activation of MAPK cascades and defense-related transcription factors (Zipfel, 2009; Macho and Zipfel, 2014; Li et al., 2016).

One of the most studied microbe-associated molecular patterns (MAMPs) in PTI is flg22, a peptide present in the bacterial flagellar protein (Felix et al., 1999; Gomez-Gomez et al., 1999). Flg22 is detected by FLAGELLIN SENSITIVE2 (FLS2), a transmembrane receptor kinase with an extracellular leucine-rich repeat (LRR) domain (Gomez-Gomez and Boller, 2000). The detection of flg22 leads to the FLS2 to form a complex with the co-receptor BRASSINOISTERIOD INSENSITVE-ASSOCIATED KINASE1 (BAK1) (Chinchilla et al., 2007; Heese et al., 2007). This FLS2-BAK1 complex initiates a signaling pathway that leads to the induction of ROS bursts, activation of MAPK cascade, and callose deposition all hallmarks of PTI (Felix et al., 1999; Gomez-Gomez et al., 1999; Lu et al., 2010; Kadota et al., 2014; Li et al., 2014).

Plant Defense – Hormone Signaling

Phytohormones are small molecules that are integral for plant development processes as well as signaling molecules for defense and immunity (Shigenaga and Argueso, 2016). There are two major defense phytohormones pathways in plants. They are the salicylic acid (SA) pathway and the jasmonic acid (JA)/ethylene (ET) pathway (Delaney et al., 1994; Penninckx et al., 1998; Shigenaga and Argueso, 2016). The SA pathway provides defense against both biotrophs and hemibiotrophs while the JA/ET pathway provides defense against both necrotrophic pathogens and chewing herbivores (Glazebrook, 2005; Berens et al., 2017).

SA is synthesized in plants from chorismate through two different pathways, the phenylalanine ammonium lyase (PAL) pathway and the isochorismate (IC) pathway (Dempsey et al., 2011). The synthesis of SA in the PAL pathway is found in the cytosol while the IC pathway SA synthesis occurs in the chloroplasts (Catinot et al., 2008; Shine et al., 2016; Berens et al., 2017). Plants employ the IC pathway to generate SA in response to pathogens (Dempsey et al., 2011). When SA levels increase, SA binds to NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1), a SA receptor and the central regulator of SA signaling (Wu et al., 2012; Li et al., 2019b). The NPR1 paralogues NPR3 and NPR4 are also SA receptors that bind SA with different affinities (Fu et al., 2012). The binding of SA to NPR1, leads to NPR1 undergoing a conformational change from oligomer to monomer (Mou et

al., 2003). The now active monomers are able to translocate to the nucleus to activate transcription factors (Kinkema et al., 2000). NPR1 lacks a DNA binding domain so it cannot bind to the DNA directly, but its transactivation domain can interact with transcription factors like the TGACG transcription factor family that are members of basic leucine zipper (bZIP) transcription factors to initiate plant defense responses (Despres et al., 2000; Johnson et al., 2003)

ET is a gaseous hormone that is produced from the amino acid methionine. Methionine undergoes multiple reactions known as the Yang cycle to produce ET (Bradford, 2008). When ET is accumulated, it is recognized by multiple ET endoplasmic reticulum (ER)-localized receptors which act as negative regulators in the ethylene signaling pathway (Ju and Chang, 2015; Li et al., 2019b). Once ET binds, the key positive regulator of the ET pathway, ETHYLENE-INSENSITIVE2 (EIN2), is dephosphorylated due to the inactivation of CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) (Alonso et al., 1999; Ju et al., 2012; Qiao et al., 2012). The dephosphorylated EIN2's C-terminal domain is released to enter the nucleus to activate the transcription factor *ETHYLENE-INSENSITIVE3* (*EIN3*) for activation of ET-regulated genes (Merchante et al., 2013; Shigenaga and Argueso, 2016).

ET was identified to work synergistically with JA in immune responses through expression analyses of the JA defense marker gene *PLANT DEFENSIN1.2* (*PDF1.2*) (Pre et al., 2008). *PDF1.2* was induced by the infection of necrotrophic pathogens as well as exogenous applications of either ET or JA

(Penninckx et al., 1996; Penninckx et al., 1998). The application of both hormones at the same time led to the higher expression of ET and JA-related genes than either hormone alone (Penninckx et al., 1998). ET and JA pathways can work independently of each other. The JA induced gene *VEGETATIVE STORAGE PROTEIN2* (*VSP2*), was found expressed independent of ET relying on the transcription factor *MYC2* pathway instead (Norman-Setterblad et al., 2000).

JA is generated through the conversion of α-linoleic acid from chloroplast membranes that is oxygenated by Lipoxygenase (LOX) enzymes (Wasternack and Hause, 2013). JA levels are controlled in plants through various modifications like hydroxylation, decarboxylation, glycosylation, and methylation (Staswick and Tiryaki, 2004; Suza and Staswick, 2008; Li et al., 2019b). The biologically active conjugate of JA in plants is jasmonic acid-isoleucine (JA-Ile) that is produced from the enzyme JA AMIDO SYNTHETASE1 (JAR1) (Staswick and Tiryaki, 2004).

In the absence of JA-Ile, JASMONATE ZIM-DOMAIN (JAZ) proteins are bound to transcription factor *MYC2*, repressing the JA signaling pathway. JAZ also binds to the bridging protein NOVEL INTERACTOR OF JAZ (NINJA) that recruits another repressor of JA protein, TOPLESS (TPL) (Pauwels et al., 2010). Upon JA induction, JA-Ile binds to the E3 ubiquitin ligase CORONATINE INSENSITIVE1 (COI1) initiating the formation of the COI1-JAZ complex. Ubiquitylated JAZ undergoes degradation via the 26S proteasome (Thines et al.,

2007). Once the JAZ proteins are degraded, MYC2 is now active to initiate JA-responsive genes (Shigenaga and Argueso, 2016; Li et al., 2019b).

There is extensive crosstalk between the different phytohormone signaling pathways. Plant defense responses to pests and pathogens are energy consuming. To account for this, plants employ a specific defense for distinct pest or pathogen (Li et al., 2019b). There is no single plant hormone for mediating all plant immune responses to pests or pathogens, but rather, an interconnected response referred to as hormonal crosstalk (Shigenaga and Argueso, 2016). The two main defense pathways, SA and ET/JA are mostly known for being antagonistic to each other although some cooperation between the two pathways exists. For example, expression of the SA induced transcription factor, *WRKY70*, suppressed the JA-induced *PDF1.2* expression (Li et al., 2004; Li et al., 2006). JA can also lead to the suppression of SA. Deletion of the JA receptor, *COI1*, led to increased accumulation of SA and enhanced resistance to *Pseudomonas syringae* (Spoel and Dong, 2008; Li et al., 2019b).

In contrast, SA and JA have been reported to have synergistic effects with each other. This is especially true at lower concentrations. In both Arabidopsis (*Arabidopsis thaliana*) and tobacco (*Nicotiana tabacum*) when treated with low concentrations of SA and JA, marker genes for both pathways (*PR1* and *PDF1.2*) were more highly expressed. This synergy was lost, and antagonism returned at higher concentrations of the phytohormones (Mur et al., 2006). In tobacco co-application of SA and JA analogs led to higher expression of the SA pathway

gene *PR1* (Xu et al., 1994). Transcriptomic analysis in Arabidopsis using SA and JA analogs also identified synergy between the pathways. About 55 genes were co-induced by both SA or JA treatment while only 8 genes were found to upregulated by one and repressed by the other (Schenk et al., 2000).

Perception of Aphid MAMPs/Elicitors

Plants also utilize PTI to defend themselves against aphids and other herbivores. The PRRs that detect aphids are currently unknown, but it is known that the co-receptor *BAK1* is required to detect aphids or aphid symbiont-derived patterns (Chaudhary et al., 2014; Prince et al., 2014). *BAK1* was found to be necessary for the perception of the *B. aphidicola* derived HAMP, GroEL (Chaudhary et al., 2014). *B. aphidicola* GroEL was identified as an elicitor of plant defense after being detected in the potato aphid saliva. The secretion of GroEL was unexpected as it was not predicted for secretion and is not originating from the aphid but from its primary endosymbiont (Chaudhary et al., 2015). Even more surprising than its presence in the saliva was the discovery that it was working against the aphid triggering plant defense responses against the aphid (Chaudhary et al., 2014; Elzinga et al., 2014). Expressing GroEL in tomato (*Solanum lycopersicum*) and Arabidopsis, the fecundity of both the potato aphid and green peach aphid, pests of tomato and Arabidopsis, respectively, decreased (Chaudhary et al., 2014). Infiltration of GroEL into wildtype Arabidopsis produced multiple hallmarks of PTI including callose deposition,

ROS bursts, and defense gene upregulation. These hallmarks were lost in mutant Arabidopsis plants lacking *BAK1* (*bak1*) (Chaudhary et al., 2014). When aphid survival assays were performed on *bak1* mutants, aphid species that Arabidopsis is normally a non-host for, survived longer than they did on wildtype plants. This suggests that the *bak1* mutants lacked or had reduced PTI responses to the aphid (Prince et al., 2014).

Besides GroEL, aphid-derived elicitors of plant defense have been identified by evaluating host PTI responses to aphid saliva or by expressing salivary gland transcripts *in planta*. Most of these elicitors have been identified from studies with the model plant, Arabidopsis. One of the first identified aphid elicitors was found by collecting green peach aphid saliva *in vitro* and syringe infiltrating it into Arabidopsis leaves (De Vos and Jander, 2009). The effects were evaluated by monitoring aphid life performance as well expression of key defense marker genes. Using size fractionated saliva, it was found that the size fraction between 3-10 kDa led to decreased aphid fecundity and activated *O-methyltransferase* genes that could be synthesizing glucosinolates as an aphid-repellent.

Additional aphid elicitors were identified from the green peach aphid by bioinformatic analysis of salivary gland expressed sequence tags (ESTs) and transiently expressing them *in planta* (Bos et al., 2010). About 48 candidate salivary gland ESTs were cloned into binary vectors and expressed in *N. benthamiana* leaves using agrobacterium-mediated transient overexpression.

Overexpression of one of these ESTs, Mp10, led to chlorosis, indicating a role as an elicitor. This role was confirmed by evaluating aphid fecundity on leaf disks overexpressing Mp10 which led to lower aphid fecundity compared to empty vector controls (Bos et al., 2010). The chlorosis phenotype and enhanced resistance to aphids suggests an enhanced immune response which was shown not to be aphid specific. *Nicotiana benthamiana* plants transiently expressing Mp10 had a decreased susceptibility to the oomycete plant pathogen *Phytophthora capsica* (Rodriguez et al., 2014). Both confocal microscopy of leaves overexpressing transiently N-terminal GFP tagged, GFP-Mp10, or using immunogold-labeling of Mp10 antibody in ultrathin sections of aphid infested leaves, found that Mp10 is localized to the plant cell cytoplasm (Rodriguez et al., 2014; Mugford et al., 2016).

Another aphid derived elicitor is Mp42. Similar to Mp10, agrobacterium-mediated transient overexpression of Mp42 in *N. benthamiana* also decreased aphid fecundity. Mp42 did this more subtly, as its overexpression did not lead to chlorosis (Bos et al., 2010). The contrast between Mp10 and Mp42 is also seen in plant signaling. While Mp10 led to upregulation of defense marker genes in both the SA and JA signaling pathways, Mp42 did not upregulate any of the defense-related genes tested. Subcellular localization of N-terminal GFP tagged Mp42, GFP-Mp42, found it to mostly localize to the plant cell plasma membrane and partly colocalizing to the ER (Rodriguez et al., 2014). The differences observed between transient overexpression of Mp10 and Mp42 indicate that they

are triggering different types of defense responses (Bos et al., 2010; Rodriguez et al., 2014).

Two more aphid elicitors were identified from a screen of nine green peach aphid salivary proteins. The proteins were originally identified from the green peach aphid's salivary proteome (Harmel et al., 2008). In transient agrobacterium-mediated overexpression in tobacco, no change in aphid fecundity was found for the majority of the proteins. However, three of the proteins screened, Mp57, Mp58, and the known aphid HAMP GroEL, were found to decrease aphid fecundity (Chaudhary et al., 2014; Elzinga et al., 2014). These results were further confirmed by expressing each of these candidate proteins by stable transformation of Arabidopsis (Elzinga et al., 2014). The result with Mp58, the green peach aphid homolog for the potato aphid effector Me10, was unexpected because expression of Me10 in tomato had been shown to lead to an increase in potato aphid fecundity (Atamian et al., 2013). This indicates that there is a difference in the tools utilized by different aphid species to manipulate their species-specific hosts.

Aphids do not only interface with plants through feeding, they also excrete waste products onto plants known as honeydew (Auclair, 1963). Honeydew consists of mainly sugars and amino acids. A recent study, using 2D-PAGE gels and protein sequencing, showed that aphid honeydew also contains proteins derived from both the aphid and symbiotic bacteria. The identified bacterial proteins were from the primary endosymbiont *B. aphidicola* as well as secondary

symbionts like *Serratia symbiotica* (Sabri et al., 2013). Of the over 140 protein spots of the honeydew visualized on the 2D-PAGE gel, two of the most notable proteins identified were the bacterial proteins, flagellin and Ef-tu, the two highly documented microbial elicitors of plant defense (Kunze et al., 2004; Chinchilla et al., 2006; Sabri et al., 2013). The known *Buchnera*-derived HAMP GroEL was also identified in the honeydew (Sabri et al., 2013).

Plant Signaling and Defenses to Aphids

Recognition of pests and pathogens by PRR requires co-receptors and membrane localized or associated proteins. One of the membrane-associated protein kinases that involved in aphid and microbial pathogen immune responses, is *BOTRYTIS-INDUCED KINASE1* (*BIK1*). At the resting state, another protein, NUCLEAR SHUTTLE PROTEIN INTERACTING KINASE (NIK1), associates with either BAK1 or FLS2 to prevent autoimmune responses (Li et al., 2019a). After dimerization, BAK1 autophosphorylates and transphosphorylates BIK1 which in turn transphosphorylates the BAK1/FLS2 complex leading to a signaling cascade that results in oxidative burst and defense genes induction (Lu et al., 2010; Lin et al., 2014). In addition to its role against microbial plant pathogens, *BIK1* was found to act as a negative regulator of defense responses against aphids (Lei et al., 2014). Arabidopsis*, bik1* and *bak1* mutants, have displayed opposite aphid growth phenotypes. The *bak1* mutants were more susceptible to green peach aphids as well as to the non-host

pea aphids, compared to wild type (Prince et al., 2014). In contrast, the *bik1* mutants were more resistant to green peach aphids, exhibiting lower aphid fecundity levels compared to wildtype (Lei et al., 2014; Prince et al., 2014). The heightened resistance in the *bik1* mutants is likely due to *bik1* having enhanced H_2O_2 production and increased cell death phenotypes. This *bik1* heightened aphid resistance was found to be dependent on *PHYTOALEXIN DEFICIENT4* (*PAD4*) (Lei et al., 2014). In the *bik1 pad4* double mutant plants, the resistance seen in the *bik1* plants to the green peach aphid was lost (Lei et al., 2014). *PAD4* encodes a lipase-like protein and its molecular partner *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) are well known for their role in defense against microbial pathogens and aphids (Jirage et al., 1999; Pegadaraju et al., 2005; Pegadaraju et al., 2007; Louis et al., 2012; Louis and Shah, 2015).

In Arabidopsis, both *BAK1* dependent and independent defense responses exist. *PHYTOALEXIN DEFICIENT3* (*PAD3*) was identified to induce defenses that are independent of *BAK1* (Prince et al., 2014). The identification of *PAD3* occurred while monitoring the effect aphid infestations on microRNA pathways (Kettles et al., 2013). *PAD3* encodes a cytochrome P450 that catalyzes the conversion of dihydrocamalexic acid to camalexin, the major phytoalexin in Arabidopsis (Schuhegger et al., 2006). Green peach aphids had increased fecundity on multiple Arabidopsis mutants that were unable to produce camalexin such as *pad3* and *cyp79b2/cyp79b3* (deficient in camalexin and indole glucosinolate). Camalexin was confirmed to be the inhibiting compound when

aphids feeding on artificial diets supplemented with camalexin led to a decrease in aphid fecundity (Kettles et al., 2013).

Secondary messengers like Ca^{2+}, present throughout eukaryotes, have been found to play an important role in perception of aphids by plants (Knight et al., 1991; Blume et al., 2000; Will and van Bel, 2006; Vincent et al., 2017). The real time Ca^{2+} dynamics have been recorded in Arabidopsis after green peach aphid infestation using the fluorescent calcium biosensor, GCamp3 (Vincent et al., 2017). The transient rise in cytosolic Ca^{2+} first occurred during aphid probing with its stylets. This rise in cytosolic Ca^{2+} was amplified through BAK1 perception of the aphid leading to Ca^{2+} entering cells from the apoplast through membrane ion channels, GLUTAMATE RECEPTOR-LIKE3.3 and 3.6 (GLR3.3, GLR3.6), and from within the cell from the vacuolar endomembrane channel, TWO-PORE CHANNEL1 (TPC1) (Mousavi et al., 2013; Vincent et al., 2017).

Ca^{2+} signaling in the Fabaceae plant family leads to sieve element occlusion through P-proteins bodies called forisomes (Peters et al., 2006). Forisomes, when not in use are in narrow spindle shape, but Ca^{2+} induced signaling led to 30% reduction in length and a 2-fold increase in width of the forisomes to form a plug (Knoblauch and Peters, 2004). When monitoring aphid feeding on fava bean (*Vicia faba*) through electrical penetration graphs (EPGs), forisome occlusion was detected from multiple aphid species. Two generalist aphids, potato aphid and green peach aphid, triggered forisome occlusion leading to the eventual withdrawal of the aphid stylets (Medina-Ortega and

Walker, 2013). However, forisome occlusion was not detected when the specialized pea aphid fed on fava bean. The pea aphid was able to readily ingest phloem sap, suggesting that during feeding the pea aphid is actively suppressing occlusion, possibly through its saliva (Will et al., 2007; Walker and Medina-Ortega, 2012; Medina-Ortega and Walker, 2013; Nalam et al., 2019).

Synthesis of callose, a β-1, 3-glucan, is another PTI hallmark that occurs through aphid infestation (Verma and Hong, 2001). Like forisomes, deposition of callose occurs through Ca^{2+} signaling and acts as a plug for the sieve element (Kauss, 1987; Nalam et al., 2019). Interestingly, callose deposition occurs in response to leaf infiltrations of both aphid body extracts and aphid saliva (Elzinga et al., 2014; Prince et al., 2014; Chaudhary et al., 2015; Naessens et al., 2015). In addition, callose deposition has been reported in aphid resistance. Resistant melon plants with the *Vat* resistance gene for the melon aphid (*Aphis gossypii*), develops faster callose deposits after aphid infestation compared to susceptible melon plants without *Vat* (Villada et al., 2009).

Aphid infestation of plants also influences phytohormone accumulation. The influence on phytohormones is complex and contradictory in different studies. This is most likely due to specific plant-aphid interactions, the length of the plant-aphid interaction studied, and the intricate crosstalk between the phytohormone pathways. In some studies aphid feeding has been found to induce SA phytohormone accumulation contrary to other insect species (Walling, 2000; Züst and Agrawal, 2016). Typically, SA is more associated with biotrophic

pathogens while insects, particular chewing insects, induce JA phytohormone accumulation (Glazebrook, 2005; Berens et al., 2017). As SA and JA are natural antagonists, aphids could be inducing SA to get a more favorable response from the plant (Walling, 2000; Züst and Agrawal, 2016). There are contradictory reports on the effects of exogenous application of SA analogs on aphids. One study reports the exogenous application of SA analogs lead to no enhanced aphid resistance (Moran and Thompson, 2001). In contrast, other studies have found that exogenous application of SA analogs negatively altered aphid behavior by increasing dispersal and in field testing SA was an effective treatment for reducing aphid populations (Elhamahmy et al., 2016; Coppola et al., 2018). There are also contradictory reports about the effect of Arabidopsis hormone related mutants on aphids. One study reported no effect on green peach aphid population fed on *npr1* mutant compared to wild type, while another study found that it led to a smaller population (Moran and Thompson, 2001; Mewis et al., 2005). Exogenous application of JA has been found to be detrimental to aphids, impairing population growth (Cooper and Goggin, 2005). Arabidopsis mutants with constitutive JA signaling were more resistant to green peach aphids compared to wild type while mutants that were insensitive to JA signaling were more susceptible (Ellis et al., 2002). In tomato, both SA and JA pathways were upregulated after potato aphid infestation. The JA pathway related gene was upregulated early after infestation (6 and 12 hours) while the SA pathway related gene was upregulated at later times (24 and 48 hours)

(Martinez de Ilarduya et al., 2003). This supports the crosstalk between the phytohormone pathways being integral in the plant-aphid interaction and the complexity of phytohormones being induced by the aphid.

In addition to hallmarks of PTI and phytohormones, aphid perception by plants leads to the synthesis of multiple secondary defense metabolites (Dreyer et al., 1985; Güntner et al., 1997; Halkier, 2006; Kim and Jander, 2007; Züst and Agrawal, 2016; Nalam et al., 2019). Among these secondary metabolites produced in response to aphids are cardiac glycosides (cardenolides). Cardenolides are steroidal compounds that are inhibitors of animal Na^+/K^+ ATPases and are present in the phloem (Agrawal, 2004). Interestingly, though toxic, some aphid species have found a way to utilize these cardenolides for their own defenses against parasitoids (Desneux et al., 2009).

Another secondary metabolite that plants utilize to defend themselves against aphids are alkaloids. Alkaloids are diverse nitrogen containing compounds present in 20-30% of higher plant species (Roberts and Wink, 1998). Alkaloids have negative impacts on DNA replication, protein synthesis, and neurotransmission (Dreyer et al., 1985; Güntner et al., 1997; Züst and Agrawal, 2016). Different alkaloids have been found to have different effects on aphids. Aphids feeding on artificial diet containing pyrrolizidine had little effect, while indolizidine and quinolizidine were both found to strongly deter aphids (Dreyer et al., 1985). Ingestion of both cardenolides and alkaloids by aphids are similarly processed; the apolar cardenolides and alkaloids accumulate inside aphids while

polar cardenolides and alkaloids are excreted in the honeydew (Züst et al., 2015; Züst and Agrawal, 2016).

Benzoxazinoids are another type of secondary metabolite synthesized by plants to deter aphids. Unlike cardenolides and alkaloids, benzoxazinoids require activation for them to be toxic. Benzoxazinoids are enzymatically activated when plant tissue damage occurs (Zúñiga et al., 1983). 2, 4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (DIMBOA-Glc) is a benzoxazinoid, that is highly abundant in maize, wheat (*Triticum* spp.) and other members of the Poaceae family (Meihls et al., 2013), and is broken down by glucosidases into different insect deterrent compounds (Grambow et al., 1986; Meihls et al., 2013; Kokubo et al., 2017). Not only is DIMBOA toxic for aphids, but also acts as a signaling molecule for callose deposition (Ahmad et al., 2011).

Like benzoxazinoids, glucosinolates are a plant secondary metabolite that are not present in a toxic form. Glucosinolates are a major defensive compound found in the plant family Brassicaceae and are activated by a myrosinase (Kim and Jander, 2007). Green peach aphids feeding on the glucosinolate singrin, in an artificial diet, led to no effect on the aphids. When the enzyme myrosinase was included in the diet with singrin, the aphid fecundity decreased (Kim and Jander, 2007). Unlike singrin, an aliphatic glucosinolate, indole glucosinolates are less stable and do not require an enzyme (myrosinase) to activate. These glucosinolates negatively affect green peach aphids in the absence of myrosinase (Kim and Jander, 2007; Kim et al., 2008). Interestingly, plant

secondary metabolites like benzoxazinoids and glucosinolates that require activation seem to be less efficient in defending against aphids than ones that are already in their active form (Züst and Agrawal, 2016).

A metabolite that is emerging as a novel defense signaling molecule in plants is methylglyoxal (Hoque et al., 2016; Li, 2016; Mostofa et al., 2018). Originally, methylglyoxal was known as a toxic metabolite at high concentrations, but it was found to be acting as a signaling molecule at lower concentrations for a number of abiotic stresses (Yadav et al., 2005; Borysiuk et al., 2018). Only recently has methylglyoxal levels been discovered to increase in response to various types of biotic stresses, including to viruses, bacteria, and fungi (Melvin et al., 2017). Another one of these biotic stresses that has been found to have an effect on methylglyoxal accumulation is the cowpea aphid. This work is described in Chapter One.

Methylglyoxal

1. Generation

Methylglyoxal is a highly toxic α,β-dicarbonyl ketoaldehyde that leads to a number of adverse effects in cells. Carbonyls such as methylglyoxal are reactive and lead to the formation of advanced glycation end products (AGEs) (Li et al., 2019a). Methylglyoxal acts as a glycating agent leading to the formation of AGEs through modification of basic positive amino acids like arginine (Thornalley, 1996; Bilova et al., 2016). Increased methylglyoxal and AGEs lead to irreversible

damage of the proteome and in humans are associated with the effects of aging and a number of diseases (Thornalley, 2006; Ahmed and Thornalley, 2007; Rabbani et al., 2016). Similarly, in plants, the increased methylglyoxal and AGEs generate irreversible damage of the proteome that can contribute to growth retardation if the methylglyoxal level exceeds the plant's detoxification capabilities (Borysiuk et al., 2018). Like other secondary metabolites, plants have a mechanism to prevent self-toxicity from methylglyoxal. The primary route of methylglyoxal detoxification in plants is through the glyoxalase pathway (Thornalley, 1990).

Formation of methylglyoxal in plants occurs through multiple different ways. The most prevalent method of methylglyoxal generation is by the spontaneous breakdown of the triose phosphates generated in glycolysis, glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) (Phillips and Thornalley, 1993; Richard, 1993; Kaur et al., 2014; Li, 2016). Both G3P and DHAP are unstable even at physiological conditions and beta eliminations of the phosphate groups lead to the formation of methylglyoxal (Richard, 1993; Sousa Silva et al., 2013; Li, 2016). During stress, the rate of glycolysis is increased, forming more G3P and DHAP that can spontaneously breakdown and increase the methylglyoxal concentration (Li, 2016). Not only is methylglyoxal generated spontaneously by G3P and DHAP, but it is also generated as a by-product through the enzymatic interconversion of G3P and

DHAP by triosephosphate isomerase (TPI) (Phillips and Thornalley, 1993; Richard, 1993).

Methylglyoxal can also be formed by additional multiple enzymatic reactions. One of these reactions generates methylglyoxal from DHAP though the activity of methylglyoxal synthase (Hopper and Cooper, 1971; Li, 2016). Acetone monooxygenase is another enzyme that generates methylglyoxal while converting acetone to acetol (Casazza et al., 1984). The last enzymatic pathway that leads to the generation of methylglyoxal is the aminoacetone pathway, where semicarbazide-sensitive amine oxidase (SSAO) converts aminoacetone into methylglyoxal (Yu, 1990). Besides enzymatic generation, methylglyoxal is also generated in the cell without enzymes is by the auto-oxidation of either ketone bodies or sugars through the Maillard reaction (Sousa Silva et al., 2013; Mostofa et al., 2018).

2. Signaling

While methylglyoxal is cytotoxic at high concentrations, it has recently been discovered to act as a signaling molecule for abiotic and biotic stresses (Hoque et al., 2016; Li, 2016; Mostofa et al., 2018). Methylglyoxal was first found to accumulate under multiple abiotic stresses like drought and salt stresses (Yadav et al., 2005). Recently, methylglyoxal was discovered to also accumulate in response to multiple biotic stresses including to viral, bacterial and fungal infections (Melvin et al., 2017). The signaling basis of methylglyoxal was

explored by exogenously applying methylglyoxal to rice (*Oryza sativa*) and evaluating gene expression using a microarray analysis (Kaur et al., 2015). Almost half of the genes that were differentially regulated were transcription factors, including upregulation of transcription factors known to be active in plant defense to abiotic and biotic stresses, like, the WRKY transcription factors (Chen et al., 2012; Kaur et al., 2015).

Besides gene transcription, methylglyoxal can induce secondary messengers to initiate signaling cascades, including the well-known plant defense signaling molecule ROS (Torres et al., 2006). The generation of ROS by methylglyoxal is done in two ways, by directly generating ROS by acting as a Hill oxidant in photosystem I (Saito et al., 2011; Hoque et al., 2016), and indirectly, through the inhibition of mitochondrial electron transfer chain and other antioxidant enzymes (Chang et al., 2005; Desai et al., 2010; Saito et al., 2011; Hoque et al., 2016; Li, 2016). The increased oxidative stress caused by methylglyoxal accumulation can lead to Ca^{2+} oscillations in the guard cells, leading to stomatal closures (McAinsh et al., 1995; Hoque et al., 2012a; Hoque et al., 2012b).

Previously, methylglyoxal had only been explored for its toxicity in plants, but its beneficial roles were only recently been examined (Li et al., 2017a; Li et al., 2017b; Mostofa et al., 2018). Application of methylglyoxal to wheat seeds before exposure to salt stress alleviated the inhibitory effects of the salt. This tolerance is attributed to increased activities of glyoxalases as well as multiple

antioxidant enzymes due to methylglyoxal exposure (Li et al., 2017a). Similarly, priming with methylglyoxal was also found to alleviate cadmium toxicity in wheat (Li et al., 2017b).

3. Detoxification

The glyoxalase pathway is present in a multitude of organisms, ranging from plants, yeast, mammals, and even protozoa (Mannervik et al., 1982; Rhee et al., 1986; Norton et al., 1990; Maiti et al., 1997; Bito et al., 1999). The role of the glyoxalase pathway is to detoxify various dicarbonyls and toxic aldehydes, but the primary substrate is believed to be the toxic methylglyoxal (Thornalley, 1990). Methylglyoxal is cytotoxic at high concentrations and to deal with this cytotoxicity, plants utilize the glyoxalase system to detoxify methylglyoxal (Thornalley, 1990). The glyoxalase system consists of two different pathways, a glutathione (GSH) dependent pathway and a GSH independent pathway. The GSH dependent pathway consists of two enzymes, glyoxalase I (GLXI) and glyoxalase II (GLXII). The spontaneous reaction of methylglyoxal and GSH forms hemithioacetal, which then can be converted by GLXI to form S-lactoylglutathione. GLXII then converts S-lactoylglutathione to D-lactate, releasing the GSH. The D-lactate generated is oxidized into pyruvate and undergoes cellular respiration (Engqvist et al., 2009; Wienstroer et al., 2012; Hoque et al., 2016).

There are 11 GLXI-like proteins in Arabidopsis but only three of these genes encode active GLXI (Kaur et al., 2013; Jain et al., 2016; Schmitz et al.,

2017). *GLXI*'s are classified based on the metal ion they are dependent on (Schmitz et al., 2017; Schmitz et al., 2018). Two of the active GLXIs, GLXI;1 and GLXI;2, are in the first subclass of GLXI and are Ni^{2+} dependent. The third GLXI, GLXI;3, is in the second subclass and is Zn^{2+} dependent (Schmitz et al., 2017). GLXI;1 is localized to the chloroplasts, while the other two GLXI isoforms are localized to the cytosol. While all three active GLXIs are able to detoxify methylglyoxal, the predominant isoform involved in detoxification was identified to be GLXI;3 through the use of T-DNA mutants (Schmitz et al., 2017).

Another Arabidopsis GLXI has been identified as a putative regulator of methylglyoxal (GLXI;4) and cross talk between SA and JA hormone pathways. While GLXI;4 has no detoxifying activity, and a *glxi;4* T-DNA mutant was found to still accumulate methylglyoxal (Proietti et al., 2019; Proietti et al., 2018), *GLXI;4* expression is induced in response to multiple abiotic and biotic stresses (Mustafiz et al., 2011; Proietti et al., 2018). Additionally, when the *glxi;4* mutant was exposed sequentially to methyl-jasmonate (MeJA) and SA, it was found to be insensitive to SA (Proietti et al., 2019). The wildtype plants exposed to MeJA and SA led to suppression of the JA-dependent gene, *PDF1.2*, but *PDF1.2* was not suppressed in the *glxi;4* mutant (Proietti et al., 2018). Moreover, the *glxi;4* mutant exhibited no infectivity difference, compared to wildtype, to the SA inducing bacterial pathogen *Pseudomonas syringae pv. tomato* (*Pst*) DC3000 (Proietti et al., 2019). Interestingly, the mutant was discovered to behave differently when infected with JA inducing pathogens. While the *glxi;4* mutant was more

susceptible to *Plectosphaerella cucumerina*, it was more resistant to *Botrytis cinerea,* both necrotrophic fungal pathogens (Proietti et al., 2018; Proietti et al., 2019).

In Arabidopsis, there are five genes that encode for the second gene in the methylglyoxal detoxification pathway (*GLXII*) (Maiti et al., 1997; Marasinghe et al., 2005; Schmitz et al., 2017). Two of the five GLXII-like proteins, GLXII;1 and GLXII;3, do not have GLXII activity. GLXII;1 has β-lactamase activity and responds to abiotic stress conditions while GLXII;3, has been found to be a persulfide dioxygenase (Holdorf et al., 2012; Devanathan et al., 2014). The other three genes have been confirmed to be active GLXIIs. GLXII;2 is localized to the cytosol while both GLXII;4 and GLXII;5 are localized to the chloroplasts and mitochondria (Schmitz et al., 2017). Like GLXI, GLXIIs depend on a metal ion; GLXII;2 contains a Zn^{2+} center and GLXII;5 has a $Fe^{3+}Zn^{2+}$ center (Crowder et al., 1997; Maiti et al., 1997; Marasinghe et al., 2005). GLXII;4 is predicted to also have a Zn^{2+} center based on sequence similarities, but no studies have yet confirmed this.

The GSH independent methylglyoxal detoxification pathway consists of a single glyoxalase III (GLXIII) enzyme. GLXIII is able to directly convert methylglyoxal into D-lactate without the need for GSH or a metal ion (Misra et al., 1995). Instead, GLXIII depends on an active site that is composed of a conserved amino acid triad, his-cys-glu (Misra et al., 1995; Subedi et al., 2011). In Arabidopsis there are six *GLXIII*-like genes that encode 11 proteins (Ghosh et

al., 2016). All Arabidopsis GLXIII-like proteins contain two DJ-1 domains differing from *E. coli*, Drosophila, and human GLXIII-like proteins that contain only one DJ-1 domain (Ghosh et al., 2016). In Arabidopsis, *GLXIII*-like gene *AtDJ-1A* is induced by multiple abiotic stresses (Xu et al., 2010). Purified AtDJ-1A showed activity for both methylglyoxal and glyoxal (Xu et al., 2010; Kwon et al., 2013). The importance of *AtDJ-1A* was demonstrated with loss of function T-DNA mutants that had a phenotype of accelerated cell death in aging plants (Xu et al., 2010). The enzymatic activity of the other AtDJ-1 proteins was also explored. Of all the DJ-1 proteins purified and screened, AtDJ-1D was found to have the highest specific activity for both methylglyoxal and glyoxal. The other AtDJ-1 proteins that had any activity with both substrates, were AtDJ-1A and AtDJ-1B but they were less active than AtDJ-1D and had more specificity for glyoxal. Functional activity of the AtDJ-1D was confirmed in a heterologous complementation assay. Expressing AtDJ-1D in an *E. coli* strain lacking endogenous glyoxalases, the complemented *E. coli* strain grew on methylglyoxal and glyoxal infused media similar to wildtype, indicating successful complementation of the glyoxalase phenotype (Kwon et al., 2013).

In addition to the glyoxalase pathways, NAD(P)H dependent oxidoreductases and dehydrogenases can detoxify methylglyoxal by conversion to either acetol or lactaldehyde (Yamauchi et al., 2011; Sengupta et al., 2015). The acetol or lactaldehyde can undergo further enzymatic reactions that result to pyruvate like products at the end of the glyoxalase system (Engqvist et al., 2009;

Wienstroer et al., 2012; Hoque et al., 2016; Welchen et al., 2016). Two additional enzymes are known to detoxify methylglyoxal, methylglyoxal reductase and methylglyoxal dehydrogenase but both of these enzymes have not been yet reported in plants (Ray and Ray, 1982; Inoue et al., 1988; Mostofa et al., 2018). One of these NAD(P)H dependent oxidoreductases is diacetyl/L-xylulose reductase (DCXR). In mammals, *DCXR* is highly expressed in the kidney and plays a role in removal of renal carbonyl compounds (Nakagawa et al., 2002; Odani et al., 2008; Ebert et al., 2015; Perco et al., 2019).

Diacetyl/L-Xylulose Reductase (DCXR)

DCXR is a multifunctional enzyme that is member of the short-chain dehydrogenase/reductases that is able to catalyze multiple carbohydrates including xylitol and is able to detoxify toxic carbonyls (Nakagawa et al., 2002; Ebert et al., 2015; Yang et al., 2017). DCXR was first discovered when defective DCXR in humans was identified as the cause of the benign condition of pentosuria (high excretion of L-xylulose in urine) (Wang and Van Eys, 1970; Ebert et al., 2015; Perco et al., 2019). In mammals, DCXR plays a key role in glucuronic acid/uronate cycle by the interconversion of L-xylulose and xylitol, that after additional reactions can be utilized in the pentose phosphate pathway (Sochor et al., 1979; Yang et al., 2017). Besides carbohydrate metabolism, DCXR is involved in the detoxification of multiple toxic carbonyls including methylglyoxal, glyoxal, and 3-deoxygluxosone (Odani et al., 2008; Ebert et al.,

2015). The accumulation of the carbonyls leads to the formation of AGEs which are detrimental and contribute to multiple diseases in humans, like chronic kidney disease (Nakagawa et al., 2002; Ebert et al., 2015; Perco et al., 2019). In chronic kidney disease, patients with lower expression of *DCXR* had worse prognosis and more severe disease (Perco et al., 2019). Interestingly, DCXR was discovered in the saliva of the cowpea aphid by LC-MS/MS analysis. To check if the cowpea aphid homolog of DCXR (AcDCXR) was able to detoxify methylglyoxal, it was expressed in *E. coli* and the purified AcDCXR was used *in vitro* enzymatic analyses. This work is described in Chapter One.

Besides methylglyoxal detoxification, DCXR also catalyzes carbohydrates including polyols (sugar alcohols). Polyols are the reduced forms of aldose and ketose sugars. They are low weight, non-reducing and can be cyclic or linear (Lewis and Smith, 1967; Noiraud et al., 2001). In plants, polyols serve as carbon skeletons for long distance translocation from the source to sink organs to be used for energy. They are stable and are able to be translocated via the phloem without modification or degradation (Noiraud et al., 2001; Kalliampakou et al., 2011). Polyols accumulate in response to multiple abiotic stresses and can accumulate to high concentrations, up to millimolar range, in the cell without generating damage (Noiraud et al., 2001; Das et al., 2017).

A polyol that occurs naturally in some plants is xylitol. Xylitol can be converted to xylulose to enter the pentose phosphate pathway. Once in the pentose phosphate pathway, it can be used as a source of energy or for

production of pentoses and NADPH, depending on the needs of the cell (Kruger and von Schaewen, A. 2003). Since DCXRs are able to catalyze polyols, it was speculated that AcDCXR is able to catalyze xylitol, and in doing so may be providing the aphid with an additional source of energy. Chapter One describes expression of AcDCXR in *E. coli* and the use of the purified product in *in vitro* analysis to test its ability to catalyze xylitol to xylulose.

Aphid Effectors

To avert the various PTI responses by the plant, pests/pathogens deposit effectors inside the plant to suppress PTI. This suppression of PTI leads to effector triggered susceptibility (ETS) (Jones and Dangl, 2006). Saliva is the main means by which aphid effectors are deposited into the plant. Proteinaceous effectors have been identified through salivary gland transcriptomics and proteomics of the saliva (Mutti et al., 2008; Bos et al., 2010; Carolan et al., 2011; Atamian et al., 2013; Pitino and Hogenhout, 2013; Elzinga et al., 2014; Chaudhary et al., 2015; Thorpe et al., 2016; Boulain et al., 2018). Functional characterization of effectors has been performed by either altering effector expression by RNAi or through overexpression of the candidate effector in the plant hosts and assessing aphid performance (Mutti et al., 2008; Bos et al., 2010; Atamian et al., 2013; Pitino and Hogenhout, 2013; Elzinga et al., 2014; Abdellatef et al., 2015; Naessens et al., 2015; Wang et al., 2015b; Guy et al., 2016; Kettles and Kaloshian, 2016). Multiple aphid life performance parameters have been

studied, including changes in aphid lifespan (growth rate or survival) and aphid fecundity (reproduction). The effect on one of these parameters by the modification of candidate's expression level is compared to a control group to see if it led to an altered phenotype.

C002 is the first aphid effector that was discovered from the pea aphid (Mutti et al., 2006). The exact function of C002 in the plant remains unknown but when its expression is knocked down in the aphid with RNAi, EPG analysis showed that the feeding of the aphid is disrupted, leading to diminished aphid survival and fecundity (Mutti et al., 2006; Mutti et al., 2008; Pitino and Hogenhout, 2013; Zhang et al., 2015). Since the initial identification in pea aphid, *C002* homologs have been identified in the saliva of multiple aphid species (Harmel et al., 2008; Carolan et al., 2009; Nicholson et al., 2012; Chaudhary et al., 2015; Loudit et al., 2018). C002 was found to be undergoing positive evolutionary selection further confirming its role as an effector. This was determined by evaluating the ratio of the number of non-synonymous substitutions to the number of synonymous substitutions per site (DN/DS) (Thorpe et al., 2016). The role of C002 as an effector was further demonstrated by *in planta* analysis. Overexpression of C002 *in planta,* using agrobacterium-mediated transient expression, led to increased aphid fecundity. This increase in number of progeny was only seen in a species-specific manner, as the *C002* homolog of an aphid species only increased the fecundity of that species and not to any other species (Pitino and Hogenhout, 2013; Elzinga et al., 2014).

However, transient overexpression of C002 did not always enhance pea aphid performance; transient overexpression of C002 in pea (*Pisum sativum*) did not alter pea aphid fecundity (Guy et al., 2016). Using immunogold labeling of ultrathin sections of aphid infested plants, C002 was localized near the aphid stylets with the salivary sheaths inside the plant being more strongly labeled than other tissues in the plant. This has led to the speculation that C002 has role in the gelling saliva, likely functioning in the plant apoplast, and its presence in the watery saliva is due to its abundance and not being completely captured during the gelling process (Mugford et al., 2016).

Similar to C002, Mp1 and Mp2 are two aphid effectors that have been found to increase aphid fecundity in a species-specific manner when transiently overexpressed (Pitino and Hogenhout, 2013). Homologs of *Mp1* and *Mp2* have been discovered in several aphid species since their first discovery in the green peach aphid (Bos et al., 2010; Pitino and Hogenhout, 2013). Knockdown of these two aphid genes through plant mediated RNAi led to decreased aphid fecundity (Pitino and Hogenhout, 2013; Coleman et al., 2015). Mp1 is one of the best studied aphid effectors to date. Similar to C002, Mp1 is also localized to the area surrounding the stylets salivary sheath inside the plant tissues (Mugford et al., 2016). Unlike most aphid effectors, the function of Mp1 in the plant has been identified. Through yeast-two-hybrid (Y2H) screens, the Arabidopsis protein target of Mp1 was identified as the VACUOLAR PROTEIN SORTING ASSOCIATED PROTEIN52 (VPS52) (Rodriguez et al., 2017). While

overexpression of *Mp1* led to an increase in green peach aphid fecundity, overexpression of VPS52 reduced the aphid fecundity by about 40% (Pitino and Hogenhout, 2013; Rodriguez et al., 2017). This same interaction was not seen with Mp1 homologs from other aphid species and their host VPS52, indicating that Mp1-VPS52 interaction/binding is specific to green peach aphid and Arabidopsis (Rodriguez et al., 2017).

The only other aphid effector that has its function partially elucidated is Me10 (Atamian et al., 2013; Chaudhary et al., 2019). *Me10* was identified in the transcriptome of the potato aphid salivary glands as well as in the potato aphid saliva (Atamian et al., 2013; Chaudhary et al., 2015). Transient overexpression of Me10 was shown to increase the fecundity of green peach aphid and the potato aphid on *N. benthamiana* and tomato hosts, respectively (Atamian et al., 2013). In contrast, Mp58 the green peach aphid homolog of Me10, has been shown to lead to a decrease in green peach aphid fecundity when expressed in Arabidopsis (Elzinga et al., 2014). This difference is likely due to difference in overcoming different host plant defenses and the use of different infestation strategies. Nevertheless, DN/DS ratio indicated that Me10 is undergoing positive selection providing further evidence for its role as an effector (Thorpe et al., 2016).

Using Me10 specific polyclonal antibody in Western blot analysis, Me10 was shown to accumulate in aphid infested tomato leaves (Chaudhary et al., 2019). The tomato target of Me10 was also identified using a Y2H screen. Me10

was shown to interact with the scaffolding protein tomato 14-3-3 isoform 7 (TFT7) that is known to function in plant defense as a scaffold for a MAP kinase signaling cascade (Oh and Martin, 2011). Using tobacco rattle virus (TRV)-based silencing of *TFT7* in tomato led to an increased longevity and fecundity to a non-host cotton melon aphid (*Aphis gossypii*). This increase of a non-host aphid performance on *TFT7* silenced plants demonstrated the role *TFT7* in aphid defense and the secretion of Me10 by the potato aphid to disrupt TFT7 involved host defense (Chaudhary et al., 2019).

Another aphid effector first identified in the potato aphid is Me23. *Me23* transcripts were identified in the potato aphid salivary gland transcriptome and peptides of Me23 were also detected in the potato aphid saliva (Atamian et al., 2013; Chaudhary et al., 2015). Me23 has a conserved enzymatic domain encoding a glutathione peroxidase. Transient expression of Me23 increased the fecundity of the green peach aphid on *N. benthiamana*. Unlike Me10, Me23 was unable to increase the fecundity of potato aphid when expressed in tomato (Atamian et al., 2013).

Similar to Me23, Me47 is among the few aphid effectors with predicted enzymatic functions. *Me47* transcripts were also identified in the potato aphid salivary gland transcriptome and peptides of Me47 were detected in the potato aphid saliva (Atamian et al., 2013; Chaudhary et al., 2015). Me47 contains a glutathione-S-transferase (GST) domain and was predicted to have GST activity. This activity was confirmed after its expression in *E. coli,* purification, and the use

of the purified enzyme in *in vitro* assays demonstrating that Me47 has the ability to detoxify multiple types of isothiocyanates, highly toxic compounds to insect herbivores present in Brassicaceae (Wadleigh and Yu, 1988). Expression of Me47 *in planta* led to increased potato aphid fecundity on tomato and green peach aphid on *N. benthamiana*. However, expression of Me47 in Arabidopsis led to a decrease in green peach aphid fecundity, indicating that Me47 is necessary for only specific host-aphid interactions and the product it detoxifies is likely not a Brassicaceae isothiocyanate but an unidentified plant compound (Kettles and Kaloshian, 2016).

Mp55 is a salivary protein first identified from the green peach aphid transcriptome. Mp55 transient overexpression in tobacco or stable overexpression in Arabidopsis led to increased green peach aphid fecundity. RNAi knockdown of Mp55 in multiple plant species led to reduced fecundity of the green peach aphid demonstrating its importance for the green peach aphid as an effector to overcome host plant defenses (Elzinga et al., 2014). Consistently, aphids feeding on Arabidopsis constitutively expressing Mp55 were found to induce lower plant defense responses, such as generating lower callose deposits, H_2O_2 production, and lower 4-methoxyindol-3-ylmethylglucosinoalte levels compared to a negative control (Elzinga et al., 2014).

An aphid effector that was identified based of its predicted function was Armet. Armet is a predicted Ca^{2+} binding protein, transcripts of which were highly expressed in the pea aphid salivary glands (Carolan et al., 2011). In mammals,

Armet is distributed in different organ tissues and found both inside the cell, as part of the unfolded protein response in the lumen of endoplasmic reticulum, or extracellularly as a neurotrophic factor (Lindholm et al., 2007; Mizobuchi et al., 2007; Apostolou et al., 2008). Both roles have been also been identified in *Drosophila* (Palgi et al., 2009; Palgi et al., 2012). Using polyclonal antibody in Western blot analysis, Armet was detected in host plant tissues infested with aphids, and when knocked down using RNAi aphid injections, the survival rate of the aphids was decreased (Wang et al., 2015a). Both transgenic expression and infiltration of purified pea aphid Armet in plants led to an increase of SA levels in the plant as well as upregulation of SA-dependent marker genes. The activation of the SA pathway did not affect aphid performance but did confer resistance to the bacterial pathogen *P. syringae*. Armet inducing SA as way to inhibit a JA response through natural antagonism, could be a way the aphids are deceiving the host plant to enable them to successfully establish feeding (Cui et al., 2019).

Macrophage Migration Inhibitory Factor 1 (MIF1) is another aphid effector that was discovered based on predicted molecular functions determined by homologs present in other organisms. The secretion of MIFs has been reported in parasitic species like nematodes and plasmodium and are associated with manipulation of host immune responses (Augustijn et al., 2007; Cho et al., 2007). *MIF1* was first identified in the genome of the pea aphid, and since then, MIF1 has been detected in the saliva of multiple aphid species (IAGC, 2010; Dubreuil et al., 2014; Vandermoten et al., 2014; Naessens et al., 2015). Knockdown of

MIF1 with RNAi aphid injections, altered pea aphid feeding ability and lowered both the survival and fecundity of the aphid confirming it importance as an aphid effector (Naessens et al., 2015).

A group of aphid effectors, Angiotensin-Converting Enzymes (ACEs), were first identified in pea aphid saliva and later in pea aphid salivary gland transcriptome (Carolan et al., 2009; Carolan et al., 2011). *ACE* transcripts have also been detected in other plant pest salivary glands like the whitefly (Su et al., 2012). Two *ACE*s (*ACE1* and *ACE2*) are highly expressed in the salivary glands of pea aphids with predictions for secretion (Carolan et al., 2011; Wang et al., 2015b; Boulain et al., 2018). Only ACE1 was detected in pea aphid saliva (Carolan et al., 2009; Boulain et al., 2018). Surprisingly, knockdown of either *ACE1* or *ACE2* with RNAi aphid injections led to no difference in pea aphid survival compared to control dsGFP injections. However, when both *ACE1* and *ACE2* were knocked down together, the aphid survival was decreased (Wang et al., 2015b). The double knockdown of *ACE1* and *ACE2* also altered the aphid feeding behavior observed by EPG analysis. The disturbed feeding behavior differed from what had been previously observed with other knocked down aphid effectors. The reduction in *ACE1 and ACE2* levels led to a longer time of phloem sap ingestion by aphids, in contrast to when other aphid effectors are knocked down phloem sap ingestion time was significantly reduced (Mutti et al., 2006; Mutti et al., 2008; Wang et al., 2015a; Wang et al., 2015b). In addition to longer sap ingestion, the aphids had a shorter probe time to reach the phloem. The

combination of enhanced feeding while increased mortality, implicate the ACEs exert negative effects on aphid feeding behavior. These results show that the two ACEs have redundant functions and have an important role in regulating aphid feeding behavior (Wang et al., 2015b).

Another aphid effector identified from the pea aphid is Ap25 (ACYPI009919). Ap25 is an unknown pea aphid salivary protein that was identified in the pea aphid salivary gland transcriptomic analysis. Ap25 was found to have similar features to the well-known aphid effector C002 (Carolan et al., 2011; Guy et al., 2016). Like *C002*, *Ap25* is a single copy gene and its orthologues only exist in the Aphididae family (Guy et al., 2016; Boulain et al., 2018). When transiently overexpressed with agrobacterium in pea, it increased pea aphid fecundity compared to the GFP control (Guy et al., 2016).

As indicated earlier, aphid secrete two types of saliva; gelling and liquid. Most of the reported salivary protein work have been about the liquid saliva and limited information exists about the gelling saliva. Gelling saliva is thought to be mostly compromised of a cysteine rich protein, SHP (Will et al., 2012; Will and Vilcinskas, 2015). SHP was first identified through proteomic analysis of the pea aphid saliva (Carolan et al., 2009). The gelling of the SHP is likely due to the disulfide bonds present between the cysteine residues (Carolan et al., 2009; Will et al., 2012). Both plant-mediated RNAi and RNAi injections of aphids designed to knockdown *SHP,* have led to decreased aphid fecundity and survival showing its necessity to the aphid (Abdellatef et al., 2015; Will and Vilcinskas, 2015). In

addition, RNAi injection of aphids altered the morphology of the salivary sheath and likely disrupted its function. Typical salivary sheaths, collected from artificial feeding chambers, had a necklace-like structure but this structure was not found in the RNAi treated aphids (Will and Vilcinskas, 2015). Interestingly, not only did the aphids that directly fed on the plant mediated RNAi demonstrate reduction in *SHP*, up to 6 more generations of their offspring had diminished *SHP* expression levels (Abdellatef et al., 2015).

Recently, a long noncoding RNA (lncRNA) was identified as a putative aphid effector (Chen et al., 2020). lncRNA do not contain any protein translation coding like mRNA's but do require traditional RNA polymerase and undergo the same modifications including 5' cap, splicing, and polyadenylated (Karapetyan et al., 2013; Zaynab et al., 2018). The *Ya* gene family was identified when monitoring differential gene expression of green peach aphids when they were exposed to different host plants and at least one member, *Ya1*, is a lncRNA (Mathers et al., 2017; Chen et al., 2020). *Ya1* transcripts could be detected in the plant tissues infested with aphids. When *Ya1* was overexpressed in Arabidopsis, there was an increase in green peach aphid fecundity compared to wild type and GFP overexpressed plants. Confirming *Ya1*'s role as an effector, plant-mediated RNAi knockdown of *Ya1* in Arabidopsis led to lower aphid fecundity compared to the GFP control (Chen et al., 2020).

Aphid Derived Effectors

As described earlier, aphids utilize multiple effectors, from aphid origin, as agents to disrupt plant defense responses and effect aphid life performance. Additionally, aphids utilize aphid viruses to manipulate host responses for their advantage (Lu et al., 2019; Patton et al., 2020). One of these viruses is the Acyrthosiphon pisum virus (APV), a symbiotic RNA virus that is found in pea aphids (van den Heuvel et al., 1997; Lu et al., 2019). The virus can only propagate in the aphid host. APV is secreted in the plant host but does not replicate in the plant but is detected in the host for at least 7 days after aphid removal from infested plants (Lu et al., 2019). Interestingly, APV is mainly transmitted to aphids horizontally through feeding on infected plant tissues though vertical transmission from mother to daughter does happen at low frequency. APV was found to increase pea aphid survival when feeding on a non-adapted host plant (*Vicia villosa*). This improved survival by APV infected pea aphids was found to be the result of phytohormone manipulation of the host plant. Pea aphids without APV induced higher JA levels in their host plant, while APV infected pea aphids induced higher SA levels (Lu et al., 2019). By inducing higher SA levels APV infected aphids alter JA levels through the natural antagonistic relationship between the two phytohormones benefiting the aphid.

In addition to aphid viruses, plant viruses transmitted by aphids have also been shown to improve aphid life performance. The potato leafroll virus (PLRV) is circulative-nonpropagative RNA virus that is a member of the luteovirus group

(Kawchuk et al., 1990; Patton et al., 2020). Circulative nonpropagative viruses are acquired through insect feeding, pass into the hemolymph of the insect before entering the salivary glands for transmission (Casteel and Falk, 2016; Patton et al., 2020). These viruses do not propagate in the insect vector. PLRV is transmitted by multiple aphid species, and aphids feeding on PLRV infected plants have higher fecundity than those feeding on non-infected plants (Castle and Berger, 1993; Srinivasan and Alvarez, 2007). Agrobacterium-mediated transient overexpression of the different PLRV proteins identified the viral proteins, P0, P1, and P7, responsible for the enhanced aphid fecundity. When these three proteins were overexpressed and the plants were infested with aphids, lower levels of all three major defense hormones, SA, JA and ET, were detected compared to the wild type plants infested with aphids. In the absence of aphids, overexpression of the viral proteins did not yield constitutive elevated levels of these hormones. This work indicates that plant viruses assist their vectors by manipulating defense phytohormones adding another layer of complexity to the tritrophic pest-virus-plant system (Patton et al., 2020).

Plant Resistance Genes to Aphids

Once pests/pathogens evade the first line of plant defense (PTI) using their various tools (effectors), plants must rely on their second line of defense known as effector triggered immunity (ETI) to protect themselves. PTI focuses largely on transmembrane PRRs to recognize the threat and respond, while ETI consists of

intracellular resistance (R) proteins. These R proteins have evolved to recognize directly or indirectly the effectors from pest/pathogen to initiate a strong immune response (Jones and Dangl, 2006). The largest class of R proteins contain multiple domains including a nucleotide-binding (NB) and a leucine rich repeat (LRR) domains (Takken and Goverse, 2012). For direct recognition, R proteins can act as receptor to cognate effectors and R-effector binding initiate ETI responses (Jones and Dangl, 2006). There are several models for the indirect recognition. The guard model, in which the R protein is not monitoring the effector, but it is monitoring the effector's target protein and any modifications made to it (Dangl and Jones, 2001). Another model of indirect recognition is the decoy model. In the decoy model, the plant contains a protein that mimics the effectors true target that is monitored by the R protein. By targeting the decoy, the effector is trapped, leading the R protein to recognize its action and initiate ETI (van der Hoorn and Kamoun, 2008). An extension to the decoy model is the integrated decoy model. In this model the decoy is another domain on the R protein itself (Cesari et al., 2014).

A limited number of *R* genes have been identified that confer resistance to hemipterans and only a few have been successfully cloned (Eenink et al., 1982; Githiri et al., 1996; Boyko et al., 2006; Kim et al., 2010; Ahman et al., 2019; Nalam et al., 2019). The cloned *R* genes have a diverse array of R protein structures including those with NB-LRR motifs representing the largest class of R proteins in plants.

Plant resistance to hemipterans or other insect herbivores has been classified into three different categories: antibiosis, antixenosis, and tolerance (Smith, 1989). Antibiosis resistance effects the insect biology through negative effects on mortality, growth, longevity, and fecundity (Painter, 1951; Smith, 2005). Antixenosis resistance affects the insect behavior leading to the insects having a non-preference for that particular host (Painter, 1951; Smith, 2005; Koch et al., 2016). The last category of resistance, tolerance, has no effect on the insect pest but is the ability of the plant to withstand insect damage (Smith, 2005; Koch et al., 2016).

Three of the *R* genes cloned against hemipteran insects confer resistance to aphids. The first of these is the *Mi-1.2* gene from tomato confers resistance to the potato aphid (Rossi et al., 1998; Vos et al., 1998). The resistance conferred by *Mi-1.2* to the potato aphids involves both antibiosis and antixenosis (Wu et al., 2015). Interestingly, *Mi-1.2* not only confers resistance to potato aphids, it also confers resistance two additional hemipteran insects, whiteflies (*Bemisia tabaci*) and psyllids (*Bactericera cockerelli*), as well as to three species of root-knot nematodes (*Meloidogyne arenaria, M. javanica and M. incognita*) (Kaloshian et al., 1995; Milligan et al., 1998; Nombela et al., 2003; Casteel et al., 2006).

The *Vat* gene of melon (*Cucumis melo*) is another cloned *R* gene that confers resistance to the cotton-melon aphid (*Aphis gossypii*) (Pitrat and Lecoq, 1982; Dogimont et al., 2014). Similar to *Mi-1.2, Vat*-mediated resistance also involves both antibiosis and antixenosis (Boissot et al., 2016). In addition to

aphids, *Vat* also confer resistance to melon viruses transmitted by the cotton-melon aphid including Cucumber mosaic virus , Papaya ringspot virus, Watermelon mosaic virus, and Zucchini yellow mosaic virus (Pitrat and Lecoq, 1982; Boissot et al., 2016). Additionally, both *Mi-1.2* and *Vat* genes encode a coiled-coil NB-LRR (CC-NB-LRR; CNL) proteins belonging to the largest *R* gene family in plants represented by hundreds of diverse genes per genome (Milligan et al., 1998; Dogimont et al., 2014).

The most recent identified and cloned *R* gene to aphids is the Arabidopsis *SIEVE ELEMENT-LINING CHAPERONE1* (*SLI1*) that confers resistance to the green peach aphid (Kloth et al., 2017). Unlike *Mi-1.2* and *Vat*, *SLI1* encodes a small heat shock-like gene that conferred resistance to the green peach aphid, most likely through lining the plant sieve element and obstructing the ability of the aphid to feed (Kloth et al., 2017; Ahman et al., 2019). As of now, *SLI1*-mediated resistance is characterized for antibiosis but since it is only recently discovered, it is possible additional characteristics could be associated with this resistance.

In addition to the above described *R* genes against aphids, several rice (*Oryza sativa*) *R* genes have been identified and cloned that confer resistance to another hemipteran, the brown planthopper (*Nilaparvata lugens*). Two of these *R* genes, *Bph14,* and *Bph26*, encode CNLs, similar to *Vat* and *Mi-1.2* (Hwang et al., 2000; Du et al., 2009; Dogimont et al., 2014; Tamura et al., 2014). Another *R* gene to the brown planthopper, *Bph18*, also encodes a CNL but has an extra nucleotide binding domain (CC-NB-NB-LRR) (Ji et al., 2016). Not all rice *R* genes

to the brown planthopper encode a CNL type proteins. The *Bph17 R* locus is comprised of a cluster of three genes and all three-encoding membrane–localized lectin receptor kinases: *OsLecRK1*, *OsLecRK2*, and *OsLecRK3* (Liu et al., 2015; Hu et al., 2016). Another cloned rice resistance gene to brown planthopper, inherited in recessive manner, is *Bph29*. *Bph29* encodes a B3 DNA-binding protein that is expressed in vascular tissues after brown planthopper infestations (Wang et al., 2015c; Hu et al., 2016).

Resistance to the cowpea aphid has been evaluated in multiple different plant species including *Medicago truncatula,* soybean and cowpea land races (Kamphuis et al., 2012; Souleymane et al., 2013; Mai et al., 2016). Resistance to cowpea aphid was identified in an African cowpea breeding line IT97K-556-6 (Souleymane et al., 2013). This resistance was further explored through recombinant inbred lines (RILs) developed between IT97K-556-6 and a susceptible cowpea line California blackeye 27 (CB27). Screening these RILs with cowpea aphids, the source of the resistance was localized to two different quantitative trait loci (QTL), a major *QAC-vu7.1* and a minor *QAC-vu1.1* QTLs (Huynh et al., 2015). The characterization of the resistance conferred by these two QTLs is described in Chapters Two and Three.

Additional sources of resistance in cowpea to the cowpea aphid have also been identified. In a recent study, 373 different lines of cowpea from the International Institute of Tropical Agriculture (IITA) were screened for resistance to the cowpea aphid. Of these 373, 21 were found to have some level of

resistance with only three being classified as resistant to cowpea aphids. Biochemical analyses of these cowpea lines found the susceptible cowpea lines to have higher sucrose content than the resistant lines (Togola et al., 2020). Sucrose is necessary for some aphid species to fed on and ingest artificial diets and its higher presence in the cowpea susceptible lines indicates that cowpea aphids prefer plants with higher sugar content (Mittler, 1967; Togola et al., 2020). The same study identified the resistant lines to have a higher content of the polyphenols kaempferol and quercetin. Both polyphenols have been previously identified in cowpea and wild *Vigna* species and implicated in resistance to cowpea aphid as well as to the black bean aphid (*Aphis fabae*) (Lattanzio et al., 2000; Togola et al., 2020).

Resistance to cowpea aphids has also been identified in soybean and in the model legume *M. truncatula* (Kamphuis et al., 2012; Mai et al., 2016). In a soybean cultivar, tolerance to cowpea aphids was found to have dependence on multiple peroxidases (Mai et al., 2016). A resistant *M. truncatula* genotype, was identified through a screen of the *M. truncatula* core collection of South Australian Research and Development Institute (SARDI). The resistant genotype exhibited both antibiosis and antixenosis to cowpea aphids and was mapped to a major QTL on chromosome 2. Through EPG analysis, the resistance was determined to be phloem based (Kamphuis et al., 2012).

Objectives of the Dissertation Research

The goal of my research has been to better understand the nature of the cowpea aphid interaction with its host plant cowpea. In order to develop a sustainable resistance source of cowpea to cowpea aphids, an intimate knowledge of host-pest interactions is needed. Aphid saliva is the main interface between the insect pest and the plant host, and an increasing body of evidence has demonstrated that saliva disrupts the plant defense responses and modifies plant metabolic processes for the aphid's advantage. At the start of this project, there was no available information on the protein composition of the cowpea aphid saliva. Therefore, the first objective, described in Chapter One, was to identify the cowpea aphid salivary proteome through LC-MS/MS. The proteins identified were compared to other aphid species salivary profiles to identify species-specific novel salivary proteins. This investigation led to the identification of AcDCXR, a diacetyl/L-xylulose reductase. AcDCXR was characterized for enzymatic activity *in vitro* and for effector function *in planta*.

The second objective of my research was to better comprehend the cowpea response to cowpea aphid infestation. Cowpea-cowpea aphid interactions are atypical and result in more damage to the plant than other aphid-host interactions. A detailed description of the susceptible cowpea phenotypic response to cowpea aphid infestations is documented in Chapter Two. Chapter two also describes the results of the third objective of my research regarding characterization of the mechanism of cowpea resistance to the cowpea aphid

mediated by the two QTLs identified in Huynh et al. (2015). A near-isogenic line pair (NILs), generated from the African resistance source and a susceptible California Blackeye cultivar (CB46), were used in a set of biological assays as well as in electrical penetration graphs (EPGs) analysis, to determine the nature of the resistance. The last objective of my work is described in Chapter Three. RNAseq analysis of the resistant and susceptible NILs, infected and uninfected, were performed to determine differential gene expression and to infer the molecular mechanisms of the resistance.

References

AATF, A.A.T.F. (2012). "Potentials and constraints: cowpea for food and poverty alleviation". The Maruca-Resistant Cowpea Project).

Abdellatef, E., Will, T., Koch, A., Imani, J., Vilcinskas, A., and Kogel, K.H. (2015). Silencing the expression of the salivary sheath protein causes transgenerational feeding suppression in the aphid *Sitobion avenae*. *Plant Biotechnol J* 13(6)**,** 849-857.

Agrawal, A.A. (2004). Plant defense and density dependence in the population growth of herbivores. *Am Nat* 164(1)**,** 113-120.

Agunbiade, T.A., Sun, W., Coates, B.S., Djouaka, R., Tamo, M., Ba, M.N., et al. (2013). Development of reference transcriptomes for the major field insect pests of cowpea: a toolbox for insect pest management approaches in west Africa. *PLoS One* 8(11)**,** e79929.

Ahmad, S., Veyrat, N., Gordon-Weeks, R., Zhang, Y., Martin, J., Smart, L., et al. (2011). Benzoxazinoid metabolites regulate innate immunity against aphids and fungi in maize *Plant Physiol* 157(1)**,** 317-327.

Ahman, I., Kim, S.Y., and Zhu, L.H. (2019). Plant Genes Benefitting Aphids-Potential for Exploitation in Resistance Breeding. *Front Plant Sci* 10**,** 1452.

Ahmed, N., and Thornalley, P.J. (2007). Advanced glycation endproducts: what is their relevance to diabetic complications? *Diabetes Obes Metab* 9(3)**,** 233-245.

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S., and Ecker, J.R. (1999). EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. *Science* 284(5423)**,** 2148-2152.

Andolfo, G., and Ercolano, M.R. (2015). Plant Innate Immunity Multicomponent Model. *Front Plant Sci* 6.

Apostolou, A., Shen, Y., Liang, Y., Luo, J., and Fang, S. (2008). Armet, a UPR-upregulated protein, inhibits cell proliferation and ER stress-induced cell death. *Exp Cell Res* 314(13)**,** 2454-2467.

Atamian, H.S., Chaudhary, R., Dal Cin, V., Bao, E., Girke, T., and Kaloshian, I. (2013). *In planta* expression or delivery of potato aphid *Macrosiphum*

euphorbiae effectors Me10 and Me23 enhances aphid fecundity. *Mol Plant Microbe Interact* 26(1)**,** 67-74.

Auclair, J. (1963). Aphid feeding and nutrition. *Annu Rev Entomol* 8**,** 439-490.

Augustijn, K.D., Kleeman, R., Thompson, J., Kooistra, T., Crawford, C., Reece, S.E., et al. (2007). Functional characterization of the *Plasmodium falciparum* and *P. berghei* homologues of macrophage migration inhibitory factor. *Infect Immun* 75(3)**,** 1116-1128.

Berens, M.L., Berry, H.M., Mine, A., Argueso, C.T., and Tsuda, K. (2017). Evolution of Hormone Signaling Networks in Plant Defense. *Annu Rev Phytopathol* 55**,** 401-425.

Bilova, T., Lukasheva, E., Brauch, D., Greifenhagen, U., Paudel, G., Tarakhovskaya, E., et al. (2016). A Snapshot of the Plant Glycated Proteome: STRUCTURAL, FUNCTIONAL, AND MECHANISTIC ASPECTS. *J Biol Chem* 291(14)**,** 7621-7636.

Bito, A., Haider, M., Briza, P., Strasser, P., and Breitenbach, M. (1999). Heterologous expression, purification, and kinetic comparison of the cytoplasmic and mitochondrial glyoxalase II enzymes, Glo2p and Glo4p, from *Saccharomyces cerevisiae*. *Protein Expr Purif* 17(3)**,** 456-464.

Blackman, R.L., and Eastop, V.F. (1989). *Aphids on the World's Crops: An Identification Guide.* New York: John Wiley & Sons.

Blackman, R.L., and Eastop, V.F. (2000). *Aphids on the world's crops.* New York, NY: John Wiley & Sons Inc.

Blackman, R.L., and Eastop, V.F. (2007). "Taxonomic Issues," in *Aphids as Crop Pests,* eds. H.F. van Emden & R. Harrington. (Wallingford, United Kingdom: CABI), 1-29.

Blume, B., Nurnberger, T., Nass, N., and Scheel, D. (2000). Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. *Plant Cell* 12(8)**,** 1425-1440.

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**,** 1420.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu Rev Plant Biol* 60**,** 379-406.

Borysiuk, K., Ostaszewska-Bugajska, M., Vaultier, M.N., Hasenfratz-Sauder, M.P., and Szal, B. (2018). Enhanced formation of methylglyoxal-derived advanced glycation end products in arabidopsis under ammonium nutrition. *Front Plant Sci* 9.

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 Genetics* 6(11).

Boulain, H., Legeai, F., Guy, E., Morliere, S., Douglas, N.E., Oh, J., et al. (2018). Fast evolution and lineage-specific gene family expansions of aphid salivary effectors driven by interactions with host-plants. *Genome Biol Evol* 10(6)**,** 1554-1572.

Boulain, H., Legeai, F., Jaquiery, J., Guy, E., Morliere, S., Simon, J.C., et al. (2019). Differential Expression of Candidate Salivary Effector Genes in Pea Aphid Biotypes With Distinct Host Plant Specificity. *Front Plant Sci* 10**,** 1301.

Boyko, E.V., Smith, C.M., Thara, V.K., Bruno, J.M., Deng, Y., Starkey, S.R., et al. (2006). Molecular basis of plant gene expression during aphid invasion: wheat Pto- and Pti-like sequences are involved in interactions between wheat and Russian wheat aphid (Homoptera: Aphididae). *J Econ Entomol* 99(4)**,** 1430-1445.

Bradford, K.J. (2008). Shang Fa Yang: Pioneer in plant ethylene biochemistry. *Plant Sci* 175**,** 2-7.

Brouwer, C., and Heibloem, M. (1986). "CROP WATER NEEDS ", in: *Irrigation Water Management: Irrigation Water Needs.* (ed.) J. van Dijk. (Rome, Italy: FAO).

Burd, J. (2002). Physiological Modification of the Host Feeding Site by Cereal Aphids (Homoptera: Aphididae). *J Econ Entom* 95(2)**,** 463-468.

CABI. (2019). Aphis craccivora (groundnut aphid) [Online]. CABI. Available: https://www.cabi.org/isc/datasheet/6192 [Accessed 12-9-20].

Carolan, J.C., Caragea, D., Reardon, K.T., Mutti, N.S., Dittmer, N., Pappan, K., et al. (2011). Predicted effector molecules in the salivary secretome of the pea aphid (*Acyrthosiphon pisum*): a dual transcriptomic/proteomic approach. *J Proteomics Res* 10(4)**,** 1505-1518.

Carolan, J.C., Fitzroy, C.I.J., Ashton, P.D., Douglas, A.E., and Wilkinson, T.L. (2009). The secreted salivary proteome of the pea aphid *Acyrthosiphon pisum* characterised by mass spectrometry. *Proteomics* 9(9)**,** 2457-2467.

Casazza, J.P., Felver, M.E., and Veech, R.L. (1984). The metabolism of acetone in rat. *J Biol Chem* 259(1)**,** 231-236.

Casteel, C., and Falk, B. (2016). Plant virus-vector interactions: More than just for virus transmission. *Current research topics in plant virology***,** 217-240.

Casteel, C.L., Walling, L.L., and Paine, T.D. (2006). Behavior and biology of the tomato psyllid, Bactericera cockerelli, in response to the *Mi-1.2* gene. *Entomol Exp Appl* 121(1)**,** 67-72.

Castle, S.J., and Berger, P.H. (1993). Rates of growth and increase of *Myzus persicae* on virus-infected potatoes according to type of virus-vector. *Entomol Exp Appl* 69(1)**,** 51-60.

Catinot, J., Buchala, A., Abou-Mansour, E., and Metraux, J.P. (2008). Salicylic acid production in response to biotic and abiotic stress depends on isochorismate in *Nicotiana benthamiana*. *FEBS Lett* 582(4)**,** 473-478.

Cesari, S., Bernoux, M., Moncuquet, P., Kroj, T., and Dodds, P.N. (2014). A novel conserved mechanism for plant NLR protein pairs: the "integrated decoy" hypothesis. *Front Plant Sci* 5**,** 606.

Chan, C.K., Forbes, A.R., and Raworth, D.A. (1991). *Aphid-transmitted viruses and their vectors of the world.* Ottawa: Research Branch, Agriculture Canada.

Chang, T., Wang, R., and Lu, R. (2005). Methylglyoxal-induced nitric oxide and peroxynitrite production in vascular smooth muscle cells. *Free Radic Biol Med* 38**,** 286-293.

Chaudhary, R., Atamian, H.S., Shen, Z., Briggs, S.P., and Kaloshian, I. (2014). GroEL from the endosymbiont *Buchnera aphidicola* betrays the aphid by triggering plant defense. *Proc Natl Acad Sci USA* 111(24)**,** 8919-8924.

Chaudhary, R., Atamian, H.S., Shen, Z., Briggs, S.P., and Kaloshian, I. (2015). Potato aphid salivary proteome: enhanced salivation using resorcinol and identification of aphid phosphoproteins. *J Proteomics Res* 14(4)**,** 1762-1778.

Chaudhary, R., Peng, H.C., He, J.M., MacWilliams, J., Teixeira, M., Tsuchiya, T., et al. (2019). Aphid effector Me10 interacts with tomato TFT7, a 14-3-3 isoform involved in aphid resistance. *New Phytol* 221(3)**,** 1518-1528.

Chen, L., Song, Y., Li, S., Zhang, L., Zou, C., and Yu, D. (2012). The role of WRKY transcription factors in plant abiotic stresses. *Biochimica et Biophysica Acta* 1819**,** 120-128.

Chen, Y., Singh, A., Kaithakottil, G., Mathers, T.C., Gravino, M., Mugford, S.T., et al. (2020). An aphid RNA transcript migrates systemically within plants and is a virulence factor. *Proc Natl Acad Sci USA* 117(23)**,** 12763-12771.

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T., and Felix, G. (2006). The Arabidopsis receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. *Plant Cell* 18(2)**,** 465-476.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, T., Jones, J.D., et al. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448(7152)**,** 497-500.

Cho, Y., Jones, B.F., Vermeire, J.J., Leng, L., DiFedele, L., Harrison, L.M., et al. (2007). Structural and functional characterization of a secreted hookworm Macrophage Migration Inhibitory Factor (MIF) that interacts with the human MIF receptor CD74. *J Biol Chem* 282(32)**,** 23447-23456.

Coleman, A.D., Wouters, R.H.M., Mugford, S.T., and Hogenhout, S.A. (2015). Persistence and transgenerational effect of plant-mediated RNAi in aphids *J Exp Bot* 66(2)**,** 541-548.

Cooper, W.R., Dillwith, J.W., and Puterka, G.J. (2010). Salivary proteins of Russian wheat aphid (Hemiptera: Aphididae). *Environ Entomol* 39(1)**,** 223-231.

Cooper, W.R., Dillwith, J.W., and Puterka, G.J. (2011). Comparisons of salivary proteins from five aphid (Hemiptera: Aphididae) species. *Environ Entomol* 40(1)**,** 151-156.

Cooper, W.R., and Goggin, F.L. (2005). Effects of jasmonate-induced defenses in tomato on the potato aphid, *Macrosiphum euphorbiae*. *Entomol Exp Appl* 115(1)**,** 107-115.

Coppola, M., Manco, E., Vitiello, A., Lelio, I.D., Giorgini, M., Rao, R., et al. (2018). Plant response to feeding aphids promotes aphid dispersal. *Entomol Exp Appl* 166(5).

Crowder, M.W., Maiti, M.K., Banovic, L., and Makaroff, C.A. (1997). Glyoxalase II from *A. thaliana* requires Zn(II) for catalytic activity *FEBS Lett* 418(3).

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. *Philos Trans R Soc Lond B Biol Sci* 374(1767)**,** 20180314
.

Dangl, J.L., and Jones, J.D. (2001). Plant pathogens and integrated defence responses to infection. *Nature* 411(6839)**,** 826-833.

Das, A., Rushton, P.J., and S., R.J. (2017). Metabolomic profiling of soybeans (*Glycine max* L.) reveals the importance of sugar and nitrogen metabolism under drought and heat stress plants. *Plants* 6(21).

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

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., et al. (1994). A central role of salicylic acid in plant disease resistance. *Science* 266(5188)**,** 1247-1250.

Dempsey, D.A., Vlot, A.C., Wildermuth, M.C., and Klessig, D.F. (2011). Salicylic Acid biosynthesis and metabolism. *Arabidopsis Book* 9**,** e0156.

Desai, K.M., Chang, T., Wang, H., Banigesh, A., Dhar, A., Liu, J.H., et al. (2010). Oxidative stress and aging: is methylglyoxal the hidden enemy? *Canadian Journal of Physiology and Pharmacology* 88**,** 273-284.

Desneux, N., Barta, R.J., Hoelmer, K.A., Hopper, K.R., and Heimpel, G.E. (2009). Multifaceted determinants of host specificity in an aphid parasitoid. *Oecologia* 160(2)**,** 387-398.

Despres, C., DeLong, C., Glaze, S., Liu, E., and Fobert, P.R. (2000). The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a

subgroup of the TGA family of bZIP transcription factors. *Plant Cell* 12(2)**,** 279-290.

Devanathan, S., Erban, A., Perez-Torres, R., Kopka, J., and Makaroff, C.A. (2014). *Arabidopsis thaliana* glyoxalase 2-1 is required during abiotic stress but is not essential under normal plant growth *PLoS One* 9(4)**,** e95971.

Dixon, A.F.G. (2012). *Aphid Ecology An optimization approach.* New York: Springer.

Dixon, A.F.G., and Dewar, A.M. (1974). The time of determination of gynoparae and males in the bird cherry-oat aphid, *Rhopalosiphum padi*. *Ann Appl Biol* 78(1)**,** 1-6.

Dogimont, C., Chovelon, V., Pauquet, J., Boualem, A., and Bendahmane, A. (2014). The *Vat* locus encodes for a CC-NBS-LRR protein that confers resistance to *Aphis gossypii* infestation and *A. gossypii*-mediated virus resistance. *Plant J* 80(6)**,** 993-1004.

Douglas, A.E. (1993). The nutritional quality of phloem sap utilized by natural aphid populations. *Ecol Evol* 18**,** 31-38.

Douglas, A.E. (1998). Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria Buchnera. *Annu Rev Entomol* 43**,** 17-37.

Dreyer, D.L., Jones, K.C., and Molyneux, R.J. (1985). Feeding deterrency of some pyrrolizidine, indolizidine, and quinolizidine alkaloids towards Pea aphid (*Acyrthosiphon pisum*) and evidence for phloem transport of indolizidine alkaloid swainsonine. *J Chem Ecol* 11(8)**,** 1045-1051.

Du, B., Zhang, W., Liu, B., Hu, J., Wei, Z., Shi, Z., et al. (2009). Identification and characterization of *Bph14,* a gene conferring resistance to brown planthopper in rice. *Proc Natl Acad Sci USA* 106(52)**,** 22163-22168.

Dubreuil, G., Deleury, E., Crochard, D., Simon, J.C., and Coustau, C. (2014). Diversification of MIF immune regulators in aphids: link with agonistic and antagonistic interactions. *BMC Genomics* 15**,** 762.

Ebert, B., Kisiela, M., and Maser, E. (2015). Human DCXR - another 'moonlighting protein' involved in sugar metabolism, carbonyl detoxification, cell adhesion and male fertility? *Biol Rev* 90(1)**,** 254-278.

Eenink, A.H., Groenwold, R., and Dieleman, F.L. (1982). Resistance of lettuce (*Lactuca*) to the leaf aphid *Nasonovia ribis nigri*. 1. Transfer of resistance from *L. virosa* to *L. sativa* by interspecific crosses and selection of resistant breeding lines. *Euphytica* 31**,** 291-299.

Elhamahmy, M., Mahmoud, M.F., and Bayoumi, T.Y. (2016). The Effect of Applying Exogenous Salicylic Acid on Aphid Infection and its Influence on Histo-Physiological Traits and Thermal Imaging of Canola. *AGRONOMIC RESEARCH IN MOLDAVIA* 2166**,** 67-85.

Ellis, C., Karafyllidis, I., and Turner, J.G. (2002). Constitutive activation of jasmonate signaling in an Arabidopsis mutant correlates with enhanced resistance to *Erysiphe cichoracearum*, *Pseudomonas syringa*e, and *Myzus persicae*. *Mol Plant Microbe Interact* 15(10)**,** 1025-1030.

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(7)**,** 747-756.

Engqvist, M., Drincovich, M.F., Flügge, U.I., and Maurino, V.G. (2009). Two D-2-hydroxy-acid dehydrogenases in *Arabidopsis thaliana* with catalytic capacities to participate in the last reactions of the methylglyoxal and beta-oxidation pathways. *J Biol Chem* 284**,** 25026-250237.

Felix, G., Duran, J.D., Volko, S., and Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J* 18(3)**,** 265-276.

Feng, H., Edwards, N., Anderson, C.M.H., Althaus, M., Duncan, R.P., Hsu, Y.C., et al. (2019). Trading amino acids at the aphid-*Buchnera* symbiotic interface. *Proc Natl Acad Sci USA* 116(32)**,** 16003-16011.

Fu, Z.Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486(7402)**,** 228-232.

Ghosh, A., Kushwaha, H.R., Hasan, M.R., Pareek, A., Sopory, S.K., and Singla-Pareek, S.L. (2016). Presence of unique glyoxalase III proteins in plants indicates the existence of shorter route for methylglyoxal detoxification. *Sci Rep* 6**,** 18358.

Githiri, S.M., Ampong-Nyarko, K., Osir, E.O., and Kimani, P.M. (1996). Genetics of resistance to *Aphis craccivora* in cowpea. *Euphytica* 89**,** 371-376.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu Rev Phytopathol* 43**,** 205-227.

Goggin, F.L., Quisenberry, S., and Ni, X. (2017). "Feeding Injury," in *Aphids as Crop Pests,* eds. H.F. van Emden & R. Harrington. 2nd ed: CAB International), 303-322.

Gomez-Gomez, L., and Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Mol Cell* 5(6)**,** 1003-1011.

Gomez-Gomez, L., Felix, G., and Boller, T. (1999). A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *Plant J* 18(3)**,** 277-284.

Grambow, H.J., Lueckge, J., Klausener, A., and Mueller, E. (1986). Occurrence of 2-(2-Hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3-one)-beta-D-glucopyranoside in *Triticum aestivum* leaves and its conversion into 6-methoxy-benzoxazolinone. *Z Naturforsch. C* 41**,** 684-690.

Güntner, C., González, A., Reis, R.D., González, G., Vázquez, A., Ferreira, F., et al. (1997). Effect of *Solanum* glycoalkaloids on potato aphid, *Macrosiphum euphorbiae*. *J Chem Ecol* 23**,** 1651-1659.

Guy, E., Boulain, H., Aigu, Y., Le Pennec, C., Chawki, K., Morliere, S., et al. (2016). Optimization of agroinfiltration in *Pisum sativum* provides a new tool for studying the salivary protein functions in the pea aphid complex. *Front Plant Sci* 7**,** 1171.

Hales, F.D., Wilson, A.C., Sloane, M.A., Simon, J.C., le Gallic, J.F., and Sunnucks, P. (2002). Lack of detectable genetic recombination on the X chromosome during the parthenogenetic production of female and male aphids. *Genet Res* 79(3)**,** 203-209.

Halkier, B.A. (2006). BIOLOGY AND BIOCHEMISTRY OF GLUCOSINOLATES. *Annu Rev Plant Biol* 57**,** 303-330.

Hall, A.E. (2004). Breeding for adaptation to drought and heat in cowpea. *European Journal of Agronomy* 21(4)**,** 447-454.

Hall, A.E., Ismail, A.M., Ehlers, J.D., Marfo, K.O., Cisse, N., Thiaw, S., et al. (2002). "Breeding cowpeas for tolerance to temperature extremes and adaptation to drought," in *Challenges and Opportunities for Enhancing Sustainable Cowpea Production,* eds. C.A. Fatokun, S.A. Tarawali, B.B.

Singh, P.M. Kormawa & M. Tamo. (Ibadan, Nigeria: Intl Inst Tropical Agric), 14-21.

Hall, A.E., Singh, B.B., and Ehlers, J.D. (1997). "Cowpea Breeding," in *Plant Breeding Reviews,* ed. J. Janick. (New York: John Wiley & Sons), 215-274.

Hansen, A.K., and Moran, N.A. (2011). Aphid genome expression reveals host-symbiont cooperation in the production of amino acids. *Proc Natl Acad Sci USA* 108(7)**,** 2849-2854.

Harmel, N., Letocart, 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(2)**,** 165-174.

Heese, A., Hann, D.R., Gimenez-Ibanez, S., Jones, A.M., He, K., Li, J., et al. (2007). The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proc Natl Acad Sci USA* 104(29)**,** 12217-12222.

Holdorf, M.M., Owen, H.A., Lieber, S.R., Yuan, L., Adams, N., Dabney-Smith, C., et al. (2012). Arabidopsis *ETHE1* encodes a sulfur dioxygenase that is essential for embryo and endosperm development. *Plant Physiol* 160(1)**,** 226-236.

Hopper, D.J., and Cooper, R.A. (1971). The regulation of *Escherichia coli* methylglyoxal synthase; a new control site in glycolysis? *FEBS Lett* 13(4)**,** 213-216.

Hoque, M.A., Uraji, M., Torii, A., Banu, M.N., Mori, I.C., Nakamura, Y., et al. (2012a). Methylglyoxal inhibition of cytosolic ascorbate peroxidase from *Nicotiana tabacum*. *J Biochem Mol Toxicol* 26(8)**,** 315-321.

Hoque, T.S., Hossain, M.A., Mostofa, M.G., Burritt, D.J., Fujita, M., and Tran, L.S. (2016). Methylglyoxal: an emerging signaling molecule in plant abiotic stress responses and tolerance. *Front Plant Sci* 7**,** 1341.

Hoque, T.S., Uraji, M., Tuya, A., Nakamura, Y., and Murata, Y. (2012b). Methylglyoxal inhibits seed germination and root elongation and up-regulates transcription of stress-responsive genes in ABA-dependent pathway in *Arabidopsis*. *Plant Biol* 14(5)**,** 854-858.

Hu, J., Xiao, C., and He, Y. (2016). Recent progress on the genetics and molecular breeding of brown planthopper resistance in rice. *Rice (N Y)* 9(1)**,** 30.

Hutchinson, L.A., and Bale, J.S. (1994). Effects of sublethal cold stress on the aphid *J Applied Ecol* 31(1)**,** 102-108.

Huynh, B.L., Ehlers, J.D., Ndeve, A., Wanamaker, S., Lucas, M.R., Close, T.J., et al. (2015). Genetic mapping and legume synteny of aphid resistance in African cowpea (*Vigna unguiculata* L. Walp.) grown in California. *Mol Breed* 35**,** 36.

Hwang, C.F., Bhakta, A.V., Truesdell, G.M., Pudlo, W.M., and Williamson, V.M. (2000). Evidence for a role of the N terminus and leucine-rich repeat region of the *Mi* gene product in regulation of localized cell death. *Plant Cell* 12**,** 1319-1329.

Inoue, Y., Rhee, H., Watanabe, K., Murata, K., and Kimura, A. (1988). Metabolism of 2-oxoaldehyde in mold. *European Journal of Biochemistry* 171**,** 213-218.

IITA. (2009). "IITA research to nourish Africa-Cowpea". (Ibadan, Nigeria).

International Aphid Genomics Consortium. (2010). Genome sequence of the pea aphid *Acyrthosiphon pisum*. *PLoS Biol* 8(2)**,** e1000313.

Jackai, L., and Daoust, R. (1986). Insect Pests of Cowpeas. *Annu Rev Entomol* 31**,** 95-119.

Jackson, K.A., McCord, J.S., and White, J.A. (2017). A window of opportunity: Subdominant predators can use suboptimal prey. *Ecol Evol* 7(14)**,** 5269-5275.

Jain, M., Batth, R., Kumari, S., and Mustafiz, A. (2016). *Arabidopsis thaliana* Contains Both Ni^{2+} and Zn^{2+} Dependent Glyoxalase I Enzymes and Ectopic Expression of the Latter Contributes More towards Abiotic Stress Tolerance in E. coli. *PLoS One* 11(7)**,** e0159348.

Jaouannet, M., Rodriguez, P.A., Thorpe, P., Lenoir, C.J., MacLeod, R., Escudero-Martinez, C., et al. (2014). Plant immunity in plant-aphid interactions. *Front Plant Sci* 5**,** 663.

Jaquiery, J., Stoeckel, S., Rispe, C., Mieuzet, L., Legeai, F., and Simon, J.C. (2012). Accelerated evolution of sex chromosomes in aphids, an x0 system. *Mol Biol Evol* 29(2)**,** 837-847.

Ji, H., Kim, S.R., Kim, Y.H., Suh, J.P., Park, H.M., Sreenivasulu, N., et al. (2016). Map-based Cloning and Characterization of the BPH18 Gene from Wild Rice Conferring Resistance to Brown Planthopper (BPH) Insect Pest. *Sci Rep* 6**,** 34376.

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., et al. (1999). *Arabidopsis thaliana* PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. *Proc Natl Acad Sci USA* 96(23)**,** 13583-13588.

Johnson, B. (1965). Wing polymorphism in aphids II. Interaction between aphids. *Entomol Exp Appl* 8**,** 49-64.

Johnson, C., Boden, E., and Arias, J. (2003). Salicylic acid and NPR1 induce the recruitment of trans-activating TGA factors to a defense gene promoter in Arabidopsis. *Plant Cell* 15(8)**,** 1846-1858.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. *Nature* 444(7117)**,** 323-329.

Ju, C., and Chang, C. (2015). Mechanistic Insights in Ethylene Perception and Signal Transduction. *Plant Physiol* 169(1)**,** 85-95.

Ju, C., Yoon, G.M., Shemansky, J.M., Lin, D.Y., Ying, Z.I., Chang, J., et al. (2012). CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in Arabidopsis. *Proc Natl Acad Sci USA* 109(47)**,** 19486-19491.

Kadota, Y., Sklenar, J., Derbyshire, P., Stransfeld, L., Asai, S., Ntoukakis, V., et al. (2014). Direct regulation of the NADPH oxidase RBOHD by the PRR-associated kinase BIK1 during plant immunity. *Mol Cell* 54(1)**,** 43-55.

Kalliampakou, K.I., Kouri, E.D., Boleti, H., Pavli, O., Maurousset, L., Udvardi, M.K., et al. (2011). Cloning and functional characterization of LjPLT4, a plasma membrane xylitol H(+)- symporter from Lotus japonicus. *Mol Membr Biol* 1**,** 1-13.

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. *Proc Natl Acad Sci USA* 92(2)**,** 622-625.

Kaloshian, I., and Walling, L.L. (2016). Hemipteran and dipteran pests: Effectors and plant host immune regulators. *J Integr Plant Biol* 58(4)**,** 350-361.

Kamphuis, L.G., Gao, L., and Singh, K.B. (2012). Identification and characterization of resistance to cowpea aphid (*Aphis craccivora* Koch) in *Medicago truncatula*. *BMC Plant Biol* 12**,** 101.

Kaplan, I., Sardanelli, S., Rehill, B.J., and Denno, R.F. (2011). Toward a mechanistic understanding of competition in vascular-feeding herbivores: an empirical test of the sink competition hypothesis. *Oecologia* 166(3)**,** 627-636.

Karapetyan, A.R., Buiting, C., Kuiper, R.A., and Coolen, M.W. (2013). Regulatory Roles for Long ncRNA and mRNA. *Cancers (Basel)* 5(2)**,** 462-490.

Kaur, C., Ghosh, A., Pareek, A., Sopory, S.K., and Singla-Pareek, S.L. (2014). Glyoxalases and stress tolerance in plants. *Biochem Soc Trans* 42**,** 485-490.

Kaur, C., Kushwaha, H.R., Mustafiz, A., Pareek, A., Sopory, S.K., and Singla-Pareek, S.L. (2015). Analysis of global gene expression profile of rice in response to methylglyoxal indicates its possible role as a stress signal molecule. *Front Plant Sci* 6**,** 682.

Kaur, C., Vishnoi, A., Ariyadasa, T.U., Bhattacharya, A., Singla-Pareek, S.L., and Sopory, S.K. (2013). Episodes of horizontal gene-transfer and gene-fusion led to co-existence of different metal-ion specific glyoxalase I. *Sci Rep* 3**,** 3076.

Kauss, H. (1987). Some Aspects of Calcium-Dependent Regulation in Plant Metabolism. *Annu Rev Plant Physiol* 38**,** 47-72.

Kawchuk, L.M., Martin, R.R., and McPherson, J. (1990). Resistance in Transgenic Potato Expressing the Potato Leafroll Virus Coat Protein Gene. *Mol Plant Microbe Interact* 3(5)**,** 301-307.

Kettles, G.J., Drurey, C., Schoonbeek, H.J., 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 Phytol* 198(4)**,** 1178-1190.

Kettles, G.J., and Kaloshian, I. (2016). The potato aphid salivary effector Me47 Is a glutathione-S-transferase involved in modifying plant responses to aphid infestation. *Front Plant Sci* 7**,** 1142.

Kim, J.H., and Jander, G. (2007). *Myzus persicae* (green peach aphid) feeding on Arabidopsis induces the formation of a deterrent indole glucosinolate. *Plant J* 49(6)**,** 1008-1019.

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(6)**,** 1015-1026.

Kim, K.S., Bellendir, S., Hudson, K.A., Hill, C.B., Hartman, G.L., Hyten, D.L., et al. (2010). Fine mapping the soybean aphid resistance gene Rag1 in soybean. *Theor Appl Genet* 120(5)**,** 1063-1071.

Kindlmann, P., and Dixon, A.F.G. (1989). Developmental Constraints in the Evolution of Reproductive Strategies: Telescoping of Generations in Parthenogenetic Aphids. *Funct Ecol* 3(5)**,** 531-537.

Kinkema, M., Fan, W., and Dong, X. (2000). Nuclear localization of NPR1 is required for activation of PR gene expression. *Plant Cell* 12(12)**,** 2339-2350.

Kloth, K.J., Busscher-Lange, J., Wiegers, G.L., Kruijer, W., Buijs, G., Meyer, R.C., et al. (2017). SIEVE ELEMENT-LINING CHAPERONE1 Restricts Aphid Feeding on Arabidopsis during Heat Stress. *Plant Cell* 29(10)**,** 2450-2464.

Knight, M.R., Campbell, A.K., Smith, S.M., and Trewavas, A.J. (1991). Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* 352(6335)**,** 524-526.

Knoblauch, M., and Peters, W.S. (2004). Forisomes, a novel type of Ca2+-dependent contractile protein motor. *Cell Motility and the Cytoskeleton* 58**,** 137-142.

Koch, K.G., Chapman, K., Louis, J., Heng-Moss, T., and Sarath, G. (2016). Plant Tolerance: A Unique Approach to Control Hemipteran Pests. *Front Plant Sci* 13.

Kokubo, Y., Nishizaka, M., Ube, N., Yabuta, Y., Tebayashi, S.I., Ueno, K., et al. (2017). Distribution of the tryptophan pathway-derived defensive secondary metabolites gramine and benzoxazinones in Poaceae. *Biosci Biotechnol Biochem* 81(3)**,** 431-440.

Kruger, N.J., and von Schaewen, A. (2003). The oxidative pentose phosphate pathway: structure and organisation. *Curr Opin Plant Biol* 6(3)**,** 236-246.

Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T., and Felix, G. (2004). The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. *Plant Cell* 16(12)**,** 3496-3507.

Kwon, K., Choi, D., Hyun, J.K., Jung, H.S., Baek, K., and Park, C. (2013). Novel glyoxalases from *Arabidopsis thaliana*. *FEBS J* 280**,** 3328-3339.

Lattanzio, V., Arpaia, S., Cardinali, A., Di Venere, D., and Linsalata, V. (2000). Role of endogenous flavonoids in resistance mechanism of *Vigna* to aphids. *J Agric Food Chem* 48(11)**,** 5316-5320.

Lei, J., Finlayson, S.A., Salzman, R.A., Shan, L., and Salzman, K.Z. (2014). BOTRYTIS-INDUCED KINASE1 Modulates Arabidopsis Resistance to Green Peach Aphids via PHYTOALEXIN DEFICIENT4 *Plant Physiol* 165(4)**,** 1657-1670.

Lemoine, R., La Camera, S., Atanassova, R., Dedaldechamp, F., Allario, T., Pourtau, N., et al. (2013). Source-to-sink transport of sugar and regulation by environmental factors. *Front Plant Sci* 4**,** 272.

Lenhart, P.A., Jackson, K.A., and White, J.A. (2018). Heritable variation in prey defence provides refuge for subdominant predators. *Proc Biol Sci* 285(1879).

Lewis, D.H., and Smith, D.C. (1967). Sugar alcohols (polyols) in fungi and green plants. *New Phytol* 66**,** 185-204.

Li, B., Ferreira, M.A., Huang, M., Camargos, L.F., Yu, X., Teixeira, R.M., et al. (2019a). The receptor-like kinase NIK1 targets FLS2/BAK1 immune complex and inversely modulates antiviral and antibacterial immunity. *Nat Commun* 10(1)**,** 4996.

Li, B., Meng, X., Shan, L., and He, P. (2016). Transcriptional Regulation of Pattern-Triggered Immunity in Plants. *Cell Host Microbe* 19(5)**,** 641-650.

Li, J., Brader, G., Kariola, T., and Palva, E.T. (2006). WRKY70 modulates the selection of signaling pathways in plant defense. *Plant J* 46(3)**,** 477-491.

Li, J., Brader, G., and Palva, E.T. (2004). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *Plant Cell* 16(2)**,** 319-331.

Li, L., Li, M., Yu, L., Zhou, Z., Liang, X., Liu, Z., et al. (2014). The FLS2-associated kinase BIK1 directly phosphorylates the NADPH oxidase RbohD to control plant immunity. *Cell Host Microbe* 15(3)**,** 329-338.

Li, N., Han, X., Feng, D., Yuan, D., and Huang, L.J. (2019b). Signaling Crosstalk between Salicylic Acid and Ethylene/Jasmonate in Plant Defense: Do We Understand What They Are Whispering? *Int J Mol Sci* 20(3).

Li, Z.G. (2016). Methylglyoxal and glyoxalase system in plants: old players, new concepts. *Bot Rev* 82**,** 183-203.

Li, Z.G., Duan, X.Q., Min, X., and Zhou, Z.H. (2017a). Methylglyoxal as a novel signal molecule induces the salt tolerance of wheat by regulating the glyoxalase system, the antioxidant system and osmolytes. *Protoplasma* 254(5)**,** 1995-2006.

Li, Z.G., Duan, X.Q., Xia, Y.M., Wang, Y., Zhou, Z.H., and Min, X. (2017b). Methylglyoxal alleviates cadmium toxicity in wheat (*Triticum aestivum* L). *Plant Cell Rep* 36**,** 367-370.

Lin, W., Li, B., Lu, D., Chen, S., Zhu, N., He, P., et al. (2014). Tyrosine phosphorylation of protein kinase complex BAK1/BIK1 mediates Arabidopsis innate immunity. *Proc Natl Acad Sci USA* 111(9)**,** 3632-3237.

Lindholm, P., Voutilainen, M.H., Lauren, J., Peranen, J., Leppanen, V.M., Andressoo, J.O., et al. (2007). Novel neurotrophic factor CDNF protects and rescues midbrain dopamine neurons *in vivo*. *Nature* 448(7149)**,** 73-77.

Liu, Y.Q., Wu, H., Chen, H., Liu, Y.L., He, J., Kang, H.Y., et al. (2015). A gene cluster encoding lectin receptor kinases confers broad-spectrum and durable insect resistance in rice. *Nat Biotechnol* 33(3)**,** 301-305.

Loudit, S.M.B., Bauwens, J., and Francis, F. (2018). Cowpea aphid-plant interactions: endosymbionts and related salivary protein patterns. *Entomol Exp Appl* 166(6)**,** 460-473.

Louis, J., Mondal, H.A., and Shah, J. (2012). Green peach aphid infestation induces Arabidopsis PHYTOALEXIN-DEFICIENT4 expression at site of insect feeding. *Plant Signal Behav* 7(11)**,** 1431-1433.

Louis, J., and Shah, J. (2015). Plant defence against aphids: the PAD4 signalling nexus. *J Exp Bot* 66(2)**,** 449-454.

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L., and He, P. (2010). A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. *Proc Natl Acad Sci USA* 107(1)**,** 496-501.

Lu, H., Zhu, J., Yu, J., Chen, X., Kang, L., and Cui, F. (2019). A Symbiotic Virus Facilitates Aphid Adaptation to Host Plants by Suppressing Jasmonic Acid Responses. *Mol Plant Microbe Interact* 33(1)**,** 55-65.

Macho, A.P., and Zipfel, C. (2014). Plant PRRs and the activation of innate immune signaling. *Mol Cell* 54(2)**,** 263-272.

Mai, V.C., Tran, N.T., and Nguyen, D.S. (2016). The involvement of peroxidases in soybean seedlings' defense against infestation of cowpea aphid. *Arthropod-Plant Interactions* 10**,** 283-292.

Maiti, M.K., Krishnasamy, S., Owen, H.A., and Makaroff, C.A. (1997). Molecular characterization of glyoxalase II from *Arabidopsis thaliana*. *Plant Mol Biol* 35(4)**,** 471-481.

Mannervik, B., Aronsson, A.C., and Tibbelin, G. (1982). Glyoxalase I from human erythrocytes. *Methods Enzymol* 90 Pt E**,** 535-541.

Marasinghe, G.P., Sander, I.M., Bennett, B., Periyannan, G., Yang, K.W., Makaroff, C.A., et al. (2005). Structural studies on a mitochondrial glyoxalase II. *J Biol Chem* 280(49)**,** 40668-40675.

Martinez de Ilarduya, O., Xie, Q., and Kaloshian, I. (2003). Aphid-induced defense responses in *Mi-1*-mediated compatible and incompatible tomato interactions. *Mol Plant Microbe Interact* 16(8)**,** 699-708.

Mathers, T.C., Chen, Y., Kaithakottil, G., Legeai, F., Mugford, S.T., Baa-Puyoulet, P., et al. (2017). Rapid transcriptional plasticity of duplicated gene clusters enables a clonally reproducing aphid to colonise diverse plant species. *Genome Biol* 18(1)**,** 27.

McAinsh, M.R., Webb, A., Taylor, J.E., and Hetherington, A.M. (1995). Stimulus-Induced Oscillations in Guard Cell Cytosolic Free Calcium. *Plant Cell* 7(8)**,** 1207-1219.

McGavin, G.C. (1993). *Bugs of the World.* New York: Blandford Press.

Medina-Ortega, K.J., and Walker, G.P. (2013). Does aphid salivation affect phloem sieve element occlusion *in vivo*? *J Exp Bot* 64**,** 5525-5535.

Meihls, L.N., Handrick, V., Glauser, G., Barbier, H., Kaur, H., Haribal, M.M., et al. (2013). Natural variation in maize aphid resistance is associated with 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside methyltransferase activity. *Plant Cell* 25(6)**,** 2341-2355.

Melvin, P., Bankapalli, K., D'Silva, P., and Shivaprasad, P.V. (2017). Methylglyoxal detoxification by a DJ-1 family protein provides dual abiotic and biotic stress tolerance in transgenic plants. *Plant Mol Biol* 94(4-5)**,** 381-397.

Merchante, C., Alonso, J.M., and Stepanova, A.N. (2013). Ethylene signaling: simple ligand, complex regulation. *Curr Opin Plant Biol* 16(5)**,** 554-560.

Mewis, I., Appel, H.M., Hom, A., Raina, R., and Schultz, J.C. (2005). Major signaling pathways modulate Arabidopsis glucosinolate accumulation and response to both phloem-feeding and chewing insects. *Plant Physiol* 138(2)**,** 1149-1162.

Miles, P.W. (1999). Aphid saliva. *Biol Rev* 74(1)**,** 41-85.

Milligan, S.B., Bodeau, J., Yaghoobi, J., Kaloshian, I., Zabel, P., and Williamson, V.M. (1998). The root knot nematode resistance gene *Mi* from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes. *Plant Cell* 10(8)**,** 1307-1319.

Misra, K., Banerjee, A.B., Ray, S., and Ray, M. (1995). Glyoxalase III from *Escherichia coli*: a single novel enzyme for the conversion of methylglyoxal into D-lactate without reduced glutathione. *Biochem J* 305 (Pt 3)**,** 999-1003.

Mittler, T.E. (1967). EFFECT OF AMINO ACID AND SUGAR CONCENTRATIONS ON THE FOOD UPTAKE OF THE APHID *MYZUS PERSICAE*. *Entomol Exp Appl* 10(1)**,** 39-51.

Mizobuchi, N., Hoseki, J., Kubota, H., Toyokuni, S., Nozaki, J.I., Naitoh, M., et al. (2007). ARMET is a soluble ER protein induced by the unfolded protein response via ERSE-II element. *Cell Struct Funct* 32(1)**,** 41-50.

Moran, N.A. (1992). The evolution of aphid lifecycles. *Annu Rev Entomol* 37**,** 321-348.

Moran, P.J., and Thompson, G.A. (2001). Molecular Responses to Aphid Feeding in Arabidopsis in Relation to Plant Defense Pathways. *Plant Physiol* 125(2)**,** 1074-1085.

Morgan, J.K., Luzio, G.A., Ammar el, D., Hunter, W.B., Hall, D.G., and Shatters, R.G., Jr. (2013). Formation of Stylet Sheaths in aere (in air) from eight species of phytophagous hemipterans from six families (Suborders: Auchenorrhyncha and Sternorrhyncha). *PLoS One* 8(4)**,** e62444.

Mostofa, M.G., Ghosh, A., Li, Z.G., Siddiqui, M.N., Fujita, M., and Tran, L.P. (2018). Methylglyoxal - a signaling molecule in plant abiotic stress responses. *Free Radic Biol Med* 122**,** 96-109.

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113(7)**,** 935-944.

Mousavi, S.A., Chauvin, A., Pascaud, F., Kellenberger, S., and Farmer, E.E. (2013). GLUTAMATE RECEPTOR-LIKE genes mediate leaf-to-leaf wound signalling. *Nature* 500(7463)**,** 422-426.

Mugford, S.T., Barclay, E., Drurey, C., Findlay, K.C., and Hogenhout, S.A. (2016). An Immuno-Suppressive Aphid Saliva Protein Is Delivered into the Cytosol of Plant Mesophyll Cells During Feeding. *Mol Plant Microbe Interact* 29(11)**,** 854-861.

Mur, L.A., Kenton, P., Atzorn, R., Miersch, O., and Wasternack, C. (2006). The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiol* 140(1)**,** 249-262.

Mustafiz, A., Singh, A.K., Pareek, A., Sopory, S.K., and Singla-Pareek, S.L. (2011). Genome-wide analysis of rice and Arabidopsis identifies two glyoxalase genes that are highly expressed in abiotic stresses *Funct Integr Genomics* 11(2)**,** 293-305.

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. *Proc Natl Acad Sci USA* 105(29)**,** 9965-9969.

Mutti, N.S., Park, Y., Reese, J.C., and Reeck, G.R. (2006). RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*. *J Insect Sci* 6**,** 1-7.

Naessens, E., Dubreuil, G., Giordanengo, P., Baron, O.L., Minet-Kebdani, N., Keller, H., et al. (2015). A secreted MIF cytokine enables aphid feeding

and represses plant immune responses. *Current Biology* 25(14)**,** 1898-1903.

Nakagawa, J., Ishikura, S., Asami, J., Isaji, T., Usami, N., Hara, A., et al. (2002). Molecular characterization of mammalian dicarbonyl/L-xylulose reductase and its localization in kidney. *J Biol Chem* 277(20)**,** 17883-17891.

Nalam, V., Louis, J., and Shah, J. (2019). Plant defense against aphids, the pest extraordinaire. *Plant Sci* 279**,** 96-107.

FAO. (2020). *FAOSTAT Data* [Online]. Available: http://www.fao.org/faostat/en/#data/QC [Accessed 8-13-20].

Nicholson, S.J., Hartson, S.D., and Puterka, G.J. (2012). Proteomic analysis of secreted saliva from Russian Wheat Aphid (*Diuraphis noxia* Kurd.) biotypes that differ in virulence to wheat. *J Proteomics* 75(7)**,** 2252-2268.

Nicholson, S.J., Nickerson, M.L., Dean, M., Song, Y., Hoyt, P.R., Rhee, H., et al. (2015). The genome of *Diuraphis noxia*, a global aphid pest of small grains. *BMC Genomics* 16**,** 429.

Nicholson, S.J., and Puterka, G.J. (2014). Variation in the salivary proteomes of differentially virulent greenbug (*Schizaphis graminum* Rondani) biotypes. *J Proteomics* 105**,** 186-203.

Noiraud, N., Maurousset, L., and Lemoine, R. (2001). Identification of a Mannitol Transporter, AgMaT1, in Celery Phloem. *Plant Cell* 13**,** 695-705.

Nombela, G., Williamson, V.M., and Muniz, M. (2003). The root-knot nematode resistance gene *Mi-1.2* of tomato is responsible for resistance against the whitefly *Bemisia tabaci*. *Mol Plant Microbe Interact* 16(7)**,** 645-649.

Norman-Setterblad, C., Vidal, S., and Palva, E.T. (2000). Interacting signal pathways control defense gene expression in Arabidopsis in response to cell wall-degrading enzymes from *Erwinia carotovora*. *Mol Plant Microbe Interact* 13(4)**,** 430-438.

Norton, S.J., Talesa, V., Yuan, W.J., and Principato, G.B. (1990). Glyoxalase I and glyoxalase II from Aloe vera: purification, characterization and comparison with animal glyoxalases. *Biochem Int* 22(3)**,** 411-418.

Obeng-Ofori, D. (2007). "Pests of grain legumes," in *Major pests of food and selected fruit and industrial crops in West Africa,* ed. D. Obeng-Ofori. (Accra: The City Publishers Limited), 81-112.

Obopile, M., and Ositile, B. (2010). Life table and population parameters of cowpea aphid, *Aphis craccivora* Koch (Homoptera: Aphididae) on five cowpea *Vigna unguiculata* (L. Walp.) varieties. *J Pest Sci* 83**,** 9-14.

Odani, H., Asami, J., Ishi, A., Oide, K., Sudo, T., Nakamura, A., et al. (2008). Suppression of renal alpha-dicarbonyl compounds generated following ureteral obstruction by kidney-specific alpha-dicarbonyl/L-xylulose reductase. *Annals of the New York Academy of Sciences* 1126**,** 320-324.

Ofuya, T. (1995). Studies on the capability of *Cheilomenes lunata* (Fabricius) (Coleoptera: Coccinellidae) to prey on the cowpea aphid, *Aphis craccivora* Koch (Homoptera: Aphididae) in Nigeria *Agriculture, Ecosystems and Environment* 52**,** 35-38.

Ogawa, K., and Miura, T. (2014). Aphid polyphenisms: trans-generational developmental regulation through viviparity. *Front Physiol* 5.

Oh, C.S., and Martin, G.B. (2011). Tomato 14-3-3 protein TFT7 interacts with a MAP kinase kinase to regulate immunity-associated programmed cell death mediated by diverse disease resistance proteins. *J Biol Chem* 286(16)**,** 14129-14136.

Omoigui, L.O., Ekeuro, G.C., Kamara, A.Y., Bello, L.L., Timko, M.P., and Ogunwolu, G.O. (2017). New sources of aphids [*Aphis craccivora* (Koch)] resistance in cowpea germplasm using phenotypic and molecular marker approaches. *Euphytica* 213**,** 178.

Painter, R.H. (1951). *Insect Resistance in Crop Plants.* New York: MacMillan.

Palgi, M., Greco, D., Lindstrom, R., Auvinen, P., and Heino, T.I. (2012). Gene expression analysis of *Drosophilaa Manf* mutants reveals perturbations in membrane traffic and major metabolic changes. *BMC Genomics* 13**,** 134.

Palgi, M., Lindstrom, R., Peranen, J., Piepponen, T.P., Saarma, M., and Heino, T.I. (2009). Evidence that DmMANF is an invertebrate neurotrophic factor supporting dopaminergic neurons. *Proc Natl Acad Sci USA* 106(7)**,** 2429-2434.

Patton, M.F., Bak, A., Sayre, J.M., Heck, M.L., and Casteel, C.L. (2020). A polerovirus, Potato leafroll virus, alters plant-vector interactions using three viral proteins. *Plant Cell Environ* 43(2)**,** 387-399.

Pauwels, L., Barbero, G.F., Geerinck, J., Tilleman, S., Grunewald, W., Perez, A.C., et al. (2010). NINJA connects the co-repressor TOPLESS to jasmonate signalling. *Nature* 464(7289)**,** 788-791.

Pegadaraju, V., Knepper, C., Reese, J., and Shah, J. (2005). Premature leaf senescence modulated by the Arabidopsis PHYTOALEXIN DEFICIENT4 gene is associated with defense against the phloem-feeding green peach aphid. *Plant Physiol* 139(4)**,** 1927-1934.

Pegadaraju, V., Louis, J., Singh, V., Reese, J.C., Bautor, J., Feys, B.J., et al. (2007). Phloem-based resistance to green peach aphid is controlled by Arabidopsis PHYTOALEXIN DEFICIENT4 without its signaling partner ENHANCED DISEASE SUSCEPTIBILITY1. *Plant J* 52(2)**,** 332-341.

Penninckx, I.A., Eggermont, K., Terras, F.R., Thomma, B.P., De Samblanx, G.W., Buchala, A., et al. (1996). Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. *Plant Cell* 8(12)**,** 2309-2323.

Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P., and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. *Plant Cell* 10(12)**,** 2103-2113.

Perco, P., Ju, W., Kerschbaum, J., Leierer, J., Menon, R., Zhu, C., et al. (2019). Identification of dicarbonyl and L-xylulose reductase as a therapeutic target in human chronic kidney disease. *JCI Insight* 4(12).

Peters, W.S., van Bel, A.J., and Knoblauch, M. (2006). The geometry of the forisomes-sieve-element- sieve plate complex in the phloem of *Vicia faba* L. leaflets. *J Exp Bot* 57(12)**,** 3091-3098.

Phillips, S.A., and Thornalley, P.J. (1993). Formation of methylglyoxal and D-lactate in human red blood cells *in vitro*. *Biochem Soc Trans* 21(2)**,** 163S.

Pitino, M., and Hogenhout, S.A. (2013). Aphid protein effectors promote aphid colonization in a plant species-specific manner. *Mol Plant Microbe Interact* 26(1)**,** 130-139.

Pitrat, M., and Lecoq, H. (1982). Genetic relations between non-acceptance and antibiosis resistance to *Aphis gossypii* in melon: search for linkage with other genes. *Agronomie* 2**,** 503-508.

Pre, M., Atallah, M., Champion, A., De Vos, M., Pieterse, C.M., and Memelink, J. (2008). The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. *Plant Physiol* 147(3)**,** 1347-1357.

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(4)**,** 2207-2219.

Proietti, S., Caarls, L., Coolen, S., Van Pelt, J.A., Van Wees, S.C.M., and Pieterse, C.M. (2018). Genome-wide association study reveals novel players in defense hormone crosstalk in *Arabidopsis*. *Plant Cell Environ* 41(10)**,** 2342-2356.

Proietti, S., Falconieri, G.S., Bertini, L., Baccalli, I., Paccosi, E., Belardo, A., et al. (2019). GLYI4 Plays A Role in Methylglyoxal Detoxificationand Jasmonate-Mediated Stress Responses in *Arabidopsis thaliana*. *Biomolecules* 9(635).

Qiao, H., Shen, Z., Huang, S.S., Schmitz, R.J., Urich, M.A., Briggs, S.P., et al. (2012). Processing and subcellular trafficking of ER-tethered EIN2 control response to ethylene gas. *Science* 338(6105)**,** 390-393.

Rabbani, N., Xue, M., and Thornalley, P.J. (2016). Dicarbonyls and glyoxalase in disease mechanisms and clinical therapeutics. *Glycoconj J* 33(4)**,** 513-525.

Rao, S.A.K., Carolan, J.C., and Wilkinson, T.L. (2013). Proteomic profiling of cereal aphid saliva reveals both ubiquitous and adaptive secreted proteins. *Plos One* 8(2)**,** e57413.

Ray, S., and Ray, M. (1982). Purification and characterization of NAD and NADP-linked alphaketoaldehyde dehydrogenases involved in catalyzing the oxidation of methylglyoxal to pyruvate. *J Biol Chem* 257(18)**,** 10566-10570.

Remaudiere, G., and Remaudiere, M. (1997). *Catalogue des Aphididae du monde (Homoptera Aphidoidea).* Paris: Paris Institut national de la recherche agronomique.

Reynolds, D.R. (1999). *Capnodium citri*: The sooty mold fungi comprising the taxon concept. *Mycopathologia* 148**,** 141-147.

Rhee, H., Murata, K., and Kimura, A. (1986). Purification and characterization of glyoxalase I from Pseudomonas putida. *Biochem Biophys Res Commun* 141(3)**,** 993-999.

Richard, J.P. (1993). Mechanism for the formation of methylglyoxal from triosephosphates. *Biochem Soc Trans* 21(2)**,** 549-553.

Roberts, M., and Wink, M. (1998). *Alkaloids: biochemistry, ecology, and medicinal applications.* New York, NY: Plenum Press.

Rodriguez, P.A., Escudero-Martinez, C., and Bos, J.I.B. (2017). An aphid effector targets trafficking protein VPS52 in a host-specific manner to promote virulence. *Plant Physiol* 173(3)**,** 1892-1903.

Rodriguez, P.A., Stam, R., Warbroek, T., and Bos, J.I. (2014). Mp10 and Mp42 from the aphid species *Myzus persicae* trigger plant defenses in Nicotiana benthamiana through different activities. *Mol Plant Microbe Interact* 27(1)**,** 30-39.

Rossi, M., Goggin, F.L., Milligan, S.B., Kaloshian, I., Ullman, D.E., and Williamson, V.M. (1998). The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. *Proc Natl Acad Sci USA* 95(17)**,** 9750-9754.

Sabri, A., Vandermoten, S., Leroy, P.D., Haubruge, E., Hance, T., Thonart, P., et al. (2013). Proteomic investigation of aphid honeydew reveals an unexpected diversity of proteins. *PLoS One* 8(9)**,** e74656.

Saito, R., Yamamoto, H., Makino, A., Sugimoto, T., and Miyake, C. (2011). Methylglyoxal functions as hill oxidant and stimulates the photoreduction of O(2) at photosystem I: a symptom of plant diabetes. *Plant, Cell & Environ* 34(9)**,** 1454-14564.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., et al. (2000). Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. *Proc Natl Acad Sci USA* 97(21)**,** 11655-11660.

Schmitz, J., Dittmar, I.C., Brockmann, J.D., Schmidt, M., Hudig, M., Rossoni, A.W., et al. (2017). Defense against Reactive Carbonyl Species Involves at Least Three Subcellular Compartments Where Individual Components of the System Respond to Cellular Sugar Status. *Plant Cell* 29(12)**,** 3234-3254.

Schmitz, J., Rossoni, A.W., and Maurino, V.G. (2018). Dissecting the Physiological Function of Plant Glyoxalase I and Glyoxalase I-Like Proteins. *Front Plant Sci* 9**,** 1618.

Schuhegger, R., Nafisi, M., Mansourova, M., Petersen, B.L., Olsen, C.E., Svatos, A., et al. (2006). CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis. *Plant Physiol* 141(4)**,** 1248-1254.

Schulze, B., Mentzel, T., Jehle, A.K., Mueller, K., Beeler, S., Boller, T., et al. (2010). Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. *J Biol Chem* 285(13)**,** 9444-9451.

Schwessinger, B., Roux, M., Kadota, Y., Ntoukakis, V., Sklenar, J., Jones, A., et al. (2011). Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. *PLoS Genet* 7(4)**,** e1002046.

Sengupta, D., Naik, D., and Reddy, A.R. (2015). Plant aldo-keto reductases (AKRs) as multi-tasking soldiers involved in diverse plant metabolic processes and stress defense: a structure-function update. *J Plant Phys* 179**,** 40-55.

Shigenaga, A.M., and Argueso, C.T. (2016). No hormone to rule them all: Interactions of plant hormones during the responses of plants to pathogens. *Semin Cell Dev Biol* 56**,** 174-189.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., and Ishikawa, H. (2000). Genome sequence of the endocellular bacterial symbiont of aphids Buchnera sp. APS. *Nature* 407(6800)**,** 81-86.

Shine, M.B., Yang, J.W., El-Habbak, M., Nagyabhyru, P., Fu, D.Q., Navarre, D., et al. (2016). Cooperative functioning between phenylalanine ammonia lyase and isochorismate synthase activities contributes to salicylic acid biosynthesis in soybean. *New Phytol* 212(3)**,** 627-636.

Singh, B.B. (2014). *Cowpea: The Food Legume of the 21st Century.* Madison, WI: Crop Science Society of America.

Singh, B.B., Chambliss, O.L., and Sharma, B. (1996). "Recent advances in cowpea breeding " in *Advances in Cowpea Research,* eds. B.B. Singh, D.R. Mohan Raj, K.E. Dashiell & L.E.N. Jackai. (Ibadan, Nigeria: International Institute of Tropical Agriculture), 30-49.

Singh, B.B., Ehlers, J.D., Sharma, B., and Freire, F.R. (Year). "Recent progress in cowpea breeding", in: *Proceedings of the World Cowpea Conference III*, eds. C.A. Fatokun, B.B. Tarawali, B.B. Singh, P.M. Kormawa & M. Tamo), 22-40.

Smith, C.M. (1989). *Plant Resistance to Insects: A Fundamental Approach.* New York: John Wiley & Sons.

Smith, C.M. (2005). *Plant Resistance to Arthropods.* Netherlands: Springer.

Sochor, M., Baquer, N.Z., and McLean, P. (1979). Glucose overutilization in diabetes: evidence from studies on the changes in hexokinase, the pentose phosphate pathway and glucuronate-xylulose pathway in rat kidney cortex in diabetes. *Biochem Biophys Res Commun* 86(1)**,** 32-339.

Sorenson, J. (2009). "Aphids," in *Encyclopedia of Insects,* eds. V. Resh & R. Carde. Second ed (San Diego, CA: Academic Press), 27-30.

Souleymane, A., Aken'Ova, M.E., Fatokun, C.A., and Alabi, O.Y. (2013). Screening for resistance to cowpea aphid (*Aphis craccivora* Koch) in wild and cultivated cowpea (*Vigna unguiculata* L. Walp.) accessions. *International Journal of Science, Environment and Technology* 2(4)**,** 611-621.

Sousa Silva, M., Gomes, R.A., Ferreira, A.E., Ponces Freire, A., and Cordeiro, C. (2013). The glyoxalase pathway: the first hundred years... and beyond. *Biochemical Journal* 453(1)**,** 1-15.

Spoel, S.H., and Dong, X. (2008). Making sense of hormone crosstalk during plant immune responses. *Cell Host Microbe* 3(6)**,** 348-351.

Srinivasan, R., and Alvarez, J.M. (2007). Effect of mixed viral infections (potato virus Y-potato leafroll virus) on biology and preference of vectors *Myzus persicae* and *Macrosiphum euphorbiae* (Hemiptera: Aphididae). *J Econ Entom* 100(3)**,** 646-655.

Staswick, P.E., and Tiryaki, I. (2004). The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. *Plant Cell* 16(8)**,** 2117-2127.

Su, Y.L., Li, M.J., Luan, J.B., Ye, X.D., Wang, X.W., and Liu, S.S. (2012). Transcriptomic analysis of the salivary glands of an invasive whitefly. *PLoS One* 7(6)**,** e39303.

Subedi, K.P., Choi, D., Kim, I., Min, B., and Park, C. (2011). Hsp31 of *Escherichia coli* K-12 is glyoxalase III. *Mol Microbiol* 81(4)**,** 926-936.

Suza, W.P., and Staswick, P.E. (2008). The role of JAR1 in Jasmonoyl-L: -isoleucine production during Arabidopsis wound response. *Planta* 227(6)**,** 1221-1232.

Takken, F.L., and Goverse, A. (2012). How to build a pathogen detector: structural basis of NB-LRR function. *Curr Opin Plant Biol* 15(4)**,** 375-384.

Tamura, Y., Hattori, M., Yoshioka, H., Yoshioka, M., Takahashi, A., Wu, J., et al. (2014). Map-based cloning and characterization of a brown planthopper resistance gene BPH26 from Oryza sativa L. ssp. indica cultivar ADR52. *Sci Rep***,** 5872.

Teixeira, M.A., Sela, N., Atamian, H.S., Bao, E., Chaudhary, R., MacWilliams, J., et al. (2018). Sequence analysis of the potato aphid *Macrosiphum euphorbiae* transcriptome identified two new viruses. *PLoS One* 13(3)**,** e0193239.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., et al. (2007). JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. *Nature* 448(7154)**,** 661-665.

Thornalley, P.J. (1990). The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. *Biochem J* 269(1)**,** 1-11.

Thornalley, P.J. (1996). Pharmacology of methylglyoxal: formation, modification of proteins and nucleic acids, and enzymatic detoxification--a role in pathogenesis and antiproliferative chemotherapy. *Gen Pharmacol* 27(4)**,** 565-573.

Thornalley, P.J. (2006). Advanced glycation end products in renal failure. *J Ren Nutr* 16(3)**,** 178-184.

Thorpe, P., Cock, P.J., and Bos, J. (2016). Comparative transcriptomics and proteomics of three different aphid species identifies core and diverse effector sets. *BMC Genomics* 17**,** 172.

Thorpe, P., Escudero-Martinez, C.M., Cock, P.J.A., Eves-van den Akker, S., and Bos, J.I.B. (2018). Shared transcriptional control and disparate gain and loss of aphid parasitism genes. *Genome Biol Evol* 10(10)**,** 2716-2733.

Tjallingii, W.F. (2006). Salivary secretions by aphids interacting with proteins of phloem wound responses. *J Exp Bot* 57(4)**,** 739-745.

Tjallingii, W.F., and Esch, T.H. (1993). Fine-structure of aphid stylet routes in plant-tissues in correlation with EPG signals. *Physiol Entomol* 18(3)**,** 317-328.

Togola, A., Boukar, O., Servent, A., Chamarthi, S., Tamo, M., and Fatokun, C. (2020). Identification of sources of resistance in cowpea mini core accessions to *Aphis craccivora* Koch (Homoptera: Aphididae) and their biochemical characterization. *Euphytica* 216(6)**,** 88.

Torres, M.A., Jones, J.D., and Dangl, J.L. (2006). Reactive oxygen species signaling in response to pathogens. *Plant Physiol* 141(2)**,** 373-378.

van den Heuvel, J., Hummelen, H., Verbeek, M., Dullemans, A., and van der Wilk, F. (1997). Characteristics of acyrthosiphon pisum virus, a newly identified virus infecting the pea aphid. *J Invertebr Pathol* 70**,** 169-176.

van der Hoorn, R.A., and Kamoun, S. (2008). From Guard to Decoy: a new model for perception of plant pathogen effectors. *Plant Cell* 20(8)**,** 2009-2017.

Vandermoten, S., Harmel, N., Mazzucchelli, G., De Pauw, E., Haubruge, E., and Francis, F. (2014). Comparative analyses of salivary proteins from three aphid species. *Insect Mol Biol* 23(1)**,** 67-77.

Verma, D.P., and Hong, Z. (2001). Plant callose synthase complexes. *Plant Mol Biol* 47(6)**,** 693-701.

Villada, E.S., Gonzalez, E.G., Lopez-Sese, A.I., Castiel, A.F., and Gomez-Guillamon, M.L. (2009). Hypersensitive response to *Aphis gossypii* Glover in melon genotypes carrying the *Vat* gene. *J Exp Bot* 60(11)**,** 3269-3277.

Vincent, T.R., Avramova, M., Canham, J., Higgins, P., Bilkey, N., Mugford, S.T., et al. (2017). Interplay of Plasma Membrane and Vacuolar Ion Channels, Together with BAK1, Elicits Rapid Cytosolic Calcium Elevations in Arabidopsis during Aphid Feeding. *Plant Cell* 29(6)**,** 1460-1479.

Vos, P., Simons, G., Jesse, T., Wijbrandi, J., Heinen, L., Hogers, R., et al. (1998). The tomato *Mi-1* gene confers resistance to both root-knot nematodes and potato aphids. *Nat Biotechnol* 16(13)**,** 1365-1369.

Wadleigh, R.W., and Yu, S.J. (1988). Detoxification of isothiocyanate allelochemicals by glutathione transferase in three lepidopterous species. *J Chem Ecol* 14(4)**,** 1279-1288.

Walker, G.P., and Medina-Ortega, K.J. (2012). Penetration of faba bean sieve elements by pea aphid does not trigger forisome dispersal. *Entomol Exp Appl* 144**,** 326-355.

Walling, L.L. (2000). The Myriad Plant Responses to Herbivores. *J Plant Growth Regul* 19(2)**,** 195-216.

Walling, L.L. (2008). Avoiding effective defenses: strategies employed by phloem-feeding insects. *Plant Physiol* 146(3)**,** 859-866.

Wang, W., Dai, H.E., Zhang, Y., Chandrasekar, R., Luo, L., Hiromasa, Y., et al. (2015a). Armet is an effector protein mediating aphid-plant interactions. *Faseb Journal* 29(5)**,** 2032-2045.

Wang, W., Luo, L., Lu, H., Chen, S., Kang, L., and Cui, F. (2015b). Angiotensin-converting enzymes modulate aphid–plant interactions. *Sci Rep* 5**,** 8885.

Wang, Y., Cao, L., Zhang, Y., Cao, C., Liu, F., Huang, F., et al. (2015c). Map-based cloning and characterization of BPH29, a B3 domain-containing recessive gene conferring brown planthopper resistance in rice. *J Exp Bot* 66(19)**,** 6035-6045.

Wang, Y.M., and Van Eys, J. (1970). The enzymatic defect in essential pentosuria. *N Engl J Med* 282(16)**,** 892-896.

Wasternack, C., and Hause, B. (2013). Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. *Ann Bot* 111(6)**,** 1021-1058.

Welchen, E., Schmitz, J., Fuchs, P., Garcia, L., Wagner, S., Wienstroer, J., et al. (2016). d-Lactate Dehydrogenase Links Methylglyoxal Degradation and Electron Transport through Cytochrome c *Plant Physiol* 172(2)**,** 901-912.

Wenger, J.A., Cassone, B.J., Legeai, F., Johnston, J.S., Bansal, R., Yates, A.D., et al. (2017). Whole genome sequence of the soybean aphid, *Aphis glycines*. *Insect Biochem Mol Biol*.

White, J.A., McCord, J.M., Jackson, K.A., Dehnel, A.K., and Lenhart, P.A. (2017). Differential aphid toxicity to ladybeetles is not a function of host plant or facultative bacterial symbionts. *Funct Ecol* 31**,** 334-339.

Wienstroer, J., Engqvist, M., Kunz, H., Flügge, U.I., and Maurino, V.G. (2012). D-Lactate dehydrogenase as a marker gene allows positive selection of transgenic plants. *FEBS Lett* 586**,** 36-40.

Will, T., Steckbauer, K., Hardt, M., and van Bel, A.J. (2012). Aphid gel saliva: sheath structure, protein composition and secretory dependence on stylet-tip milieu. *PLoS One* 7(10)**,** e46903.

Will, T., Tjallingii, W.F., Thonnessen, A., and van Bel, A.J.E. (2007). Molecular sabotage of plant defense by aphid saliva. *Proc Natl Acad Sci USA* 104(25)**,** 10536-10541.

Will, T., and van Bel, A.J.E. (2006). Physical and chemical interactions between aphids and plants. *J Exp Bot* 57**,** 729-733.

Will, T., and Vilcinskas, A. (2015). The structural sheath protein of aphids is required for phloem feeding. *Insect Biochem Mol Biol* 57**,** 34-40.

Wu, C., Avila, C.A., and Goggin, F.L. (2015). The ethylene response factor Pti5 contributes to potato aphid resistance in tomato independent of ethylene signalling. *J Exp Bot* 66(2)**,** 559-570.

Wu, Y., Zhang, D., Chu, J.Y., Boyle, P., Wang, Y., Brindle, I.D., et al. (2012). The Arabidopsis NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep* 1(6)**,** 639-647.

Xu, X.M., Lin, H., Maple, J., Björkblom, B., Alves, G., Larsen, J.P., et al. (2010). The *Arabidopsis* DJ-1a protein confers stress protection through cytosolic SOD activation. *J Cell Sci* 123**,** 1644-1651.

Xu, Y., Chang, P., Liu, D., Narasimhan, M.L., Raghothama, K.G., Hasegawa, P.M., et al. (1994). Plant Defense Genes Are Synergistically Induced by Ethylene and Methyl Jasmonate. *Plant Cell* 6(8)**,** 1077-1085.

Yadav, S.K., Singla-Pareek, S.L., Ray, M., Reddy, M.K., and Sopory, S.K. (2005). Methylglyoxal levels in plants under salinity stress are dependent on glyoxalase I and glutathione. *Biochem Biophys Res Commun* 337(1)**,** 61-67.

Yamauchi, Y., Hasegawa, A., Taninaka, A., Mizutani, M., and Sugimoto, Y. (2011). NADPH-dependent reductases involved in the detoxification of reactive carbonyls in plants. *J Biol Chem* 286(9)**,** 6999-7009.

Yang, S., Jan, Y.H., Mishin, V., Heck, D.E., Laskin, D.L., and Laskin, J.D. (2017). Diacetyl/l-Xylulose reductase mediates chemical redox cycling in lung epithelial cells. *Chem Res Toxicol* 30(7)**,** 1406-1418.

Yang, Z., Ma, L., Francis, F., Yang, Y., Chen, H., Wu, H., et al. (2018). Proteins identified from saliva and salivary glands of the Chinese gall aphid *Schlechtendalia chinensis*. *Proteomics* 18(9)**,** e1700378.

Yu, P.H. (1990). Oxidative deamination of aliphatic amines by rat aorta semicarbazide-sensitive amine oxidase. *J Pharm Pharmacol* 42(12)**,** 882-884.

Zaynab, M., Fatima, M., Abbas, S., Umair, M., Sharif, Y., and Raza, M.A. (2018). Long non-coding RNAs as molecular players in plant defense against pathogens. *Microbial Pathogenesis* 121**,** 277-282.

Zhang, Y., Fan, J., Sun, J.R., and Chen, J.L. (2015). Cloning and RNA interference analysis of the salivary protein C002 gene in *Schizaphis graminum*. *J Integrative Agriculture* 14(4)**,** 698-705.

Zipfel, C. (2009). Early molecular events in PAMP-triggered immunity. *Curr Opin Plant Biol* 12(4)**,** 414-420.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., et al. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. *Cell* 125(4)**,** 749-760.

Zúñiga, G.E., Argandoña, V.H., Niemeyer, H.M., and Corcuera, L.J. (1983). Hydroxamic acid content in wild and cultivated graminae. *Phytochemistry* 22(12)**,** 2665-2668.

Züst, T., and Agrawal, A.A. (2016). Mechanisms and evolution of plant resistance to aphids. *Nat Plants* 2**,** 15206.

Züst, T., Agrawal, A.A., and Lee, K.P. (2015). Population growth and sequestration of plant toxins along a gradient of specialization in four aphid species on the common milkweed *Asclepias syriac*. *Funct Ecol* 30(4)**,** 547-556.

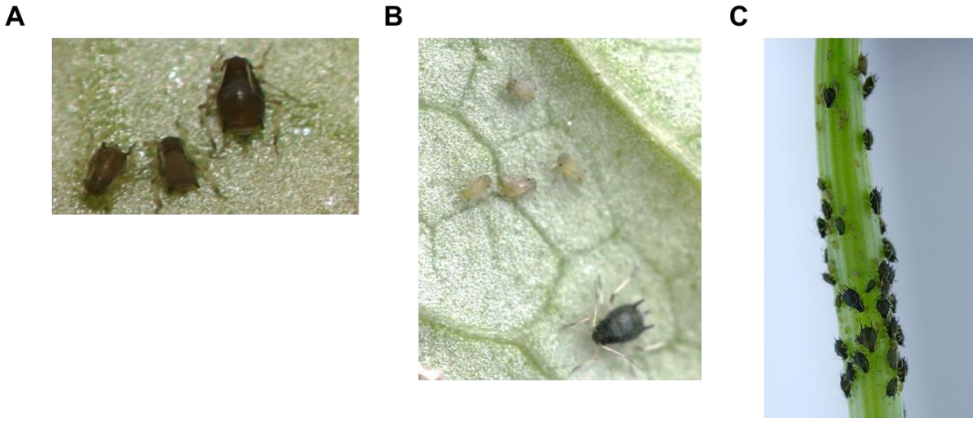

Fig 0.1. Cowpea aphids feeding on cowpea. **A)** A cowpea aphid adult and nymphs. **B)** A cowpea aphid adult with newborn nymphs. **C)** Cowpea aphid infestation on a cowpea stem.

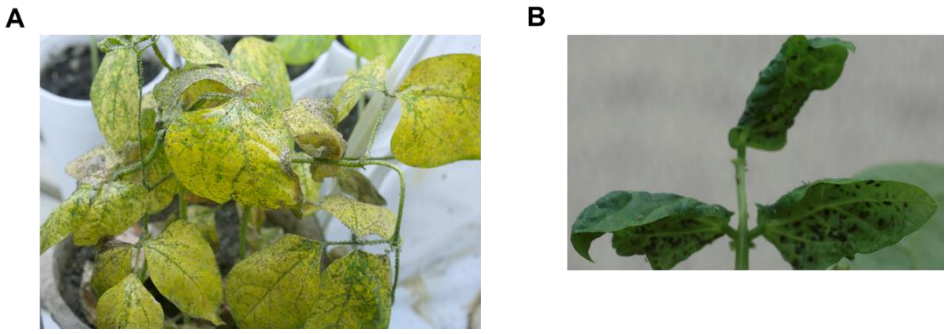

Fig 0.2. Cowpea aphid generated damage on cowpea. **A)** Cowpea plants infested with cowpea aphids displaying chlorosis and necrosis symptoms. **B)** A cowpea trifoliate infested with cowpea aphids displaying the pseudogalling/leaf rolling symptom.

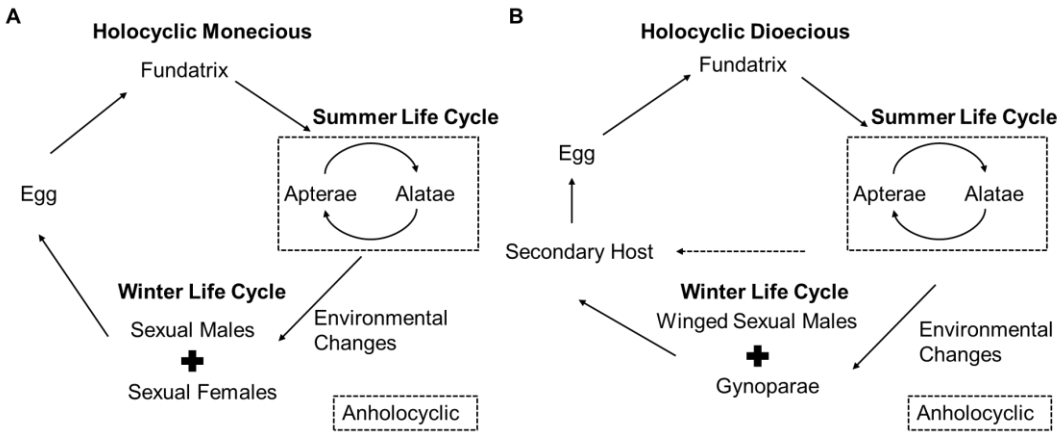

Fig 0.3. Diagram of aphid life cycles. Dotted lines indicate parts of the life cycle that are present in the anholocyclic life cycle. **A)** Monecious life cycle for holocyclic and anholocyclic aphids. **B)** Dioecious lifecycle for holocyclic and anholocyclic aphids.

Chapter One

AcDCXR is a Cowpea Aphid Effector with Putative Roles in Altering Host Immunity and Physiology

Abstract

Cowpea, *Vigna unguiculata*, is a crop that is essential to semiarid areas of the world like Sub-Sahara Africa. Cowpea is highly susceptible to cowpea aphid, *Aphis craccivora,* infestation that can lead to major yield losses. Aphids feeds on their host plant by inserting their hypodermal needlelike flexible stylets into the plant to reach the phloem sap. During feeding, aphids secrete saliva, containing effector proteins, into the plant to disrupt plant immune responses and alter the physiology of the plant to their own advantage. Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to identify the salivary proteome of the cowpea aphid. About 150 candidate proteins were identified including diacetyl/L-xylulose reductase (DCXR), a novel enzyme previously unidentified in aphid saliva. DCXR is a member of short-chain dehydrogenases/reductases with dual enzymatic functions in carbohydrate and dicarbonyl metabolism. To assess whether cowpea aphid DCXR (AcDCXR) has similar functions, recombinant AcDCXR was purified and assayed enzymatically. For carbohydrate metabolism, the oxidation of xylitol to xylulose was tested. The dicarbonyl reaction involved the reduction of methylglyoxal, an α-β-dicarbonyl ketoaldehyde, known as an abiotic and biotic stress response molecule causing cytotoxicity at high concentrations. To assess whether cowpea aphids induce methylglyoxal in plants, we measured methylglyoxal levels in both cowpea and pea (*Pisum sativum*) plants and found them elevated transiently after aphid infestation.

Agrobacterium-mediated transient overexpression of AcDCXR in pea resulted in an increase of cowpea aphid fecundity. Taken together, our results indicate that AcDCXR is an effector with a putative ability to generate additional sources of energy to the aphid and to alter plant defense responses. In addition, this work identified methylglyoxal as a potential novel aphid defense metabolite adding to the known repertoire of plant defenses against aphid pests.

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Introduction

Cowpea (*Vigna unguiculata*) is one of the most important agronomic plant species grown in semiarid tropical regions of the world. Cowpea is well adapted to biotic and abiotic stresses and provides an excellent source of nutrition (Singh et al., 2002; Timko and Singh, 2008). However, a stress that is a limiting factor in cowpea production is infestation by the cowpea aphid, *Aphis craccivora* (Jackai and Daoust, 1986). Cowpea aphid infestation can cause devastating effects; it has been reported that young plants of highly susceptible cowpea cultivars were killed by an infestation of cowpea aphids initiated with fewer than ten aphids (Ofuya, 1995). Cowpea aphid feeding induced damage includes chlorosis, leaf curling, and stunted growth resulting in a decrease in yield (Blackman and Eastop, 2000; Kamphuis et al., 2012; Choudhary et al., 2017). In addition to cowpea aphid being a deadly pest, this aphid species is also known to vector over 50 plant viruses (Chan et al., 1991).

There are about 4500 species of aphids reported to date (Remaudiere and Remaudiere, 1997; Blackman and Eastop, 2000; Sorenson, 2009). Of these species, only 100 are considered to have an economic impact and 14 are considered to be serious pests, among which is the cowpea aphid (Sorenson, 2009). Aphids feed differently from chewing insects, which generate massive mechanical tissue damage. Aphids insert their specialized and flexible mouthparts, the stylets, through plant tissues to reach their source of food, the phloem sap, thus avoiding much of the mechanical tissue damage (Tjallingii and

Esch, 1993; Tjallingii, 2006). *En route* to the phloem, aphids puncture cells and deposit saliva in the plant apoplast and the punctured cells to facilitate feeding and interfere with plant defenses (Miles, 1999; Will et al., 2007). Aphid feeding and colonization damage the plant, and aphids are categorized based on the type of damage they incur onto their hosts. Aphids that cause extensive direct damage are considered phytotoxic, whereas others that cause indirect damage – for example, by transmitting viruses – are considered non-phytotoxic (Nicholson et al., 2012). Phytotoxic aphids, such as the Russian wheat aphid (*Diuraphis noxia*) and greenbug (*Schizaphis graminum*), cause damage in low numbers and are believed to secrete salivary proteins into the plant that are responsible for the increased manifestation of the damage symptoms. In contrast, the non-phytotoxic aphids, like the pea aphid (*Acyrthosiphon pisum*) and potato aphid (*Macrosiphum euphorbiae*), do not cause damage at low numbers and secrete salivary proteins to enhance feeding and interfere with plant defenses (Nicholson et al., 2012; Nicholson and Puterka, 2014; Chaudhary et al., 2015).

Aphid saliva has been shown to contain effector proteins that are necessary for successful aphid colonization (Mutti et al., 2006; Mutti et al., 2008; Bos et al., 2010; Atamian et al., 2013; Pitino and Hogenhout, 2013; Elzinga et al., 2014; Naessens et al., 2015; Wang et al., 2015; Will and Vilcinskas, 2015; Guy et al., 2016; Kaloshian and Walling, 2016). To characterize aphid salivary protein content, the saliva of several aphid species has been investigated with liquid chromatography tandem mass spectrometry (LC-MS/MS) (Harmel et al., 2008;

Carolan et al., 2009; Cooper et al., 2010; 2011; Rao et al., 2013; Vandermoten et al., 2014; Chaudhary et al., 2015; Thorpe et al., 2016; Boulain et al., 2018; Loudit et al., 2018; Yang et al., 2018). These studies have identified numerous conserved salivary proteins common among the different aphid species as well as some that have only been identified in a single aphid species. The conserved proteins are presumed to be a core set of aphid effectors that are used by aphids to facilitate feeding or disrupt general plant defenses, while the unique proteins identified in only a single aphid species or biotype, act in a species-specific host-aphid interaction (Thorpe et al., 2016). This recent wealth of salivary protein identification stems from the release of additional aphid genomes and transcriptomes. Since the first aphid genome was released for the pea aphid, five additional aphid genomes are publicly available (International Aphid Genomics, 2010; Nicholson et al., 2015; Mathers et al., 2017; Wenger et al., 2017; Thorpe et al., 2018). Numerous aphid transcriptomes are also available including a transcriptome for the cowpea aphid (Agunbiade et al., 2013). Three main criteria have been used to identify putative aphid effectors: (1) expression of the candidate transcripts in aphid heads or salivary glands with prediction for secretion, (2) presence in saliva, and (3) sequence similarity to previously identified aphid effectors.

In general, microbial, nematode and pest effectors are diverse, lacking consensus sequences and features, making it difficult to predict effectors. This has led to reporting of mostly specific subclasses of effectors. For example,

effectors from plant pathogenic fungi are small sized proteins with high cysteine content while those from Phytophthora contain a RXLR motif (Jiang et al., 2008; Stergiopoulos and de Wit, 2009; Petre and Kamoun, 2014; Sperschneider et al., 2015). To enhance plant fungal effector predictions, EffectorP was developed as a machine-learned predictor for fungal effectors that does not rely only on predetermined thresholds based on criteria including protein size and cysteine content (Sperschneider et al., 2016; Sperschneider et al., 2018). It is therefore likely that the repertoire of aphid effectors can be enhanced with the development of machine learned effector identification programs.

Numerous studies have functionally characterized aphid effectors. These included overexpression of the candidate effector *in planta* or silencing it, through plant-mediated RNAi or injection with RNAi constructs, in the aphid and determining aphid performance on the plants. Of the effectors experimentally tested, about a dozen have shown altered aphid colonization phenotypes (Mutti et al., 2006; Mutti et al., 2008; Bos et al., 2010; Atamian et al., 2013; Pitino and Hogenhout, 2013; Elzinga et al., 2014; Abdellatef et al., 2015; Naessens et al., 2015; Wang et al., 2015; Will and Vilcinskas, 2015; Guy et al., 2016; Kettles and Kaloshian, 2016). The altered survival/colonization phenotypes determined by some of these effectors act in species-specific and host-specific manner (Atamian et al., 2013; Pitino and Hogenhout, 2013; Elzinga et al., 2014; Rodriguez et al., 2017).

To date, the plant targets for only Mp1 and Me10 aphid effectors have been identified and the mechanism of effector function partially elucidated (Rodriguez et al., 2017; Chaudhary et al., 2019). The function of two additional aphid effectors MIF1 (Naessens et al., 2015) and Armet (Wang et al., 2015) have been predicted based on the function of homologous sequences from other organisms. Both MIF1 and Armet are highly conserved proteins in the animal kingdom. MIF1 encodes a macrophage migration inhibitory factor that is a cytokine deposited in aphid saliva during feeding (Calandra, 2003; Naessens et al., 2015). Armet in mammalian systems and in Drosophila has been reported in the cell as part of the unfolded protein response and extracellularly as a neurotrophic factor (Lindholm et al., 2007; Lindholm et al., 2008; Palgi et al., 2009; Palgi et al., 2012). Both MIF1 and Armet are important for the pea aphid survival as knockdown of their expressions results in shortened lifespan (Naessens et al., 2015; Wang et al., 2015). The function of an additional effector, Me47 encoding a Glutathione S-transferase (GST), was shown based on its GST enzymatic activity and its ability to detoxify isothiocyanates that are implicated in herbivore defense (Kettles and Kaloshian, 2016).

Here we report the salivary proteome of a California population of the cowpea aphid using LC-MS/MS and publicly available aphid genomes and transcriptomes. We also characterize the function of a novel salivary protein, diacetyl/L-xylulose reductase (DCXR). DCXR is a member of short-chain dehydrogenases/reductases (Nakagawa et al., 2002). Mammalian orthologs of

DCXR are involved in NADPH-dependent reduction of both carbohydrates and dicarbonyls (Nakagawa et al., 2002; Ishikura et al., 2003; Ebert et al., 2015). The reversible oxidative reduction of the carbohydrates, xylitol and L-xylulose can lead to an additional energy source through the pentose phosphate pathway (Sochor et al., 1979; Nakagawa et al., 2002). The reduction of dicarbonyls detoxifies and prevents the formation of advanced glycation end-products (AGEs), also known as glycotoxins, associated with development of numerous degenerative human diseases (Chen et al., 2009; Gkogkolou and Bohm; Kizer et al., 2014). In plants, the build-up of dicarbonyls leads to oxidative stress and cell death resulting in stunted growth (Hoque et al., 2012; Ray et al., 2013; Sankaranarayanan et al., 2015; Li, 2016). One of these dicarbonyls, generated through multiple pathways in plants and animals, is methylglyoxal (Yadav et al., 2005a; Yadav et al., 2005b; Hoque et al., 2016; Mostofa et al., 2018). Depending on concentration, methylglyoxal can act as defense signaling molecule or as a cytotoxin during abiotic stress in plants (Li, 2016). Recently methylglyoxal has also been implicated in plant defense against biotic stresses (Melvin et al., 2017). Here we report the identification of DCXR in cowpea aphid saliva. We show that the recombinant cowpea aphid DCXR, AcDCXR, is able to catalyze the reversible xylitol to xylulose reaction as well as to utilize methylglyoxal as substrate. We also demonstrate that aphid feeding induced methylglyoxal accumulation and that expression of AcDCXR *in planta* enhanced aphid fecundity contributing to the success of the aphid as a pest.

Materials and Methods

Plants and Growth Condition

Cowpea California blackeye cultivar 46 (CB46) and pea (*Pisum sativum*) cv ZP1130 were grown in UC Mix 3 soil (https://agops.ucr.edu/soil-mixing) in 32 oz plastifoam cups in a pesticide free room at 22-24 °C with a 16:8 light:dark photoperiod. Plants were fertilized weekly with MiracleGro (18−18−21; Stern's MiracleGro Products).

Aphid Colony

A colony of cowpea aphids, collected from a field in Riverside, California, in summer of 2016, was reared on cowpea cv CB46. A second colony, taken from the cowpea plants, was reared on pea cv ZP1130 for 3 months before use. The colonies were maintained separately in insect cages in growth chambers at 26−30°C with a 16:8 light:dark photoperiod. The colony on cowpea was used for aphid saliva collection and the colony on pea was used for aphid bioassays.

Saliva Collection

Cowpea aphid saliva was collected by feeding mixed developmental stages of the aphid on a water diet as previously described (Chaudhary et al., 2015). About 100-200 mixed stage aphids were loaded in a feeding chamber, consisting of a plastic cylinder with one end containing the diet inside a parafilm sachet, and the

other end secured with a cheesecloth. Aphids were allowed to feed on the 200 µL of ultrapure autoclaved water for 16 hours under yellow light. The components of the chamber were sterilized or treated with alcohol and all materials were handled in a laminar flow hood using aseptic conditions. After feeding the diet was collected aseptically using a pipet and stored at −80 °C. A new cohort of aphids were used for each overnight collection and saliva was collected from an estimated 10,000 aphids over a three-month period.

Saliva Preparation for MS/MS

Saliva was vacuum concentrated down to protein pellets and dissolved in 100 µL trypsin buffer (50 mM ammonium bicarbonate, pH 8.0, 10% v/v acetonitrile) containing 1 µg trypsin and treated overnight at 37°C. After trypsin digestion, the sample was centrifuged, the supernatant was collected, pelleted with a speedvac concentrator and suspended in 24 µL 0.1% formic acid for LC-MS/MS analysis.

LC-MS/MS

A MudPIT approach was employed to analyze the trypsin-treated samples. A two-dimension nanoAcquity UPLC (Waters) and an Orbitrap Fusion MS (ThermoFisher Scientific) were configured together to perform online 2D-nano LC-MS/MS analysis. The 2D-nanoLC was operated with a 2D-dilution method that was configured with nanoAcquity UPLC. Two mobile phases for the first dimension LC fractionation were 20 mM ammonium formate (pH 10) and

acetonitrile, respectively. Online fractionation was achieved by 5-min elution off a NanoEase trap column (PN# 186003682; Waters) using stepwise-increased concentration of acetonitrile. A total of five fractions were generated with 13%, 18%, 21.5%, 27%, and 50% of acetonitrile, respectively. A final flushing step used 80% acetonitrile to clean up the trap column. Each and every fraction was then analyzed online using a second dimension LC gradient.

For the second-dimension LC, a BEH130 C18 column (1.7 μm particle, 75 μm i.d., 20 cm long, PN# 186003544; Waters) was used for peptide separation. A Symmetry C18 (5 μm particle, 180 μm i.d., 20 mm long, PN# 186003514; Waters) served as a trap/guard column for desalting and pre-concentrating the peptides for each MudPIT fraction. The solvent components for peptide separation were as follows: mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. The separation gradient was as follows: at 0 to 1 min, 3% B; at 2 min, 8% B; at 50 min, 45% B; at 52-55 min, 85% B; at 56-70 min, 3% B. The nano-flow rate was set at 0.3 μl/min without flow-splitting.

Spectra were obtained using Orbitrap Fusion MS (Thermo Fisher Scientific). The Orbitrap Fusion MS was in positive ion mode with an ion transfer tube temperature of 275°C. The isolation window used was 2 Da. Three different types of dissociation were used: Collision Induced Dissociation (CID), High-energy Collision Induced Dissociation (HCD), and Electron Transfer Dissociation

(ETD). The energy for each of these was 30%. Three scan ranges were used (300-1800, 300-2000 400-1400 Da) with 30 second dynamic exclusion.

Proteome Data Analysis

The MS/MS spectra were filtered for high confident peptides with strict FDR (1%), with enhanced peptide and protein annotations using the software Proteome Discoverer v2.3 (Thermo Fisher). Spectra with peptide sequences less than 6 residues were removed. The search parameters allowed for 0.5 Da mass tolerance and 2 missed cleavage sites. The following modifications were included: modification of Met Oxidation ±15.99492 D, Lys Acetyl ±42.01057 D, Ser, Thr, Tyr Phospho ±79.966333 D, N-Terminus Formyl ±27.99492 Da, Pyro-Glu ±17.02655 Da, N-Terminus Acetyl ±42.01057 Da. The identified peptides were then searched against an aphid proteome database compiled from every aphid genome available on NCBI and AphidBase (Pea aphid, Russian wheat aphid, soybean aphid (*Aphis glycines),* bird cherry-oat aphid (*Rhopalosiphum padi*), green peach aphid (*Myzus persicae*), and black cherry aphid (*Myzus cerasi*) and other aphid proteins deposited in NCBI in 2017. These other proteins included six-frame translations of a cowpea aphid transcriptome and the transcriptome of the potato aphid). The 13,330 PSMs identified corresponded to 2,119 proteins and were further filtered to 721 protein group hits. Only high confidence (99%) were considered further filtering the protein groups to 521 protein groups. Spectra that came up when filtering out possible contaminants

with a FASTA file containing common contaminants. To accept proteins, they needed to have at least 3 peptides in at least 2 of the 3 replicates (CID, HCD, ETD). The raw peptide spectra were deposited in the Mass Spectrometry Interactive Virtual Environment (MassIVE) repository with the proteome ID: PXD017323.

Annotation

The MS/MS identified proteins were annotated with BLASTP using OmicsBox (V 1.1.135 Hotfix) and the NCBI nonredundant protein database with the taxonomy filter for aphids, Aphidomorpha (3380) (e value = 1e-3) (Gotz et al., 2008). The proteins were then subjected to BLASTP to the pea aphid annotation v2.1b proteins on Aphidbase to identify the corresponding ACYPI homologs (BIPAA, 2017). Gene ontology (GO) was determined for molecular function, biological process, and cellular component using InterProScan (v5.36-75.0) (Gotz et al., 2008; Jones et al., 2014). The identified proteins were screened with SignalP (V3.0 and V5.0) and SecretomeP 1.0 using eukaryote and mammalian filters, respectively, and by TMHMM V2.0 (Krogh et al., 2001; Bendtsen et al., 2004a; Bendtsen et al., 2004b; Armenteros et al., 2019). The proteins were further analyzed using EffectorP 2.0 (Sperschneider et al., 2018).

Clone Construction

RNA was extracted from 10 mixed developmental stage aphids using Trizol (Invitrogen), and cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) according to manufactures instructions. Using *AcDCXR* (MN855408) gene-specific Gateway recombination primers (DCXRF-ACAAGTTTGTACAAAAAAGCAGGCTCCATGGAAGAATTCTTTGTCGGAAAAA AGTTCAT, DCXRR-GGGGACCACTTTGTACAAGAAAGCTGGGTCACTGGCCAAAAATCCACCATC) , the DCXR coding region, excluding the secretion signal peptide, was amplified using Q5® High-Fidelity DNA Polymerase (New England Biolabs) with the following conditions: an initial 98°C for 30 sec, 98°C for 7 sec, 54°C for 20 sec, 72°C for 30 sec, for 30 cycles and a final cycle of 72°C for 3 min. DCXR was purified using GeneJET PCR Purification Kit (Thermo Scientific) and recombined into vector pDONR207 (Invitrogen) using BP Clonase (Invitrogen). Following Sanger sequencing pDONR207-DCXR was recombined into the expression vectors pDEST17 (Invitrogen; pDEST17-DCXR), pEAQ-HT-DEST1 (Sainsbury et al., 2009; pEAQ-HT-DEST1-AcDCXR), or pCAMBIA1300-GW-mScarlet (pCAMBIA1300-AcDCXR-mScarlet). pCAMBIA1300-GW-mScarlet was developed by modifying pCAMBIA1300 using parts from pGWB614 and p#128060 by restriction digestion and ligations. After transformation into *E. coli* strain DH5α and the purified pDEST17-DCXR was transformed into *E. coli* strain ArcticExpress (Agilent) while pEAQ-HT-DEST1-AcDCXR and pCAMBIA1300-

AcDCXR-mScarlet were transformed into *Agrobacterium tumefaciens* strains AGL01 and GV3101, respectively.

Protein Purification

The pDEST17-AcDCXR was purified in a similar manner as previously described for the aphid effector Me47 (Kettles and Kaloshian, 2016). Briefly, pDEST17-AcDCXR (N-terminal 6xHis tag) in ArcticExpress was grown in LB media at 37°C to an OD_{600} of 0.8 and the expression induced by adding of 0.5 mM IPTG followed by incubation at 10°C for 16 h. After centrifugation (6,000 x g for 20 min) the cells were resuspended in chilled lysis buffer (300mM NaCl, 50 mM NaH_2PO_4/Na_2HPO_4, pH 7.0). The cells were lysed using sonication (4 x 15 sec pulses), the soluble protein fraction was separated by centrifugation (10,000 x g for 45 min) and incubated with Ni- NTA agarose beads (Qiagen) for 1 h at 4°C with gentle agitation. The column was washed with the lysis buffer containing 40 mM imidazole to remove non-specifically bound proteins. After four washes, DCXR was eluted with three washes of lysis buffer containing 150, 200, and 200 mM of imidazole, respectively. The eluted fractions were concentrated with VivaSpin 500 Centrifugal Concentrator PES (Sartorius, United Kingdom) and monitored using Bradford assay with BSA as the standard. The recombinant DCXR was analyzed on a 12% SDS-PAGE using Coomassie Brilliant Blue G-250 staining.

AcDCXR Enzyme Activity Assays

Oxidation of xylitol to xylulose by recombinant DCXR was measured through the reduction of $NADP^+$ to NADPH as previously described (Yang et al., 2017) with minor modification. A 0.5 mL reaction mixture containing 10 µg AcDCXR 100 mM glycine buffer, pH 9.5, 3 mM $MgCl_2$, $NADP^+$, and 200 mM xylitol were used in 1 mL cuvettes and a Beckman Coulter Du® 730 Life Sciences spectrophotometer. Reactions began after the addition of AcDCXR, and changes in absorbance at 340 nm were monitored. The reaction rates were calculated based on the $NADP^+$ concentrations.

Methylglyoxal reduction by recombinant DCXR was measured through the oxidation of NADPH to $NADP^+$ using 1 mL cuvettes as previously described (Misra et al., 1996) and the Beckman Coulter Spectrophotometer. The 0.5 mL reaction was composed of 10 µg DCXR, 100 µM sodium phosphate buffer (NaH_2PO_4/Na_2HPO_4, pH 6.5), 200 µM NADPH and methylglyoxal. The reaction was initiated with the addition of NADPH and monitored by the decrease in absorbance at 340 nm. The reaction rates were calculated based on the methylglyoxal concentrations.

Transient Expression in Pea and Western Blot Analysis

Agrobacterium tumefaciens strain AGL01, carrying either pEAQ-HT-GFP or pEAQ-HT-DEST1-AcDCXR, were used in transient expression of pea, *Pisum sativum,* cv. ZP1130 as described previously (Guy et al., 2016). Bacterial cells,

grown up overnight in YEP media, were harvested, washed three times in infiltration buffer (10 mM $MgCl_2$, 10 mM MES pH 5.6, and 150 µM acetosyringone) and resuspended at a final OD_{600} of 0.5. The youngest expanded leaf of a 2-week-old plant was infiltrated with a needleless syringe.

The duration of GFP expression in pEAQ-HT-GFP infiltrated leaves was monitored with Western blot analysis. Three 1 cm diameter leaf discs were cut from the same agroinfiltrated leaf using a cork borer after 2, 3, 5, 7, 8, 9, and 10 days post infiltration. Protein was extracted from the leaf discs by grinding in 200 µl lysis buffer (6M Urea, 2M Thiourea, 1% Protease inhibitor cocktail [Sigma P9599]). Samples were centrifuged at 14,000 x g for 5 min and the supernatant was resuspended in equal volume 2x loading buffer (100 mM Tris-HCl pH 6.8, 100 mM DTT, 10% glycerol, 2% SDS, 0.01% bromophenol blue). About 25 µg of protein were loaded per sample on 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with mouse anti-GFP antibody (Sigma) and secondary antibody, goat anti-mouse HRP-conjugated (Santa Cruz Biotechnology). Primary antibody was used at 1:2000 and secondary antibody was used at 1:2000 dilution. Pierce ECL Western Blotting Substrate (Thermo Scientific) was used to detect the signal with autoradiography film (Denville Scientific Inc).

In Planta Localization of AcDCXR

Agrobacterium tumefaciens GV3101 carrying pCAMBIA1300-DCXR-mScarlet or pCAMBIA1300-GFP were grown and prepared as previously described for transient agroexpression. At an OD_{600} = 0.5 each, the constructs were co-infiltrated in *Nicotiana benthamiana* leaves. Three days post infiltration, leaf epidermal cells were analyzed using a Leica SP5 confocal microscope. GFP and mScarlet were excited by 488 nm and 543 nm filters, respectively, and images were collected through band emission filters at 498-520 nm and 553-650 nm, respectively.

Aphid Bioassays

A day after agroinfiltration, five adult cowpea aphids were caged onto the adaxial side of an agroinfiltrated leaf of 2-week-old pea plants. After 24 h (i.e., 2 days post infiltration; dpi), the adult aphids were removed, and 5 to 6 new-born nymphs were left on the leaf with both the adaxial and abaxial sides of the leaf accessible to the aphids. Eight days later (10 dpi), the surviving aphids were counted and transferred to a new infiltration site on a plant infiltrated 2 days earlier. The fecundity of these aphids was monitored two and five days later (i.e., when the aphids were 12 and 15 day-old). The nymphs were removed after each counting. This experiment was performed three times. Each experiment consisted of 13-15 plants per construct. All experiments were conducted at 22°C, 16:8 light:dark photoperiod.

Determining Methylglyoxal Levels

Methylglyoxal levels were evaluated in 2-week-old cowpea and pea plants following the protocol by Borysiuk et al (2018). Highly infested leaves were harvested at day 1, 2, and 3 after infestation. Briefly, samples were homogenized in 5% perchloric acid and centrifuged at 13,000 x g for 10 min at 4°C. The supernatant was decolorized with charcoal and neutralized with 1 M potassium carbonate. After centrifuging at 13,000 x g at 4°C the supernatant was used to estimate the methylglyoxal concentration in sodium dihydrogen phosphate buffer (pH 7.0). The absorbance was recorded after 10 min incubation with N-acetyl-L-cysteine to monitor the N-a-acetyl-S-(1-hydroxy-2-oxo-prop-1-yl)cysteine formation (Wild et al., 2012). Methylglyoxal concentration was determined using a standard curve of known methylglyoxal concentrations. The experiment with pea was performed once and with cowpea was performed twice.

Statistical Analyses

We used generalized linear models (GLM) with a likelihood ratio and chi-square test to assess whether AcDCXR expression had an effect on aphid survival and fecundity. Data on aphid survival were analyzed with GLM following a binomial distribution and data on aphid fecundity were assumed to follow a Poisson distribution. The fit of all generalized linear models was checked by inspecting residuals and QQ plots. Methylglyoxal levels in plants were analyzed using a

nested ANOVA (biological replicates treated as random factor) (package R: ‘nlme’). When a significant effect was detected, a pairwise comparison using multiple comparisons of the means (package R: ‘multcomp’) (Tukey contrasts, p-values adjustment with ‘fdr’ method) at the 0.05 significance level was used to test for differences between days after infestation. Statistical analyses were performed using the R software (version 3.6.0) (R Core Team, 2019).

Results

Aphid Salivary Proteome Analyses and Annotation

To identify the protein composition of the cowpea aphid saliva, aphid saliva was collected in parafilm feeding pouches containing water. The contents of the pouches were concentrated and subjected to proteome analyses. The peptides identified by LC-MS/MS were searched against a custom aphid protein database. The database was composed of proteomes based on all aphid genomes available in the summer of 2017, as well as cowpea aphid-specific transcriptome and a transcriptome from the potato aphid, both with six-frame translations. Around 175 candidate proteins were identified with at least three peptides from at least two replicates and having at least one unique peptide (Supplemental Table 1). The identified proteins were then annotated using BLASTP with OmicsBox (TaxID: Aphididiae 27482). Among these annotated proteins, 18/175 (10.29%) were uncharacterized. In addition, functional redundancies were recorded among the proteins with annotations. To eliminate these redundancies, the proteins were subjected to BLASTP on AphidBase to identify their corresponding ACYPI homolog using the pea aphid protein database annotation v2.1b. Among these proteins, 47/175 (26.86%) shared one of 21 ACYPI top hits. Although these 47 proteins had at least one unique peptide, we grouped them as 21 proteins, resulting in a total of 149 salivary proteins (Supplemental Table 1).

Annotation of these proteins presented a wide range of functional attributes to the cowpea aphid salivary proteins. Among the 149 identified proteins, 33 proteins with similar functional annotations have been previously reported in the saliva of a cowpea aphid population from Gabon, Africa (Loudit et al., 2018). Among these 33 proteins are glucose dehydrogenases, carbonic anhydrases and a trehalase (Supplemental Table 1).

Of the 149 identified cowpea aphid proteins, gene ontology (GO) assigned 123 proteins with at least one GO term in the three most common ontological designations: molecular function, biological process and cellular component. The three most abundant biological process designations were carbohydrate metabolic process (19%), translation (16%) and catabolic process (11%) (Fig 1.1). The three most abundant molecular function designations were oxidoreductase activity (20%), structural constituent of ribosome (16%) and ATP binding (13%) (Fig 1.1). As for the most abundant cellular component designations, they were for protein-containing complexes (33%) and cytosol (29%) (Fig 1.1).

Effector Prediction

Since the cowpea aphid genome has not been sequenced, homologous proteins from the different aphid species or those based on cowpea aphid transcriptome, used in our custom database, were used for these analyses. Multiple bioinformatics tools were harnessed to screen the identified salivary proteins for

putative effector function. First, the salivary proteins were evaluated for secretion using tools that predict classical and non-classical secretions, SignalP and SecretomeP (Bendtsen et al., 2004a; Bendtsen et al., 2004b; Armenteros et al., 2019), respectively. Using SignalP, a secretion signal was detected in 29 (19.46%) proteins, while SecretomeP predicted the secretion of an additional 23 (15.44%) of the 149 salivary proteins (Table 1.1). To eliminate proteins with transmembrane domains, presence of transmembrane helices was evaluated using TMHMM (Sonnhammer et al., 1998). Six of these predicted secreted proteins contained transmembrane helices.

A machine learning approach was recently used to develop novel prediction program for fungal effectors (Sperschneider et al., 2016; Sperschneider et al., 2018). We wondered whether this tool, EffectorP, could be used to predict aphid effectors. To test this, we first subjected known aphid effectors for EffectorP analysis. We tested the C002 effector, identified first in pea aphid (Mutti et al., 2008), and Me10, identified in potato aphid (Atamian et al., 2013). Both C002 and Me10 were identified as effectors by EffectorP indicating that EffectorP can be utilized as a tool to screen for aphid effectors. Using EffectorP, 20/149 (13.4%) of the cowpea aphid salivary proteins were identified as putative effectors (Table 1.1). Only eight of the 20 putative effectors were identified for secretion by SignalP or SecretomeP. Taken together 58 proteins were predicted for secretion or for effector function encoding a wide range of functions with eight being unknowns (Fig 1.2; Table 1.1)

Selection and *In Vitro* Characterization of AcDCXR

A set of criteria were applied to choose a putative effector protein identified by EffectorP for functional characterization. These included a previously unidentified effector predicted for secretion or with secretion signal peptide, a protein with predicted enzymatic activity, and high abundance in cowpea aphid saliva based on the SEQUEST score. Based on these criteria, DCXR was selected for further analysis.

Sequence prediction indicated that cowpea aphid DCXR (AcDCXR; GAJW01000401.1) consists of at least 263 amino acids, with the first 23 amino acids encoding a predicted signal peptide, and a conserved enzymatic domain for short-chain dehydrogenases/reductases (Fig 1.3). Using AcDCXR in BLASTP searches identified DCXR homologs in seven aphid species. Interestingly, only the DCXR from cotton melon aphid (*Aphis gossypii;* XP_027848224.1) contains a secretion signal peptide (Fig 1.3). Consistent with this information, DCXR has been reported previously from other aphid species but has not been previously identified in aphid saliva (Nguyen et al., 2008; Nguyen et al., 2009; Pinheiro et al., 2014).

DCXR is a multifunctional enzyme. Mammalian orthologs of DCXR have been shown to function in the glucuronic acid/uronate cycle, in a reversible reaction either oxidizing or reducing xylitol and xylulose, respectively (Sochor et al., 1979; Yang et al., 2017), as well as having α-β-dicarbonyl reductase activity to metabolize toxic carbonyls like methylglyoxal (Ebert et al., 2015). Direct

comparison between AcDCXR and XP_027848224.1 showed 100% identity at the amino acid level with perfect conservation of the enzyme active site (Fig 1.3). To test whether AcDCXR has similar functions as the mammalian orthologs, we expressed recombinant AcDCXR and performed enzymatic assays.

AcDCXR, amplified from cDNAs developed from the whole bodies of mixed stages of the aphid, was cloned into the pDEST17 expression vector and expressed in *E. coli* strain ArcticExpress. Purified AcDCXR (Fig 1.4) was used in two distinct enzymatic assays to check its functionality. To verify whether AcDCXR is able to oxidize xylitol to xylulose, AcDCXR was assayed using xylitol as the substrate and $NADP^+$ as co-substrate. The reduction of $NADP^+$ to NADPH was spectroscopically monitored by the increase of absorbance at 340 nm. AcDCXR was able to oxidize xylitol to xylulose in a $NADP^+$ concentration-dependent manner (Fig 1.5A). Analysis of the Lineweaver-Burke plot data determined the enzymatic constants to be: k_{cat} = 1.85 s^{-1}, a Km = 0.56 mM and a Vmax = 79.4 µM/min (Fig 1.5B).

To determine whether AcDCXR was able to use methylglyoxal as a substrate, we tested the reduction of methylglyoxal by spectroscopically measuring the decrease in absorption of concomitant NADPH oxidation at 340 nm. We found that AcDCXR was able to reduce methylglyoxal in a concentration-dependent manner (Fig 1.6A). Analysis of the Lineweaver-Burke plot data determined the enzymatic constants to be: k_{cat} = 0.23 s^{-1}, a Km = 1.3 mM and a Vmax = 13.8 µM/min (Fig 1.6B). The control reactions, in the presence of

AcDCXR and absence of a substrate, showed neither oxidation nor reduction (Figs 1.5A, 1.6A). Similarly, the control reactions in the absence of the enzyme showed neither oxidation nor reduction, indicating the AcDCXR's presence was necessary to complete the reactions (Figs 1.5A, 1.6A). The kinetic constants in AcDCXR show that, *in vitro,* it was more efficient oxidizing xylitol with a k_{cat}/K_m of 3.32 mM^{-1} s^{-1} compared to reducing methylglyoxal that had only a k_{cat}/K_m of 0.174 mM^{-1} s^{-1}, nearly a 20-fold difference in activity.

Functional Analysis of AcDCXR *In Planta*

To functionally evaluate the role of AcDCXR on cowpea aphid colonization, AcDCXR was cloned into the binary vector pEAQ-DEST1 for Agrobacterium-mediated transient expression. Since Agrobacterium-mediated transient expression in cowpea has not yet been developed, pea plants were used for this experiment. Pea is a host for cowpea aphid and has been previously used successfully in transient expression experiments for evaluation of aphid effectors (Guy et al., 2016). Using the same cultivar of pea cv ZP1130, we first transiently expression GFP using *A. tumefaciens* strain AGL01. Monitoring GFP expression by western blot analysis, GFP was detected as early as 2 days after agroinfiltration and lasted at least for 10 days (Fig 1.7). Based on the GFP expression in pea, a cowpea aphid bioassay was developed.

Aphid bioassays were performed to evaluate the effect of AcDCXR overexpression in pea plants on cowpea aphid. Plants were agroinfiltrated with

AcDCXR or GFP control constructs as described earlier for the western blot analysis. A day post infiltration (dpi), adult cowpea aphids, maintained on pea cv ZP1130, were placed on a leaf, at the site of the infiltration, in a clip cage. After 24 hours (2 dpi), all adult aphids were removed, and six newborn nymphs were left at the infiltration site. At ten dpi, similar number of aphids were counted on GFP and AcDCXR infiltrated leaves indicating no effect on nymph survival rate (GLM, Chisq = 0.034, $P = 0.854$) (Fig 1.8A). To evaluate the fecundity of these aphids, one aphid per cage was transferred to a freshly agroinfiltrated (2 dpi) plant, with the same construct, and aphid survival and fecundity was monitored 4 and 7 days later. Sixteen days after initiation of the aphid bioassay, no difference in adult survival was detected between aphids feeding on AcDCXR compared to those feeding on the GFP infiltrated leaves (GLM, Chisq = 0.367, $P = 0.544$) (Fig 1.8B). However, a significant difference (GLM, Chisq = 16.901, $P < 0.001$) in aphid fecundity was observed between the aphids feeding on AcDCXR compared to those feeding on the GFP control indicating a role for AcDCXR in cowpea aphid colonization (Fig 1.8C).

To determine the subcellular localization of AcDCXR *in planta*, AcDCXR was cloned into the binary vector pCAMBIA-1300-mScarlet and used in Agrobacterium-mediated transient expression in *N. benthiamana*. pCAMBIA-1300-AcDCXR-mScarlet was co-infiltrated with a GFP construct. As expected, GFP was detected throughout the cell including the nucleus, while AcDCXR-mScarlet was localized to the cytoplasm (Fig 1.9).

Aphid Induce Methylglyoxal Accumulation

Methylglyoxal has been shown to accumulate in multiple plant species when exposed to abiotic stresses (Yadav et al., 2005a; Hossain et al., 2009; Mustafiz et al., 2014). Recently, it was also shown that methylglyoxal accumulates in plants exposed to biotic stresses (Melvin et al., 2017). To assess if methylglyoxal also accumulates by aphid infestation, methylglyoxal levels in cowpea and pea plants were monitored. A day after infestation of cowpea plants to cowpea aphids, a significantly higher (multiple comparisons, $z = 2.812$, $P = 0.015$) levels of methylglyoxal were detected in the infested leaves compared to the uninfested control leaves (Fig 1.10A). Methylglyoxal levels remained significantly higher (multiple comparisons, $z = 3.832$, $P < 0.001$) on day 2 but reduced to pre-infective levels on day 3 (multiple comparisons, $z = 1.479$, $P = 0.208$) (Fig1.10A). A similar trend of methylglyoxal accumulation was detected in pea leaves exposed to cowpea aphids indicating that cowpea aphid feeding induces methylglyoxal levels irrespective of the host species (Fig 1.10B).

Discussion

Cowpea Aphid Salivary Proteome

We carried out proteomics analysis to identify the salivary protein composition of a population of cowpea aphid from California. The identified proteins had a diverse range of functions including some that are uncharacterized. We were conservative in assessing the salivary proteome and used strict cut-off measures to identify the proteins. Nevertheless, we identified 149 non-redundant proteins. Previously, the salivary proteome from an African cowpea aphid population was reported (Loudit et al., 2018). The majority of the proteins identified in our study were not reported from this African population suggesting that our approach allowed us to identify higher numbers of proteins. While the cowpea aphid saliva in this work was collected in water, the African cowpea aphid saliva was collected in a sucrose-based diet and required clean up steps before undergoing mass spectrometry and that could have contributed to the low number of proteins identified in the saliva. Interestingly, both studies did not identify a set of functionally characterized aphid effectors such as Armet, Me23, Ap25, Mp2, Mp55 (Atamian et al., 2013; Pitino and Hogenhout, 2013; Elzinga et al., 2014; Wang et al., 2015; Guy et al., 2016). While in our study we identified Me10/Mp58 and SHP, the structural sheath protein, these two proteins were not identified in the African cowpea aphid saliva (Carolan et al., 2009; Chaudhary et al., 2015). The well characterized effector C002, was reported in the African population and

not in this work (Mutti et al., 2006; Mutti et al., 2008; Pitino and Hogenhout, 2013; Elzinga et al., 2014; Loudit et al., 2018). Although peptides for C002 and two additional effectors, Mp1 and MIF1, were detected in the saliva of the California cowpea aphids, this work, they did not fulfil the criteria used in our selection (Harmel et al., 2008; Naessens et al., 2015).

Unlike the salivary proteome of the African cowpea aphid, there were no proteins identified from secondary symbionts in the California cowpea aphid saliva (Loudit et al., 2018). The only bacterial proteins identified in the California cowpea aphid salivary proteome were from the primary endosymbiont *Buchnera aphidicola*, the chaperonin GroEL and GroES. GroEL has been previously identified in the saliva of several aphid species including the cowpea aphid (Chaudhary et al., 2014; Vandermoten et al., 2014; Chaudhary et al., 2015; Loudit et al., 2018). GroEL is an aphid-associated molecular pattern triggering immune responses in plants (Chaudhary et al., 2014).

Our work was limited by the absence of a cowpea aphid genome and a gland/head specific transcriptome that could have been used for the peptide searches. In addition, homologous sequences from different aphid species were used in the secretion prediction analyses including some originating from transcriptomes that could have been truncated. Therefore, the number of proteins predicted for secretion, 46 out of 149 (30.9%), based on the bioinformatic programs SignalP and SecretomeP, are likely an underestimate (Table 1.1). Previous work describing salivary proteome from aphids with

genome sequences and gland/head specific RNAseq generated sequences, also identified a large number of proteins from aphid saliva, collected in sugar and amino acid-based diets, with no prediction for secretion (Thorpe et al., 2016; Boulain et al., 2018). Boulain et al. (2018) reported 37/51 (72.5%) of the pea aphid salivary proteins with a secretion prediction. Thorpe et al. (2016), studying three different aphid species, green peach aphid, black cherry aphid, and bird cherry-oat aphid, reported only 61/204 (30%) secretion prediction of the identified salivary proteins. Taken together, this information indicates that the current bioinformatic prediction programs are likely limited in their ability to identify aphid secreted proteins.

Effector Prediction

Here we reported the use of a machine learning plant-pathogenic fungi effector prediction program, EffectorP, for prediction of aphid effectors (Sperschneider et al., 2016; Sperschneider et al., 2018). We confirmed the use of EffectorP as a possible program for identifying aphid effector proteins by successfully subjecting the well-characterized aphid effectors C002 and Me10 to EffectorP analysis (Mutti et al., 2008; Atamian et al., 2013; Pitino and Hogenhout, 2013; Chaudhary et al., 2019). Interestingly, EffectorP predicted 20/149 of the cowpea aphid proteins as effectors. Among these 20 proteins, is the functionally characterized Me10 effector and three proteins which have been predicted for effector function (Atamian et al., 2013; Elzinga et al., 2014; Thorpe et al., 2016; Chaudhary et al.,

2019). Orthologs of Me10 have been identified in multiple aphid species. Me10 has been detected in plant tissues fed on by aphids and expression of Me10 in plants has been shown to enhance the performance of potato aphid on tomato and green peach aphid on *N. benthamiana* (Atamian et al., 2013; Chaudhary et al., 2015; Chaudhary et al., 2019). In addition, Me10 was shown to interact with the tomato scaffold protein Fourteen-Three-Three isoform 7 (TFT7) and predicted to interfere with a mitogen-activated protein kinase defense signaling pathway (Chaudhary et al., 2019).

The remaining three previously predicted putative effectors are carbonic anhydrase, superoxide dismutase, and peptidyl-prolyl cis-trans isomerase (PPIase). The latter two proteins were identified in the proteomes of the pea aphid salivary glands (Carolan et al., 2011). While carbonic anhydrases have been identified in aphid saliva, superoxide dismutase and PPIase have not been previously reported in aphid saliva (Rao et al., 2013; Nicholson and Puterka, 2014; Chaudhary et al., 2015; Loudit et al., 2018). A carbonic anhydrase and a superoxide dismutase have been shown to be under positive selection further implicating these proteins as effectors (Thorpe et al., 2016). While clear roles for carbonic anhydrases and PPIases have not been characterized in plant immune responses, superoxide dismutases are attributed to detoxify reactive oxygen species (ROS), the well-known defense signaling molecule.

Among the EffectorP identified putative effector proteins, that had not been previously identified in aphid saliva or as a putative effector, is AcDCXR

(Table 1.1, Supplemental Table 1). DCXR has been identified in the pea aphid salivary gland but has not been reported in the saliva of this aphid species (Carolan et al., 2011; Boulain et al., 2018). Interestingly, pea aphid homolog of AcDCXR as well as homologs from five additional aphid species with genome sequences, do not have a secretion signal peptide. The homolog from the cotton melon aphid does have a secretion signal suggesting that DCXR is one of the differential pest arsenals utilized by a subset of aphid species. An increase in DCXR accumulation was reported in a virulent biotype of greenbug infesting resistant wheat (Pinheiro et al., 2014). Additionally, enhanced accumulation of DCXR in response to heat/UV stress as well as predation by parasitoids in the potato aphid were reported from whole insects (Nguyen et al., 2008; Nguyen et al., 2009). Taken together, this information suggest that aphids may have evolved different roles for DCXR to deal with stress conditions in the plant and within the aphid itself.

Diacetyl/L-xylulose reductases

In mammals DCXRs are reported to be oxidoreductases for monosaccharides and dicarbonyls. Human DCXR was first discovered while investigating the disease pentosuria and found that an enzymatic defect in DCXR was the cause of the high excretion of L-xylulose. This lead to the conclusion that L-xylulose is a possible substrate of DCXR (Wang and Van Eys, 1970). DCXR has been shown also to catalyze reactions with other sugars. For example, xylitol is a sugar

alcohol that is transported through the phloem as a carbon source (Lewis and Smith, 1967; Lemoine et al., 2013). Xylitol can be converted to xylulose and be used in the pentose phosphate pathway to generate glycolytic intermediates as a source of energy. Since the AcDCXR catalyzes the reversible reaction between xylulose and xylitol, the enzyme may provide the aphid an additional mode of generating energy.

DCXR also participates in the reductive metabolism of carbonyls. In this role, the enzyme is considered as a defense mechanism against harmful carbonyls (Nakagawa et al., 2002; Ebert et al., 2015; Yang et al., 2017). These molecules lead to formation of AGEs by reacting with lysine, cysteine and arginine, thus inactivating proteins (Thornalley, 2006; Ahmed and Thornalley, 2007). One of these harmful carbonyls is methylglyoxal which is reactive α-β-dicarbonyl ketoaldehyde. Interestingly, methylglyoxal has been shown to accumulate in a number of plant species under various abiotic stresses (Yadav et al., 2005a; Hossain et al., 2009; Mustafiz et al., 2014; Rahman et al., 2015; Borysiuk et al., 2018). Recently, methylglyoxal has also been implicated in biotic stresses. Increases in methylglyoxal levels were detected in tobacco plants exposed to the bacterium *Pseudomonas syringae*, or the *Mungbean yellow mosaic virus,* or to the fungus *Alternaria alternata* (Melvin et al., 2017). In addition, exogenous application of methylglyoxal in wheat and rice plants upregulated antioxidant and defense-related genes indicating a role for methylglyoxal in plant defense (Kaur et al., 2015; Li et al., 2017). In this work we

showed that aphid feeding also enhanced accumulation of methylglyoxal in cowpea and pea, suggesting methylglyoxal also functions in aphid defense. Since methylglyoxal levels in aphid infested leaves were mostly transient, this suggests that aphids are able to counteract methylglyoxal accumulation possibly through AcDCXR activity.

Transient expression of AcDCXR indicates that this enzyme is localized in the plant cell cytoplasm. Likewise, both AcDCXR substrates tested in this study, methylglyoxal and xylitol/xylulose, are also located in the cell cytoplasm. In plants, the pentose phosphate pathway where xylitol/xylulose are used, takes place in both the cytoplasm and plastids. Methylglyoxal is generated in multiple pathways in the cytoplasm and in various organelles (Phillips and Thornalley, 1993; Dennis and Blakeley, 2000; Kruger and von Schaewen, 2003).

The transient expression of AcDCXR increased the fecundity of the cowpea aphid most likely due to its effect on one or both of these two substrates; either by increasing the obtained nutrient content and/or through diminishing defense responses. This increase in fecundity was seen despite no differences in the survival of both adult and nymphal stages of the aphid. Transient or stable overexpression of a number of aphid effectors in various plant species including, Arabidopsis, tomato, pea and *N. benthamiana* also yielded increases in aphid fecundity but no effect on aphid survival suggesting that overexpression of multiple effectors may be needed to observe a pronounced change in aphid survival.

In this work, using a classical and a novel bioinformatics programs, SignalP and EffectorP, respectively, we identified a novel aphid effector, AcDCXR. The functional annotation of DCXR and *in vitro* biochemical analysis of AcDCXR lead us to identify methylglyoxal as a potential novel metabolite involved in aphid defense. Therefore, identification of novel effectors may lead to the discovery of yet unknown defense pathways that may lead to novel approaches to engineer pest/pathogen resistance in crops.

References

Abdellatef, E., Will, T., Koch, A., Imani, J., Vilcinskas, A., and Kogel, K.H. (2015). Silencing the expression of the salivary sheath protein causes transgenerational feeding suppression in the aphid *Sitobion avenae*. *Plant Biotechnol J* 13(6), 849-857.

Agunbiade, T.A., Sun, W., Coates, B.S., Djouaka, R., Tamo, M., Ba, M.N., et al. (2013). Development of reference transcriptomes for the major field insect pests of cowpea: a toolbox for insect pest management approaches in west Africa. *PLoS ONE* 8(11), e79929.

Ahmed, N., and Thornalley, P.J. (2007). Advanced glycation endproducts: what is their relevance to diabetic complications? *Diabetes Obes Metab* 9(3), 233-245.

Armenteros, J.J.A., Tsirigos, K.D., Sonderby, C.K., Peterson, T.N., Winther, O., Brunak, S., et al. (2019). SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nat Biotechnol* 37, 420-423.

Atamian, H.S., Chaudhary, R., Dal Cin, V., Bao, E., Girke, T., and Kaloshian, I. (2013). In planta expression or delivery of potato aphid *Macrosiphum euphorbiae* effectors Me10 and Me23 enhances aphid fecundity. *Mol Plant Microbe Interact.* 26(1), 67-74.

Bendtsen, J.D., Jensen, L.J., Blom, N., von Heijne, G., and Brunak, S. (2004a). Feature based prediction of non-classical and leaderless protein secretion. *Protein Eng Des Sel* 17(4), 349-356.

Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S. (2004b). Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* 340(4), 783-795.

BIPAA (2017). *AphidBase* [Online]. Available: https://bipaa.genouest.org/is/aphidbase/ [Accessed 6-27-2017 2017].

Blackman, R.L., and Eastop, V.F. (2000). *Aphids on the world's crops.* New York, NY: John Wiley & Sons Inc.

Borysiuk, K., Ostaszewska-Bugajska, M., Vaultier, M.N., Hasenfratz-Sauder, M.P., and Szal, B. (2018). Enhanced formation of methylglyoxal-derived advanced glycation end products in arabidopsis under ammonium nutrition. *Front Plant Sci* 9, 667.

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(11).

Boulain, H., Legeai, F., Guy, E., Morliere, S., Douglas, N.E., Oh, J., et al. (2018). Fast evolution and lineage-specific gene family expansions of aphid salivary effectors driven by interactions with host-plants. *Genome Biol Evol* 10(6), 1554-1572.

Calandra, T. (2003). Macrophage migration inhibitory factor and host innate immune responses to microbes. *Scand J Infect Dis.* 35(9), 573-576.

Carolan, J.C., Caragea, D., Reardon, K.T., Mutti, N.S., Dittmer, N., Pappan, K., et al. (2011). Predicted effector molecules in the salivary secretome of the pea aphid (*Acyrthosiphon pisum*): a dual transcriptomic/proteomic approach. *J Proteome Res* 10(4), 1505-1518.

Carolan, J.C., Fitzroy, C.I.J., Ashton, P.D., Douglas, A.E., and Wilkinson, T.L. (2009). The secreted salivary proteome of the pea aphid *Acyrthosiphon pisum* characterised by mass spectrometry. *Proteomics* 9(9), 2457-2467.

Chan, C.K., Forbes, A.R., and Raworth, D.A. (1991). *Aphid-transmitted viruses and their vectors of the world.* Ottawa: Research Branch, Agriculture Canada.

Chaudhary, R., Atamian, H.S., Shen, Z., Briggs, S.P., and Kaloshian, I. (2014). GroEL from the endosymbiont *Buchnera aphidicola* betrays the aphid by triggering plant defense. *Proc Natl Acad Sci USA* 111(24), 8919-8924.

Chaudhary, R., Atamian, H.S., Shen, Z., Briggs, S.P., and Kaloshian, I. (2015). Potato aphid salivary proteome: enhanced salivation using resorcinol and identification of aphid phosphoproteins. *J Proteome Res* 14(4), 1762-1778.

Chaudhary, R., Peng, H.C., He, J.M., MacWilliams, J., Teixeira, M., Tsuchiya, T., et al. (2019). Aphid effector Me10 interacts with tomato TFT7, a 14-3-3 isoform involved in aphid resistance. *New Phytol* 221(3), 1518-1528.

Chen, L., Wang, T., Wang, X., Sun, B.B., Li, J.Q., Liu, D.S., et al. (2009). Blockade of advanced glycation end product formation attenuates bleomycin-induced pulmonary fibrosis in rats. *Respir Res* 10(55).

Choudhary, A., Hussein, A., Samota, R.G., and Nehra, S. (2017). Effect of biotic and abiotic factors on the incidence of aphid, *Aphis craccivora* Koch on cowpea. *J Pharmacogn Phytochem* 6(4), 1587-1590.

Cooper, W.R., Dillwith, J.W., and Puterka, G.J. (2010). Salivary proteins of Russian wheat aphid (Hemiptera: Aphididae). *Environ Entomol* 39(1), 223-231.

Cooper, W.R., Dillwith, J.W., and Puterka, G.J. (2011). Comparisons of salivary proteins from five aphid (Hemiptera: Aphididae) species. *Environ Entomol* 40(1), 151-156.

Dennis, D.T., and Blakeley, S.D. (2000). "Carbohydrate Metabolism," in *Biochemistry & Molecular Biology of Plants,* eds. B.B. Buchanan, W. Gruissem & R.L. Jones. (Waldorf, MD: American Society of Plant Physiologists), 630-675.

Ebert, B., Kisiela, M., and Maser, E. (2015). Human DCXR - another 'moonlighting protein' involved in sugar metabolism, carbonyl detoxification, cell adhesion and male fertility? *Biol Rev* 90(1), 254-278.

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(7), 747-756.

Gkogkolou, P., and Bohm, M. (2012). Advanced glycation end products: key players in skin aging? *Dermatoendocrinol* 4(3), 259-270.

Gotz, S., Garcia-Gomez, J.M., Terol, J., Williams, T.D., Nagaraj, S.H., Nueda, M.J., et al. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res* 36(10), 3420-3435.

Guy, E., Boulain, H., Aigu, Y., Le Pennec, C., Chawki, K., Morliere, S., et al. (2016). Optimization of agroinfiltration in *Pisum sativum* provides a new tool for studying the salivary protein functions in the pea aphid complex. *Front. Plant Sci* 7, 1171.

Harmel, N., Letocart, 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(2), 165-174.

Hoque, M.A., Uraji, M., Torii, A., Banu, M.N., Mori, I.C., Nakamura, Y., et al. (2012). Methylglyoxal inhibition of cytosolic ascorbate peroxidase from *Nicotiana tabacum*. *J Biochem Mol Toxicol* 26(8), 315-321.

Hoque, T.S., Hossain, M.A., Mostofa, M.G., Burritt, D.J., Fujita, M., and Tran, L.S. (2016). Methylglyoxal: an emerging signaling molecule in plant abiotic stress responses and tolerance. *Front Plant Sci* 7, 1341.

Hossain, M.A., Z., H.M., and F., F. (2009). Stress-induced changes of methylglyoxal level and glyoxalase I activity in pumpkin seedlings and cDNA cloning of glyoxalase I gene. *J Crop Sci* 3(2), 53.

International Aphid Genomics, C. (2010). Genome sequence of the pea aphid *Acyrthosiphon pisum*. *PLoS Biol* 8(2), e1000313.

Ishikura, S., Usami, N., El-Kabbani, O., and Hara, A. (2003). Structural determinant for cold inactivation of rodent L-xylulose reductase. *Biochem Biophys Res Comm* 308(1), 68-72.

Jackai, L., and Daoust, R. (1986). Insect Pests of Cowpeas. *Annu Rev Entomol* 31, 95-119.

Jiang, R.H., Tripathy, S., Govers, F., and Tyler, B.M. (2008). RXLR effector reservoir in two Phytophthora species is dominated by a single rapidly evolving superfamily with more than 700 members. *Proc Natl Acad Sci USA* 105(12), 4874-4879.

Jones, P., Binns, D., Chang, H.Y., Fraser, M., Li, W., McAnulla, C., et al. (2014). InterProScan 5: genome-scale protein function classification. *Bioinformatics* 30(9), 1236-1240.

Kaloshian, I., and Walling, L.L. (2016). Hemipteran and dipteran pests: Effectors and plant host immune regulators. *J Integr Plant Biol* 58(4), 350-361.

Kamphuis, L.G., Gao, L., and Singh, K.B. (2012). Identification and characterization of resistance to cowpea aphid (*Aphis craccivora* Koch) in *Medicago truncatula*. *BMC Plant Biol* 12, 101.

Kaur, C., Kushwaha, H.R., Mustafiz, A., Pareek, A., Sopory, S.K., and Singla-Pareek, S.L. (2015). Analysis of global gene expression profile of rice in response to methylglyoxal indicates its possible role as a stress signal molecule. *Front Plant Sc.* 6, 682.

Kettles, G.J., and Kaloshian, I. (2016). The potato aphid salivary effector Me47 is a glutathione-S-transferase involved in modifying plant responses to aphid infestation. *Front Plant Sci* 7, 1142.

Kizer, J.R., Benkeser, D., Arnold, A.M., Ix, J.H., Mukamal, K.J., Djousse, L., et al. (2014). Advanced glycation/glycoxidation endproduct carboxymethyl-lysine and incidence of coronary heart disease and stroke in older adults. *Atherosclerosis* 235(1), 116-121.

Krogh, A., Larsson, B., von Heijne, G., and Sonhammer, E.L.L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* 305(3), 567-580.

Kruger, N.J., and von Schaewen, A. (2003). The oxidative pentose phosphate pathway: structure and organisation. *Curr Opin Plant Biol* 6(3), 236-246.

Lemoine, R., La Camera, S., Atanassova, R., Dedaldechamp, F., Allario, T., Pourtau, N., et al. (2013). Source-to-sink transport of sugar and regulation by environmental factors. *Front Plant Sci* 4, 272.

Lewis, D.H., and Smith, D.C. (1967). Sugar alcohols (polyols) in fungi and green plants. *New Phytol* 66, 185-204.

Li, Z.G. (2016). Methylglyoxal and glyoxalase system in plants: old players, new concepts. *Bot Rev* 82, 183-203.

Li, Z.G., Duan, X.Q., Min, X., and Zhou, Z.H. (2017). Methylglyoxal as a novel signal molecule induces the salt tolerance of wheat by regulating the glyoxalase system, the antioxidant system and osmolytes. *Protoplasma* 254(5), 1995-2006.

Lindholm, P., Peranen, J., Andressoo, J.O., Kalkkinen, N., Kokaia, Z., Lindvall, O., et al. (2008). MANF is widely expressed in mammalian tissues and differently regulated after ischemic and epileptic insults in rodent brain. *Mol Cell Neurosci* 39(3), 356-371.

Lindholm, P., Voutilainen, M.H., Lauren, J., Peranen, J., Leppanen, V.M., Andressoo, J.O., et al. (2007). Novel neurotrophic factor CDNF protects and rescues midbrain dopamine neurons *in vivo*. *Nature* 448(7149), 73-77.

Loudit, S.M.B., Bauwens, J., and Francis, F. (2018). Cowpea aphid-plant interactions: endosymbionts and related salivary protein patterns. *Entomol Exp Appl* 166(6), 460-473.

Marchler-Bauer, A., Bo Y., Han, L., He, J., Lanczycki, C.J., Lu, S., et al. (2017). CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res* 45(D1), 200-203.

Mathers, T.C., Chen, Y., Kaithakottil, G., Legeai, F., Mugford, S.T., Baa-Puyoulet, P., et al. (2017). Rapid transcriptional plasticity of duplicated gene clusters enables a clonally reproducing aphid to colonise diverse plant species. *Genome Biol* 18(1), 27.

Melvin, P., Bankapalli, K., D'Silva, P., and Shivaprasad, P.V. (2017). Methylglyoxal detoxification by a DJ-1 family protein provides dual abiotic and biotic stress tolerance in transgenic plants. *Plant Mol Biol* 94(4-5), 381-397.

Miles, P.W. (1999). Aphid saliva. *Biol. Rev.* 74(1), 41-85.

Misra, K., Banerjee, A.B., Ray, S., and Ray, M. (1996). Reduction of methylglyoxal in *Escherichia coli* K12 by an aldehyde reductase and alcohol dehydrogenase. *Mol Cell Biochem.* 156(2), 117-124.

Mostofa, M.G., Ghosh, A., Li, Z.G., Siddiqui, M.N., Fujita, M., and Tran, L.P. (2018). Methylglyoxal - a signaling molecule in plant abiotic stress responses. *Free Radic Biol Med* 122, 96-109.

Mustafiz, A., Ghosh, A., Tripathi, A.K., Kaur, C., Ganguly, A.K., Bhavesh, N.S., et al. (2014). A unique Ni2+-dependent and methylglyoxal-inducible rice glyoxalase I possesses a single active site and functions in abiotic stress response. *Plant J* 78(6), 951-963.

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. *Proc Natl Acad Sci USA* 105(29), 9965-9969.

Mutti, N.S., Park, Y., Reese, J.C., and Reeck, G.R. (2006). RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*. *J Insect Sci* 6, 1-7.

Naessens, E., Dubreuil, G., Giordanengo, P., Baron, O.L., Minet-Kebdani, N., Keller, H., et al. (2015). A secreted MIF cytokine enables aphid feeding and represses plant immune responses. *Curr Biol* 25(14), 1898-1903.

Nakagawa, J., Ishikura, S., Asami, J., Isaji, T., Usami, N., Hara, A., et al. (2002). Molecular characterization of mammalian dicarbonyl/L-xylulose reductase and its localization in kidney. *J Biol Chem* 277(20), 17883-17891.

Nguyen, T.T., Boudreault, S., Michaud, D., and Cloutier, C. (2008). Proteomes of the aphid *Macrosiphum euphorbiae* in its resistance and susceptibility responses to differently compatible parasitoids. *Insect Biochem Mol Biol* 38(7), 730-739.

Nguyen, T.T., Michaud, D., and Cloutier, C. (2009). A proteomic analysis of the aphid *Macrosiphum euphorbiae* under heat and radiation stress. *Insect Biochem Mol Biol* 39(1), 20-30.

Nicholson, S.J., Hartson, S.D., and Puterka, G.J. (2012). Proteomic analysis of secreted saliva from Russian Wheat Aphid (*Diuraphis noxia* Kurd.) biotypes that differ in virulence to wheat. *J Proteomics* 75(7), 2252-2268.

Nicholson, S.J., Nickerson, M.L., Dean, M., Song, Y., Hoyt, P.R., Rhee, H., et al. (2015). The genome of *Diuraphis noxia*, a global aphid pest of small grains. *BMC Genomics* 16, 429.

Nicholson, S.J., and Puterka, G.J. (2014). Variation in the salivary proteomes of differentially virulent greenbug (*Schizaphis graminum* Rondani) biotypes. *J Proteomics* 105, 186-203.

Ofuya, T. (1995). Studies on the capability of *Cheilomenes lunata* (Fabricius) (Coleoptera: Coccinellidae) to prey on the cowpea aphid, *Aphis craccivora* Koch (Homoptera: Aphididae) in Nigeria. *Agric Ecosyst Environ* 52, 35-38.

Palgi, M., Greco, D., Lindstrom, R., Auvinen, P., and Heino, T.I. (2012). Gene expression analysis of *Drosophilaa Manf* mutants reveals perturbations in membrane traffic and major metabolic changes. *BMC Genomics* 13, 134.

Palgi, M., Lindstrom, R., Peranen, J., Piepponen, T.P., Saarma, M., and Heino, T.I. (2009). Evidence that DmMANF is an invertebrate neurotrophic factor supporting dopaminergic neurons. *Proc Natl Acad Sci USA.* 106(7), 2429-2434.

Petre, B., and Kamoun, S. (2014). How do filamentous pathogens deliver effector proteins into plant cells? *PLoS Biol* 12(2), e1001801.

Phillips, S.A., and Thornalley, P.J. (1993). Formation of methylglyoxal and D-lactate in human red blood cells *in vitro*. *Biochem Soc Trans* 21(2), 163S.

Pinheiro, P., Bereman, M.S., Burd, J., Pals, M., Armstrong, S., Howe, K.J., et al. (2014). Evidence of the biochemical basis of host virulence in the greenbug aphid, *Schizaphis graminum* (Homoptera: Aphididae). *J Proteome Res* 13(4), 2094-2108.

Pitino, M., and Hogenhout, S.A. (2013). Aphid protein effectors promote aphid colonization in a plant species-specific manner. *Mol Plant Microbe Interact.* 26(1), 130-139.

R Core Team (2019). R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rahman, A., Mostofa, M.G., Alam, M.M., Nahar, K., Hasanuzzaman, M., and Fujita, M. (2015). Calcium mitigates arsenic toxicity in rice seedlings by reducing arsenic uptake and modulating the antioxidant defense and glyoxalase systems and stress markers. *BioMed Res Int* 12.

Rao, S.A.K., Carolan, J.C., and Wilkinson, T.L. (2013). Proteomic profiling of cereal aphid saliva reveals both ubiquitous and adaptive secreted proteins. *Plos ONE* 8(2), e57413.

Ray, A., Ray, S., Mukhopadhyay, S., and Ray, M. (2013). Methylglyoxal with glycine or succinate enhances differentiation and shoot morphogenesis in *Nicotiana tabacum* callus. *Biol Plant* 57(2), 219-223.

Remaudiere, G., and Remaudiere, M. (1997). *Catalogue des Aphididae du monde (Homoptera Aphidoidea).* Paris: Paris Institut national de la recherche agronomique.

Rodriguez, P.A., Escudero-Martinez, C., and Bos, J.I.B. (2017). An aphid effector targets trafficking protein VPS52 in a host-specific manner to promote virulence. *Plant Physiol* 173(3), 1892-1903.

Sainsbury, F., Thuenemann, E.C., Lomonossoff, G.P. (2009) pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant Biotechnol J* 7(7), 682-693.

Sankaranarayanan, S., Jamshed, M., and Samuel, M.A. (2015). Degradation of glyoxalase I in *Brassica napus* stigma leads to self-incompatibility response. *Nat Plants* 1, 15185.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., et al., (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* 7 539.

Singh, B.B., Ehlers, J.D., Sharma, B., and Freire, F.R. (2002). "Recent progress in cowpea breeding", in: *Proceedings of the World Cowpea Conference III*,

eds. C.A. Fatokun, B.B. Tarawali, B.B. Singh, P.M. Kormawa & M. Tamo), 22-40.

Sochor, M., Baquer, N.Z., and McLean, P. (1979). Glucose overutilization in diabetes: evidence from studies on the changes in hexokinase, the pentose phosphate pathway and glucuronate-xylulose pathway in rat kidney cortex in diabetes. *Biochem Biophys Res Commun* 86(1), 32-39.

Sonnhammer, E.L., von Heijne, G., and Krogh, A. (1998). A hidden Markov model for predicting transmembrane helices in protein sequences. *Proceedings of the 6th International Conference on Intelligent Systems for Molecular Biology* 6, 175-182.

Sorenson, J. (2009). "Aphids," in *Encyclopedia of Insects,* eds. V. Resh & R. Carde. Second ed (San Diego, CA: Academic Press), 27-30.

Sperschneider, J., Dodds, P., Gardiner, D., Singh, K.B., and Taylor, J.M. (2018). Improved prediction of fungal effector proteins from secretomes with EffectorP 2.0. *Mol Plant Path* 19(9), 2094-2110.

Sperschneider, J., Dodds, P.N., Gardiner, D.M., Manners, J.M., Singh, K.B., and Taylor, J.M. (2015). Advances and challenges in computational prediction of effectors from plant pathogenic fungi. *PLoS Pathog* 11(5), e1004806.

Sperschneider, J., Gardiner, D.M., Dodds, P.N., Tini, F., Covarelli, L., Singh, K.B., et al. (2016). EffectorP: predicting fungal effector proteins from secretomes using machine learning. *New Phytol* 210(2), 743-761.

Stergiopoulos, I., and de Wit, P.J. (2009). Fungal effector proteins. *Annu. Rev. Phytopathol.* 47, 233-263. doi: 10.1146/annurev.phyto.112408.132637.

Thornalley, P.J. (2006). Advanced glycation end products in renal failure. *J Ren Nutr* 16(3), 178-184.

Thorpe, P., Cock, P.J., and Bos, J. (2016). Comparative transcriptomics and proteomics of three different aphid species identifies core and diverse effector sets. *BMC Genomics* 17, 172.

Thorpe, P., Escudero-Martinez, C.M., Cock, P.J.A., Eves-van den Akker, S., and Bos, J.I.B. (2018). Shared transcriptional control and disparate gain and loss of aphid parasitism genes. *Genome Biol Evol* 10(10), 2716-2733.

Timko, M., and Singh, B.B. (2008). "Cowpea, a Multifunctional Legume," in *Genomics of Tropical Crop Plants,* eds. P.H. Moore & R. Ming. (New York, NY: Springer), 227-258.

Tjallingii, W.F. (2006). Salivary secretions by aphids interacting with proteins of phloem wound responses. *J Exp Bot* 57(4), 739-745.

Tjallingii, W.F., and Esch, T.H. (1993). Fine-structure of aphid stylet routes in plant-tissues in correlation with EPG signals. *Physiol Entomol* 18(3), 317-328.

Vandermoten, S., Harmel, N., Mazzucchelli, G., De Pauw, E., Haubruge, E., and Francis, F. (2014). Comparative analyses of salivary proteins from three aphid species. *Insect Mol Biol* 23(1), 67-77.

Wang, W., Dai, H.E., Zhang, Y., Chandrasekar, R., Luo, L., Hiromasa, Y., et al. (2015). Armet is an effector protein mediating aphid-plant interactions. *Faseb J* 29(5), 2032-2045.

Wang, Y.M., and Van Eys, J. (1970). The enzymatic defect in essential pentosuria. *N Engl J Med* 282(16), 892-896.

Wenger, J.A., Cassone, B.J., Legeai, F., Johnston, J.S., Bansal, R., Yates, A.D., et al. (2017). Whole genome sequence of the soybean aphid, *Aphis glycines*. *Insect Biochem Mol Biol.*

Wild, R., Ooi, L., Srikanth, V., and Munch, G. (2012). A quick, convenient and economical method for the reliable determination of methylglyoxal in millimolar concentrations: the N-acetyl-L-cysteine assay. *Anal Bioanal Chem* 403(9), 2577-2581.

Will, T., Tjallingii, W.F., Thonnessen, A., and van Bel, A.J.E. (2007). Molecular sabotage of plant defense by aphid saliva. *Proc Natl Acad Sci USA* 104(25), 10536-10541.

Will, T., and Vilcinskas, A. (2015). The structural sheath protein of aphids is required for phloem feeding. *Insect Biochem Mol Biol* 57, 34-40.

Yadav, S.K., Singla-Pareek, S.L., Ray, M., Reddy, M.K., and Sopory, S.K. (2005a). Methylglyoxal levels in plants under salinity stress are dependent on glyoxalase I and glutathione. *Biochem Biophys Res Commun* 337(1), 61-67.

Yadav, S.K., Singla-Pareek, S.L., Reddy, M.K., and Sopory, S.K. (2005b). Transgenic tobacco plants overexpressing glyoxalase enzymes resist an increase in methylglyoxal and maintain higher reduced glutathione levels under salinity stress. *FEBS Lett* 579(27), 6265-6271.

Yang, S., Jan, Y.H., Mishin, V., Heck, D.E., Laskin, D.L., and Laskin, J.D. (2017). Diacetyl/l-Xylulose reductase mediates chemical redox cycling in lung epithelial cells. *Chem Res Toxicol* 30(7), 1406-1418.

Yang, Z., Ma, L., Francis, F., Yang, Y., Chen, H., Wu, H., et al. (2018). Proteins identified from saliva and salivary glands of the Chinese gall aphid *Schlechtendalia chinensis*. *Proteomics* 18(9), e1700378.

Table 1.1. Cowpea aphid salivary proteins identified for secretion or effector function using bioinformatic programs.

Accession	ACYPI	Description (BLASTP)	SignalP	SecretomeP	EffectorP
GAJW01000730.1_3	009769	glyceraldehyde-3-phosphate dehydrogenase [*Aphis gossypii*]			X
AG009127-PA	088094	histone H4-like, partial [*Sipha flava*]		X	X
NP_240299.1	085083	protein lin-28 homolog isoform X1 [*Sipha flava*]			X
AG010231-PA	23752	carbonic anhydrase 2-like [*Aphis gossypii*]	X		X
GAJW01000939.1_3	006727	triosephosphate isomerase [*Aphis gossypii*]			X
GAJW01000401.1_4	000057	putative diacetyl/L-xylulose reductase [*Aphis citricidus*]	X		X
Rpa07060.t1-protein	000028	nucleoside diphosphate kinase-like isoform X1 [*Rhopalosiphum maidis*]			X
Rpa13763.t1-protein	004622	probable pseudouridine-5'-phosphatase [*Rhopalosiphum maidis*]			X
GAJW01000399.1_1	001643	ribosomal protein S19e-like [*Acyrthosiphon pisum*]		X	X
NP_239859.1	000693	co-chaperonin GroES [*Buchnera aphidicola* str. APS (*Acyrthosiphon pisum*)]		X	X
GAJW01002612.1_4	002607	ras-related protein Rab-7a-like [*Rhopalosiphum maidis*]			X
Rpa11900.t1-protein	003541	peptidyl-prolyl cis-trans isomerase-like [*Aphis gossypii*]			X
GAJW01001525.1_4	008224	Uncharacterized protein LOC114123729 [*Aphis gossypii*] (Me10/Mp58)	X		X
GAJW01005083.1_5	006909	dihyropteridine reductase *[Aphis gossypii*]			X
GAJW01000315.1_5	000041	cytochrome c-like [*Aphis gossypii*]			X
GAJW01000773.1_5	000058	twinstar [*Acyrthosiphon pisum*]		X	X
GAJW01001573.1_3	I007471	Superoxide dismutase [Cu-Zn] [*Aphis gossypii*]			X
AG013923-PA	006002	protein dj-1beta-like isoform X2 [*Melanaphis sacchari*]			X
AG015946-PA	26018	uncharacterized protein LOC114132136 [*Aphis gossypii*]		X	X
GAJW01000973.1_1	000031	ribosomal protein S12 [*Acyrthosiphon pisum*]			X
AG001995-PA	000422	apolipophorins-like [*Aphis gossypii*]	X		
AG007466-PA	002298	soluble trehalase [*Aphis glycines*]	X		
AG008787-PA	000288	glucose dehydrogenase [FAD, quinone]-like [*Aphis gossypii*]	X		
AG009504-PA	005582	glucose dehydrogenase [FAD, quinone]-like [*Melanaphis sacchari*]	X		
Rpa18947.t1-	009964	endochitinase [*Rhopalosiphum maidis*]	X		

protein				
AG005369-PA	000986	uncharacterized protein LOC114133079 [*Aphis gossypii*]	X	
AG011655-PA	009915	endoplasmin homolog [*Aphis gossypii*]	X	
AG005025-PA	002622	calreticulin [*Aphis gossypii*]	X	
GAJW01001996.1_3	009755	protein disulfide-isomerase [*Melanaphis sacchari*]	X	
Rpa07735.t1-protein	37407	uncharacterized protein LOC113549759 [*Rhopalosiphum maidis*]	X	
Rpa04366.t1-protein	005594	protein disulfide-isomerase A3-like [*Rhopalosiphum maidis*]	X	
AG011982-PA	004904	uncharacterized protein LOC114121223 [*Aphis gossypii*]	X	
AG008256-PA	004432	uncharacterized protein LOC114132390 [*Aphis gossypii*]	X	
Rpa01411.t1-protein	008926	protein disulfide-isomerase A6 homolog [*Rhopalosiphum maidis*]	X	
AG011980-PA	25151	uncharacterized protein LOC107882155 [*Acyrthosiphon pisum*]	X	
AG001228-PA	001479	phospholipase A1-like [*Aphis gossypii*]	X	
AG014046-PA	000975	calumenin-A-like [*Aphis gossypii*]	X	
AG001716-PA	007690	uncharacterized protein LOC100166851 precursor [*Acyrthosiphon pisum*]	X	
AG000496-PA	50398	RCC1 domain-containing protein 1 [*Aphis gossypii*]	X	
AG002007-PA	003669	puromycin-sensitive aminopeptidase isoform X2 [Aphis gossypii]	X	
AG012271-PA	000817	peroxidase-like [Aphis gossypii]	X	
GAJW01000521.1_1	008165	60S ribosomal protein L4 [Rhopalosiphum maidis]	X	
GAJW01002804.1_6	000056	calmodulin [*Bombyx mori*]		X
MYZPE13164_0_v1.0_00007220.1	086010	histone H2B [*Myzus persicae*]		X
AG009875-PA	007671	histone H3-like [*Diuraphis noxia*]		X
AG015809-PA	003625	citrate synthase 1 [*Aphis gossypii*]		X
Rpa01379.t1-protein	007342	annexin B9-like isoform X2 [*Rhopalosiphum maidis*]		X
GAJW01000299.1_6	002483	eukaryotic initiation factor 4A [*Acyrthosiphon pisum*]		X
GAOM01000916.1_4	000087	bicaudal [*Acyrthosiphon pisum*]		X
BAH71147.1	010042	40S ribosomal protein S7-like *[Acyrthosiphon pisum*]		X
AG001482-PA	002577	adenylate kinase isoenzyme 1 [*Aphis gossypii*]		X
Mca25739.t1-protein	006705	ump-cmp kinase isoform X2 [*Myzus persicae*]		X
AG001456-PA	009018	inositol monophosphatase 1-like		X

		[*Aphis gossypii*]		
AG004283-PA	21663	lipase member H-B-like isoform X1 [*Aphis gossypii*]		X
GAJW01001081.1_4	004620	complement component 1 Q subcomponent-binding protein, mitochondrial [*Aphis gossypii*]	X	
AG012940-PA	006879	importin-5 [*Aphis gossypii*]	X	
GAJW01001854.1_3	009959	protein lethal(2)essential for life [*Aphis gossypii*]	X	
Mca14412.t1-protein	000100	40s ribosomal protein s4 [*Diuraphis noxia*]	X	

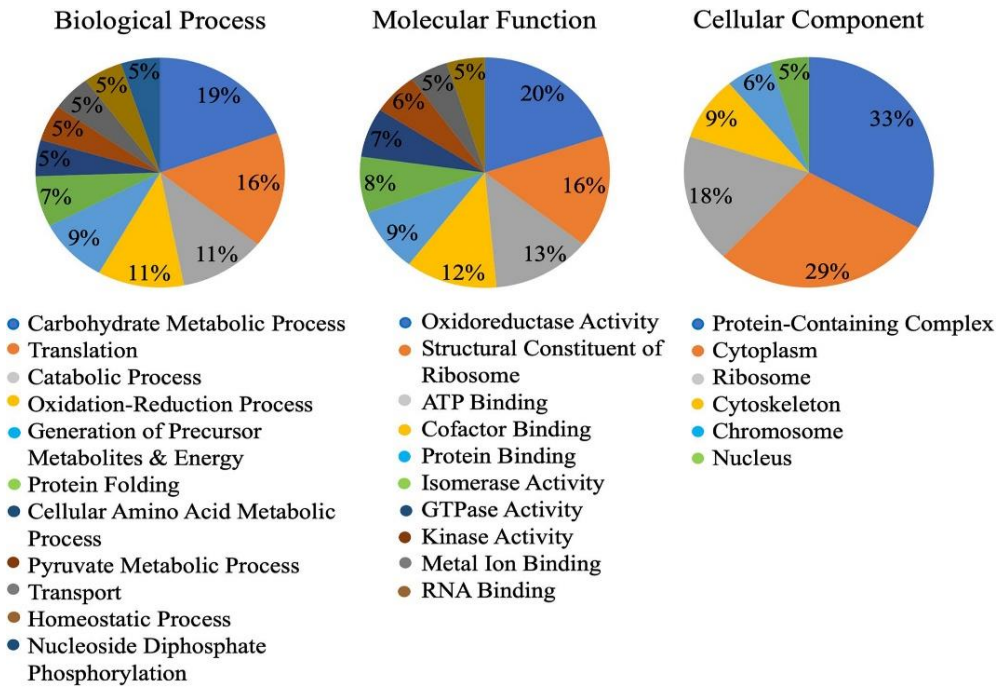

Fig 1.1. Gene ontology (GO) of the Cowpea aphid salivary proteins. Cowpea aphid salivary proteins were identified by LC-MS/MS and protein content were determined using a number of aphid genomes and the transcriptomes of cowpea aphid and potato aphid.

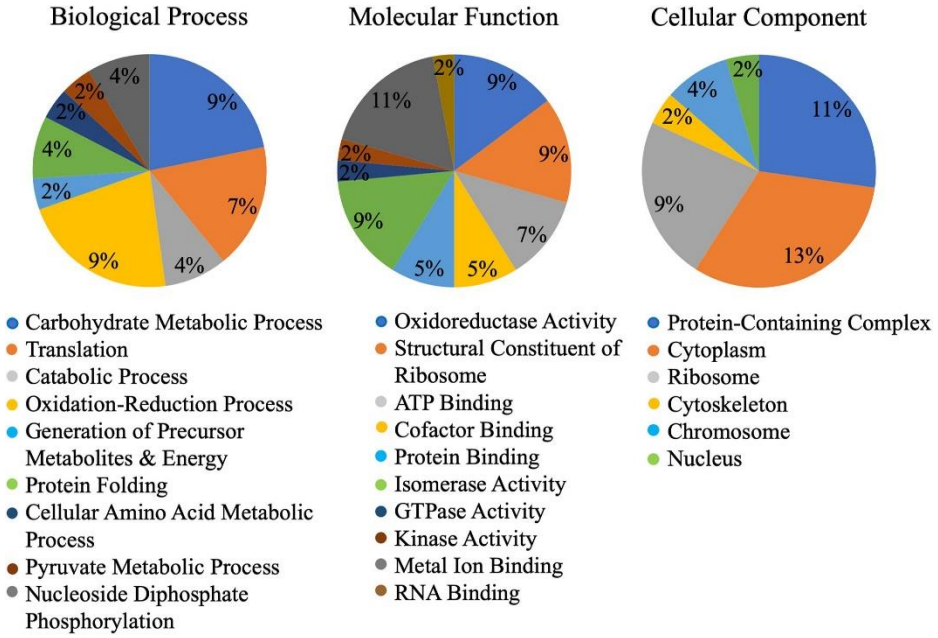

Fig 1.2. Gene ontology (GO) of putative Cowpea aphid effectors. Cowpea aphid putative effectors were identified by analyzing the salivary proteins with SignalP, SecretomeP and EffectorP.

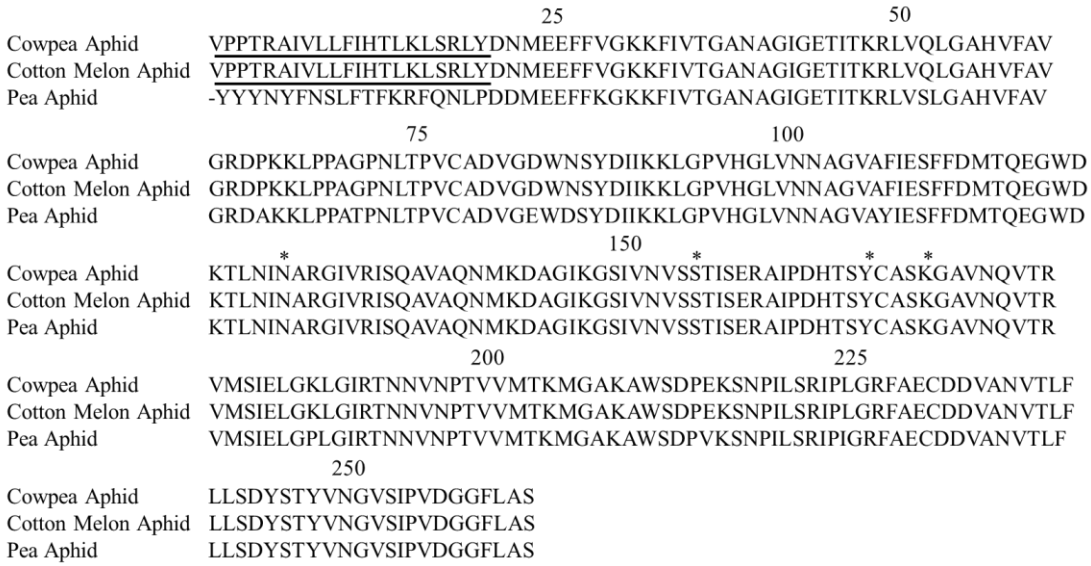

Fig 1.3. DCXR amino acid sequence alignment. Alignment of AcDCXR from cowpea aphid (*Aphis craccivora*), and orthologs from the cotton melon aphid (*Aphis gossypii*; XP_027848224.1) and the pea aphid (*Acyrthosiphon pisum*; NP_001119641.1) using Clustal Omega (Sievers et al., 2011). The secretion signal found in the cowpea aphid and cotton melon aphid using SignalP is underlined (Bendsten et al., 2004b; Armenteros et al., 2019). The active site for short-chain dehydrogenases/reductases is made up of a YXXXK motif with an upstream N and S (Marchler-Bauer et al., 2017). The residues forming the active site are indicated with the asterisks.

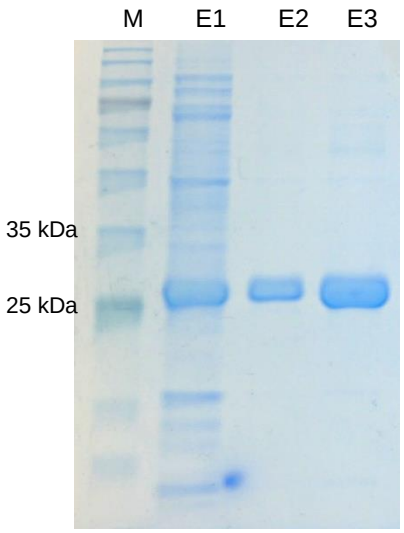

Fig 1.4. Recombinant AcDCXR purified from *E. coli*. pDEST17-AcDCXR was expressed in *E. coli* and induced with 0.5 mM IPTG. Aliquots of the purification products were run on 12% SDS−PAGE. M is the protein standard, E1 and E2 are the first (150 mM imidazole) and second (200 mM imidazole) elutions from the Ni-NTA column. The expected 6xHis tagged AcDCXR size is 28.3 kDa.

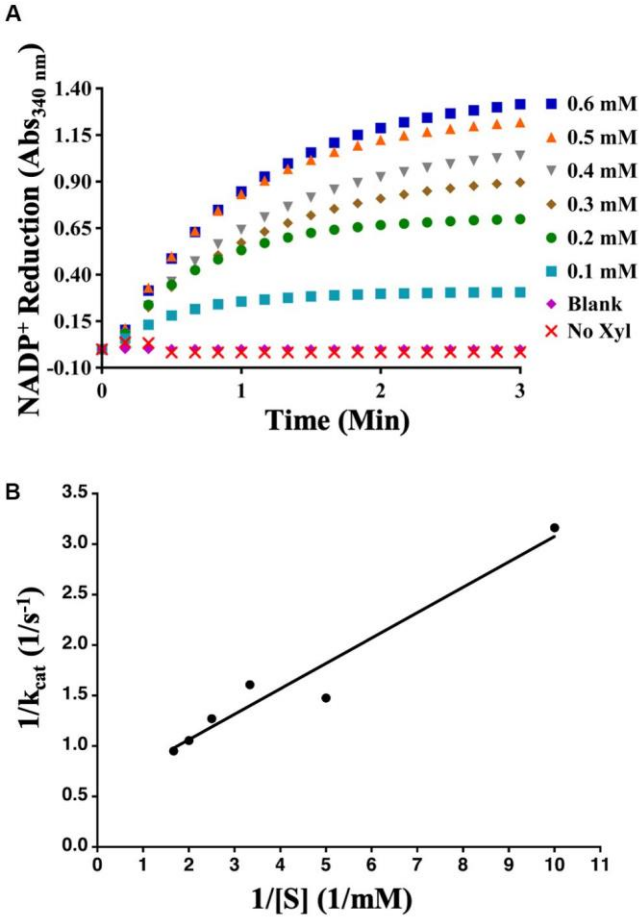

Fig 1.5. Recombinant AcDCXR oxidation activity. Xylitol oxidation by cowpea aphid recombinant AcDCXR. **A)** Various concentrations of $NADP^+$ were used to oxidize 200 mM xylitol in the presence of 10 µg of AcDCXR. Reactions containing no AcDCXR (Blank) or no xylitol (Xyl) were used as controls. **B)** Lineweaver-Burk plot of xylitol oxidation. Data represent average of two technical replicates from a single experiment. The experiment was repeated once with similar results.

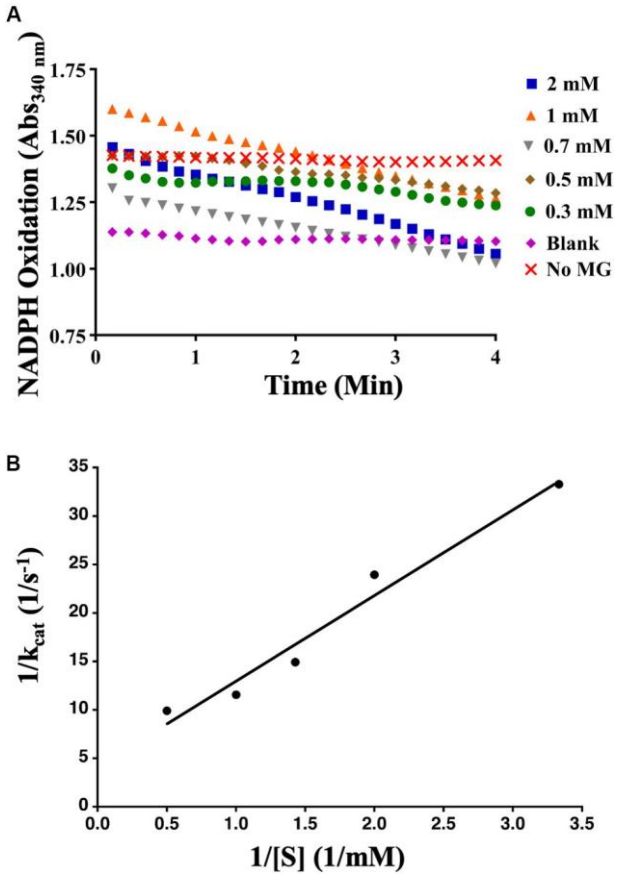

Fig 1.6. Recombinant AcDCXR reduction activity. Methylglyoxal reduction by cowpea aphid recombinant AcDCXR. **A)** Various concentrations of methylglyoxal were reduced with 200 mM NADPH in the presence of 10 µg of AcDCXR. Reactions containing no AcDCXR (Blank) or no methylglyoxal (MG) were used as controls. **B)** Lineweaver-Burk plot of methylglyoxal reduction. Data represent average of two technical replicates from a single experiment. The experiment was repeated once with similar results.

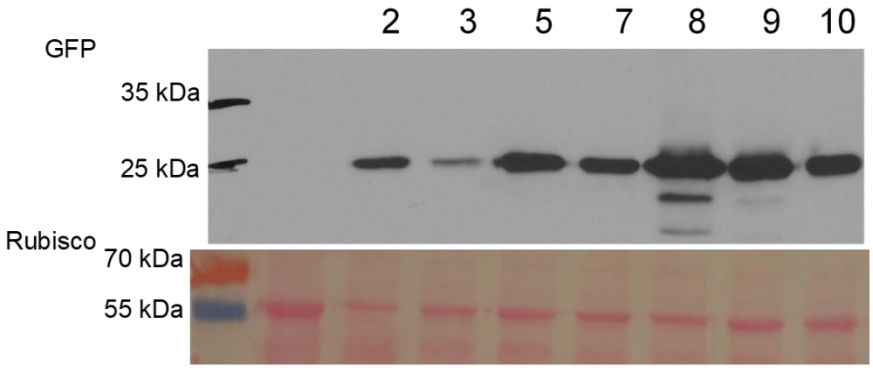

Fig 1.7. Western blot analysis of GFP expression in *Pisum sativum* cv ZP1130**.** Leaves were infiltrated with *Agrobacterium tumefaciens* containing pEAQ-DEST1-GFP and samples were collected at 2, 3, 5,7, 8, 9, 10 days post infiltration (dpi). Samples were extracted with urea-thiourea lysis buffer and 25 ug of protein was loaded onto 12% SDS-PAGE. GFP was detected with anti-GFP at 1:2000 dilution. Lower panel is Ponceau S Red staining of Rubisco to show protein loading.

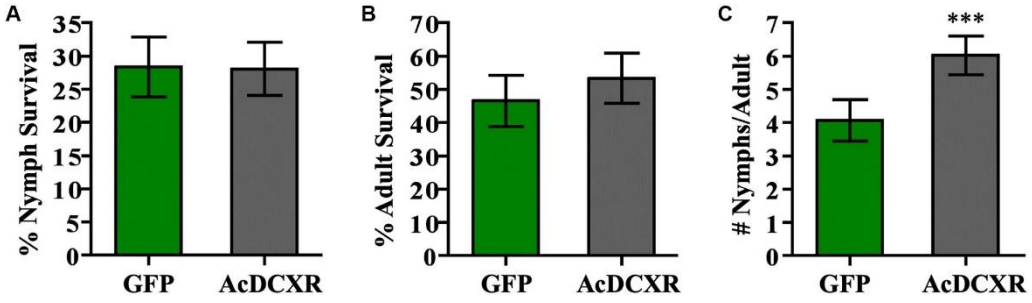

Fig 1.8. AcDCXR effect on aphid performance. *Agrobacterium tumefaciens* strain AGL01 was used to transiently express pEAQ-HT-DEST1-GFP and pEAQ-HT-DEST1-AcDCXR in *Pisum sativum* cv. ZP1130. Adult cowpea aphid adults were placed the infiltration site to lay nymphs and removed 24 hours later. **A)** The survival rate of the nymphs after 8 days on the site of infiltration. **B, C)** A single adult was transferred to a new infiltration site of the same construct and the **B)** survival of the adult and **C)** fecundity were monitored. Graphs show the mean with error bars representing ±SE of the mean for n = 43 for GFP and n = 45 for AcDCXR from three independent experiments. *** $P < 0.001$ as determined by generalized linear models (GLM).

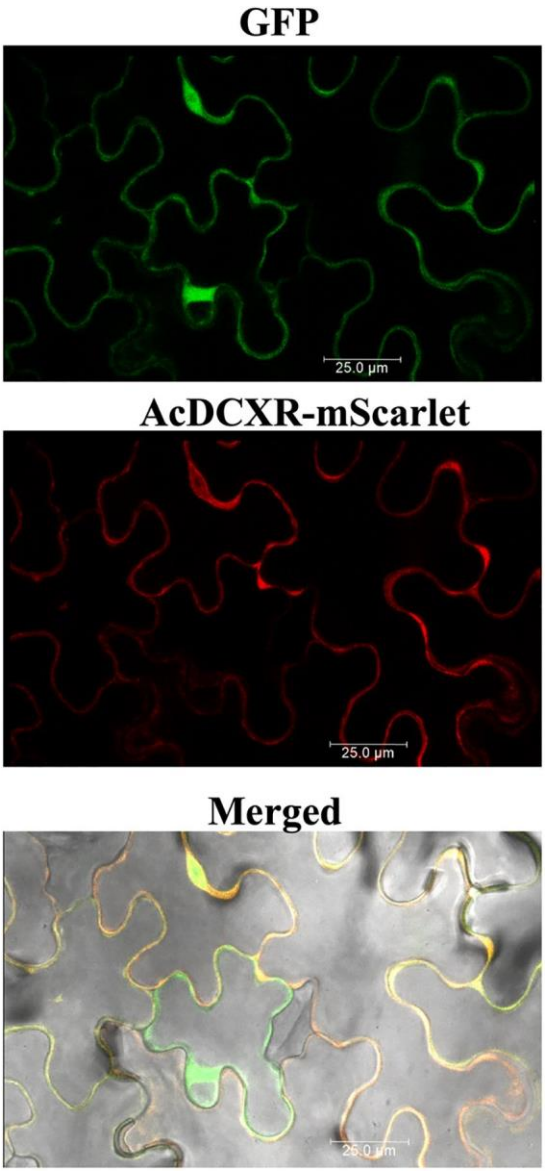

Fig 1.9. *In planta* subcellular localization of the recombinant AcDCXR. *A. tumefaciens* strain GV3101 containing pCAMBIA-1300-GFP or pCAMBIA-1300-AcDCXR-mScarlet were co-infiltrated into *N. benthamiana* leaves. Three days after agroinfiltration, leaf epidermal cells were used in confocal microscopy.

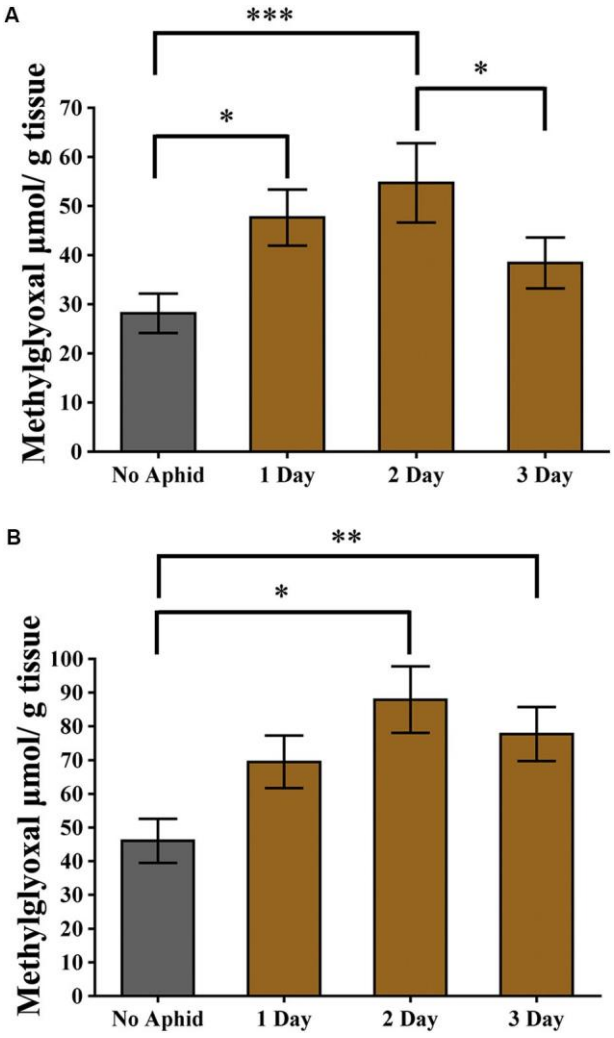

Fig 1.10. Methylglyoxal levels induced by aphid infestation. **A)** Cowpea and **B)** pea plants were exposed to a heavy infestation of cowpea aphids. Leaves were harvested at 1, 2, and 3 days post infestation. Uninfested plants of the same age were used as controls. Graphs show the mean with error bars representing ±SE of the mean of n = 6 for cowpea, from two independent experiments, and n = 3 for pea, from a single experiment, with two technical replicates each. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ as determined by nested ANOVA followed by multiple comparisons of means.

Chapter Two

Characterization of Cowpea Aphid Infestation on Susceptible and Resistant Cowpea

Abstract

One of the most important crops for semiarid areas of world is cowpea (*Vigna unguiculata*). Cowpea is able to thrive in the hot and dry growing conditions in these parts of the world. While cowpea is able to withstand these abiotic stresses, it faces a number of biotic stresses, among which is the cowpea aphid (*Aphis craccivora*). Cowpea aphid is a major pest of cowpea and infestations are highly destructive especially on young plants. Aphids can be classified based on their ability to generate damage on their host plant at low population levels (phytotoxic) or not (nonphytotoxic). To better understand cowpea aphid induced damage on susceptible cowpea, variable aphid densities and systemic versus local infestation assays were employed to compare plant damage caused by both high and low cowpea aphid densities. Results from these assays revealed presence of cowpea aphid damage at low aphid density infestations, indicating cowpea aphids are phytotoxic to cowpea. In cowpea, resistance to the cowpea aphid has been previously identified in an African cowpea germplasm and near isogenic lines (NILs) were generated in the California blackeye cultivar background. To better characterize this resistance, aphid performance was compared between the two NILs. Using no choice assays, the resistance was found to affect aphid survival, fecundity and growth. Using choice assays, a preference by cowpea aphids for the susceptible NIL was observed. Electrical penetration graph (EPG) analysis revealed that the resistance is partially phloem based, with aphids feeding on the resistant plants struggling to ingest phloem

sap. Taken together, these results indicate that cowpea aphids are phytotoxic and the resistance originating from an African breeding line can be described as having both antibiosis and antixenosis components.

Introduction

Cowpea (*Vigna unguiculata*) is one of the most important sources of food in semi-arid regions of the world (Singh et al., 2002). Not only is cowpea an important source of food in these areas of the world, it also contributes as an important cover crop and as fodder (Langyintuo et al., 2003; Timko and Singh, 2008). Cowpea is vital to this region because it is able to withstand the harsh growing conditions such as heat and drought stress (Hall et al., 2002; Hall, 2004). Cowpea can also withstand other negative growth conditions such as low soil fertility and has a better tolerance to soil pH ranges compared to other legumes (Elawad and Hall, 1987; Fery, 1990).

While cowpea has a robust ability to withstand abiotic stressors, it faces numerous biotic stresses. The cowpea aphid (*Aphis craccivora*) is one of the most important of these biotic stresses. Aphids feed by navigating their flexible hypodermic needlelike stylets through plant tissues to reach the phloem sap, with minimal mechanical damage to the plant (Tjallingii and Esch, 1993; Tjallingii, 2006). Though it afflicts minimal mechanical damage, cowpea aphid infestation can lead to both direct and indirect damage. Direct plant damage caused by cowpea aphids is manifested as chlorosis, stunted growth and pseudogalling of the leaves (Jackai and Daoust, 1986; Goggin et al., 2017; Omoigui et al., 2017). The indirect damage occurs in two major ways. The first is by excreting honeydew, or insect waste product, onto above ground plant parts leading to the growth of sooty molds which block photosynthesis and reduce marketable

product value (Reynolds, 1999). The second indirect damage is by acting as viral vectors and transmitting plant viruses. Cowpea aphids act as viral vectors for over 50 plant viruses including the notorious cowpea-aphid-borne mosaic virus (Chan et al., 1991; Singh, 2014).

Aphids can be classified based on the amount of damage they generate in their host plants. Aphids that cause little or no observable damage to plant under low population can be classified as nonphytotoxic. Nonphytotoxic aphids include the pea aphid (*Acyrthosiphon pisum*) and the green peach aphid (*Myzus persicae*). Alternatively, phytotoxic aphids are able to generate damage in their host plants at low population. Two of the most economically important phytotoxic aphids are the Russian wheat aphid (*Diuraphis noxia*) and greenbug (*Schizaphis graminum*) (Miles, 1999; Nicholson et al., 2012; Nicholson and Puterka, 2014). Both phytotoxic aphid species are able to generate damage like chlorosis and pseudogalling at low densities eventually leading to reduced grain yield (Burd and Burton, 1992; Burd et al., 1993).

To control aphid populations, experts recommend the use of chemical insecticides; however, in the areas of the world where cowpea is grown insecticide use is not always an option (Singh and Allen, 1980; Obopile, 2006; Souleymane et al., 2013). The limiting factors for pesticide use include cost, loss of beneficial insects, and adverse effects on the environment (Souleymane et al., 2013). Without the use of pesticides, prolonged cowpea aphid infestations can cause over 50% losses in crop yield (Obopile, 2006). This highlights the need for

identifying endogenous cowpea resistance to cowpea aphids to better protect cowpea from aphid infestations. An endogenous source of cowpea aphid resistance was previously identified in an African breeding line (IT97K-556-6) (Souleymane et al., 2013). Using recombinant inbred lines (RILs), derived from a susceptible California blackeye cultivar (CB27) and the resistant African breeding line (IT97K-556-6), the sources of the resistance were mapped to two quantitative trait loci (QTLs). The resistance QTLs were found to be a major QTL, *QAC-vu7.1,* and a minor QTL, *QAC-vu1.1* (Huynh et al., 2015). The mechanism of the resistance mediated by these two QTLs remains unknown.

Plant resistance to insects has been classified into three different subcategories: antibiosis, antixenosis, and tolerance (Smith, 1989). Resistance with antibiosis effects the insect biology and growth, while antixenosis type resistance effects the insect behavior. Tolerance on the other hand is the ability of the plant to withstand insect damage.

Taking advantage of aphids being phloem feeders, an electrical penetration graph (EPG) could be employed to measure aphid feeding behavior within the plant tissues through electrical waveforms (Tjallingii, 1988). An EPG is performed by securing one electrode to an aphid and placing the other electrode in the soil next to plant roots. When the aphid feeds on the plant, the electrical circuit is complete, generating different waveforms for each step in the feeding activity of the aphid as its stylets migrate through plant tissues delivering saliva and acquiring plant sap (Tjallingii, 1988; Tjallingii et al., 2010). EPGs have been

used to characterize plant resistance to aphids by comparing aphid feeding behavior on resistant and susceptible plants. It can also be used to identify where in the leaf tissues the aphid encounters the resistance (Tjallingii, 2006; Sun et al., 2018).

In the present study, direct damage caused by cowpea aphid to susceptible cowpea and the mechanism of cowpea resistance to cowpea aphids was characterized. Susceptible cowpea responses to cowpea aphids were evaluated by using controlled and uncontrolled aphid densities and monitoring the aphid induced damage. To elucidate the mechanism of resistance to cowpea aphids conferred by the two QTLs, a pair of cowpea near-isogenic lines (NILs) 2014-008-51-82 and California blackeye 46 (CB46) were used. CB46 is a susceptible California blackeye cultivar. The NIL 2014-008-51-82 has the CB46 background plus two resistance QTLs from the resistant African breeding line (IT97K-556-6) (Huynh et al., 2015); it was developed by marker-assisted backcrossing as part of the Blackeye varietal improvement program at UC Riverside (Huynh and Roberts, pers. comm.). Therefore, these two NILs were used in aphid reproduction, behavioral and feeding assays.

Materials and Methods

Plant and aphid growth conditions

Two lines of cowpea (*Vigna unguiculata*), CB46 and its NIL 2014-008-51-82, were grown in UC Mix 3 soil (agops.ucr.edu/soil/) in 24 oz plastifoam cups and fertilized weekly with MiracleGro (18−18−21; Stern's MiracleGro Products). Plants were grown in a pesticide free plant growth room at 26-30°C and 16:8 light:dark photoperiod.

The cowpea aphids *(Aphis craccivora)* were collected from a field in Riverside, California, in the summer of 2016. Since then, the aphids were reared on cowpea line CB46 in a pesticide free greenhouse or a plant growth chamber and maintained at 26−30°C with a 16:8 light:dark photoperiod.

Aphid population damage assay

One-week old susceptible cowpea plants were infested with 15 mixed stages of 3rd instar, 4th instar, and adults of cowpea aphids or cotton melon aphids (*Aphis gossypii*) for 15 days. Plants were encased in plastic pollination bags with minute holes (Seedburo, SKU: S27) to restrict aphids to the plant and were maintained at 22–28°C with a 16:8 light:dark photoperiod for 15 days.

The cowpea aphid infestation levels were at two different aphid densities, with one density of aphids maintained at a constant 15 aphids on the entire plant (constant cowpea aphid density), and a second density where aphids were

allowed to grow and reproduce without any numeric restriction (variable cowpea aphid density). To keep the cowpea aphids at a constant density, newborn nymphs were removed daily, and the population was maintained at 15 aphids. Newborn nymphs were kept when the number of the original aphids dropped below 15. The cotton melon aphids were allowed to grow without any numeric restriction like the variable cowpea aphid density (variable cotton melon aphid density). The damage symptoms induced by the aphids were monitored periodically throughout the experiment. The damage symptoms monitored were the presence of chlorosis, pseudogalling, and/or stunted growth. For each aphid density or species, 8-10 plants were used. The experiment was performed three times.

Local vs systemic damage assay

A single unifoliate leaf of one-week old susceptible cowpea plants was infested with 15 mixed stages of 3rd instar, 4th instar, and adults of cowpea aphids using a mesh sleeve bag, at 22–28°C with a 16:8 light:dark photoperiod for 15 days. Damage symptoms including presence of chlorosis, pseudogalling, and/or stunted growth were monitored periodically throughout the 15 days on the uninfested areas of the plant. After 15 days, the mesh sleeves were removed and the symptoms on the infested leaves as well as on the entire plants were documented. Three plants were infested per experiment and the experiment was performed three times.

Electrical penetration graph

A DC-EPG system was used for the electrical penetration graph (EPG) analysis (Tjallingii, 1988). Aphids were tethered to a 12.5 µm gold wire on their dorsal abdomen using a water-based silver glue (Cervantes and Backus, 2018). After an hour-long starvation period, aphids were placed on the abaxial side of a two-week old unifoliate leaf and a second electrode was placed in the soil of the potted plant. Simultaneous recordings for eight aphids were performed on a Giga-8 DC-EPG amplifier for 8 hours. The aphid-plant systems were housed in a Faraday cage in a climate-controlled room at 24 ± 1°C. Each recording session had half of the aphids on susceptible plants and half on resistant plants. At the end, a total of 25 susceptible and 30 resistant EPGs were obtained and analyzed. The analysis of the EPG variables and waveforms were performed with PROBE 3.5 software (EPG systems, www.epgsystems.eu) naming convention based (Ebert et al., 2015). The calculations were performed with EPG-Calc 6.1 software (Giordanengo, 2014).

Aphid choice assay

A large modified petri dish arena was placed above two 2-week-old cowpea plants, one of each resistant and susceptible lines. The arena had two 2 cm holes in the bottom of the plate cut-out on either end, directly across from each other, each exposing a leaf surface from either the resistant or the susceptible lines. A third 2 cm hole was directly in the center and was where the aphids were

introduced. Twenty adult apterous cowpea aphids were collected in a modified 50 mL conical tube and starved for an hour. The modified conical tube was then inserted into the hole and fastened in the center of the arena and aphids were allowed to choose between the exposed leaves. The number of aphids feeding on each leaf was recorded at 2, 3, 6, and 24 hours after exposure to the aphids. The experiment was performed four times with 4 – 5 plant pairs used per experiment.

Aphid growth rate assay

Ten cowpea aphid adults were clip caged onto the adaxial side of a CB46 or 2014-008-51-82 leaf. After 24 hours, the adult aphids were removed, and ten newborn nymphs were left on the leaf in the clip cage. After six days, the surviving aphids were counted and weighed with a microbalance (CN HG 01.121, Sartorius).

The mean relative growth rate (MRGR) was calculated as the difference of logarithms of the mean weight of day-old aphid nymphs and the mean weight of the surviving aphid nymphs divided by the number of days (MRGR = ($\log W_{surviving}$ – $\log W_{day\text{-}old}$) / Number of days). Twelve biological replicates were used per each cowpea genotype.

Aphid fecundity assay

Age synchronized one-day old adult aphids were developed by clip-caging adult apterous aphids to the adaxial side of a susceptible cowpea plant to lay progeny. After 24 hours, the adult aphids were removed with a fine tip paint brush and the first instars were allowed to develop to maturity. A single, age synchronized, one-day-old adult cowpea aphid was transferred to a new a two-week-old unifoliate leaf of cowpea lines CB46 and 2014-008-51-82 and clip-caged on the adaxial side. The cages were monitored daily, for one week, and the survival of the adult aphid and the number of nymphs laid were recorded. After counting, the newly laid nymphs were removed. For each cowpea line, a total of 16 plants were used and each plant was infested with a single adult aphid.

Results

Damage caused by cowpea aphids

Cowpea aphids generate an inordinate amount of damage to cowpea in the field. This damage caused by cowpea aphids includes chlorosis, pseudogalling, and stunted growth. As cowpea aphid population level can reach high levels on cowpea crop, the high aphid numbers could be the reason for the damage seen rather than the phytotoxicity of the aphid. To check if cowpea aphid population density affects the damage generated in cowpea, susceptible cowpea plants underwent a population damage evaluation with cowpea aphids. Three different aphid population densities were screened to observe the effect of the damage: constant cowpea aphid density, a variable cowpea aphid density, and a variable cotton melon aphid density. The cotton melon aphid was chosen because it is phylogenetically closely related to the cowpea aphid but is not adapted to cowpea and causes no damage to the plant (Song et al., 2016).

The constant cowpea aphid density was maintained at 15 aphids (constant cowpea aphid density) on the entire plant for the duration of the infestation. The variable cowpea aphid density started at 15 aphids and was left alone to grow without any intervention to the aphid density. The cotton melon aphid was treated like the cowpea aphid variable population density.

In the variable cowpea aphid density, all three measured symptoms, chlorosis, pseudogalling, and stunted growth, were observed (Figs 2.1, 2.2).

These damage symptoms first became apparent at the end of week one and increased in severity by the end of week 2. Damage symptoms were also seen in the constant cowpea aphid density, with the majority of the damage symptoms observed as pseudogalling (Fig 2.1B). Unlike the variable cowpea aphid density, where every plant exhibited at least one of the expected symptoms, only half of the constant cowpea aphid density plants exhibited a symptom(s) (Fig 2.1D). Damage symptoms on the constant cowpea aphid density plants were localized to areas where aphids clustered (Fig 2.3).

As expected, no damage was observed on plants with the variable cotton melon aphid density (Fig 2.1). After the 15-day infestation period, the total number of aphids was calculated for the variable aphid densities. The cotton melon aphid density was found to be on average less than 15 aphids, equivalent to the number of aphids used for the constant cowpea aphid density (Fig 2.4), while the variable cowpea aphid density had more than 15-fold higher aphids than either of the other two aphid populations.

Local vs systemic damage

It is unclear whether damage symptoms, such as chlorosis, is limited to infestation sites or can be manifested systemically in uninfested tissues. To determine if the damage generated by cowpea aphid is localized to the infestation site, 15 cowpea aphids were caged to a single unifoliate leaf for 15 days. Periodically the uninfested areas of the plants were examined for damage

symptoms. No damage was observed in uninfested tissues of the plant. After the infestation period, the mesh sleeve bag was removed from the infested leaf and the entirety of the plants were examined thoroughly for damage symptoms. The only damage observed was on the infested unifoliate leaf (Fig 2.5).

Life performance

Initial no choice assays indicated a stark difference in the number of aphid population growth on resistant and susceptible cowpea lines (Fig 2.6). The cowpea aphids feeding on susceptible plants had about 5-fold higher numbers than those on the resistant plants. This difference in number could be due to changes in the aphid reproduction rate, survival, or aphid growth indicating antibiosis. To evaluate whether the cowpea resistance mediated by the two QTLs involves antibiosis, the life performance of cowpea aphids was monitored on the resistant and susceptible lines.

To explore if the antibiosis affected the adult aphids, single age-synchronized one-day-old adult aphids were clipped caged onto leaves. The aphid's fecundity and survival were monitored for a week (Fig 2.7). Adults feeding on the resistant cowpea had significantly lower fecundity than aphids feeding on the susceptible line (Fig 2.7A, GLM, Chisq = 41.704, $P < 0.001$). Adult aphid survival was also significantly lower on the resistant line compared to the susceptible (Fig 2.7B, GLM, Chisq = 8.049, $P = 0.005$).

To investigate whether cowpea resistance affected the growth rate of the aphid, ten newborn aphid nymphs were caged onto either susceptible or resistant plants. The weight of another cohort of newborn nymphs were measured and used as the baseline weight. After six days, significantly more nymphs survived on the susceptible plants compared to the resistant plants (Fig 2.8A). This is consistent with what was observed with the adult cowpea aphid survival. The growth rate of the nymphs was evaluated by calculating the mean relative growth rate (MRGR) of now six-day old nymphs. The nymphs feeding on the susceptible plants had significantly higher MRGR than the nymphs feeding on the resistant plants (Fig 2.8B). Combined these no choice life performance results showed that the resistance in cowpea line 2014-008-51-82 involves antibiosis and has negative effects on the aphid's ability to reproduce, survive, and grow.

Electrical penetration graph

An in-depth look at the cowpea aphid feeding on resistant and susceptible cowpea lines through EPG analysis identified multiple significant differences between them. Overall, the aphids feeding on the susceptible cowpea line had a significantly longer probing time than the aphids feeding on the resistant cowpea line; 30 more minutes of stylets inserted in plant tissues out of 8 hours of recordings (Figs 2.9, 2.10, Table 2.1). One of the notable differences observed between the two plant lines was the inability of over half the aphids, feeding on the resistant cowpea line, to ingest phloem sap while 96% of aphids feeding on

the susceptible cowpea line were able to do so (Fig 2.9, Table 2.1). Besides sap ingestion, additional differences were observed in the behavior of cowpea aphids feeding on the two cowpea lines. The aphids feeding on the susceptible cowpea line spent one hour less in the pathway phase than aphids feeding on the resistant cowpea line. This difference indicates that aphids on the resistant cowpea are spending more time trying to reach the phloem than those on the susceptible cowpea line.

For the cowpea aphids that were able to reach the phloem sieve element, differences were observed in both salivation and ingestion phases on the different lines. Aphids feeding on the susceptible cowpea salivated significantly more frequently than on the resistant line but not for longer periods of time (Fig 2.9, Table 2.1). Aphids feeding on the susceptible cowpea reached the phloem tissues almost 2 hours faster, ingested more frequently and fed twice longer than on resistant cowpea.

Host selection behavior

Host selection can reveal clues on the mechanism of resistance employed by the resistant plant and determine whether deterrence occurs and hence the involvement of antixenosis. To explore this, adult apterous cowpea aphids were placed in a petri dish arena and allowed to settle on either the susceptible or resistant cowpea plants (Fig 2.11). Plants were monitored at 2, 3, 6 and 24 hours. Participation to choose a plant by aphids at 2, 3 and 6 hours was low, and

aphids were equally present on resistant and susceptible leaves. However, at 24 hours, aphid participation was high, and aphids had a significant preference for the susceptible cowpea over the resistant (Fig 2.12). This indicates that aphids are either attracted to the susceptible cowpea or are deterred from the resistant. This attraction or deterrence indicates that cowpea resistance has also an antixenosis component.

Discussion

Cowpea aphid infestation leads to significant damage on susceptible cowpea plants. In the current work, all plants without aphid population control, manifested at least one of the damage symptoms (chlorosis, pseudogalling, and stunted growth) under investigation. These results are consistent with previous work with susceptible cowpea and cowpea aphids that found severe damage could be seen from infestations initiated from only 10 aphids (Ofuya, 1995). Interestingly, when the cowpea aphid population was kept at constant levels on susceptible cowpea, plants displayed at least one of the anticipated damage symptoms. Being able to generate damage at a low constant density levels, cowpea aphids should be considered as phytotoxic aphids like the Russian wheat aphid and greenbug (Miles, 1999; Nicholson et al., 2012; Nicholson and Puterka, 2014). While damage was observed on plants with constant aphid density, the damage level did not match the variable cowpea aphid density indicating the damage done does have some density dependence. Regardless on the infestation density, the damage was observed only on the infested leaves and not systemically. Taken together, the cowpea aphid is a phytotoxic aphid that becomes more phytotoxic as its population rises. However, unlike the Russian wheat aphid and greenbug, the cowpea aphid's induced symptoms are localized to the infestation sites and are not systemic (Deol et al., 2001).

Cowpea aphids did not produce damage symptoms on the resistant cowpea NIL. The resistance was originally identified in an African breeding line

through lack of damage symptoms (Souleymane et al., 2013). The QTLs that were mapped to confer this resistance were crossed and backcrossed into a known susceptible cowpea generating NILs that were used in this study (Huynh et al., 2015; Huynh and Roberts, pers. comm.). Therefore, the NILs exhibited the characteristic lack of symptoms associated with the original African breeding line further demonstrating that the two QTLs are responsible for the resistance.

Besides the phenotypic differences seen between the two cowpea NILs, major differences were observed in the cowpea aphids' behavior on these plants. One of these was the difference in the buildup of aphid population levels. After 6 days, the aphid population on the susceptible cowpea was 5-fold higher than the population on the resistant cowpea (Fig 2.6). This difference in population growth between the two cowpea lines is similar to what is reported for the soybean aphid (*Aphis glycines*) growth on susceptible and resistant soybean (*Glycine max*) over a similar time period (Studham and MacIntosh, 2013). The population difference indicates that the conferred resistance has an antibiosis component. The reduced fecundity of adult aphids and the low MRGR on resistant plants compared to susceptible (Figs 2.7, 2.8) further demonstrated a role for antibiosis. The decreased aphid growth rate observed on resistant cowpea is similar to cowpea aphid resistance identified in *Medicago truncatula* (Kamphuis et al., 2012). Taken together, the cowpea resistance has an antibiosis component and it affects the ability of the aphid to survive, reproduce and grow. The resistance was not limited to antibiosis as cowpea aphid behavior was also affected by the

resistance. Considering that aphids had significant higher preference for susceptible cowpea compared to resistant, this resistance mediated by the two QTLs also has an antixenosis component. Therefore, the mechanism of the resistance from the African breeding line conferred by the two QTLs includes both antibiosis and antixenosis.

Using EPG, significant differences were observed in a number of parameters between the aphids feeding on susceptible cowpea and those feeding on resistant cowpea. Considering the effect of the resistance on aphid development and fecundity, it is not surprising that the biggest difference in aphid feeding behavior between the two lines of cowpea was the phloem sap ingestion. Cowpea aphids feeding on susceptible cowpea were able to reach the phloem sieve element and have their first sap ingestion significantly faster than aphids feeding on the resistant cowpea. They also spent a significantly longer time ingesting sap than the aphids feeding on the resistant cowpea. This indicates that resistance conferred by these QTLs appears to be partially phloem based. The difference in phloem sap ingestion time indicates the aphids feeding on the resistant line had trouble ingesting sap. Interestingly, while there was a difference in the phloem ingestion, there was no significant difference in the salivation time. Typically, phloem-based resistances are associated with differences in salivation time (Kamphuis et al., 2012; Sun et al., 2018). However, significant differences in ingestion time, in the absence of a difference in salivation time, have been reported for the phloem-based resistance in *Medicago truncatula* to the

bluegreen aphid (*Acyrthosiphon kondoi*) (Klinger et al., 2005). Aphids feeding on susceptible cowpea also spent longer time in the plant with a shorter time in the pathway phase, indicating that some aspect of the resistance is encountered before the aphid reaches the phloem sieve element.

EPG analyses also indicated a difference in the amount of time aphids spent feeding in the plant (Fig 2.9, Table 2.1). Aphids feeding on the susceptible cowpea had a longer probing time than the aphids feeding on the resistant cowpea. Taken together, the cowpea aphids had a more significant preference for the susceptible than resistant cowpea and fed longer on susceptible plants. Further investigations of the cowpea NILs, using global gene expression analysis and defense hormone profiles, may shed light on the defense signaling pathways and molecular mechanisms of the antibiosis and antixenosis.

References

Burd, J., and Burton, R.L. (1992). Characterization of plant damage caused by Russian Wheat Aphid (Homoptera: Aphididae). *J Econ Entom* 85(5)**,** 2017-2022.

Burd, J., Burton, R.L., and Webster, J.A. (1993). Evaluation of Russian Wheat Aphid (Homoptera: Aphididae) Damage on Resistant and Susceptible Hosts with Comparisons of Damage Ratings to Quantitative Plant Measurements. *J Econ Entom* 86(3)**,** 974-980.

Cervantes, F.A., and Backus, E.A. (2018). EPG waveform library for *Graphocephala atropunctata* (Hemiptera: Cicadellidae): effect of adhesive, input resistor, and voltage levels on waveform appearance and stylet probing behaviors. *J Insect Physiol* 109**,** 21-40.

Chan, C.K., Forbes, A.R., and Raworth, D.A. (1991). *Aphid-transmitted viruses and their vectors of the world.* Ottawa: Research Branch, Agriculture Canada.

Deol, G.S., Reese, J.C., Gill, B.S., Wilde, G.E., and Campbell, L.R. (2001). Comparative chlorophyll losses in susceptible wheat leaves fed upon by Russian wheat aphids or greenbugs (Homoptera: Aphididae). *Journal of the Kansas Entomological Society* 74(4)**,** 192-198.

Ebert, T.A., Backus, E.A., Cid, M., Fereres, A., and Rogers, M.E. (2015). A new SAS program for behavioral analysis of electrical penetration graph data. *Computers and electronics in agriculture* 116**,** 80-87.

Elawad, H.O.A., and Hall, A.E. (1987). Influences of early and late nitrogen fertilization on yield and nitrogen fixation of cowpea under well-watered and dry field conditions. *Field Crops Res* 12**,** 197-222.

Fery, R.L. (1990). The cowpea: production, utilization, and research in the United States. *Hort Rev* 12**,** 197-222.

Giordanengo, P. (2014). EPG-Calc: a PHP-based script to calculate electrical penetration graph (EPG) parameters. *Arthropod-Plant Interactions* 8(2)**,** 163-169.

Goggin, F.L., Quisenberry, S., and Ni, X. (2017). "Feeding Injury," in *Aphids as Crop Pests,* eds. H.F. van Emden & R. Harrington. 2nd ed: CAB International), 303-322.

Hall, A.E. (2004). Breeding for adaptation to drought and heat in cowpea. *European Journal of Agronomy* 21(4)**,** 447-454.

Hall, A.E., Ismail, A.M., Ehlers, J.D., Marfo, K.O., Cisse, N., Thiaw, S., et al. (2002). "Breeding cowpeas for tolerance to temperature extremes and adaptation to drought," in *Challenges and Opportunities for Enhancing Sustainable Cowpea Production,* eds. C.A. Fatokun, S.A. Tarawali, B.B. Singh, P.M. Kormawa & M. Tamo. (Ibadan, Nigeria: Intl Inst Tropical Agric), 14-21.

Huynh, B.L., Ehlers, J.D., Ndeve, A., Wanamaker, S., Lucas, M.R., Close, T.J., et al. (2015). Genetic mapping and legume synteny of aphid resistance in African cowpea (*Vigna unguiculata* L. Walp.) grown in California. *Mol Breed* 35**,** 36.

Jackai, L., and Daoust, R. (1986). Insect Pests of Cowpeas. *Annu Rev Entomol* 31**,** 95-119.

Kamphuis, L.G., Gao, L., and Singh, K.B. (2012). Identification and characterization of resistance to cowpea aphid (*Aphis craccivora* Koch) in *Medicago truncatula*. *BMC Plant Biol* 12**,** 101.

.

Klinger, J., Creasy, R., Gao, L., Nair, R.M., Jacob, H.S., Edwards, O.R., et al. (2005). Aphid resistance in *Medicago trunculata* involves antixenosis and phloem-specific, inducible antibiosis, and maps to a single locus flanked by NBS-LRR resistance gene analogs. *Plant Physiol* 137**,** 1445-1455.

Langyintuo, A.S., Lowenberg-DeBoer, J., Faye, M., Lamber, D., Ibro, G., Moussa, B., et al. (2003). Cowpea supply and demand in West and Central Africa. *Field Crops Res* 82**,** 215-231.

Miles, P.W. (1999). Aphid saliva. *Biol Rev* 74(1)**,** 41-85.

Nicholson, S.J., Hartson, S.D., and Puterka, G.J. (2012). Proteomic analysis of secreted saliva from Russian Wheat Aphid (*Diuraphis noxia* Kurd.) biotypes that differ in virulence to wheat. *J Proteomics* 75(7)**,** 2252-2268.

Nicholson, S.J., and Puterka, G.J. (2014). Variation in the salivary proteomes of differentially virulent greenbug (*Schizaphis graminum* Rondani) biotypes. *J Proteomics* 105**,** 186-203.

Obopile, M. (2006). Economic threshold and injury levels for control of cowpea aphid, *Aphis crassivora* Linnaeus (Homoptera: Aphididae), on cowpea. *African Plant Protection* 12**,** 111-115.

Ofuya, T. (1995). Studies on the capability of *Cheilomenes lunata* (Fabricius) (Coleoptera: Coccinellidae) to prey on the cowpea aphid, *Aphis craccivora* Koch (Homoptera: Aphididae) in Nigeria *Agriculture, Ecosystems and Environment* 52**,** 35-38.

Omoigui, L.O., Ekeuro, G.C., Kamara, A.Y., Bello, L.L., Timko, M.P., and Ogunwolu, G.O. (2017). New sources of aphids [*Aphis craccivora* (Koch)] resistance in cowpea germplasm using phenotypic and molecular marker approaches. *Euphytica* 213**,** 178.

Reynolds, D.R. (1999). *Capnodium citri*: The sooty mold fungi comprising the taxon concept. *Mycopathologia* 148**,** 141-147.

Singh, B.B. (2014). *Cowpea: The Food Legume of the 21st Century.* Madison, WI: Crop Science Society of America.

Singh, B.B., Ehlers, J.D., Sharma, B., and Freire, F.R. (Year). "Recent progress in cowpea breeding", in: *Proceedings of the World Cowpea Conference III*, eds. C.A. Fatokun, B.B. Tarawali, B.B. Singh, P.M. Kormawa & M. Tamo), 22-40.

Singh, S.R., and Allen, D.J. (1980). "Pests, diseases, resistance and protection in cowpea," in *Advances in legume science,* eds. R.J. Summerfield & A.H. Bunting. (Richmond, UK: Royal Botanic Gardens), 419-443.

Smith, C.M. (1989). *Plant Resistance to Insects: A Fundamental Approach.* New York: John Wiley & Sons.

Song, N., Zhang, H., Lu, H., and Cai, W. (2016). All 37 Mitochondrial Genes of Aphid *Aphis craccivora* Obtained from Transcriptome Sequencing: Implications for the Evolution of Aphids. *PLoS One* 11(6)**,** e0157857.

Souleymane, A., Aken'Ova, M.E., Fatokun, C.A., and Alabi, O.Y. (2013). Screening for resistance to cowpea aphid (*Aphis craccivora* Koch) in wild and cultivated cowpea (*Vigna unguiculata* L. Walp.) accessions. *International Journal of Science, Environment and Technology* 2(4)**,** 611-621.

Studham, M.E., and MacIntosh, G.C. (2013). Multiple phytohormone signals control the transcriptional response to soybean aphid infestation in

susceptible and resistant soybean plants. *Mol Plant Microbe Interact* 26(1)**,** 116-129.

Sun, M., Voorrips, R.E., Steenhuis-Broers, G., Van't Westende, W., and Vosman, B. (2018). Reduced phloem uptake of *Myzus persicae* on an aphid resistant pepper accession. *BMC Plant Biol* 18(1)**,** 138.

Timko, M.P., and Singh, B.B. (2008). "Cowpea, a Multifunctional Legume," in *Genomics of Tropical Crop Plants,* eds. P.H. Moore & R. Ming. (New York: Springer), 227-258.

Tjallingii, W.F. (1988). "Electrical recording of stylet penetration activities.," in *Aphids, their biology, natural enemies and control,* eds. A.K. Minks & P. Harrewijn. (Amsterdam: Elsevier Science Publishers), 95-108.

Tjallingii, W.F. (2006). Salivary secretions by aphids interacting with proteins of phloem wound responses. *J Exp Bot* 57(4)**,** 739-745.

Tjallingii, W.F., and Esch, T.H. (1993). Fine-structure of aphid stylet routes in plant-tissues in correlation with EPG signals. *Physiol Entomol* 18(3)**,** 317-328.

Tjallingii, W.F., Garzo, E., and Fereres, A. (2010). New structure in cell puncture activities by aphid stylets: a dual-mode EPG study. *Entomol Exp Appl* 135(2).

Table 2.1. Cowpea aphid feeding and probing parameters on susceptible and resistant cowpea.

		Susceptible (n=25)				**Resistant (n=30)**			**Statistical Results**
Probing time	# [1]	22.04	±	3.418		27.733	±	2.240	**Chi-sq = 17.738 ; *P* < 0.001 *****
	Duration[2]	7.068	±	0.191		6.477	±	0.190	**Chi-sq = 4.649 ; *P* = 0.031 ***
Pathway phase	#	25.04	±	3.463		30.633	±	2.210	**Chi-sq = 15.305 ; *P* < 0.001 *****
	duration	3.315	±	0.314		4.544	±	0.250	**Chi-sq = 8.853 ; *P* = 0.003 ****
Xylem	#	1	±	0		1	±	0	Not enough repetition
	duration	0.923	±	0.197		0.962	±	0.215	
	N[3]	12% (3/25)				10% (3/30)			
Sieve element salivation	#	5.64	±	0.789		3.852	±	0.416	**Chi-sq = 14.388 ; *P* < 0.001 *****
	duration	0.615	±	0.121		0.917	±	0.166	Chi-sq = 2.226; *P* = 0.136 NS
	n	100% (25/25)				90% (27/30)			*P* = 0.242 NS
Phloem sap ingestion	#	3.667	±	0.560		2.5	±	0.442	**Chi-sq = 34.286; *P* < 0.001 *****
	duration	2.899	±	0.446		1.429	±	0.394	**Chi-sq = 4.089; *P* = 0.043 ***
	n	96% (24/25)				46,7% (14/30)			***P* < 0.001 *****
	Time to first[4]	2.306	±	0.383		3.942	±	0.672	**Chi-sq = 22.619; *P* < 0.001 *****

Feeding and probing parameters measured from the aphids feeding on the different cowpea varieties, n = number of individual aphids, and # = number of phases measured. The statistics used are the following: [1] GLM (family=poisson), [2] GLM (family=Gamma), [3] Fisher's exact test, and [4] Cox model.

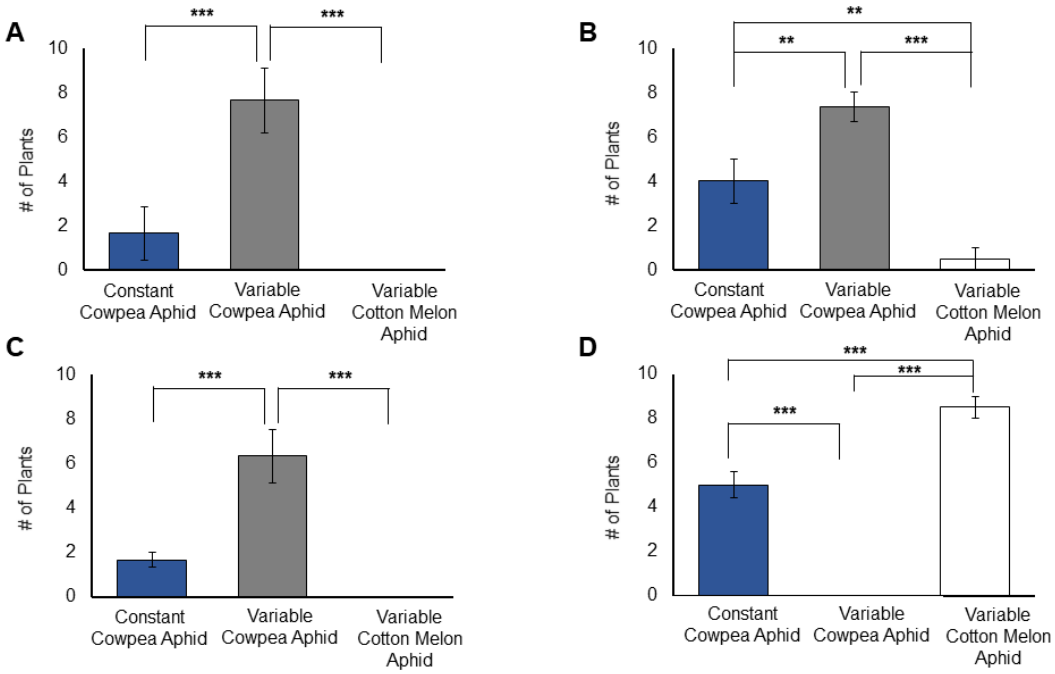

Fig 2.1. The number of cowpea plants showing damage symptoms after infestation by cowpea aphids or cotton melon aphids. The damage symptoms observed were **A)** chlorosis, **B)** pseudogalling, **C)** stunted growth, and **D)** no damage present. The constant cowpea aphid density was maintained at 15 aphids for the entirety of the experiment. The variable cowpea aphid density and cotton melon aphid density were allowed to grow without any restrictions. The experiment was performed three times with 10 plants per aphid density each time except one replicate of cotton melon aphid had only 8 plants (constant cowpea aphid n = 30, variable cowpea aphid n =30, variable cotton melon aphid n = 28). Values are expressed as the mean and SE. Analyses were performed with one-way ANOVA followed by Tukey's HSD test. Asterisks indicate significant differences ** $p < 0.01$, and *** $p < 0.001$.

Fig 2.2. Susceptible cowpea plants infested with cowpea aphids or cotton melon aphids. The constant cowpea aphid density was maintained at 15 aphids for the entirety of the experiment. The variable cowpea aphid density and cotton melon aphid were allowed to grow without any restrictions. Plants were photographed 13 days after infestation.

Fig 2.3. Damage on cowpea leaves from the constant cowpea aphid density infestation. Damage from the constant cowpea aphid density group occurred when cowpea aphids were mostly localized to one area. Both images show pseudogalling. Leaves were photographed 15 days after infestation.

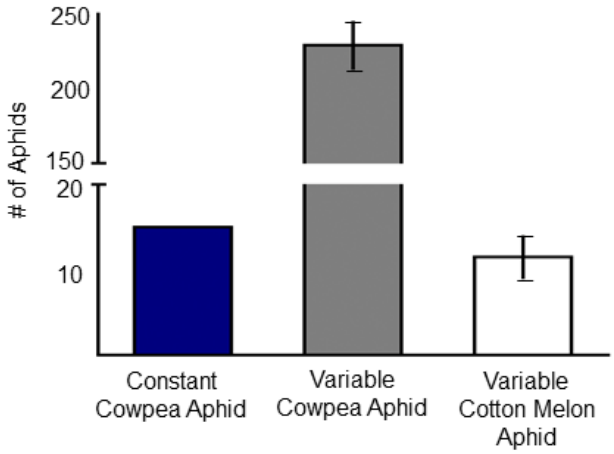

Fig 2.4. Cowpea aphid and cotton melon aphid population growth on susceptible cowpea plants. The constant cowpea aphid density was maintained at 15 aphids for the entirety of the experiment. The variable cowpea aphid density and cotton melon aphids were allowed to grow without any restrictions. Plants were infested for 15 days. The experiment was performed three times with 10 plants per aphid density each time except one replicate of cotton melon aphid that had only 8 plants (constant cowpea aphid n = 30, variable cowpea aphid n =30, variable cotton melon aphid n = 28). Values are expressed as the mean and SE.

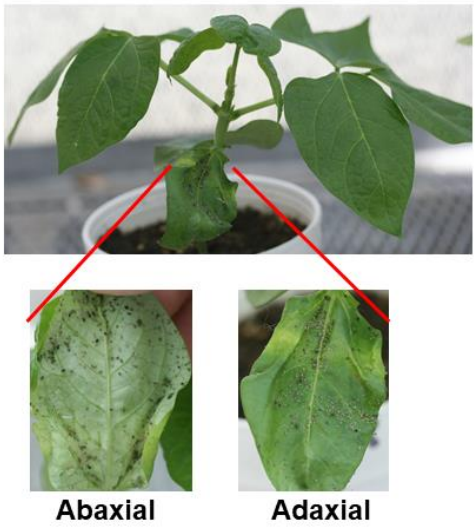

Fig 2.5. A cowpea plant infested with cowpea aphids only at a single unifoliate leaf. Fifteen cowpea aphids were caged on a single unifoliate leaf for 15 days. The only damage seen on the plant is on the infested caged unifoliate leaf.

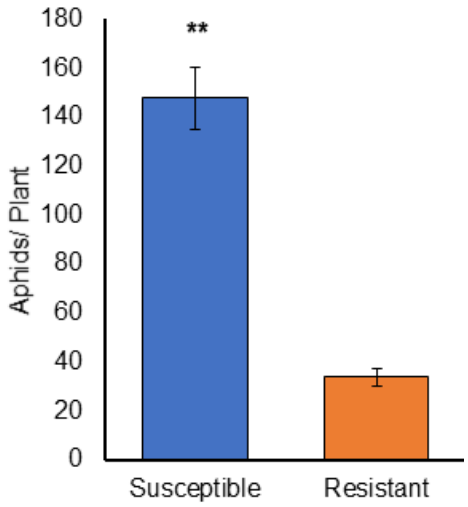

Fig 2.6. Cowpea aphid population growth on susceptible and resistant cowpea plants. Plants were infested with 20 adult aphids for 6 days. The difference in aphid population between the two plant lines (n=15) was analyzed using two-tailed Student's *t*-test. Asterisks indicate significance ** $p < 0.01$.

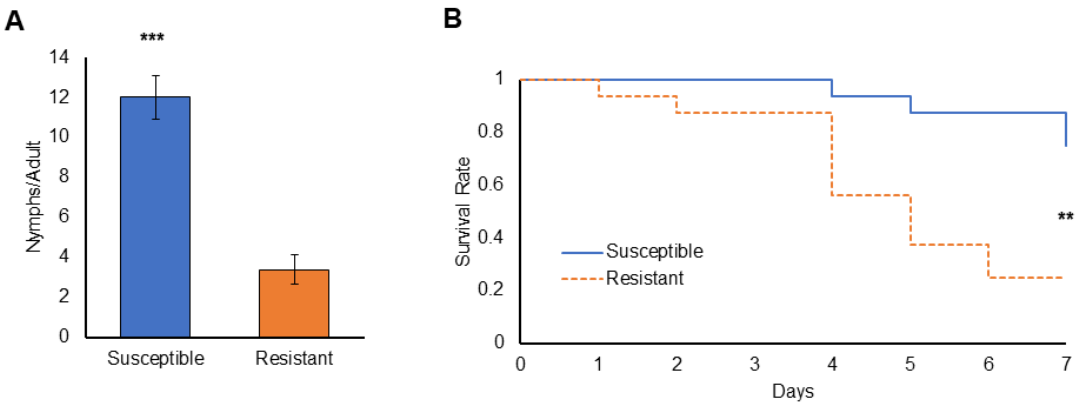

Fig 2.7. Adult cowpea aphid performance on susceptible and resistant cowpea. **A)** Fecundity of age synchronized one-day-old adult aphids on resistant or susceptible plants. Values are the means and SE of 16 biological replicates. **B)** Survival rate of one-day-old adults on susceptible or resistant cowpea. Aphid performances were monitored for 7 days. The asterisks indicate a significant difference between aphids on susceptible and resistant cowpea plants (GLM, Chi-sq = 41.704 ; *** $p < 0.001$ for the daily fecundity and Cox model, Chi-sq = 8.049; ** $p = 0.005$ for the aphid survival rate).

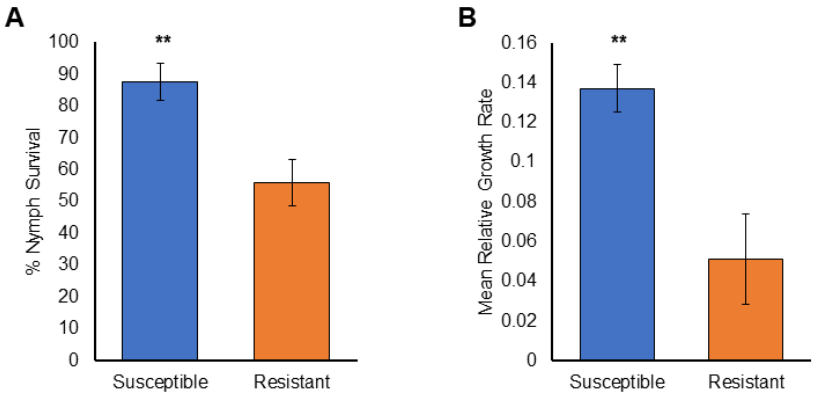

Fig 2.8. Cowpea aphid nymphal growth rate and survival on susceptible and resistant cowpea. Ten newborn nymphs were clip caged onto a single leaf of susceptible or resistant plants (n-12) for 6 days. Values are the means and SE of 12 biological replicates. **A)** Nymphal survival. **B)** Mean relative growth rate (MRGR) [MRGR = ($\log W_{surviving} - \log W_{day\text{-}old}$) / Number of days]. Average weight of 20 day-old nymphs were used as base value. Analysis was performed by Student's *t*-test. Asterisks indicate significance ** $p < 0.01$.

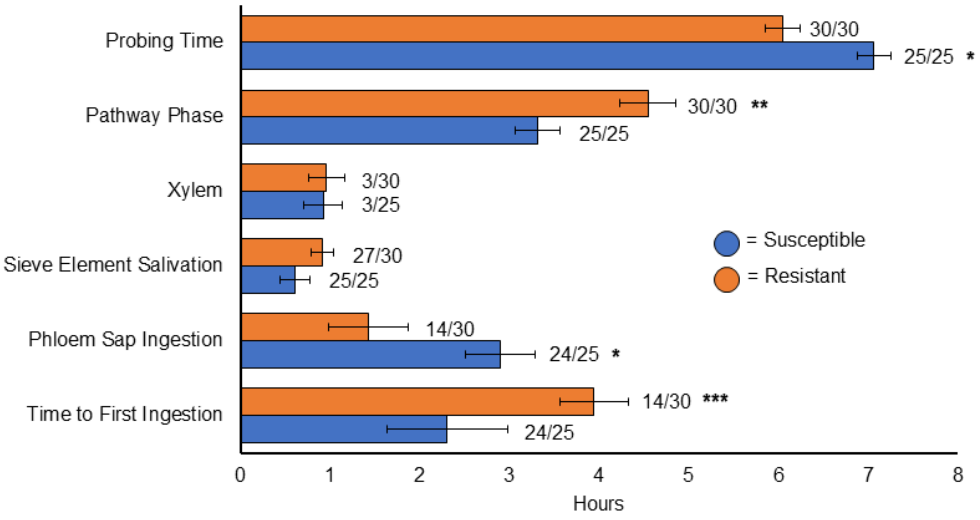

Fig 2.9. Electrical penetration graphs (EPGs) of cowpea aphids feeding on susceptible and resistant cowpea. Probing time indicates the time the aphid stylets were in the plant. The pathway phase indicates the time the aphid stylets are in the mesophyll or parenchyma cells (C phase + potential drops). The xylem phase indicates the time the aphid stylets are in the xylem (G phase). The sieve element salivation is the time the aphid is salivating into the sieve element (E1 phase). The phloem sap ingestion is the time the aphid is ingesting the plant phloem sap (E2 phase). The time to first ingestion is the average time it took for the aphid to first ingest phloem sap. Data is based on 25 and 30 aphids tested on susceptible and resistant cowpea, respectively. The asterisks indicate a significant difference between the plants (GLM models, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

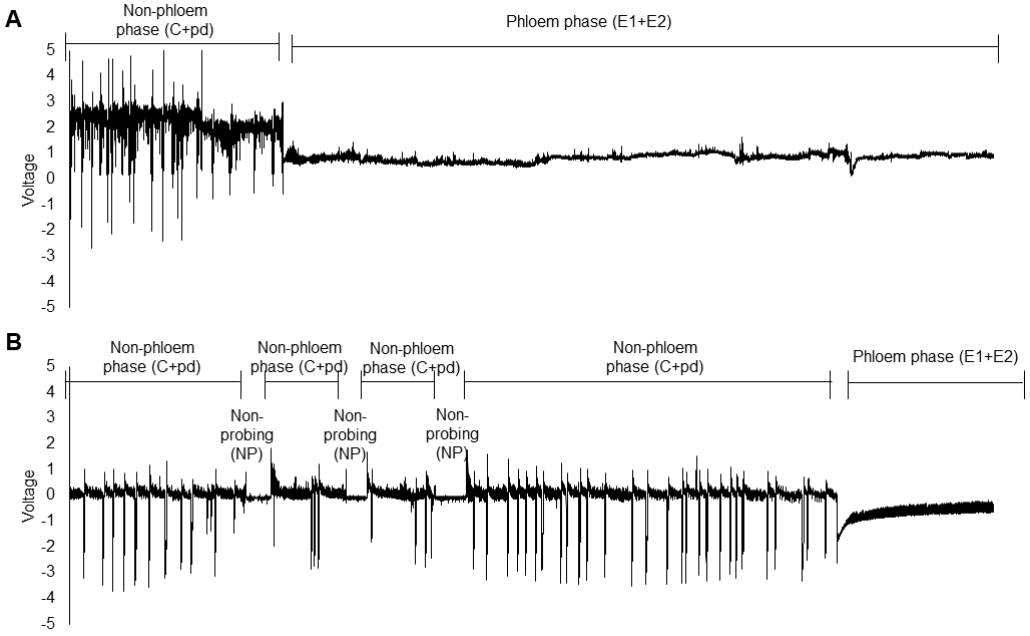

Fig 2.10. Representative EPG waveform patterns of cowpea aphids feeding on **A)** susceptible cowpea, and **B)** resistant cowpea.

Fig 2.11. Large petri dish arena used for cowpea aphid choice assays. Aphid were introduced through the hole in the center of the arena with either a susceptible or resistant cowpea leaf exposed in the holes on either side.

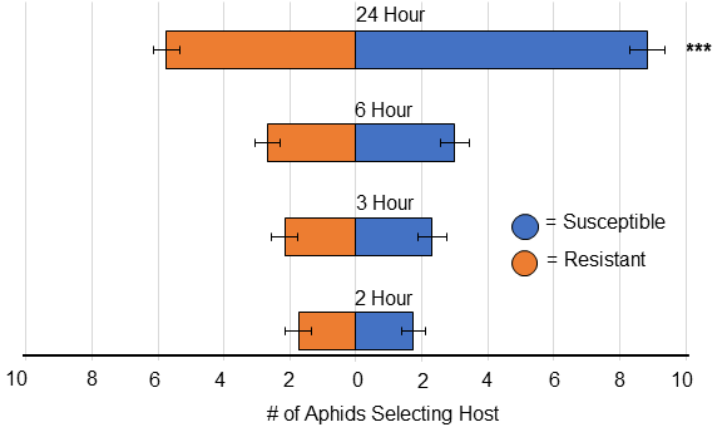

Fig 2.12. Aphid choice assays. Twenty cowpea aphids were introduced into a large petri dish arena to choose between a unifoliate leaf from susceptible or resistant cowpea lines on either side. Values are the mean and SE of 19 biological replicates. The number of adult aphids on either leaf line was documented at the indicated time points. Two-way ANOVA (genotype x time points), followed by Tukey HSD. Asterisks indicate significance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Chapter Three

Molecular Characterization of Susceptible and Resistant Cowpea Responses to Cowpea Aphid Infestation

Abstract

Cowpea (*Vigna unguiculata*) is a crucial crop for regions of the world that are prone to both heat and drought. It is able to withstand these harsh conditions, but it faces a number of other limiting factors. One of these limiting factors is the cowpea aphid (*Aphis craccivora*), a phytotoxic aphid, that generates damage in cowpea even at low population levels. Resistance to the cowpea aphid has been identified in an African cowpea line. This resistance was mapped to two quantitative trait loci (QTLs) and near isogenic lines (NILs) were generated in California blackeye cultivar background. Various aphid infestation approaches and behavioral assays identified the mechanism of the resistance as both antibiosis and antixenosis. To understand the molecular mechanism of susceptibility and resistance, the NILs were used in 1-day and 6-days aphid infestation regiment and subjected to RNAseq to identify differentially expressed genes (DEGs). Major differences between the susceptible and resistant cowpea were identified including the involvement of multiple plant hormones and defense related genes. A set of DEGs, encoding leucine-rich-repeat motifs or receptor like kinases, identified in the resistant cowpea line are leading candidates for further exploration as the source of resistance.

Introduction

Cowpea (*Vigna unguiculata*) is a grain that is grown mostly in sub-Saharan Africa. In 2018, over 6 million tonnes were produced in this region of the world alone (FAO, 2020). The importance of cowpea as a crop in this region is because it has high nutritional value and is able to withstand the harsh growing conditions of drought and heat stress (Hall et al., 2002; Hall, 2004). Cowpea has a high protein content, around 30%, and is a source of vitamins and minerals (Singh et al., 2002a; Singh, 2014).

Cowpea germplasm and current cowpea lines grown as crop, present a large phenotypic variation in plant growth and time to maturity. It can take between 50 – 160 days to complete one cycle from seed to seed (Singh, 2014). Cowpea development is similar to other beans. After planting, seeds take 4 – 7 days to germinate and germination gives the emergence of cotyledons, also known as the VE stage, before developing unifoliate leaves, also known as the VC stage. After the VC stage, the plants grow with alternating trifoliate leaves designated as the “V” followed by the number of nodes with fully developed leaves (Davis et al., 1991; Naeve, 2018) .

Despite its ability to withstand harsh growing conditions, cowpea is prone to various biotic stresses including insect attacks. An insect, the cowpea aphid (*Aphis craccivora*), is especially a devastating pest (Obeng-Ofori, 2007; Singh, 2014). Aphids feed on their host plant’s phloem sap and they reach the phloem using a piercing-sucking mouthpart or stylets. They insert their flexible

hypodermic needlelike stylets into the plant and navigating to the phloem with minimal mechanical damage (Tjallingii and Esch, 1993; Tjallingii, 2006). Not only does the cowpea aphid feeding mode minimize mechinical damage, aphids also deposit saliva on their way to the phloem and in the phloem to suppress damage. This deposition of saliva benefits the aphid as the saliva both facilitate feeding and disrupts plant defense responses and metabolism (Miles, 1999; Will et al., 2007).

Cowpea aphid induced cowpea loss can exceed over 50% of the grain yield. To deal with these enormous losses, it is recommended to use chemical pesticidies (Obopile, 2006). However, in sub-Saharan Africa where large acrage of cowpea is grown, pesticide use is not always an option. Pesticides are expensive, and have additional undesirable consequences including loss of benefical insects and contamination of the environment (Souleymane et al., 2013). Because of these limiting factors, other strategies have been pursued to control aphid populations. The most effectivie of these strategies has been host-plant resistance (Huynh et al., 2013; Souleymane et al., 2013; Huynh et al., 2015; Togola et al., 2020). Large numbers of cowpea lines have undergone screening with aphids to identify resistant lines through monitoring aphid-induced damage symptoms (Souleymane et al., 2013; Togola et al., 2020). Symptoms of cowpea aphid infestation on susceptible genotypes include chlorosis, necrosis and twisting of the leaves or pseudogalling (Fig 3.1). One of the most promising sources of cowpea aphid resistance was identifed in an African breeding line

(IT97K-556-6) (Souleymane et al., 2013). The source of the resistance was crossed with the susceptible California blackeye cultivar 27 (CB27) generating recombinant inbred lines (RILs). This RIL population underwent a field-based screen for aphid induced damage and the resistance was mapped to two quantitative trait loci (QTLs), a major QTL, *QAC-vu7.1,* and a minor QTL, *QAC-vu1.1* (Huynh et al., 2015). The IT97K-556-6 line was crossed to and backcrossed with California blackeye cultivar 46 (CB46) to the generate resistant near-isogenic line (NIL) 2014-008-51-82 (Huynh et al., 2015; Huynh and Roberts, pers. comm.).

The mechanism of this resistance was first explored in Chapter Two. Initial tests monitoring aphid-induced damage on the susceptible CB46 cowpea line, showed that damage symptoms on this genotype can be seen as early as a week after infestation. Damage symptoms can be seen with as few as 15 aphids indicating the severity of this pest. In contrast, no damage symptoms were observed on the resistant NIL 2014-008-51-82 infested with aphids monitored for 2 weeks. In addition, the number of aphids on the resistant plants were five-fold lower than those on susceptible. No choice assays showed that both aphid survival and fecundity are reduced on the resistant cowpea. Additional experiments showed that aphid growth rate was also impeded on the resistant plants indicating that the resistance involves antibiosis. Moreover, aphid choice assays demonstrated that after 24 hours, there was a significant preference for susceptible cowpea over the resistant. Furthermore, monitoring aphid feeding on

both cowpea lines by electrical penetration graphs (EPGs), demonstrated a significant difference in sap ingestion on these cowpea lines. Aphids feeding on the resistant cowpea struggled to ingest sap, indicating the resistance is at least partially phloem based. The preference of aphids for susceptible plants coupled with the EPG data, showing that aphids spent less time feeding on resistant plants, indicated the existence of also an antixenosis component to this resistance.

The first line of active defense in plants is known as pattern-triggered immunity (PTI). PTI is induced by perception of microbe/herbivore-associated molecular patterns from pathogens/pests by plasma membrane localized pattern recognition receptors (PRRs). Recognition by these receptors initiates an immune response that enables the plant to defend itself (Jones and Dangl, 2006; Boller and Felix, 2009). If pests/pathogens are able to evade PTI through use of their defense modifying effector proteins, then plants come to rely on a second line of defense known as effector-triggered immunity (ETI). PTI is the weaker of the defenses while ETI is a more robust defense and is initiated through intracellular receptors. These intracellular receptors are also known as resistance (R) proteins (Jones and Dangl, 2006). The majority of R proteins contain both nucleotide-binding (NB) and leucine rich repeat (LRR) motifs (Takken and Goverse, 2012). Pest/pathogen recognition by the R proteins can happen in a number of ways. Direct recognition, where R protein acts as a receptor for the pest/pathogen effector (Jones and Dangl, 2006). There are two models for

indirect recognition of an effector by the R protein: the guard model and the decoy model. In the guard model the R protein guards the protein target of the effector and any modifications made to it (Dangl and Jones, 2001). Similar to the guard model, in the decoy model the R protein monitors a plant protein that mimics the effector's target. If the effector protein binds to the decoy, it is trapped, and recognized by the R protein initiating ETI (van der Hoorn and Kamoun, 2008). A variation of the decoy model is known as the integrated decoy model that has a decoy as a domain within the R protein (Cesari et al., 2014).

The cowpea resistant loci, QTL, *QAC-vu7.1,* and QTL, *QAC-vu1.1,* encompass large genomic regions and the identity of the gene(s) contributing to this resistance remain unknown (Huynh et al., 2015). Although multiple resistance genes have been identified to aphids in different plant species, to date only three of these resistance genes have been cloned (Ahman et al., 2019; Nalam et al., 2019). The first cloned aphid resistance gene was the tomato (*Solanum lycopersicum*) *Mi-1.2* gene, originating from the wild tomato species *Solanum peruvianum,* confers resistance to the potato aphid (*Macrosiphum euphorbiae*) (Rossi et al., 1998; Vos et al., 1998). Another cloned *R* gene is *Vat* from melon (*Cucumis melo*) that confers resistance to the cotton-melon aphid (*Aphis gossypii*) (Pitrat and Lecoq, 1982; Dogimont et al., 2014). Both *R* genes encode classical R proteins with coiled-coil NB-LRR (CC-NB-LRR; CNL) domains. Interestingly, both genes have been found to confer more than just resistance to an aphid species. Whereas *Mi-1.2* confers also resistance to three

species of root-knot nematodes (*Meloidogyne arenaria, M. incognita and M. javanica*), whiteflies (*Bemisia tabaci*), and psyllids (*Bactericera cockerelli*) (Kaloshian et al., 1995; Milligan et al., 1998; Nombela et al., 2003; Casteel et al., 2006), the *Vat* gene confers also resistance to multiple viruses transmitted by the cotton-melon aphid (Pitrat and Lecoq, 1982; Boissot et al., 2016). The most recent identified and cloned *R* gene is the Arabidopsis (*Arabidopsis thaliana*) *SIEVE ELEMENT-LINING CHAPERONE1 (SL1)*. *SLI1* confers resistance to the green peach aphid (*Myzus persicae*) and encodes a small heat shock like protein that likely functions by lining the sieve elements and obstructing the ability of the aphid to feed (Kloth et al., 2017; Ahman et al., 2019).

To better understand the resistance in cowpea, this chapter explores aphid behavior through dispersal assays and the molecular mechanisms of this resistance through gene expression analysis using RNAseq. For the RNAseq analysis early (1-day) and late (6-day) infestation timepoints were used to explore both the initial and late effects. These timepoints were chosen to both accommodate for cowpea aphid non-preference for resistant cowpea, identified with the EPGs and choice assays, and the effect of prolonged aphid feeding on both resistant and susceptible cowpea. The number of differentially expressed genes (DEGs) indicate that transcription activity is higher at the early timepoint in the susceptible line while this high activity is present at the late time point in the resistant line. The DEGs in the early timepoint in the susceptible line indicate increases in protein biosynthesis activities among others. The DEGs in the late

timepoint in the resistant line indicate increased activity in signal transduction and defense related processes.

Materials and Methods

Plants and Aphids Growth Conditions

The cowpea aphids were collected in the summer of 2016 from a field in Riverside, California. The aphids have been reared on cowpea line California blackeye 46 (CB46) in a pesticide free greenhouse or a plant growth chamber (Conviron) at 26−30°C with a 16:8 light:dark photoperiod.

Both cowpea lines, CB46 and the nearly isogenic line (NIL) 2014-008-51-82, were grown in UC Mix 3 soil (agops.ucr.edu/soil/) in 24 oz plastifoam cup and fertilized weekly with MiracleGro (18−18−21; Stern's MiracleGro Products). Plants were maintained in a pesticide free plant growth room at 26-30°C with a16:8 light:dark photoperiod.

RNAseq Experimental Design

Two-week-old plants from both cowpea lines were infested with 20 adult apterous aphids on a single unifoliate leaf and enclosed in a mesh sleeve bag. Control non-infested plants received an empty mesh sleeve bag. Samples were collected at 1 and 6 days after infestation. Each sample consisted of three unifoliate leaves pooled from three plants. Five biological replicates, each consisting of three pooled plants, per line were used. For both plant lines, the number of aphids were counted on the six-day before harvest. The cowpea aphids were removed by immersing the leaves in water and gently removing any

remaining aphids with a fine-tip paintbrush. Non-infested control plants were treated the same way. Leaves were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA Extraction and Library Preparation

Leaf tissues were ground to a fine powder in liquid nitrogen with a mortar and pestle. Total RNA from leaves was isolated using NucleoSpin RNA Plant kit (Macherey-Nagel). RNA was quality checked and quantified by Bioanalyzer RNA Nano Kit (Agilent, Santa Clara, CA, USA). RNA samples were prepared for Illumina sequencing using the NEB Ultra II RNAseq stranded kit (New England Biolabs, Ipswich, MA, USA) with unique dual index adapters following manufacturers recommendations. Individual libraries were pooled based on BioAnalzyer molarities (Agilent, Santa Clara, CA, USA) and sent to UCLA for 2x150 bp sequencing on a NovaSeq 4000 (Illumina, San Diego, CA, USA) and demultiplexed with BCL2FASTQ**.**

Analysis of RNAseq

Raw RNA-Seq reads were assessed for quality FastQC; (Andrews, 2010) and edited using BBDuk (sourceforge.net/projects/bbmap/). Remaining adapter artifacts were trimmed using default settings (from the right with k=23, mink=11, hdist=1). Reads were mapped to the annotated cowpea reference genome (*V. unguiculata* v1.2; Phytozome) using hisat2.1.0 with a k value of 5, minimum

intron length of 30 bases, and maximum intron length of 3000 bases (Kim et al., 2019). Reads ranged from 69-94% alignment but averaged 87% (Table 3.1), a high rate of alignment given the repetitive nature of the genome (Lonardi et al., 2019). Counts obtained from Hisat were filtered to remove zero and low count genes using filterByExpr (min.count=20), and remaining genes were compared for differential gene expression using EdgeR-limma (Robinson et al., 2009; Ritchie et al., 2015; Law et al., 2016) The linear model y~0+trt was used to compare all treatment combinations. Counts were normalized using voomWithQualityWeights to account for sample heterogeneity among treatment conditions (Supplemental File 1).

Genes were determined significant at the adjusted *P* value (FDR) <0.05 and logFC ≥0.6 or ≤-0.6 (fold change ≥1.5 or ≤-1.5). Genes of interest were extracted from contrasts after multiple testing for each contrast separately (decideTests: method = “separate”, i.e., topTable). Contrasts of treatments and their combinations were made as defined in Table 3.2. GO analyses were performed following (Bonnot et al., 2019) where best hit *Arabidopsis thaliana* IDs were used to identify GO terms in Panther (Mi et al., 2018). Categories were separated by up or down-regulated genes per contrast and considered enriched at FDR <0.05. Enriched families were clustered by semantic similarity in REVIGO (Supek et al., 2011) using default settings to visualize GO terms among contrasts and reduce redundancy by extracting terms with “dispensability” (d) ≤ 0.1. Most unique terms are highlighted using d ≤ 0.05. A subset of the DEGs were further

annotated using their matching *A. thaliana* IDs in TAIR and UniProt to identify molecular function (Berardini et al., 2015; UniProt Consortium, 2019). Predicted cowpea protein sequences of others were further annotated using TMHMM V2.0 and NCBI Conserved Domain searches (Marchler-Bauer et al., 2017; Krogh et al., 2001).

Results

Analysis of DEGs

To determine what genes underlie resistance to cowpea aphid, the transcriptional response of *V. unguiculata* lines CB46 and 2014-008-51-82, were surveyed after 1-day and 6-days of aphid infestation. These timepoints were selected based on previous observations how aphids behave on resistant plants. Cowpea aphids do not prefer resistant cowpea and feed significantly less on this genotype based on EPG and choice tests. Therefore, the 1-day time point will capture these early interactions. The 6-day time point was selected based on aphid growth studied where a significant increase in aphid population and appearance of damage symptoms was observed on the susceptible compared to the resistant line. This time point will also capture the effect of prolonged aphid feeding on both resistant and susceptible cowpea. Each treatment had non-infested controls and 5 biological replicates, each consisting of 3 pooled leaves from different plants. The average number of reads for each sample was 68.2 million, with 87% alignment to the cowpea genome (Table 3.1). DEGs were identified from multiple contrasts with transcripts having a false discovery rate (FDR) of < 5% and a log-fold change (LFC) ≥ 0.6 or ≤ -0.6 (Table 3.2).

The resistant cowpea line had 480 unique upregulated DEGs and 594 unique downregulated DEGs at 1-day and 6-day timepoints combined (Fig 3.2). The majority of these DEGs were found at the 6-day timepoint with only 31 and

78 genes upregulated and downregulated, respectively, at 1-day. In the susceptible cowpea line, there were 776 unique DEGs upregulated and 271 unique DEGs downregulated the at 1-day and 6-day timepoints combined. Unlike the resistant cowpea line, the majority of the DEGs in the susceptible cowpea line were at the 1-day timepoint with 552 genes upregulated and 223 downregulated.

Gene Ontology (GO) Analysis

GO enrichment analysis was performed to elucidate the function of the DEGs. At 1-day, there was a high number of DEGs from a number of different GO classifications in the susceptible line while there were no GO classifications from the resistant line because of the very low number of DEGs at this timepoint (Fig 3.3). There were three GO classifications that were in the highest LFC category. These were biosynthetic process (GO:0009058), nitrogen compound metabolic process (GO:0006807), and ribosome biogenesis (GO:0042254). At a slightly lower LFC were cellular component organization or biogenesis processes (GO:0071840), cellular metabolic process (GO:0044237), cellular process (GO:0009987), metabolic process (GO:0008152), and ncRNA metabolic process (GO:0034660).

The GO classifications for downregulated genes were much less in the susceptible line at the 1-day time point, with only 3 GO classifications: regulation of multicellular organismal development (GO:2000026), response to oxygen-

containing compound (GO:1901700), response to stimulus (GO:0050896). Similarly, in the resistant cowpea line at 1-day there was also only 3 GO classifications for the downregulated DEGs: chloroplast relocation (GO:0009902), phenylpropanoid metabolic process (GO:009698), and response to blue light (GO:0009637).

In the susceptible line, there were less DEGs and GO classifications at 6-day than at 1-day. The 5 GO classifications present at 6-day were immune system process (GO:0002379), interspecies interaction between organisms (GO:0044419), leaf abscission (GO:0060866), response to hypoxia (GO:0001666), and response to stimulus (GO:0050896). Similar to the resistant cowpea line at 1-day, that lacked upregulated GO classification, the susceptible cowpea line at 6-day lacked downregulated GO classification because of the low number of DEGs in this treatment and time point.

In the resistant cowpea at 6-day, similar to the number of DEGs, there were more downregulated GO classifications than upregulated. There were only 4 GO classifications in the upregulated DEGs: interspecies interaction between organisms (GO:0044419), protein phosphorylation (GO:0006468), response to chemical (GO:0042221), and response to stimulus (GO:0050896). The downregulated GO classifications interestingly had one overlap with upregulated, response to stimulus (GO:0050896). There was also an overlap with the upregulated 1-day susceptible with the cellular process (GO:0009987). While cellular process (GO:0009987) involved genes were upregulated at a higher LFC

at the 1-day timepoint in the susceptible line, cellular process genes were downregulated at the 6-day timepoint in the resistant line (Fig 3.3). The other GO classifications in the 6-day downregulated resistant cowpea were: cellular lipid metabolic process (GO:0044255), circadian rhythm (GO:0007623), inorganic anion transport (GO:0015698), localization (GO:0051179), organic hydroxy compound metabolic process (GO:1901615), photosynthesis (GO:0015979), photosynthesis light reactions (GO:0019684), plastid organization (GO:0009657), regulation of biological control (GO:0065008), response to light stimulus (GO:0009416), and rhythmic process (GO:0042254).

Upregulated DEGs in Susceptible and Resistant Cowpea

The DEG analysis in both susceptible and resistant cowpea identified major gaps in the gene annotation. Because cowpea is a non-model organism and its genome was only released in 2019, many of the DEGs identified lacked annotation (Lonardi et al., 2019). To address this, manual functional annotation was performed for a subset of the DEGs. These DEGs were those upregulated in only one of the cowpea genotypes at the same time point. Initial analysis based on the Phytozome genome resources, the upregulated DEGs in the susceptible cowpea were 599 and 225 for 1-day and 6-day, respectively, and the upregulated DEGs in the resistant cowpea were 47 and 511 for 1-day and 6-day, respectively. At the two genotype/time combinations with the most DEGs, susceptible 1-day (120 DEGs) and resistant 6-day (162 DEGs), had the most DEGs without

annotations. These genes did not have *A. thaliana* IDs and could not be used in TAIR analysis (Berardini et al., 2015). For further analysis, these DEGs, susceptible 1-day and resistant 6-day, as well as DEGs without annotation and *A. thaliana* IDs in susceptible 6-day (85 DEGs) and resistant 1-day (8 DEGs) were excluded from further analysis as described in Table 3.3.

Overwhelmingly in susceptible 1-day, the DEGs corresponded to components of protein biosynthesis. This was reflected in the GO analysis in the biosynthetic process and ribosome biogenesis classifications (Fig 3.2). Of the original 599 DEGs, 206 were part of the protein biosynthesis process. This indicates that aphid infestation of susceptible plants leads to high level of protein biosynthesis. These DEGs, involved in proteins biosynthesis, were excluded from further analysis. Additional DEGs, such as histone-related and those involved in DNA replication were also excluded from further analysis. After the DEG exclusions for the susceptible line, there were 220 and 121 for 1-day and 6-day respectively, and for resistant line there were 31 and 307 for 1-day and 6-day respectively.

The molecular function of the remaining genes from each contrast was surveyed in TAIR and UniProt and assigned to categories (Fig 3.4). In the susceptible 1-day, there was about an equal distribution of transcription factors (5%), ubiquitin related genes (5.5%), methylation related genes (6%), and genes involved in secondary metabolites (4.5%) (Fig 3.4A). The highest percentage of these DEGs (57%), were involved in numerous different functions and could not

be categorized. Inspection of phytohormone related genes did not identify a dominant phytohormone pathway. The two well-known defense hormones, salicylic acid (SA) (2%) and jasmonic acid (JA) (1.5%) associated genes were found at similar proportions. Further examination of the phytohormone DEGs revealed that the majority were involved in either biosynthesis or regulated by the phytohormone, and there were a few DEGs that acted as regulators of or catabolized the phytohormone (Fig 3.5).

In the susceptible 6-day, there was a higher percentage of transcription factors (15.7%), and ubiquitin related genes (14.9%) (Fig 3.4B). In the susceptible 6-day, there was also a high level of cell wall/cytoskeleton related genes present (14.9%). The high level of ubiquitin related genes for both susceptible 1-day and 6-day suggests the existence of high level of protein turnover due to aphid infestation. Similar to susceptible 1-day, there was no dominant defense phytohormone pathway identified with both SA and JA associated genes found at about equal percentage, around 5%. There were however higher percentage of other phytohormone associated genes. Ethylene (ET) and abscisic acid (ABA) associated genes were the highest phytohormone associated genes, each present at 7.4%. Auxin (IAA) (4.1%) and cytokinin (CK) (1%) associated genes were also present. Similar to the susceptible 1-day, the majority of the DEGs were involved in either biosynthesis or regulated by the phytohormone. Less DEGs present at this treatment/time acted as regulators or catabolized the phytohormone (Fig 3.5).

The resistant 1-day had the fewest total DEGs. Because of the lack of DEGs (Fig 3.2) it was omitted from molecular function analysis. In contrast, the resistant 6-day had the most DEGs present after exclusion of the genes as indicated above and in Table 3.3. The two most prevalent categories for the resistant line at 6-day besides the other category (28.7%) were kinases (11.8%) and transcription factors (16%) (Fig 3.4C). This suggests the existence of a high signal transduction activity in these plants. Transcription factors were also represented at high percentage in the susceptible 6-day (15.7%) and similar families of transcription factors, such as ethylene response factor (ERF) genes and zinc finger transcription factors, were present in both resistant and susceptible 6-day. However, the individual transcription factor family member was different between the two cowpea lines. In contrast, one of the well-known defense-related transcription factors, the WRKY transcription factors, were not present in the susceptible 6-day but were present in the resistant 6-day (Table 3.4).

Like the susceptible line, no dominant defense phytohormone pathway could be identified in the resistant 6-day. The SA (2.6%) and JA (3.3%) associated genes were at similar percentages. Though there were more DEGs involved in negative regulation and catabolism of these phytohormones than in the susceptible line at either timepoint, there were still more DEGs involved in biosynthesis and regulated by the phytohormone in the resistant 6-day (Fig 3.5).

Within in these categories in the resistant 6-day, there were identifiable defense related categories including mitogen activated protein kinases (MAPKs), WRKY transcription factors, and LRR encoding genes, all with known roles in plant defense to microbial pathogens and some have been also implicated in aphid defense. Interestingly, the MAPK category was not identified in the susceptible interaction (Table 3.4). In contrast, one of the WRKY transcription factors in the resistant 6-day, *WRKY70*, with known roles in aphid resistance in tomato, is also present in both resistant 6-day and susceptible 1-day (Atamian et al., 2012).

There were multiple types of LRR encoding genes among the upregulated DEGs among the different contrasts. The predicted proteins of these LRR encoding genes were further annotated using NCBI Conserved Domain search and TMHMM V2.0 to identify additional domains in the proteins (Marchler-Bauer et al., 2017; Krogh et al., 2001). In the susceptible 1-day, two of the LRR proteins had kinase domains and transmembrane domains (Vigun09g096400.1, Vigun09g054500.1) indicating that both are receptor-like kinases (RLKs) (Table 3.4). In susceptible 6-day, only one LRR protein (Vigun04g017600.1) had a kinase domain and transmembrane domain and therefore is an RLK. The other LRR proteins (Vigun02g097700.1, Vigun03g273200.1) in susceptible 6-day encoded proteins with CNL domains. In resistant 6-day, there were six LRR encoding genes among the upregulated DEGs. Of these six, five (Vigun03g371200.1, Vigun03g350500.1, Vigun09g096400.1, Vigun03g097900.1,

Vigun03g435500.1) had kinase domains and transmembrane domains and are RLKs. Interestingly, one of these RLKs (Vigun09g096400.1) overlapped with the susceptible 1-day. The sixth LRR encoding gene (Vigun02g204100.1) in the resistant 6-day did not have any other predicted domains.

Over the course of the experiment, the plants had grown from 2-week-old to almost 3-week-old. During this period, cowpea plants have developed from the VC stage, with only unifoliate leaves, to the V1 phase with trifoliate leaves and some even entering the V2 phase (Fig 3.6). Therefore, the identified DEGs were also those involved in plant growth and development. To account for these changes in growth and development, indicated by the DEG for the contrast "Time" (Table 3.2), DEGs for the later aphid infestation timepoints were normalized to both changes in control plants for each genotype as well as the initial infestation time point for each genotype. This normalization was done to remove genes involved in normal senescence that were not aphid induced, as well as only slightly induced aphid DEGs at the initial timepoint. To identify possible sources of the aphid resistance, this contrast is especially important in the resistant line. There were 202 DEGs upregulated in the resistant line in this new contrast, Aphid Res Dev. Three of these 202 DEGs were previously identified in Table 3.4 as LRR encoding genes (Vigun02g204100.1, Vigun03g371200.1, Vigun03g350500.1), with two of them being RLKs. The

upregulation of these three in both resistant 6-day and in Aphid Res Dev suggests these three are aphid upregulated and suggests they are playing a role in aphid resistance.

Discussion

In this chapter, the transcriptomes of susceptible and resistant cowpea were investigated after cowpea aphid infestation. While transcriptome analysis of cowpea infested with aphids had not been studied previously, there have been numerous studies in another legume, soybean (*Glycine max*), infested with the soybean aphid (*Aphis glycines*). Like cowpea aphids, soybean aphids cause soybean significant economic losses and damage, including pseudogalling. These losses occur during heavy infestations which can lead up to a 40% crop yield loss (Wu et al., 2004; Ragsdale et al., 2007). A number of sources of resistance to the soybean aphid have been identified in different soybean cultivars and plant introductions (PIs). Nine of these resistance sources have been characterized at some level (Hesler et al., 2013; Zhang et al., 2017). Among these is the source of the *Rag1* (*Resistance to Aphis glycines*) gene and is the one best described. *Rag1* was originally identified in the soybean cultivar Dowling and the resistance was found to involve both antibiosis and antixenosis (Hill et al., 2004; 2006a; b; Li et al., 2007; Kim et al., 2010).

Multiple transcriptomic analyses of soybean with *Rag1* have been performed. In a comparison of the resistant Dowling to the susceptible Williams 82 cultivar, the resistant plants had more than double of DEGs than the susceptible at 6 hours post infestation. By 12 hours post infestation, the DEGs were at the same level between the two cultivars. Some of the early DEGs in the resistant plants were genes involved in the defense hormones SA and JA

signaling pathways and the phenylpropanoid pathway (Li et al., 2008). A major limitation of this study was the lack of an identical genetic background of the resistant and susceptible soybean cultivars. Other transcriptomic studies of *Rag1* based resistance used related, but not NILs, LD16060 (resistant) and SD01-76R (susceptible) soybean cultivars. Interestingly, at 1-day post aphid infestation, there were no DEGs in the resistant soybean plants and only a small number of DEGs were present in the susceptible (Studham and MacIntosh, 2013). The low level of DEGs in the resistant soybean at 1-day is similar to the results of this study that had resistant cowpea 1-day with the lowest DEGs of any other contrast (Fig 3.2) (Studham and MacIntosh, 2013). The lack of DEGs in the resistant soybean in response to the aphid at 1-day is believed to be a result that either the plant response is fast, earlier than one day after infestation, or that the resistance is constitutive (Li et al., 2008; Studham and MacIntosh, 2013). However, another major limitation of these soybean studies was the use of microarray instead of RNAseq. The microarray could be missing the probe necessary to identify the DEGs involved in the resistance. In contrast, the limited DEGs in the resistant line of cowpea at 1-day in this study, is most likely due to lack of aphid feeding. Based on EPG analysis from Chapter Two, aphids struggled to feed on resistant plants during an 8-hour inspection. The lack or limited aphid feeding could explain the low DEGs at the early time point in the resistant line.

In contrast to the 1-day resistance interaction, there were notable differences between susceptible soybean and cowpea 1-day infestations with their respective aphids. In the susceptible soybean 1-day, there were only 49 DEGs upregulated in response to soybean aphid while there were 552 unique DEGs upregulated in susceptible cowpea 1-day after cowpea aphid infestation (Fig 3.2) (Studham and MacIntosh, 2013). These DEGs in the susceptible cowpea had a high representation of function in protein biosynthesis. Besides protein biosynthesis, there were DEGs for diverse molecular functions (Fig 3.4A). This high level of multiple activities could be the result of both initial plant defenses against the aphid as well as the beginning of aphid induced manipulation to generate a sink in the plant (Ahman et al., 2019).

At 7-day post aphid infestation, there were more DEGs in the susceptible soybean than at 1-day (Studham and MacIntosh, 2013). In contrast, in susceptible cowpea, there were fewer DEGs in the susceptible 6-day than in the susceptible 1-day (Fig 3.2). This loss of DEGs at an advanced timepoint after infestation is not unique to the cowpea system as after 4-days of corn infestation with the corn leaf aphid (*Rhopalosiphum maidis*) DEGs were also closer to controls than to earlier infestations (Tzin et al., 2015).

The susceptible soybean plants were found to have a high level of ABA and ET associated genes after 7-day aphid infestation (Studham and MacIntosh, 2013). A similar trend was observed in the 6-day timepoint in the susceptible cowpea. Of the phytohormones, ABA and ET had the highest numbers of

associated genes (Fig 3.4B). There were also higher IAA associated DEGs in susceptible 6-day than 1-day. The susceptible 6-day had the highest level of cell wall/cytoskeleton associated DEGs than any of the contrasts. The differential regulation of both cytoskeletal genes and those associated in IAA, a hormone known to be involved in the formation of true galls by gall forming insects (Kutsukake et al., 2019), could be initiating the formation of the pseudogalls (Fig 3.1). In line with this, at 6-days, upregulation of both ET and IAA pathways were seen in susceptible barley (*Hordeum vulgare*) infested with the Russian wheat aphid (*Diuraphis noxia*) which also generates pseudogalling in its hosts including barley (Marimuthu and Smith, 2012).

In the resistant cowpea 6-day, there was a high number of transcription factors and kinases suggesting signal transduction and gene regulation was induced (Fig 3.4C). Most notably, in this signal transduction category were a number of known defense pathway genes including MAPKs, LRRs, and WRKYs (Table 3.4). There were LRRs and WRKYs in the susceptible cowpea but only a few overlapped with those in the resistant. There was no overlap however with MAPKs.

Three of the most known gene families that have roles in plant defense are MAPKs, WRKYs and those that include an LRR motif (Asai et al., 2002; Pedley and Martin, 2005). MAPKs are a well-conserved eukaryotic signaling cascade proteins that regulate a number of cellular functions including defense signaling (Widmann et al., 1999). Pest/pathogen recognition by a receptor leads

to the phosphorylation of MAPK kinase kinase (MAPKKK) that in turn phosphorylates MAPK kinase (MAPK) which ultimately phosphorylates MAPK. Once MAPK is activated, it then phosphorylates other downstream proteins leading to cellular defenses such as production of radical oxygen species (Rossi et al., 1998) (Pedley and Martin, 2005). The presence of multiple MAPKs in the resistant 6-day, and their absence in the susceptible line suggests that MAPK cascades are an important component of this aphid resistance (Table 3.4).

A number of transcription factors are known to regulate plant defense including the WRKY family (Singh et al., 2002b). Members of the WRKY transcription factor family contain ~60 amino acid domains that bind to the W-box promoter region in response to a pest or pathogen (Eulgem et al., 2000; Atamian et al., 2012). Several WRKY genes have been identified to be involved in resistance to aphids. In tomato, both *SlWRKY72a* and *SlWRKY72b* are involved in *Mi-1*-mediated resistance against potato aphids and root-knot nematodes (Bhattarai et al., 2010). Another *WRKY* gene identified in wheat (*Triticum aestivum*), *TaWRKY53*, is implicated in resistance against the Russian wheat aphid (Van Eck et al., 2010).

The Arabidopsis *WRKY70* was discovered to have a complex role in plant defense integrating signals from both SA- and JA-regulated defenses (Li et al., 2004; Li et al., 2006). *WRKY70* is involved in both promoting the SA pathway and suppressing the JA pathway (Li et al., 2004; AbuQamar et al., 2006; Li et al., 2006; Knoth et al., 2007). This relationship with SA and JA was also observed in

tomato as *WRKY70* was upregulated in response to SA and suppressed in response to methyl-jasmonate (Atamian et al., 2012). In *Mi-1* resistant tomato, *WRKY70* is induced in response to both potato aphids and root-knot nematodes. Silencing *WRKY70* through virus-induced gene silencing attenuated the *Mi-1*-mediated resistance (Atamian et al., 2012). Interestingly, *WRKY70* was upregulated in both susceptible and resistant cowpea lines (Table 3.4). The upregulation happened at different time points though, early in the susceptible and later in the resistant. While some of this difference in timepoints could be from the delayed onset of aphid feeding on the resistant line, it also suggests aphids maybe manipulating their host to alter plant defense responses in the susceptible cowpea.

Proteins with LRRs motif are also important component of plant defense. A majority of R proteins are intracellular LRRs that have NB domains to recognize pest/pathogen effector proteins. Recognition of effectors activates R proteins which frequently results in hypersensitive response, a well characterized programmed cell death (Heath, 2000; Balint-Kurti, 2019). Interestingly, the two LRR encoding genes (Vigun02g097700.1, Vigun03g273200.1) that had predicted proteins with domains consistent with R proteins were upregulated only in the susceptible 6-day and not in the resistant 6-day (Table 3.4). The lack of upregulation of these two LRR encoding genes in the resistant line suggests they are not necessary for resistance to the cowpea aphid. Alternatively, these two LRR encoding genes could be upregulated at different timeline and transiently in

the resistant cowpea and therefore were not detected in the time points chosen for this study.

The resistant 6-day had six LRR encoding genes among the upregulated DEGs. The majority of these were RLKs which are important component of plant defense. RLKs interact with a diverse group of proteins in order to coordinate specify signaling responses (Afzal et al., 2008). Like *WRKY70*, an RLK (Vigun09g096400.1) was also upregulated in both the resistant 6-day and the susceptible 1-day. The difference in the temporal upregulation of Vigun09g096400.1 between the two cowpea lines, could be from the delayed aphid interaction in the resistance or it could be due to manipulation by the aphids in the susceptible line. Two of the RLKs (Vigun03g371200.1, Vigun03g350500.1) in the resistant 6-day were also identified when growth and development were accounted for in the Aphid Res Dev contrast (Table 3.4). Interestingly, the third LRR encoding gene present in Aphid Res Dev contrast had no other domains besides the LRR domain (Vigun02g204100.1). These three LRR encoding genes are top candidates for further exploration on cowpea aphid resistance.

References

AbuQamar, S., Chen, X., Dhawan, R., Bluhm, B., Salmeron, J., Lam, S., et al. (2006). Expression profiling and mutant analysis reveals complex regulatory networks involved in Arabidopsis response to Botrytis infection. *Plant J* 48(1)**,** 28-44.

Afzal, A.J., Wood, A.J., and Lightfoot, D.A. (2008). Plant Receptor-Like Serine Threonine Kinases: Roles in Signaling and Plant Defense. *Mol Plant Microbe Interact* 21(5), 507-517.

Ahman, I., Kim, S.Y., and Zhu, L.H. (2019). Plant Genes Benefitting Aphids-Potential for Exploitation in Resistance Breeding. *Front Plant Sci* 10**,** 1452.

Andrews, S. (2010). *FastQC* [Online]. Available: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ [Accessed].

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., et al. (2002). MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature* 415(6875)**,** 977-983.

Atamian, H.S., Eulgem, T., and Kaloshian, I. (2012). *SlWRKY70* is required for *Mi-1*-mediated resistance to aphids and nematodes in tomato. *Planta* 235(2)**,** 299-309.

Balint-Kurti, P. (2019). The plant hypersensitive response: concepts, control and consequences. *Mol Plant Pathol* 20(8)**,** 1163-1178.

Berardini, T.Z., Reiser, L., Li, D., Mezheritsky, Y., Muller, R., Strait, E., et al. (2015). The Arabidopsis Information Resource: Making and mining the "gold standard" annotated reference plant genome. *Genesis* 53**,** 474-485.

Bhattarai, K.K., Atamian, H.S., Kaloshian, I., and Eulgem, T. (2010). *WRKY72*-type transcription factors contribute to basal immunity in tomato and Arabidopsis as well as gene-for-gene resistance mediated by the tomato *R* gene *Mi-1*. *Plant J* 63(2)**,** 229-240.

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**,** 1420.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu Rev Plant Biol* 60**,** 379-406.

Bonnot, T., Gillard, M., and Nagel, D. (2019). A Simple Protocol for Informative Visualization of Enriched Gene Ontology Terms. *BIO-PROTOCOL* 9(22).

Casteel, C.L., Walling, L.L., and Paine, T.D. (2006). Behavior and biology of the tomato psyllid, Bactericera cockerelli, in response to the *Mi-1.2* gene. *Entomol Exp Appl* 121(1)**,** 67-72.

Cesari, S., Bernoux, M., Moncuquet, P., Kroj, T., and Dodds, P.N. (2014). A novel conserved mechanism for plant NLR protein pairs: the "integrated decoy" hypothesis. *Front Plant Sci* 5**,** 606.

Dangl, J.L., and Jones, J.D. (2001). Plant pathogens and integrated defence responses to infection. *Nature* 411(6839)**,** 826-833.

Davis, D.W., Oelke, E.A., Oplinger, E.S., Doll, J.D., Hanson, C.V., and Putnam, D.H. (1991). Cowpea. *Alternative Field Crops Manual*.

Dogimont, C., Chovelon, V., Pauquet, J., Boualem, A., and Bendahmane, A. (2014). The *Vat* locus encodes for a CC-NBS-LRR protein that confers resistance to *Aphis gossypii* infestation and *A. gossypi*i-mediated virus resistance. *Plant J* 80(6)**,** 993-1004.

Eulgem, T., Rushton, P.J., Robatzek, S., and Somssich, I.E. (2000). The WRKY superfamily of plant transcription factors. *Trends Plant Sci* 5(5)**,** 199-206.

FAO (2020). *FAOSTAT Data* [Online]. Available: http://www.fao.org/faostat/en/#data/QC [Accessed 8-13-20 2020].

Hall, A.E. (2004). Breeding for adaptation to drought and heat in cowpea. *European Journal of Agronomy* 21(4)**,** 447-454.

Hall, A.E., Ismail, A.M., Ehlers, J.D., Marfo, K.O., Cisse, N., Thiaw, S., et al. (2002). "Breeding cowpeas for tolerance to temperature extremes and adaptation to drought," in *Challenges and Opportunities for Enhancing Sustainable Cowpea Production,* eds. C.A. Fatokun, S.A. Tarawali, B.B. Singh, P.M. Kormawa & M. Tamo. (Ibadan, Nigeria: Intl Inst Tropical Agric), 14-21.

Heath, M.C. (2000). Nonhost resistance and nonspecific plant defenses. *Curr Opin Plant Biol* 3**,** 315-319.

Hesler, L.S., Chiozza, M.V., O'Neal, M.E., MacIntosh, G.C., Tilmon, K.J., Chandrasena, D.I., et al. (2013). Performance and prospects of *Rag*

genes for management of soybean aphid. *Entomol Exp Appl* 147(3)**,** 201-216.

Hill, C.B., Li, Y., and Hartman, G.L. (2004). Resistance to the Soybean Aphid in Soybean Germplasm. *Crop Sci* 44(1)**,** 98-106.

Hill, C.B., Li, Y., and Hartman, G.L. (2006a). A single dominant gene for resistance to the soybean aphid in the soybean cultivar Dowling. *Crop Sci* 46**,** 1601-1605.

Hill, C.B., Li, Y., and Hartman, G.L. (2006b). Soybean aphid resistance in soybean Jackson is controlled by a single dominant gene. *Crop Sci* 46, 1606-1608.

Huynh, B.L., Ehlers, J.D., Close, T.J., Cisse, N., Drabo, I., Boukar, O., et al. (2013). "Enabling tools for modern breeding of cowpea for biotic stress resistance," in *Translational Genomics for Crop Breeding,* eds. R.V. Varshney & R. Tuberosa. (London: John Wiley & Sons), 183-199.

Huynh, B.L., Ehlers, J.D., Ndeve, A., Wanamaker, S., Lucas, M.R., Close, T.J., et al. (2015). Genetic mapping and legume synteny of aphid resistance in African cowpea (*Vigna unguiculata* L. Walp.) grown in California. *Mol Breed* 35**,** 36.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. *Nature* 444(7117)**,** 323-329.

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. *Proc Natl Acad Sci USA* 92(2)**,** 622-625.

Kim, D., Paggi, J.M., Park, C., Bennett, C., and Salzberg, S.L. (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat Biotechnol* 37(8)**,** 907-915.

Kim, K., Bellendir, S., Hudson, K.A., Hill, C.B., Hartman, G.L., Hyten, D.L., et al. (2010). Fine mapping the soybean aphid resistance gene *Rag1* in soybean. *Theor Appl Genet* 120**,** 1063-1071.

Kloth, K.J., Busscher-Lange, J., Wiegers, G.L., Kruijer, W., Buijs, G., Meyer, R.C., et al. (2017). *SIEVE ELEMENT-LINING CHAPERONE1* Restricts Aphid Feeding on Arabidopsis during Heat Stress. *Plant Cell* 29(10)**,** 2450-2464.

Knoth, C., Ringler, J., Dangl, J.L., and Eulgem, T. (2007). Arabidopsis *WRKY70* is required for full *RPP4*-mediated disease resistance and basal defense against *Hyaloperonospora parasitica*. *Mol Plant Microbe Interact* 20(2)**,** 120-128.

Krogh, A., Larsson, B., von Heijne, G., and Sonhammer, E. L. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* 305, 567–580.

Kutsukake, M., Uematsu, K., and Fukatsu, T. (2019). Plant Manipulation by Gall-Forming Social Aphids for Waste Management. *Front Plant Sci* 10**,** 933.

Law, C.W., Alhamdoosh, M., Su, S., Dong, X., Tian, L., Smyth, G.K., et al. (2016). RNA-seq analysis is easy as 1-2-3 with limma, Glimma and edgeR. *F1000Research* 5**,** 1408.

Li, J., Brader, G., Kariola, T., and Palva, E.T. (2006). *WRKY70* modulates the selection of signaling pathways in plant defense. *Plant J* 46(3)**,** 477-491.

Li, J., Brader, G., and Palva, E.T. (2004). The *WRKY70* transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *Plant Cell* 16(2)**,** 319-331.

Li, Y., Hill, C.B., Carlson, S.R., Diers, B.W., and Hartman, G.L. (2007). Soybean aphid resistance genes in the soybean cultivars Dowling and Jackson map to linkage group M. *Mol Breed* 19**,** 25-34.

Li, Y., Zou, J., Li, M., Bilgin, D.D., Vodkin, L.O., Hartman, G.L., et al. (2008). Soybean defense responses to the soybean aphid. *New Phytol* 179(1)**,** 185-195.

Lonardi, S., Munoz-Amatriain, M., Liang, Q., Shu, S., Wanamaker, S.I., Lo, S., et al. (2019). The genome of cowpea (*Vigna unguiculata* [L.] Walp.). *Plant J* 98(5)**,** 767-782.

Marchler-Bauer, A., Bo Y., Han, L., He, J., Lanczycki, C.J., Lu, S., et al. (2017). CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res* 45(D1), 200-203.

Marimuthu, M., and Smith, C.M. (2012). Barley tolerance of Russian wheat aphid (Hemiptera: Aphididae) biotype 2 herbivory involves expression of defense response and developmental genes. *Plant Signal Behav* 7(3)**,** 382-391.

Mi, H., Muruganujan, A., Ebert, D., Huang, X., and Thomas, P.D. (2018). PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic Acids Res* 47(D1).

Miles, P.W. (1999). Aphid saliva. *Biol Rev* 74(1)**,** 41-85.

Milligan, S.B., Bodeau, J., Yaghoobi, J., Kaloshian, I., Zabel, P., and Williamson, V.M. (1998). The root knot nematode resistance gene *Mi* from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes. *Plant Cell* 10(8)**,** 1307-1319.

Naeve, S.L. (2018). *Soybean growth stages* [Online]. University of Minnesota Extension. Available: https://extension.umn.edu/growing-soybean/soybean-growth-stages#vegetative-phase-%28table-1%29-539860 [Accessed 11-30-2020 2020].

Nalam, V., Louis, J., and Shah, J. (2019). Plant defense against aphids, the pest extraordinaire. *Plant Sci* 279**,** 96-107.

Nombela, G., Williamson, V.M., and Muniz, M. (2003). The root-knot nematode resistance gene *Mi-1.2* of tomato is responsible for resistance against the whitefly *Bemisia tabaci*. *Mol Plant Microbe Interact* 16(7)**,** 645-649.

Obeng-Ofori, D. (2007). "Pests of grain legumes," in *Major pests of food and selected fruit and industrial crops in West Africa,* ed. D. Obeng-Ofori. (Accra: The City Publishers Limited), 81-112.

Obopile, M. (2006). Economic threshold and injury levels for control of cowpea aphid, *Aphis crassivora* Linnaeus (Homoptera: Aphididae), on cowpea. *African Plant Protection* 12**,** 111-115.

Pedley, K.F., and Martin, G.B. (2005). Role of mitogen-activated protein kinases in plant immunity. *Curr Opin Plant Biol* 8(5)**,** 541-547.

Pitrat, M., and Lecoq, H. (1982). Genetic relations between non-acceptance and antibiosis resistance to *Aphis gossypii* in melon: search for linkage with other genes. *Agronomie* 2**,** 503-508.

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). *J Econ Entomol* 100(4)**,** 1258-1267.

Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., et al. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 47(7)**,** e47.

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2009). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26(1)**,** 139-140.

Rossi, M., Goggin, F.L., Milligan, S.B., Kaloshian, I., Ullman, D.E., and Williamson, V.M. (1998). The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. *Proc Natl Acad Sci USA* 95(17)**,** 9750-9754.

Singh, B.B. (2014). *Cowpea: The Food Legume of the 21st Century.* Madison, WI: Crop Science Society of America.

Singh, B.B., Ehlers, J.D., Sharma, B., and Freire, F.R. (Year). "Recent progress in cowpea breeding", in: *Proceedings of the World Cowpea Conference III*, eds. C.A. Fatokun, B.B. Tarawali, B.B. Singh, P.M. Kormawa & M. Tamo), 22-40.

Singh, K., Foley, R.C., and Onate-Sanchez, L. (2002b). Transcription factors in plant defense and stress responses. *Curr Opin Plant Biol* 5**,** 430-436.

Souleymane, A., Aken'Ova, M.E., Fatokun, C.A., and Alabi, O.Y. (2013). Screening for resistance to cowpea aphid (*Aphis craccivora* Koch) in wild and cultivated cowpea (*Vigna unguiculata* L. Walp.) accessions. *International Journal of Science, Environment and Technology* 2(4)**,** 611-621.

Studham, M.E., and MacIntosh, G.C. (2013). Multiple phytohormone signals control the transcriptional response to soybean aphid infestation in susceptible and resistant soybean plants. *Mol Plant Microbe Interact* 26(1)**,** 116-129.

Supek, F., Bosnjak, M., Skunca, N., and Smuc, T. (2011). Summarizes and Visualizes Long Lists of Gene Ontology Terms. *PLoS One* 6(7)**,** e21800.

Takken, F.L., and Goverse, A. (2012). How to build a pathogen detector: structural basis of NB-LRR function. *Curr Opin Plant Biol* 15(4)**,** 375-384.

Tjallingii, W.F. (2006). Salivary secretions by aphids interacting with proteins of phloem wound responses. *J Exp Bot* 57(4)**,** 739-745.

Tjallingii, W.F., and Esch, T.H. (1993). Fine-structure of aphid stylet routes in plant-tissues in correlation with EPG signals. *Physiol Entomol* 18(3)**,** 317-328.

Togola, A., Boukar, O., Servent, A., Chamarthi, S., Tamo, M., and Fatokun, C. (2020). Identification of sources of resistance in cowpea mini core accessions to *Aphis craccivora* Koch (Homoptera: Aphididae) and their biochemical characterization. *Euphytica* 216(6)**,** 88.

Tzin, V., Fernandez-Pozo, N., Richter, A., Schmelz, E.A., Schoettner, M., Schafer, M., et al. (2015). Dynamic Maize Responses to Aphid Feeding Are Revealed by a Time Series of Transcriptomic and Metabolomic Assays. *Plant Physiol* 169(3)**,** 1727-1743.

UniProt Consortium (2019). UniProt: a worldwide hub of protein knowledge. *Nucleic Acids Res* 47(D1)**,** D506-D515.

van der Hoorn, R.A., and Kamoun, S. (2008). From Guard to Decoy: a new model for perception of plant pathogen effectors. *Plant Cell* 20(8)**,** 2009-2017.

Van Eck, L., Schultz, T., Leach, J.E., Scofield, S.R., Peairs, F.B., Botha, A.M., et al. (2010). Virus-induced gene silencing of *WRKY53* and an inducible phenylalanine ammonia-lyase in wheat reduces aphid resistance. *Plant Biotechnol J* 8**,** 1023-1032.

Vos, P., Simons, G., Jesse, T., Wijbrandi, J., Heinen, L., Hogers, R., et al. (1998). The tomato *Mi-1* gene confers resistance to both root-knot nematodes and potato aphids. *Nat Biotechnol* 16(13)**,** 1365-1369.

Widmann, C., Gibson, S., Jarpe, M.B., and Johnson, G.L. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev* 79(1)**,** 143-180.

Will, T., Tjallingii, W.F., Thonnessen, A., and van Bel, A.J.E. (2007). Molecular sabotage of plant defense by aphid saliva. *Proc Natl Acad Sci USA* 104(25)**,** 10536-10541.

Wu, Z., Schenk-Hamlin, D., Zhan, W., Ragsdale, D.W., and Heimpel, G.E. (2004). The Soybean Aphid in China: A Historical Review. *Annals of the Entomological Society of America* 97(2)**,** 209-216.

Zhang, S., Zhang, Z., Bales, C., Gu, C., DiFonzo, C.D., Li, M., et al. (2017). Mapping novel aphid resistance QTL from wild soybean, *Glycine soja. Theor Appl Genet* 130(9)**,** 1941-1952.

Table 3.1. Alignment statistics for raw reads

Sequencing ID	Plant	Insect	Time	total sequences	% Aligned	M Aligned
adpJM-01	susceptible	no aphid	day one	45.2	89%	40.3
adpJM-02	susceptible	no aphid	day one	51.8	88%	45.5
adpJM-03	susceptible	no aphid	day one	52.0	87%	45.5
adpJM-04	susceptible	no aphid	day one	40.6	72%	29.0
adpJM-05	susceptible	no aphid	day one	43.2	85%	36.9
adpJM-06	resistant	no aphid	day one	52.7	85%	44.6
adpJM-07	resistant	no aphid	day one	39.7	84%	33.4
adpJM-08	resistant	no aphid	day one	45.6	88%	40.0
adpJM-09	resistant	no aphid	day one	43.6	86%	37.4
adpJM-10	resistant	no aphid	day one	36.0	91%	32.6
adpJM-11	susceptible	aphid	day one	52.0	85%	44.2
adpJM-12	susceptible	aphid	day one	68.0	69%	47.0
adpJM-13	susceptible	aphid	day one	49.3	84%	41.4
adpJM-14	susceptible	aphid	day one	32.1	83%	26.7
adpJM-15	susceptible	aphid	day one	64.8	87%	56.1
adpJM-16	resistant	aphid	day one	62.5	83%	51.8
adpJM-17	resistant	aphid	day one	76.6	86%	66.1
adpJM-18	resistant	aphid	day one	64.2	86%	55.0
adpJM-19	resistant	aphid	day one	57.1	87%	49.4
adpJM-20	resistant	aphid	day one	36.5	88%	32.0
adpJM-21	susceptible	no aphid	day six	38.6	89%	34.4
adpJM-22	susceptible	no aphid	day six	49.3	90%	44.3
adpJM-23	susceptible	no aphid	day six	47.1	89%	42.0
adpJM-24	susceptible	no aphid	day six	51.8	89%	45.9
adpJM-25	susceptible	no aphid	day six	41.6	90%	37.5
adpJM-26	resistant	no aphid	day six	65.3	88%	57.2
adpJM-27	resistant	no aphid	day six	44.3	92%	40.6
adpJM-28	resistant	no aphid	day six	39.4	90%	35.6
adpJM-29	resistant	no aphid	day six	38.3	85%	32.6
adpJM-30	resistant	no aphid	day six	47.4	88%	41.9
adpJM-31	susceptible	aphid	day six	51.1	89%	45.3
adpJM-32	susceptible	aphid	day six	82.0	92%	75.1
adpJM-33	susceptible	aphid	day six	45.0	85%	38.3
adpJM-34	susceptible	aphid	day six	78.9	82%	65.0
adpJM-35	susceptible	aphid	day six	227.6	91%	208.0
adpJM-36	resistant	aphid	day six	127.1	94%	119.1
adpJM-37	resistant	aphid	day six	128.9	85%	110.1
adpJM-38	resistant	aphid	day six	86.0	93%	79.9
adpJM-39	resistant	aphid	day six	119.5	92%	110.3
adpJM-40	resistant	aphid	day six	181.8	94%	170.9
			average	**68.2**	**87%**	**60.2**
			max	**227.6**	**94%**	**208.0**
			min	**32.1**	**69%**	**26.7**

Table 3.2. Contrasts of treatments and associated DEGs.

		-0.6 ≤ LFC ≥ 0.6		***P* (FDR) <0.05**	
Contrast Name	Contrast	**Up**	**Down**	Up	Down
Aphid	Mean Aphid – mean Control	773	496	1982	1365
Time	Mean Day 6 Control – mean Day 1 Control	3384	2900	5481	4313
Genotype	Mean Resistance – Mean Susceptible	344	1362	1617	2407
Development with Aphid	(Mean Aphid Day 6 – Mean Aphid Day 1) – (Mean Control Day 6 – Mean Control Day 1)	779	483	1211	603
Baseline Resistance	Mean Resistance Control – Mean Susceptible Control	235	473	547	688
AphidD1	Mean Aphid Day 1 – mean Control Day 1	759	574	1310	1302
AphidD6	Mean Aphid Day 6 – mean Control Day 6	876	600	1787	1024
AphidResD1	Resistance+Aphid Day 1 – Resistance Control Day 1	126	197	136	230
AphidResD6	Resistance+Aphid Day 6 – Resistance Control Day 6	597	643	944	786
AphidSuscD1	Susceptible+Aphid Day 1 – Susceptible Control Day 1	678	381	974	785
AphidSuscD6	Susceptible+Aphid Day 6 – Susceptible Control Day 6	311	105	394	121
Leaf Development	Mean Control Day 6 – Mean Control Day 1	2404	2625	3592	3453
ControlResD6	Resistance Control Day 6 – Resistance Control Day 1	1134	1558	1416	1801
ControlSusD6	Susceptible Control Day 6 – Susceptible Control Day 1	2772	2982	3714	3519
Aphid Res Dev	(Resistance+Aphid Day 6 – Resistance+Aphid Day 1) – (Resistance Control Day 6 – Resistance Control Day 1)	202	40	210	40
Aphid Sus Dev	(Susceptible+Aphid Day 6 – Susceptible+Aphid Day 1) – (Susceptible Control Day 6 – Susceptible Control Day 1)	388	333	464	356

Total numbers of up and down regulated genes at adjusted *P* (FDR) <0.05.

Table 3.3 Molecular Functional categorization of DEGs from susceptible and resistant cowpea lines at 1-day and 6-day after aphid infestation.

	Susceptible 1-day	Susceptible 6-day	Resistant 1-day	Resistant 6-day
Total upregulated	599	225	47	511
Excluded Genes				
Blank/No annotation	120	85	8	162
Unknown	14	15	1	32
Uncharacterized	2	4	4	1
Histone Related	23	0	0	3
Protein Biosynthesis	206	0	3	5
DNA Replication	14	0	0	1
Total Excluded	379	104	16	204
Genes Remaining	220	121	31	307
Biotic Stress Genes				
Kinases	9	5	0	36
LRR	3	3	0	6
Peptidases	5	3	0	10
Transcription Factors	11	19	3	49
Ubiquitin related	12	18	1	18
Cell wall related/cytoskeleton	4	18	3	19
Secondary Metabolite Related	13	4	0	18
Methylation	10	5	0	11
SA	4	6	0	8
JA	3	5	0	10
Et	3	9	0	5
ABA	6	9	0	13
Auxin	5	5	1	15
Cytokinin	6	1	1	1
Total	94	110	10	219
Other	126	11	21	88

Table 3.4. Upregulated DEGs involved in plant defense in response to cowpea aphid infestation.

Contrast	Cowpea Gene	Arabidopsis Gene	Description
Susceptible Cowpea 1-Day	Vigun09g133800.1	AT3G56400	WRKY DNA-binding protein 70 (WRKY70)
	Vigun03g069800.1	AT5G64810	WRKY DNA-binding protein 51 (WRKY51)
	Vigun09g128400.1	AT5G64810	WRKY DNA-binding protein 51 (WRKY51)
	Vigun09g096400.1	AT2G31880	Leucine-rich repeat protein kinase family protein, SUPPRESSOR OF BIR1 1 (SOBIR1), receptor like kinase (RLK)
	Vigun09g054500.1	AT3G47570	Leucine-rich repeat protein kinase family protein, receptor like kinase (RLK)
Susceptible Cowpea 6-Day	Vigun02g097700.1	AT3G07040	NB-ARC domain-containing disease resistance protein, RESISTANCE TO PSEUDOMONAS SYRINGAE 3 (RPS3), (CNL)
	Vigun04g017600.1	AT4G13340	Leucine-rich repeat (LRR) family protein, LEUCINE-RICH REPEAT/EXTENSIN 3 (LRX3), receptor like kinase (RLK)
	Vigun03g273200.1	AT5G66900	Disease resistance protein (CC-NBS-LRR class) family, (CNL)
Resistant Cowpea 6-Day	Vigun03g249400.1	AT2G17530	Protein kinase superfamily protein (MAP kinase activity)
	Vigun03g274400.1	AT5G66850	mitogen-activated protein kinase kinase kinase 5 (MAPKKK5)
	Vigun02g204100.1*	**AT1G49750***	Leucine-rich repeat (LRR) family protein
	Vigun03g371200.1*	**AT2G24230***	Leucine-rich repeat protein kinase family protein, receptor like kinase (RLK)
	Vigun03g350500.1*	**AT2G26730***	Leucine-rich repeat protein kinase family protein, receptor like kinase (RLK)
	Vigun09g096400.1	AT2G31880	Leucine-rich repeat protein kinase family protein, SUPPRESSOR OF BIR1 1 (SOBIR1), receptor like kinase (RLK)
	Vigun03g097900.1	AT5G45800	Leucine-rich repeat protein kinase family protein, MATERNAL EFFECT EMBRYO ARREST 62 (MEE62), receptor like kinase (RLK)
	Vigun03g435500.1	AT5G49760	Leucine-rich repeat protein kinase family protein, CANNOT RESPOND TO DMBQ 1 (CARD1), receptor like kinase (RLK)
	Vigun06g159600.1	AT1G62300	WRKY DNA-binding protein 6 (WRKY6)
	Vigun07g127100.1	AT2G47260	WRKY DNA-binding protein 23 (WRKY23)
	Vigun09g133800.1	AT3G56400	WRKY DNA-binding protein 70 (WRKY70)
	Vigun05g085500.1	AT5G22570	WRKY DNA-binding protein 38 (WRKY38)
	Vigun06g122600.1	AT5G24110	WRKY DNA-binding protein 30 (WRKY30)

Asterisks indicate genes that were present in Aphid Res Dev.

Fig 3.1. Cowpea aphid induced pseudogalling on cowpea. Leaves of susceptible cowpea were photographed 15-days after cowpea aphid infestation.

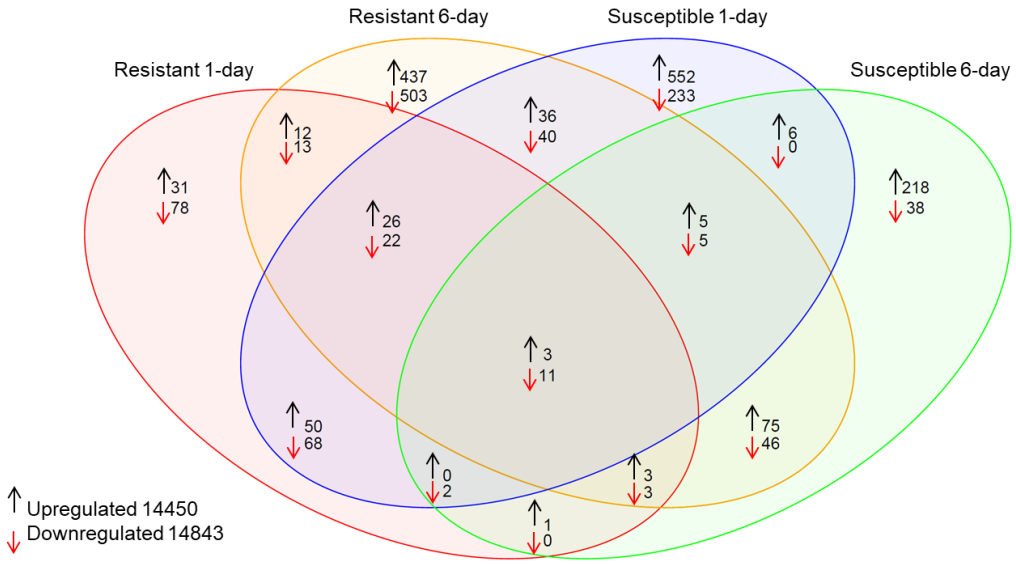

Fig 3.2. Venn diagram illustration of the number of DEGs up or downregulated by cowpea aphid infestation of susceptible and resistant cowpea, at the two different time timepoints, *P* (FDR) <0.05 and LFC ≥ 0.6 or ≤ -0.6. Resistant 1-day and Resistant 6-day are resistant cowpea with 1-day and 6-day infestations, respectively. Susceptible 1-day and Susceptible 6-day are susceptible cowpea with 1-day and 6-day infestations, respectively.

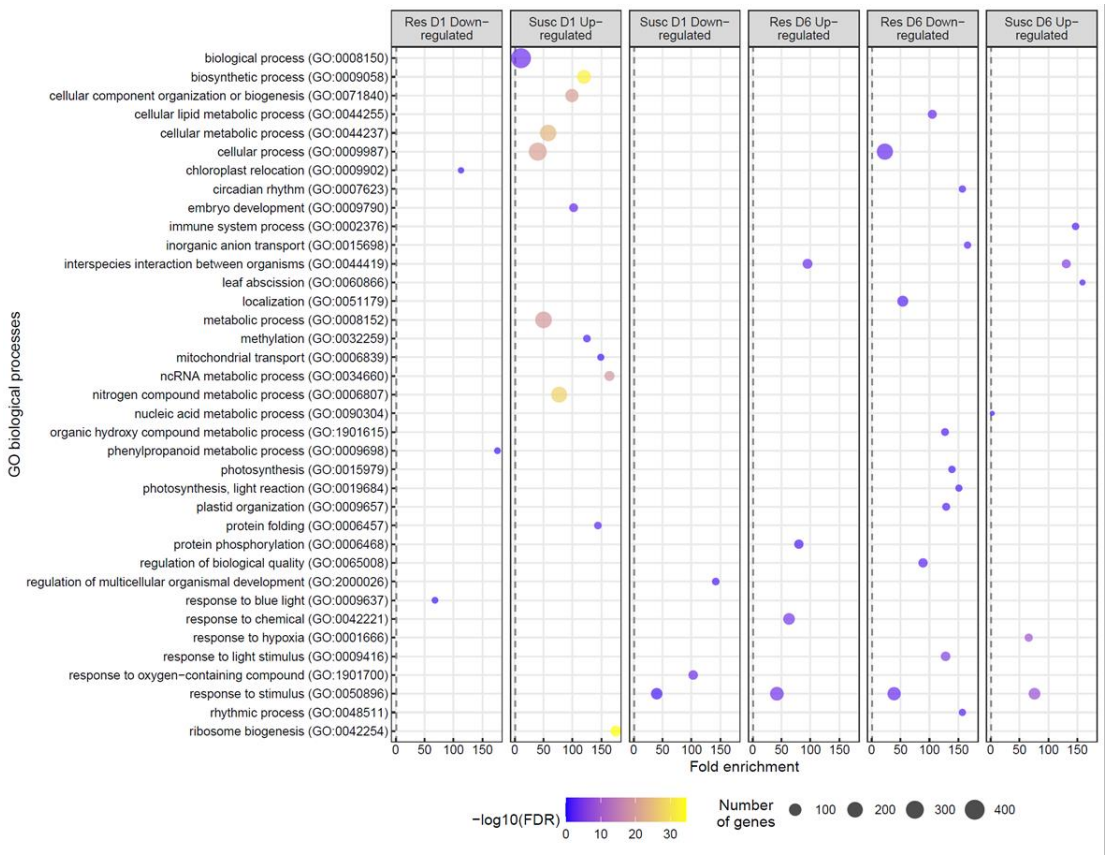

Fig 3.3. GO analysis of cowpea DEGs up or downregulated by cowpea aphid infestation. Cowpea aphids were exposed to either susceptible (Susc) or resistant (Res) cowpea for 1-day (D1) or 6-day (D6).

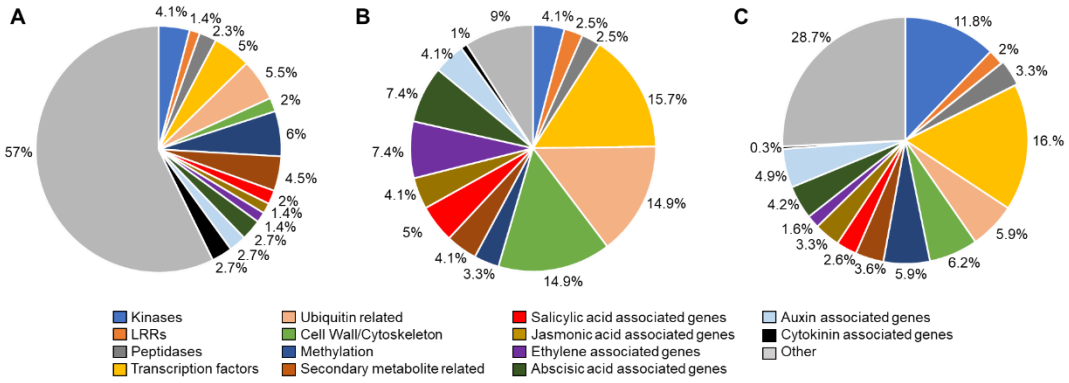

Fig 3.4. Molecular functions of upregulated DEGs present only in susceptible or resistant cowpea at the same timepoint after cowpea aphid infestation. Unknown DEGs, DEGs involved in protein biosynthesis, DNA replication, and histone related genes were excluded from this analysis. Due to a limited number of DEGs, resistant cowpea at 1-day was also not included. The remaining DEGs in susceptible **A)** 1-day (220 DEGs) and **B)** 6-day (121 DEGs) and in **C)** resistant 6-day (307 DEGs) were categorized.

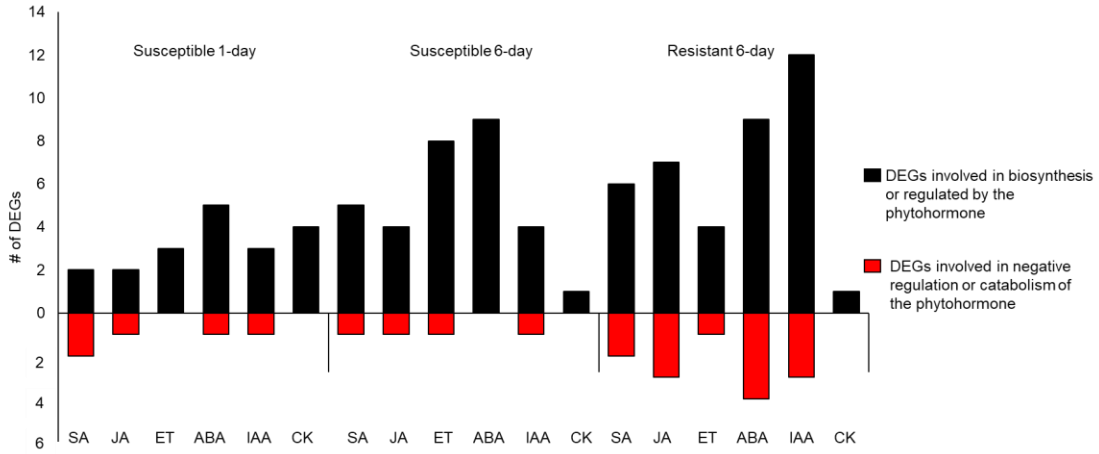

Fig 3.5. Distribution of phytohormone related genes identified from the molecular functionally categorized DEGs in susceptible (1-day, 6-day) and resistant cowpea (6-day). Genes involved in the biosynthesis or regulated by the phytohormone are in black. Genes that act as regulators or involved in the catabolism of the phytohormone are in red. The phytohormones monitored are salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), auxin (IAA), and cytokinin (CK).

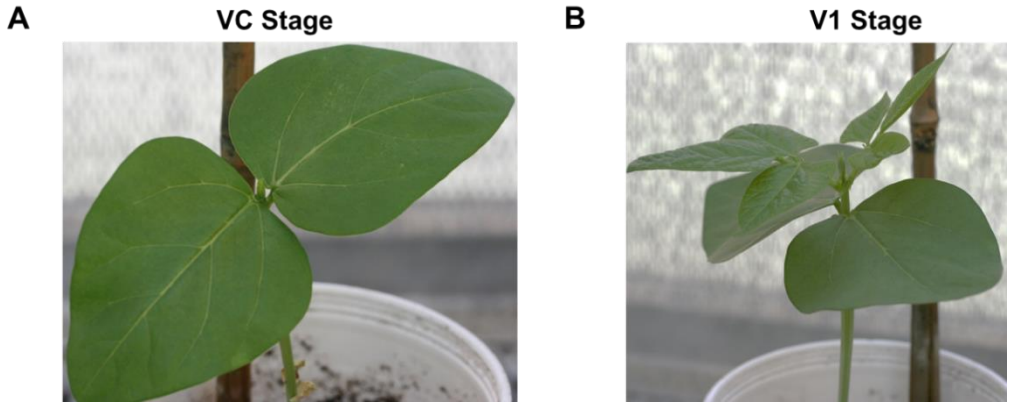

Fig 3.6. Development stages of cowpea plants used in the RNAseq experiments. During the time course of the aphid infestation, plants developed from the **A)** VC stage (1-day), with a pair of unifoliate leaves, to **B)** V1 stage (6-day), with trifoliate leaves.

General Conclusions

Aphids are plant pests from the order Hemiptera of insects. With over 5000 species, aphids feed on a wide range of plants including commercial crops (McGavin, 1993; Remaudiere and Remaudiere, 1997; Sorenson, 2009). Aphid feeding on plants, especially crops, can generate extensive damage and economic losses. The focus of this study, cowpea (*Vigna unguiculata*), can have over a 50% loss in crop yield due to cowpea aphid (*Aphis craccivora*) infestation (Obopile, 2006). Cowpea aphids have a worldwide distribution, but they are especially a problem in semiarid regions of the world like Sub-Saharan Africa (Singh, 2014). The most common control measure to aphid infestations is through the use of pesticides. However, in the regions where cowpea is grown, pesticide use is limited highlighting the need for endogenous cowpea resistance to cowpea aphids (Souleymane et al., 2013; Togola et al., 2020). In order to better control cowpea aphids, cowpea-cowpea aphid interactions need to be better studied and understood.

For this purpose, aphid saliva was collected *in vitro* and the proteome content was identified through liquid chromatography tandem mass spectrometry (LC-MS/MS) as described in Chapter One. During this analysis a putative aphid effector, diacetyl/L-xylulose reductase (DCXR), was identifed and functionally characterized in Chapter One. At the beginning of this study, there was no information available on the composition of cowpea aphid saliva. While this study was in progress, information became available on saliva of a cowpea aphid

native to Gabon, Africa (Loudit et al., 2018). While there were similarities in the salivary proteome composition of the African cowpea aphids and the cowpea aphids from California, used in this study, almost double the number of salivary proteins were identified. While a number of these proteins had been previously identified in other aphid species saliva, one protein, the salivary enzyme DCXR, had not.

DCXR is a member of short-chain dehydrogenase/reductase that is involved in NADPH-dependent reductions of carbohydrates and toxic dicarbonyls (Nakagawa et al., 2002; Ishikura et al., 2003; Ebert et al., 2015). It was shown that the cowpea aphid recombinant DCXR (AcDCXR) was able to catalyze the reversible reaction of xylitol to xylulose, generating a potential additional source of energy, as well as detoxify the cytotoxic dicarbonyl methylglyoxal. The presence of AcDCXR in cowpea aphid saliva, led to the discovery of methylglyoxal accumulation in cowpea in response to the aphid. At low concentrations, methylglyoxal has recently emerged as a plant signaling molecule to both abiotic and biotic stresses (Hoque et al., 2016; Li, 2016; Melvin et al., 2017). Cowpea aphid infestation led to a transient increase in methylglyoxal levels in multiple plant species indicating it is a conserved response to cowpea aphid infestation. Agrobacterium-mediated transient overexpression of AcDCXR on pea (*Pisum sativum*) increased aphid fecundity. This increased fecundity demonstrated AcDCXR as a putative effector, most

likely through generation of additional energy for the aphid as well as altering plant defense responses.

Cowpea aphid infestations generate an excessive amount of damage to cowpea in the field. The high level of damage generated by cowpea aphids is not seen in most plant-aphid interactions. At low populations, aphids that cause little to no damage are classified as nonphytotoxic, while aphids that generate damage at low populations are classified as phytotoxic (Miles, 1999; Nicholson et al., 2012; Nicholson and Puterka, 2014). In Chapter Two, cowpea aphids were examined to determine if they were phytotoxic. While an uncontrolled cowpea aphid population always generated at least one damage symptom, a cowpea aphid population that was kept at a low constant level was also found to generate damage symptoms. Damage symptoms present from low aphid populations demonstrated that the cowpea aphids are phytotoxic aphids.

Endogenous resistance to the cowpea aphid has been previously identified in the African cowpea breeding line IT97K-556-6 (Souleymane et al., 2013). This resistance was further explored through recombinant inbred lines (RILs), and the source of the resistance was identified as two quantitative trait loci (QTL), *QAC-vu7.1* and *QAC-vu1.1* (Huynh et al., 2015). Near isogenic lines (NILs) were developed in the California blackeye cultivar background (Huynh et al., 2015; Huynh and Roberts, pers. comm.). The resistance conferred by the two QTLs in 2014-008-51-82 was characterized in Chapters Two and Three.

The mechanism of resistance conferred by these two QTLs was identified as having both antibiosis and antixenosis components as described in Chapter Two. Cowpea aphid growth and fecundity were observed on the resistant and susceptible NILs. Cowpea aphids feeding on the resistant line had both a slower growth rate and lower fecundity than aphids feeding on the susceptible line, confirming the resistance has an effect on the aphids' development and reproduction. Monitoring aphid feeding behavior using electrical penetration graphs (EPGs) identified a significant difference in sap ingestion from the two cowpea lines. Cowpea aphids on the resistant line struggled to ingest sap indicating that the resistance is at least partially phloem based. In choice assays in Chapter Two and dispersal assays in Appendix A, cowpea aphids were found to have significant preference for the susceptible cowpea over the resistant confirming that the resistance has an effect on the aphids' behavior.

In addition to characterization of the mechanism of resistance in Chapter Two, molecular characterization of the resistance was explored in Chapter Three. This was achieved by analyzing the transcriptomes of the NILs, after 1-day and 6-day infestations with the cowpea aphids, using RNAseq. Not many differentially expressed genes (DEGs) were identified in resistant 1-day. This is most likely due to lack of interaction between the aphid and plant, as the aphids struggled to feed early after exposure to plants based on EPG analysis as described in Chapter Two. The most DEGs for a single timepoint/treatment were identified in the susceptible 1-day. A high representation in these DEGs were genes involved

in protein biosynthesis. This could be an indication of either initiation of plant defenses against the aphid or the beginnings of aphid induced host manipulation to generate a sink in the plant (Ahman et al., 2019).

In the susceptible 6-day, there was a high level of cell wall/cytoskeleton associated DEGs and genes associated with the phytohormones auxin (IAA) and ethylene (ET). The ET and IAA pathways are upregulated in barely (*Hordeum vulgare*) after Russian wheat aphid (*Diuraphis noxia*) infestation and thought to be involved in pseudogall formation (Marimuthu and Smith, 2012). The upregulation of ET and IAA pathways, as well as cell wall/cytoskeleton associated DEGs in cowpea fed on by aphids, is likely part of initiation of the pseudogalls.

In the resistant 6-day, there were a number of DEGs known to be involved in the defense related processes. One of these defense genes identified was a mitogen activated protein kinases (MAPKs). MAPKs are a well conserved signaling cascade proteins that regulate a number of cellular functions like defense (Widmann et al., 1999). No MAPKs were found upregulated in response to aphid infestation in the susceptible line suggesting their importance for the resistance. Another of these DEGs is the transcription factor *WRKY70* which has been previously implicated in tomato (*Solanum lycopersicum*) resistance to the potato aphid (*Macrosiphum euphorbiae*) (Atamian et al., 2012). In this study, *WRKY70* was upregulated in both the susceptible and resistant lines, but at different timepoints, early in susceptible and late in the resistant. The difference

in timepoints could be from the delayed onset of aphid feeding on the resistant line, but it also suggests aphids maybe manipulating their host plant defenses in the susceptible cowpea. Other defense related DEGs upregulated in resistant 6-day were those encoding leucine rich repeats (LRRs). The majority of the LRR encoding genes identified in resistant 6-day were identified as transmembrane receptor-like kinases (RLKs). RLKs are integral components of plant defense. They interact with a diverse group of proteins in order to coordinate specific signaling responses (Afzal et al., 2008). Two of the RLKs identified in resistant 6-day were also identified when accounting for growth and development. A third LRR encoding gene was also identified when accounting for growth and development, but it contained only an LRR domain. There is little other information on these three LRR encoding genes but based on their expression patterns they are the top candidates for further exploration of cowpea aphid resistance.

References

Afzal, A.J., Wood, A.J., and Lightfoot, D.A. (2008). Plant Receptor-Like Serine Threonine Kinases: Roles in Signaling and Plant Defense *Mol Plant Microbe Interact* 21(5)**,** 507-517.

Ahman, I., Kim, S.Y., and Zhu, L.H. (2019). Plant Genes Benefitting Aphids-Potential for Exploitation in Resistance Breeding. *Front Plant Sci* 10**,** 1452.

Atamian, H.S., Eulgem, T., and Kaloshian, I. (2012). *SlWRKY70* is required for *Mi-1*-mediated resistance to aphids and nematodes in tomato. *Planta* 235(2)**,** 299-309.

Ebert, B., Kisiela, M., and Maser, E. (2015). Human DCXR - another 'moonlighting protein' involved in sugar metabolism, carbonyl detoxification, cell adhesion and male fertility? *Biol Rev* 90(1)**,** 254-278.

Hoque, T.S., Hossain, M.A., Mostofa, M.G., Burritt, D.J., Fujita, M., and Tran, L.S. (2016). Methylglyoxal: an emerging signaling molecule in plant abiotic stress responses and tolerance. *Frontiers in Plant Science* 7**,** 1341.

Huynh, B.L., Ehlers, J.D., Ndeve, A., Wanamaker, S., Lucas, M.R., Close, T.J., et al. (2015). Genetic mapping and legume synteny of aphid resistance in African cowpea (*Vigna unguiculata* L. Walp.) grown in California. *Mol Breed* 35**,** 36.

Ishikura, S., Usami, N., El-Kabbani, O., and Hara, A. (2003). Structural determinant for cold inactivation of rodent L-xylulose reductase. *Biochem Biophys Res Commun* 308(1)**,** 68-72.

Li, Z.G. (2016). Methylglyoxal and glyoxalase system in plants: old players, new concepts. *Bot Rev* 82**,** 183-203.

Loudit, S.M.B., Bauwens, J., and Francis, F. (2018). Cowpea aphid-plant interactions: endosymbionts and related salivary protein patterns. *Entomol Exp Appl* 166(6)**,** 460-473.

Marimuthu, M., and Smith, C.M. (2012). Barley tolerance of Russian wheat aphid (Hemiptera: Aphididae) biotype 2 herbivory involves expression of defense response and developmental genes. *Plant Signal Behav* 7(3)**,** 382-391.

McGavin, G.C. (1993). *Bugs of the World.* New York: Blandford Press.

Melvin, P., Bankapalli, K., D'Silva, P., and Shivaprasad, P.V. (2017). Methylglyoxal detoxification by a DJ-1 family protein provides dual abiotic and biotic stress tolerance in transgenic plants. *Plant Mol Biol* 94(4-5)**,** 381-397.

Miles, P.W. (1999). Aphid saliva. *Biol Rev* 74(1)**,** 41-85.

Nakagawa, J., Ishikura, S., Asami, J., Isaji, T., Usami, N., Hara, A., et al. (2002). Molecular characterization of mammalian dicarbonyl/L-xylulose reductase and its localization in kidney. *J Biol Chem* 277(20)**,** 17883-17891.

Nicholson, S.J., Hartson, S.D., and Puterka, G.J. (2012). Proteomic analysis of secreted saliva from Russian Wheat Aphid (*Diuraphis noxia* Kurd.) biotypes that differ in virulence to wheat. *J Proteomics* 75(7)**,** 2252-2268.

Nicholson, S.J., and Puterka, G.J. (2014). Variation in the salivary proteomes of differentially virulent greenbug (*Schizaphis graminum* Rondani) biotypes. *J Proteomics* 105**,** 186-203.

Obopile, M. (2006). Economic threshold and injury levels for control of cowpea aphid, *Aphis crassivora* Linnaeus (Homoptera: Aphididae), on cowpea. *African Plant Protection* 12**,** 111-115.

Remaudiere, G., and Remaudiere, M. (1997). *Catalogue des Aphididae du monde (Homoptera Aphidoidea).* Paris: Paris Institut national de la recherche agronomique.

Singh, B.B. (2014). *Cowpea: The Food Legume of the 21st Century.* Madison, WI: Crop Science Society of America.

Sorenson, J. (2009). "Aphids," in *Encyclopedia of Insects,* eds. V. Resh & R. Carde. Second ed (San Diego, CA: Academic Press), 27-30.

Souleymane, A., Aken'Ova, M.E., Fatokun, C.A., and Alabi, O.Y. (2013). Screening for resistance to cowpea aphid (*Aphis craccivora* Koch) in wild and cultivated cowpea (*Vigna unguiculata* L. Walp.) accessions. *International Journal of Science, Environment and Technology* 2(4)**,** 611-621.

Togola, A., Boukar, O., Servent, A., Chamarthi, S., Tamo, M., and Fatokun, C. (2020). Identification of sources of resistance in cowpea mini core accessions to *Aphis craccivora* Koch (Homoptera: Aphididae) and their biochemical characterization. *Euphytica* 216(6)**,** 88.

Widmann, C., Gibson, S., Jarpe, M.B., and Johnson, G.L. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human *Physiol Rev* 79(1)**,** 143-180.

Appendix A

Cowpea Aphid Dispersal Assays

Introduction

Choice assays with resistant and susceptible plants were performed to determine whether the cowpea aphid resistance conferred by the two QTLs had an antixenosis component. Choices assays were monitored at several time points within a 24-hour period. More aphids were present on the susceptible cowpea than on the resistant after 24 hours. These experiments were described in Chapter Two. However, the actual cowpea aphid interaction with the resistant and susceptible plants was unknown since the aphids were released between the two plant genotypes and little aphid participation occurred until the final timepoint. It was not known if the aphids moved between plants and sampled them, or they simply chose the susceptible. To account for this lack of interaction, aphids were placed directly on either susceptible or resistant leaves and the aphids' dispersal to a leaf of the opposite genotype was monitored. This was compared to a baseline dispersal where two plants of the same genotype were tested next to each other.

Materials and Methods

The rate of cowpea aphid dispersal in response to resistant cowpea was measured using behavior dispersal assay as described in Mauck et al., (2010). Bioassay arenas developed from 100 x 15 mm petri dishes, with two conjoining holes (17 mm^2) cut in the center, were used. A unifoliate leaf from two cowpea plants were exposed on one side of the conjoining holes. After a 15 -minute chill at 4°C, twenty 4^{th} stage instar and adult aphids were placed on a piece of filter paper. The filter paper with the chilled aphids was then placed directly on one of the exposed leaves (Fig 4.1). The placement of the filter paper on the leaf ensured that the aphids contacted the initial leaf they were placed on before dispersal. The filter paper was removed after an hour when all aphids had dispersed and the location of the aphids were documented at 1, 2, 6, and 24 hours. The cowpea plants screened against each other included, susceptible with resistant, susceptible with susceptible and resistant with resistant.

Results

A cowpea aphid dispersal assay was utilized to test aphids' initial response to the resistant and susceptible cowpea genotypes. Cowpea aphids were introduced directly to either a susceptible or resistant leaf with a leaf of the opposite cowpea line directly next to it (Fig 4.1). As control, the same genotype was also tested next to each other and is referred to as baseline dispersal.

Monitoring cowpea aphid dispersal at different intervals for a period of 24 hours showed that aphids initially placed on the susceptible leaf had a low level of dispersion when screened with the resistant leaf and with the susceptible leaf (baseline dispersal) (Fig 4.2). Aphids remained on the initial susceptible plant for the majority of the replicated experiments. Only at 6 hours, the number of aphids on the originating leaf go below 50% (8.7/20 aphids), but the missing aphids did not go to resistant leaf, instead, they were elsewhere in the testing arena (Fig 4.2C). This movement at 6 hours was also seen in the baseline dispersal group where the aphids had left the initial leaf they were placed on but did not go to the adjoining leaf and were elsewhere in the testing arena. By 24 hours, the majority of the aphids were found on the original susceptible leaf they had been placed on. At 24 hours, only 19% (3.8/20 aphids) of the aphids were found on the adjoining resistant leaf and the baseline dispersal at 24 hours, where susceptible

leaves were tested against each other, was only 29.5% (5.9/20 aphids) (Fig 4.2D).

Lack of dispersion was also observed at 1 hour when the experiment was reversed, and cowpea aphids were initially placed on a resistant cowpea leaf (Fig 4.3A). However, after 2 hours, less than half of the aphids remained on the resistant leaf they were originally placed on (6.5/20 aphids) (Fig 4.3B). The aphids had not dispersed to the susceptible plant however and were elsewhere in the testing arena. At 24 hours, there were more aphids on the susceptible leaf than on the initial resistant leaf they were placed on (Fig 4.3D). This dispersal from the resistant to the susceptible leaf was significantly different than the baseline dispersal from resistant to resistant leaf. At 24 hours, the baseline dispersal was only 27.5% (5.5/ 20 aphids) while the dispersal of aphids placed on the resistant leaf and found on the susceptible leaf was 47.5% (9.5/20 aphids). This affirms the cowpea aphids' preference for susceptible cowpea and the involvement of antixenosis in the resistance.

References

Mauck, K.E., De Moraes, C.M., and Mescher, M.C. (2010). Deceptive chemical signals induced by a plant virus attract insect vectors to inferior hosts. *Proc Natl Acad Sci USA* 107(8)**,** 3600-3605.

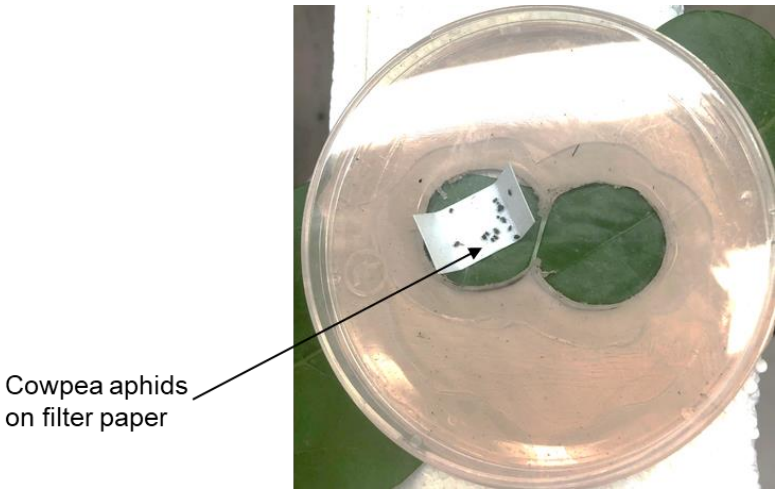

Fig 4.1. A Petri dish arena used for the cowpea aphid dispersal assays. Cowpea aphids were introduced into the arena on a filter paper on either a susceptible or a resistant leaf.

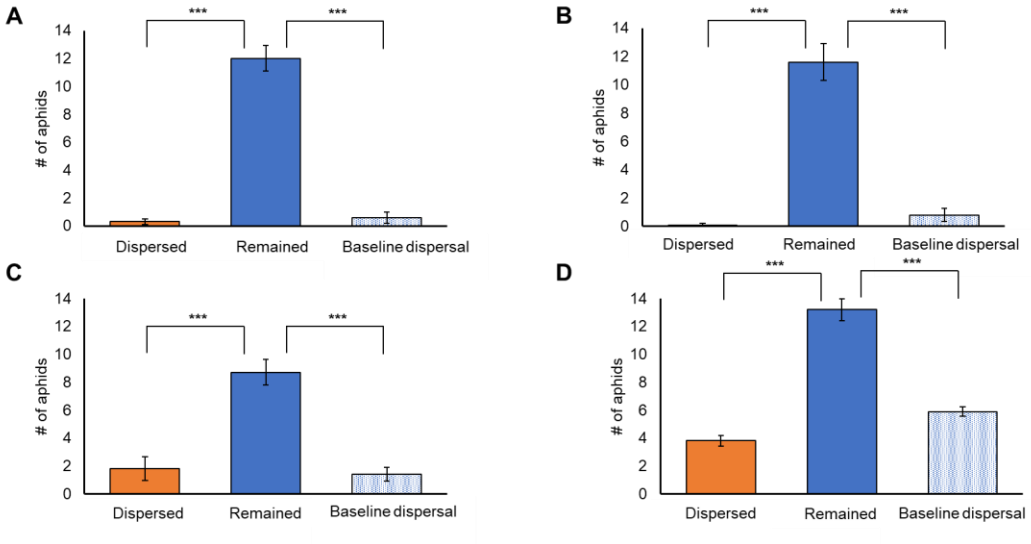

Fig 4.2. Dispersal of cowpea aphids when introduced onto a susceptible cowpea. Twenty cowpea aphids were introduced to a cowpea unifoliate leaf on a filter paper with the opposite cowpea genotype next to leaf the aphids were placed on. The location of the aphids was monitored at **A)** 1 hour, **B)** 2 hours, **C)** 6 hours, and **D)** 24 hours. The baseline dispersal was determined in the same way but using two plants of the same genotype next to each other. Dispersal level was compared to the baseline dispersal through generalized linear models (GLM) followed by Tukey's HSD test. Asterisks indicate significant differences $p < 0.001$.

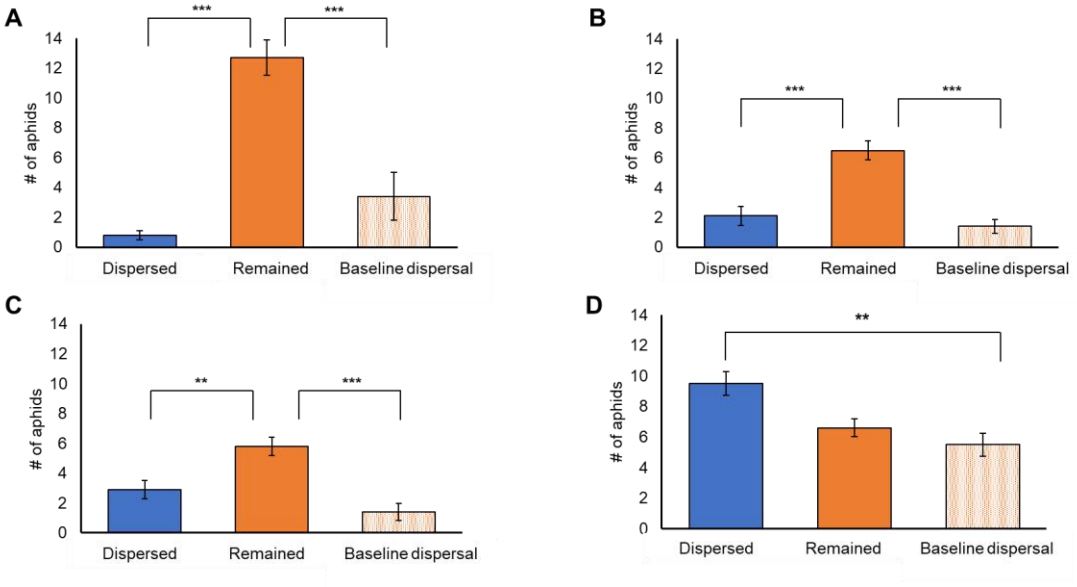

Fig 4.3. Dispersal of cowpea aphids when introduced on resistant cowpea. Twenty cowpea aphids were introduced to a cowpea unifoliate leaf on a filter paper with the opposite cowpea genotype next to leaf the aphids were placed on. The location of the aphids was monitored at **A)** 1 hour, **B)** 2 hours, **C)** 6 hours, and **D)** 24 hours. The baseline dispersal was determined in the same way but using two plants of the same genotype next to each other. Dispersal level was compared to the baseline dispersal through generalized linear models (GLM) followed by Tukey's HSD test. Asterisks indicate significant differences ** $p < 0.01$ and *** $p < 0.001$.

Appendix B

Quantification of Methylglyoxal Levels in Cowpea Leaves in Response to Cowpea Aphid Infestation

Abstract

Aphids are a serious pest of crops across the world. Aphids feed by inserting their flexible hypodermal needlelike mouthparts, or stylets, into their host plant tissues. They navigate their way to the phloem where they feed on its sap causing little mechanical damage to the plant. Additionally, while feeding, aphids secrete proteinaceous effectors in their saliva to alter plant metabolism and disrupt plant defenses to gain an advantage over the plant. Even with these arsenals to overcome plant responses, plants have evolved ways to detect and counter with defense responses to curtail aphid infestation. One of such response of cowpea to cowpea aphid infestation, is accumulation of the metabolite methylglyoxal. Methylglyoxal is an α,β-dicarbonyl ketoaldehyde that is toxic at high concentrations. Methylglyoxal levels increase modestly after exposure to a number of different abiotic and biotic stresses and has been shown to act as an emerging defense signaling molecule at low levels. Here we describe a protocol to measure methylglyoxal in cowpea leaves after cowpea aphid infestation, by utilizing a perchloric acid extraction process. The extracted supernatant was neutralized with potassium carbonate, and methylglyoxal was quantified through its reaction with N-acetyl-L-cysteine to form N-α-acetyl-S-(1-hydroxy-2-oxo-prop-1-yl)cysteine, a product that is quantified spectrophotometrically.

The text of this chapter is a reprint of the material as it appears in Bio-protocol.

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Introduction

The importance of methylglyoxal in plant response and signaling to various stresses is only just beginning to be understood. Various abiotic stresses have been shown to lead to an accumulation of methylglyoxal in plants (Yadav *et al*., 2005; Borysiuk *et al*., 2018). This accumulation suggests that methylglyoxal has a signaling role in plants (Hossain *et al.*, 2009; Hoque *et al.*, 2016; Mostofa *et al.*, 2018). Exogenous application of methylglyoxal has been found to upregulate antioxidant and defense genes in plants corroborating the role methylglyoxal has as a signaling molecule (Kaur *et al.*, 2015; Li *et al.*, 2017). The accumulation of methylglyoxal in plants has also been described in response to biotic stresses including bacterial, viral and fungal infections (Melvin *et al.*, 2017). Recently, it has been shown that cowpea aphid infestation also leads to an increase in methylglyoxal level expanding its role in defense against herbivore pests (MacWilliams *et al.*, 2020). Three methods have been established for quantification of methylglyoxal. Of these three methods, the N-acetyl-L-cysteine method by Wild *et al.* (2012) has been found to measure methylglyoxal in the most economical and safest way. The other two methods involve expensive enzyme purification or derivatization with an explosive chemical (Racker, 1951; Gilbert and Brandt, 1975). N-acetyl-L-cysteine method involves mixing methylglyoxal with N-acetyl-L-cysteine to generate N-α-acetyl-S-(1-hydroxy-2-oxo-prop-1-yl)cysteine which is detected and measured at absorbance 288 nm by a spectrophotometer (Fig 5.1). In this protocol, we combine the method by

Wild *et al.* (2012), that simply measures methylglyoxal levels, with extraction of methylglyoxal from plant tissues, and measuring its levels in cowpea (*Vigna unguiculata*) leaves after cowpea aphid infestation (*Aphis craccivora*).

Materials and Methods

1. Disposable nitrile gloves (Fisher Scientific, catalog number: 19-130-1597)

2. Lab coat

3. N95 mask (3M, catalog number: 19-033-524)

4. 32 oz (946 ml) plastifoam cups (First Street, model: SF32)

5. White printing paper letter size (8 1/2" x 11") (Office Depot, catalog number: 841195)

6. Bug domes (BioQuip)

7. Fine tip paint brush (academy 775 round size 0; Grumbacher, catalog number: 14173361639)

8. Disposable Petri dish (Fisher Scientific, catalog number: FB0875713)

9. Pipette tips 2 µl, 10 µl, 200 µl, 1,000 µl

10. Eppendorf tubes (1.5 ml)

11. Eppendorf tubes (2 ml)

12. Pellet pestles (Fisher Scientific, catalog number: 12-141-364)

13. Methacrylate disposable cuvettes (Fisher Scientific, catalog number: 14-955-127)

14. UC Mix 3 Soil (Plaster Sand 15.50 cu. ft., peat moss 11.50 cu. ft., KNO3 0.25 lb., limestone flour 1.50 lb., phosphate 1.25 lb., dolomite 3.75 lb., magnesium 0.07 lb., iron 0.13 lb., manganese 0.03 lb., zinc 0.05 lb., copper 0.11 lb.) (https://agops.ucr.edu/soil-mixing) or similar plaster sand-peat moss mix soil

15. Cowpea seeds (cv CB46)

16. Cowpea aphid colony

17. Sodium phosphate monobasic (NaH_2PO_4) (Fisher Scientific, catalog number: S369-500)

18. Methylglyoxal (Sigma-Aldrich, catalog number: M0252-25ML)

19. N-acetyl-L-cysteine (Alfa Aesar, catalog number: A15409)

20. 70% Perchloric acid (Macron Fine Chemicals, catalog number: MK-2766-500)

21. Potassium carbonate (CK_2O_3) (Fisher Scientific, catalog number: P208-500)

22. Sodium hydroxide (NaOH) (Fisher Scientific, catalog number: BP359-212)

23. Charcoal (Fisher Scientific, catalog number: C270C)

24. Alkacid wide-range test ribbons (Fisher Scientific, catalog number: A979)

25. ddH_2O

26. Ice

27. 100 mM Methylglyoxal (see Recipes)

28. 100 mM NaH_2PO_4 buffer (pH 7.0) (see Recipes)

29. 500 mM N-acetyl-L-cysteine (see Recipes)

30. 5% Perchloric acid (see Recipes)

31. 1 M CK_2O_3 (see Recipes)

32. 10 mM NaOH (see Recipes)

Equipment

1. Pipettes (P2, P20, P200, P1000)

2. Microcentrifuge (Beckman Coulter, model: Microfuge 22R Centrifuge)

3. Spectrophotometer (Beckman Coulter, model: Du 730 Life Science UV/Vis Spectrophotometer)

4. pH meter (Mettler Toledo, model: MP 220 pH Meter)

5. Electronic balance (Mettler Toledo, model: AG104 Electric Balance)

6. Plant and aphid growth room or greenhouse maintained at 28 ± 2 °C and 16 h light/8 h dark photoperiod

7. Fume hood

Procedure

A. Planting and plant growth

1. Fill up the plastifoam cups with UC Mix 3 soil. Use a minimum of three plants for each infestation time point and for non-infested control (biological replicates).

2. Plant a single cowpea seed in each cup at around 2.5 cm depth.

3. Maintain the plants at 28 ± 2 °C for 16 h light/8 h dark photoperiod and water as needed.

4. After 2 weeks, plants will have full grown unifoliate leaves and will be ready for infestation.

B. Aphid infestation

1. Using a wet fine paintbrush, collect apterous (wingless) 4th stage nymphs and adult cowpea aphids from a colony and place the aphids in a Petri dish as seen in Fig 5.2.

Note: A cowpea aphid colony is best maintained on young plants with 16 h of light at 28 ± 2 °C.

2. Move the dish with the aphids to the plant growth room where the plants will be infested and maintained.

3. Using the same paintbrush, transfer about 100 aphids to a single unifoliate leaf.

Notes:

a. Ensure that the leaf is evenly infest with aphids as seen in Fig. 5.3C.

b. For multiple time point infestations, infest the plants such that you are able to harvest the leaves, from all time points, at the same time. For example, for 24 h and 48 h time points, infest on day 1 and day 2 at a similar time of the day and harvest the leaves on day 3.

4. Make a slit in a piece of paper of about 15-mm height and 7-mm width (Fig 5.3A), and place the paper around the cowpea leaf to form a barrier, to inhibit aphids from leaving the leaf, as seen in Fig 5.3. Place a paper barrier on the control non-infested plants as well.

Note: The paper barrier discourages aphids from leaving the leaf, while limiting mechanical damage that could be caused by traditional clip cages, used to confine aphids on a given location on a leaf.

5. Move the infested plants into a bug dome and maintain at 28 ± 2 °C for 16 h light/8 h dark photoperiod.

6. Place the non-infested control plants in a different bug dome, adjacent to the bug dome containing the aphid-infested plants.

C. Methylglyoxal extraction

Notes: Because perchloric acid and methylglyoxal are toxic, before starting the procedure, take the following safety measures:

a. Wear personal protective equipment (PPE), including a lab coat, gloves and N95 mask, for handling perchloric acid and methylglyoxal.

b. Perform all steps in a fume hood.

c. Have an easily accessible designated waste container, inside the hood, to dispose everything that comes in contact with perchloric acid.

1. Move the plants and a balance, for weighing the leaves, near the fume hood.
2. Mark and weigh appropriate number of empty 1.5 ml Eppendorf tubes and record their weights.
3. Remove the paper barrier, cut the petiole of the infested leaf and immediately cut the leaf in half and place a half leaf in a 1.5 ml Eppendorf tube.
4. Weigh the tube and determine the leaf tissue weight by subtracting the weight of the empty tube.

Note: The weight of half a cowpea 2-week-old unifoliate leaf is around 300 mg.

5. Add 300 µl of 5% perchloric acid and homogenize the leaf tissue using a pestle.

Notes:

a. Use 1:1 ratio of mg leaf tissue and µl volume of 5% perchloric acid.

b. To avoid variability, steps 3-5 should be performed quickly.

6. Incubate the samples for 15 min at room temperature.
7. Centrifuge the samples at 13,000 *x g* for 10 min at 4 °C.
8. Transfer the supernatant to a new 1.5 ml tube.

9. Divide equally the supernatant from each sample into two 1.5 ml tubes to use as technical replicates.

10. Add 10 mg of charcoal to each tube to decolorize the supernatant and incubate for 15 min at room temperature.

Note: Invert the tubes a couple of times during the 15 min incubation.

11. Neutralize the solution by adding an appropriate volume of 1 M CK_2O_3.

Note: Use caution when titrating with 1 M CK_2O_3 and monitor the pH of the sample with Alkacid wide-range test ribbons to prevent over-neutralization. For 250 µl of supernatant, around 45 µl of 1 M CK_2O_3 is needed. If additional titration is needed, use small volumes of 1 M CK_2O_3.

12. Centrifuge the samples at 13,000 *x g* for 10 min at 4 °C.

Note: If the pellet is floating and is not stuck at the bottom of the tube, repeat the centrifugation.

13. Transfer the supernatant from each tube to a new 1.5 ml tube and keep on ice until ready to measure methylglyoxal concentration.

D. Methylglyoxal standard curve

1. Prepare a standard curve by adding the reagents in 2 ml tubes as described in Table 5.1.

Notes:

a. Methylglyoxal is highly toxic and should be handled in a fume hood with proper PPE. All steps including the formation and reading of N-α-acetyl-S-(1-hydroxy-2-

oxo-prop-1-yl)cysteine with the spectrophotometer should also be performed in the fume hood for maximum safety, with an easily accessible designated waste container for everything that comes in contact with methylglyoxal.

b. 100 mM methylglyoxal is made fresh in ddH_2O.

2. Add 20 µl of 500 mM N-acetyl-L-cysteine to each tube to start the reaction shown in Fig 5.1.

3. Invert the tubes to mix the reaction and incubate at room temperature for 10 min.

Notes:

a. Tubes should be inverted multiple times during the incubation period.

b. If this protocol is used for measuring methylglyoxal under different stresses or different plant species, then, observe the kinetics of the reaction of a sample to ensure that the 10 min is a long enough incubation time for the reaction to reach a plateau.

4. Move the samples into cuvettes and using a spectrophotometer detect the formation of N-α-acetyl-S-(1-hydroxy-2-oxo-prop-1-yl)cysteine by measuring the absorbance at 288 nm.

5. Plot a standard curve as shown in Fig 5.4.

E. Methylglyoxal estimation

1. For each sample, use a 2 ml tube and label accordingly. In each tube, add 930 µl of 100 mM NaH_2PO_4 buffer (pH 7.0).

Note: 100 mM NaH_2PO_4 buffer (pH 7.0) should be prepared fresh.

2. Add 50 µl of each sample to the 2 ml tube with the 100 mM NaH_2PO_4 buffer.

3. Add 20 µl of 500 mM N-acetyl-L-cysteine to each tube to start the reaction shown in Fig 5.1.

Note: 500 mM N-acetyl-L-cysteine is prepared fresh in 100 mM NaH_2PO_4 buffer (pH 7.0).

4. Invert the tubes to mix and incubate at room temperature for 10 min.

Note: Invert the tubes multiple times during the incubation period.

5. Move the samples into cuvettes and detect the formation of N-α-acetyl-S-(1-hydroxy-2-oxo-prop-1-yl)cysteine by measuring absorbance at 288 nm using a spectrophotometer.

Note: If the sample absorbance falls outside of the standard curve, dilute the sample and redo the reaction starting at Step E1.

Data Analysis

1. Using the methylglyoxal standard curve shown in Fig 5.4, determine the methylglyoxal concentration (mM) in the samples by (Abs288 – intercept)/slope.

2. Multiply the value obtained by 20 [20 being the dilution factor (1,000 µl total volume/50 µl of the sample) for the example given above].

Note: If the samples were diluted before measurement, include that dilution factor in the calculation.

3. Determine the total methylglyoxal content of a leaf sample (µmol g-1 fresh weight) by multiplying the methylglyoxal concentration (mM) by the extracted sample volume (ml) and dividing by the leaf fresh weight (g).

4. Average the two technical replicates for the value of a biological replicate. Then, determine the value of each time point by calculating the average of the different biological replicates.

Recipes

1. 100 mM Methylglyoxal (prepared fresh)

Add 15.4 µl of methylglyoxal to 984.6 µl ddH_2O

2. 100 mM NaH_2PO_4 buffer (pH 7.0) (prepared fresh)

Dissolve 0.599 g NaH_2PO_4 in 50 ml ddH_2O. Bring to pH 7 with 10 mM NaOH

3. 500 mM N-acetyl-L-cysteine (prepared fresh)

Dissolve 81.5 mg N-acetyl-L-cysteine in 1 ml NaH_2PO_4 buffer (pH 7.0)

4. 5% Perchloric Acid

Add 1.79 ml 70% Perchloric acid to 23.21 ml ddH_2O

5. 1 M CK2O3

Dissolve 6.91 g CK_2O_3 in 50 ml ddH_2O

6. 10 mM NaOH

Dissolve 0.4 g of NaOH pellets in 1,000 ml ddH_2O

References

Borysiuk, K., Ostaszewska-Bugajska, M., Vaultier, M. N., Hasenfratz-Sauder, M. P. and Szal, B. (2018). Enhanced formation of methylglyoxal-derived advanced glycation end products in *Arabidopsis* under ammonium nutrition. *Front Plant Sci* 9: 667.

Gilbert, R. P. and Brandt, R. B. (1975). Spectrophotometric determination of methylglyoxal with 2,4-dinitrophenylhydrazine. *Anal Chem* 47: 2418-2422.

Hoque, T. S., Hossain, M. A., Mostofa, M. G., Burritt, D. J., Fujita, M. and Tran, L. S. (2016). Methylglyoxal: an emerging signaling molecule in plant abiotic stress responses and tolerance. *Front Plant Sci* 7: 1341.

Hossain, M. A., Hossain, Z. and Masayuki, F. (2009). Stress-induced changes of methylglyoxal level and glyoxalase I activity in pumpkin seedlings and cDNA cloning of glyoxalase I gene. *J Crop Sci* 3(2): 53-64.

Kaur, C., Kushwaha, H. R., Mustafiz, A., Pareek, A., Sopory, S. K. and Singla-Pareek, S. L. (2015).Analysis of global gene expression profile of rice in response to methylglyoxal indicates its possible role as a stress signal molecule. *Front Plant Sci* 6: 682.

Li, Z. G., Duan, X. Q., Min, X. and Zhou, Z. H. (2017). Methylglyoxal as a novel signal molecule induces the salt tolerance of wheat by regulating the glyoxalase system, the antioxidant system, and osmolytes. *Protoplasma* 254(5): 1995-2006.

MacWilliams, J. R., Dingwall, S., Chesnais, Q., Sugio, A. and Kaloshian, I. (2020). AcDCXR Is a cowpea aphid effector with putative roles in altering host immunity and physiology. *Front Plant Sci* 11(605).

Melvin, P., Bankapalli, K., D'Silva, P. and Shivaprasad, P. V. (2017). Methylglyoxal detoxification by a DJ-1 family protein provides dual abiotic and biotic stress tolerance in transgenic plants. *Plant Mol Biol* 94(4-5): 381-397.

Mostofa, M. G., Ghosh, A., Li, Z. G., Siddiqui, M. N., Fujita, M. and Tran, L. P. (2018). Methylglyoxal - a signaling molecule in plant abiotic stress responses. *Free Radic Biol Med* 122: 96-109.

Racker, E. (1951). The mechanism of action of glyoxalase. J Biol Chem 190: 685-696.

Wild, R., Ooi, L., Srikanth, V. and Munch, G. (2012). A quick, convenient and economical method for the reliable determination of methylglyoxal in millimolar concentrations: the N-acetyl-L-cysteine assay. *Anal Bioanal Chem* 403(9): 2577-2581.

Yadav, S. K., Singla-Pareek, S. L., Ray, M., Reddy, M. K. and Sopory, S. K. (2005). Methylglyoxal levels in plants under salinity stress are dependent on glyoxalase I and glutathione. *Biochem Biophys Res Commun* 337(1): 61-67.

Table 5.1. Reagents and concentrations used for developing the methylglyoxal standard curve.

Tube	Methylglyoxal concentration	100 mM Methylglyoxal	100 mM NaH_2PO_4 buffer (pH 7.0)
1	0.5 mM	5 µl	975 µl
2	1 mM	10 µl	970 µl
3	2 mM	20 µl	960 µl
4	3 mM	30 µl	950 µl
5	5 mM	50 µl	930 µl

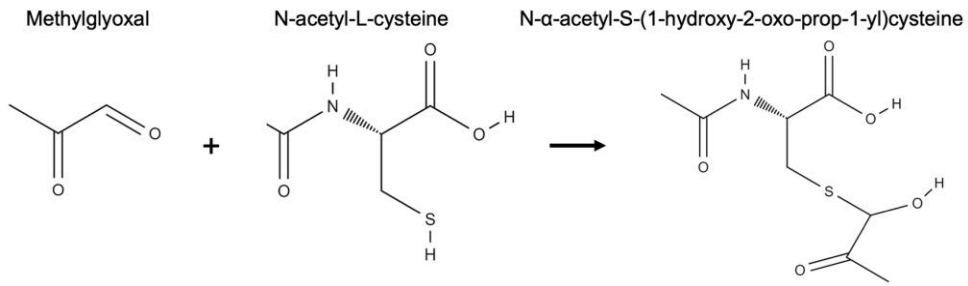

Fig 5.1. Reaction of methylglyoxal with N-acetyl-L-cysteine to form N-α-acetyl-S-(1-hydroxy- 2-oxo-prop-1-yl)cysteine. Structures were drawn using the online resource at http://molview.org.

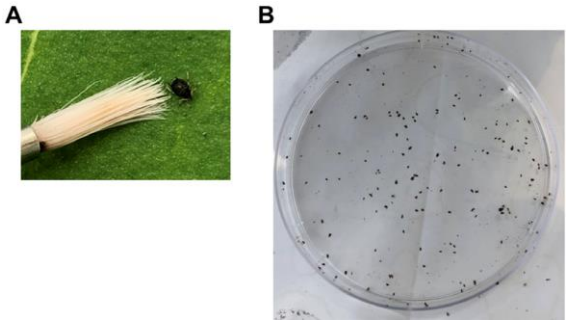

Fig 5.2. Collection of cowpea aphids. **A)** An adult cowpea aphid next to a paintbrush. **B)** Cowpea aphids in a 9-mm Petri dish.

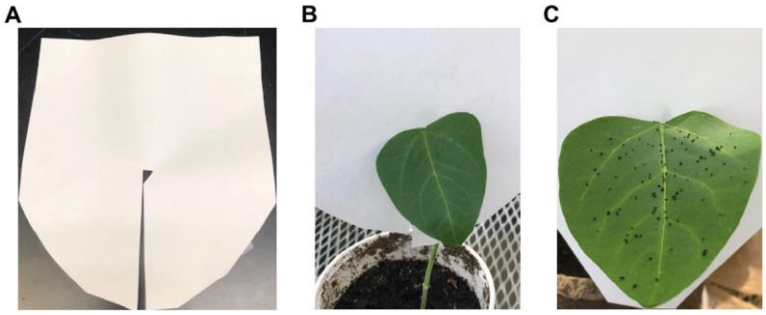

Fig 5.3. The paper barrier used to keep aphids on a single leaf. **A)** The slit in the paper allows to insert the paper around the petiole of a leaf. **B)** A cowpea plant with the paper barrier in place. **C)** A cowpea leaf evenly infested with cowpea aphids.

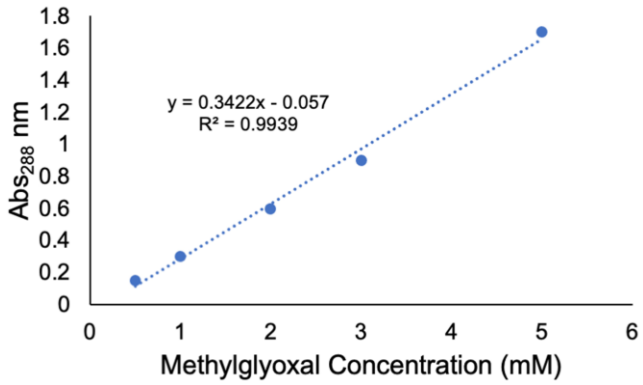

Fig 5.4. A standard curve generated by measuring absorbance at 288 nm using a spectrophotometer and the methylglyoxal concentrations and reagents in Table 5.1.

Appendix C

The Effect of Adaptation to Different Host Plants on Potato Aphid Host Choice and Endogenous Viral Load

Introduction

Herbivorous piercing-sucking insects have an intimate relationship with their host plants. A majority of herbivorous insects are limited to a specific host plant species or small range of host plants due to specific plant defenses or plant nutritional composition (Futuyma and Moreno, 1988; Forister et al., 2012). Currently, the adaption by insects to their host plants is of great interest (Simon et al., 2015; Birnbaum and Abbot, 2018). With enhanced understanding of insect adaption to their host plants, a better management strategy can be employed against crop pests.

Aphids are major crop pests with more than 5000 species known to date (Blackman and Eastop, 2000). Most aphids are specialists and have a narrow range of plant hosts while some aphids are generalists and have a wide range of plant hosts (Peccoud and Simon, 2010). Aphids feed by inserting their flexible hypodermal needle like stylets into the plant tissues navigating to the plant phloem to feed on the plant's phloem sap. On the way to the phloem and in the phloem, aphids deposit saliva to facilitate feeding, alter plant physiology and disrupt plant host defense responses (Miles, 1999; Will et al., 2007).

The pea aphid (*Acyrthosiphon pisum*) is a legume specialist feeding on different leguminous crops such as lentils, beans, peas, alfalfa, and clover (Ferrari et al., 2006; Frantz et al., 2006; Ferrari et al., 2008; Peccoud et al., 2009). The pea aphid has been chosen as the model aphid species with over 15 biotypes of the aphid documented (Peccoud et al., 2009; Peccoud et al., 2015).

Each of these biotypes is a specialist that feeds on only one or a few closely related plant hosts (Peccoud et al., 2009). These biotypes have a differing fitness level and survival rate on these different host species, but all can survive on fava bean (*Vicia faba*) (Sandström, 1994; Sandström and Pettersson, 1994; Peccoud et al., 2009).

Differential gene expression has been investigated in multiple pea aphid biotypes fed on their natural host plant compared to feeding on the common host fava bean. Interestingly, little differences in gene expression were identified among a specific biotype fed on different host plants (Eyres et al., 2016; Boulain et al., 2019). The differences in gene expression were mostly detected among the different aphid biotypes (Boulain et al., 2019). This lack of gene expression change on different plant hosts, may explain why the pea aphid is a specialist and unable to colonize a wide host range like the generalist green peach aphid (*Myzus persicae)* (Eyres et al., 2016; Mathers et al., 2017; Boulain et al., 2019). Further examination of host responses to pea aphids, found changes in plant defense hormones. When non-native pea aphid biotypes fed on different non-adapted host plants, higher levels of plant defense hormones jasmonic acid (JA) and salicylic acid (SA) were detected on these non-adapted plants compared to those fed on by the native pea aphid biotype (Sanchez-Arcos et al., 2016).

In contrast to pea aphids, the green peach aphid is a genuine generalist that is able to colonize more than 100 different plant species from 40 families (Peccoud et al., 2009; CABI, 2020). The green peach aphid is able to accomplish

this broad generalism through rapid transcriptional plasticity of aphid-specific genes (Mathers et al., 2017). Unlike pea aphids, when green peach aphids were transferred from host to host multiple gene clusters of aphid-specific genes were differentially expressed (Eyres et al., 2016; Mathers et al., 2017; Boulain et al., 2019). Of these gene clusters, cathepsin B cysteine proteases were thought to be the most important cluster for green peach aphid colonization. When the green peach aphids were transferred between host plants, different cathepsin B genes differentially expressed as early as two days after transfer. The importance of the cathepsin B genes was confirmed by knocking down the expression of a group of them through plant-mediated RNAi. Interestingly, the effect of knocking down these genes on aphid survival and fecundity were only detected on *Arabidopsis thaliana* but not on *Nicotiana benthamiana* (Mathers et al., 2017).

The potato aphid (*Macrosiphum euphorbiae*) is another generalist aphid with a wide host range of over 200 plant species from 20 different families (CABI, 2019). The potato aphid is able to survive on these many different plants, but it prefers plants from the Solanaceae family, especially potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum)* (Blackman and Eastop, 2000). The potato aphid like other aphids is known to act as a vector to a number of economically important plant viruses (Chan et al., 1991; Xu and Gray, 2020).

Several aphid-specific viruses have been identified to date including three from the potato aphid. Transcriptomic analysis of the potato aphid identified the

existence of three novel potato aphid viruses, Macrosiphum euphorbiae virus 1-3 (MeV-1, MeV-2 and MeV-3). Transmission of all three viruses is suspected to be vertically from mother to nymph (Teixeira et al., 2016; Teixeira et al., 2018). However, MeV-1 was shown to be also horizontally transmitted through its host plant as it was detected in plant tissues fed on by virus-infected aphids (Teixeira et al., 2016), and naïve aphids feeding on tomato plants previously infested with MeV-1 aphids, became infected with MeV-1 (Kaloshian, Unpublished). MeV-2 was also detected in the host plant tissue, but it is unclear if it is transmitted horizontally (Teixeira et al., 2018). The effects and roles of these three viruses on their aphid host are not well understood.

Some aphid viruses have been identified to have symbiotic effects on their aphid host, such as, the rosy apple aphid virus (RAAV), dyaphis plantaginea densovirus (DplDNV), and Acyrthosiphon pisum virus (APV) (van der Wilk et al., 1997; Ryabov et al., 2009; Lu et al., 2019). The beneficial effects of these viruses vary but each have a role in helping their host aphid. DplDNV induces winged morphs of the rosy apple aphid (*Dysaphis plantaginea*) that help disperse the aphid colony. Rosy apple aphids infected with DplDNV have a lower fecundity but, interestingly, the presence of RAAV decreases this lost fecundity (Ryabov et al., 2009). APV infection of pea aphids increased the aphid survival on the low-fitness host *Vicia villosa* through lowering the JA defense hormone levels (Lu et al., 2019).

In this study I investigated the long-term effects of adaptation of a tomato biotype of a potato aphid adapted to different host plants. I also investigated whether adaptation to new hosts affected MeV-1, MeV-2 and MeV-3 levels and the possibility that these viruses have a role in the aphid adaptation to a new host.

Materials and Methods

Potato aphid colonies

A colony of potato aphids isolate WU11, was imported from France to California and maintained on tomato for over two decades. Potato aphids were adapted to new sets of hosts from this original tomato colony. These hosts include fava bean, cotton (*Gossypium hirsutum*), and Arabidopsis. These aphids were maintained exclusively on their adapted hosts for over 2.5 years. The tomato, fava bean, and cotton colonies were maintained in a pesticide-free greenhouse at 20-30°C in separate large cages supplemented with light for a 16-hour light photoperiod. The Arabidopsis colony was maintained in a growth chamber (Conviron) at 20°C with 16-hour light photoperiod.

Plant growth

Tomato cvs. UC82 or EP7, fava bean (cv Windsor), and cotton were grown in UC Mix 3 soil (https://agops.ucr.edu/soil-mixing) in 32 oz plastifoam cups. Wild-type Arabidopsis Col-0 plants were grown in sunshine mix (SunGro). The plants were maintained in a pesticide free plant growth room at 22-24 C with 16-hour light photoperiod. Plants were fertilized weekly with MiracleGro (18-18-21; Stern's MiracleGro Products).

Potato aphid choice assay

An arena developed from a large petri dish had two 2 cm holes in the bottom of the plate, cut-out directly across from one another on either end of the plate. Two 4-week-old plants were positioned at the two holes farthest apart and a leaf from each of the two plants was made accessible to the aphids through each of these holes. A third 2 cm hole was cut-out at the center of the plate. A threaded ring was made by cutting out the center of a 50 ml conical tube cap. This threaded ring was glued onto the bottom of the dish surrounding the center hole. To hold the aphids, a 50 ml conical tube was cut 2 cm below the threads. A mesh was glued to the cut section. Twenty 4^{th} stage nymphs and adult apterous aphids were placed in this modified tube and starved for an hour.

To introduce the aphids into the arena, the modified tube was attached to the bottom of the petri dish, by screwing it into the threaded ring. Aphids were allowed to choose between the plant they were adapted to and a set of plant species including fava bean, cotton and Arabidopsis as well as the original host, tomato. Aphids were also allowed to choose between non-adapted hosts. The number of aphids on each plant was recorded at 1, 6, and 24 hours. Four to six plant pairs were used per experiment. Experiments were performed at least twice.

RNA extraction and RT-PCR

Ten aphids (4th stage nymph or adult) were collected from each colony for RNA extraction using TRIzol (Invitrogen). RNA extraction and RT-PCR were performed as described by Teixeira et al. (2016). Extracted RNA was treated with DNase I (New England Biolabs) and 2 µg RNA from each sample was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen) and oligo-dT primer. The PCR was performed with iQ SYBR Green Supermix (Bio-Rad) using gene-specific primers (Table 6.1) and amplification consisted of 3 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 57°C, and 30 s at 72°C, 15 min at 72°C, followed by the generation of a dissociation curve. The generated threshold cycle CT was used to calculate the transcript abundance relative to aphid ribosomal protein (L27) gene ($\Delta\Delta$CT). The transcript levels in aphids on the original host tomato was adjusted to 1 for comparisons.

Statistical analysis

Choice assays were analyzed using two-way ANOVAs (genotype x time points) followed by Tukey HSD, performed using GraphPad PRISM (GraphPad Software, San Diego, California USA). The comparison of the 6-hour time points was analyzed using a one-way ANOVA, followed by Tukey HSD performed using GraphPad PRISM (GraphPad Software, San Diego, California USA).

For RT-PCR statistical analysis, a one-way ANOVA followed by Tukey HSD was performed using GraphPad PRISM (GraphPad Software, San Diego, California USA).

Results

Choice assay

Potato aphid colonies adapted to three different plant species were used in choice experiments to determine if the adaptation to their new host plants altered aphid host preference. Choice assays were performed using modified petri dish arenas that allowed the choice between two plant species. Aphids were allowed to choose between the plant they were adapted to and either the original plant host tomato or other plant species. Irrespective to the plant species potato aphids were adapted to, overwhelmingly aphids chose tomato as the preferential host (Figs 6.1-6.3). Both the cotton and Arabidopsis adapted colonies significantly chose tomato by one hour after initiation of the experiment (Figs 6.1A, 6.2A). By six hours, all three adapted colonies had significantly chosen tomato irrespective of the species of the other plant (Figs 6.1-6.3). Examining the aphid choices at the six hours, between the different plant species they were adapted to and tomato, the cotton adapted colony had the highest numbers preferring tomato and the highest aphid participation (Fig 6.4; $P = 0.0022$). Taken together, these results indicate that even after successful adaptation to a new plant host, potato aphids continued to prefer their original host tomato.

In contrast to the choices performed with tomato, the different plant adapted colonies had much less of a host preference when tomato was not an option. The adapted host were screened against the other plants and two of the

three colonies tested had no preference between the plant they were adapted to and the other plant species tested after 24 hours (Figs 6.1, 6.2). The fava bean adapted colony however, when assayed with fava bean and Arabidopsis, significantly higher number of aphids chose fava beans (P <0.001) over Arabidopsis (Fig 6.3C). This difference in choice was detected as early as six hours (P = 0.0469) and it became more significant by 24 hours (P < 0.001) (Fig 6.3C). Interestingly, the cotton adapted colony had a significant preference to Arabidopsis over cotton at the 1-hour time point but this preference was no longer significant at 6 or 24 hours (Fig 6.1C).

Detection of aphid viruses

All three novel potato aphid viruses, MeV-1, MeV-2, and MeV-3 were originally identified and analyzed in the original tomato host colony of potato aphids (Teixeira et al., 2018; Teixeira et al., 2016). After the adaption of the potato aphids to the other host plants, the titer of these potato aphid viruses was examined in each colony through RT-PCR. Adult aphids and 4th stage nymphs were used to for these evaluations. There was variation in the titer levels of all three viruses within the four different colonies. There was no aphid colony that had the highest viral titers. However, the fava bean adapted colony had an overall lowest viral titer, displaying the lowest viral titers for both MeV-1 and MeV-2 and the same low level for MeV-3 as the Arabidopsis adapted colony (Fig 6.5). The other three aphid colonies had variable levels of viral titers.

The highest MeV-1 titer was found in the Arabidopsis adapted colony. The MeV-1 titer was significantly higher in the Arabidopsis adapted colony compared to both the cotton and fava bean adapted colonies (Fig 6.5A). However, the Arabidopsis adapted colony had the lowest viral titer for MeV-3 out of all the potato aphid colonies (Fig 6.5C). MeV-3 was the only virus the fava bean adapted colony did not have the lowest viral titer for. The cotton adapted colony was found to have the highest MeV-2 and MeV-3 titers (Figs 6.5B, 6.5C). In the original tomato colony, the viral titer was never the highest nor the lowest.

References

Birnbaum, S.S.L., and Abbot, P. (2018). Insect adaptations toward plant toxins in milkweed–herbivores systems – a review. *Entomol Exp Appl* 166(5)**,** 357-366.

Blackman, R.L., and Eastop, V.F. (2000). *Aphids on the world's crops.* New York, NY: John Wiley & Sons Inc.

Boulain, H., Legeai, F., Jaquiery, J., Guy, E., Morliere, S., Simon, J.C., et al. (2019). Differential Expression of Candidate Salivary Effector Genes in Pea Aphid Biotypes With Distinct Host Plant Specificity. *Front Plant Sci* 10**,** 1301.

CABI (2019). *Macrosiphum euphorbiae (potato aphid)* [Online]. CABI. Available: https://www.cabi.org/isc/datasheet/32154 [Accessed 12-9-20].

CABI (2020). *Myzus persicae (green peach aphid)* [Online]. CABI. Available: https://www.cabi.org/isc/datasheet/35642 [Accessed 12-9-20].

Chan, C.K., Forbes, A.R., and Raworth, D.A. (1991). *Aphid-transmitted viruses and their vectors of the world.* Ottawa: Research Branch, Agriculture Canada.

Eyres, I., Jaquiery, J., Sugio, A., Duvaux, L., Gharbi, K., Zhour, J.J., et al. (2016). Differential gene expression according to race and host plant in the pea aphid. *Mol Ecol* 25**,** 4197-4215.

Ferrari, J., Godfray, H.C.J., Faulconbridge, A.S., Prior, K., and Via, S. (2006). Population differentiation and genetic variation in host choice among pea aphids from eight host plant genera. *Evolution* 60**,** 1574-1584.

Ferrari, J., Via, S., and Godfray, H.C.J. (2008). Population differentiation and genetic variation inperformance on eight hosts in the pea aphid complex. *Evolution* 62**,** 2508-2524.

Forister, M.L., Dyer, L.A., Singer, M.S., Stireman, J.O., 3rd, and Lill, J.T. (2012). Revisiting the evolution of ecological specialization, with emphasis on insect-plant interactions. *Ecology* 93(5)**,** 981-991.

Frantz, A., Plantegenest, M., Mieuzet, L., and Simon, J.C. (2006). Ecological specialization correlates with genotypic differentiation in sympatric host-populations of the pea aphid. *J Evol Biol* 19**,** 392-401.

Futuyma, D.J., and Moreno, G. (1988). The Evolution of Ecological Specialization. *Annu Rev Ecol System* 19**,** 207-233.

Lu, H., Zhu, J., Yu, J., Chen, X., Kang, L., and Cui, F. (2019). A Symbiotic Virus Facilitates Aphid Adaptation to Host Plants by Suppressing Jasmonic Acid Responses. *Mol Plant Microbe Interact* 33(1)**,** 55-65.

Mathers, T.C., Chen, Y., Kaithakottil, G., Legeai, F., Mugford, S.T., Baa-Puyoulet, P., et al. (2017). Rapid transcriptional plasticity of duplicated gene clusters enables a clonally reproducing aphid to colonise diverse plant species. *Genome Biol* 18(1)**,** 27.

Miles, P.W. (1999). Aphid saliva. *Biol Rev* 74(1)**,** 41-85.

Peccoud, J., Maheo, F., de la Huerta, M., Laurence, C., and Simon, J.C. (2015). Genetic characterisation of new host-specialised biotypes and novel associations with bacterial symbionts in the pea aphid complex. *Insect Conserv Divers* 8(5)**,** 484-492.

Peccoud, J., Ollivier, A., Plantegenest, M., and Simon, J.C. (2009). A continuum of genetic divergence from sympatric host races to species in the pea aphid complex. *Proc Natl Acad Sci USA* 106**,** 7495-7500.

Peccoud, J., and Simon, J.C. (2010). The pea aphid complex as a model of ecological speciation. *Ecolog Entomol* 35(119-130).

Ryabov, E.V., Keane, G., Naish, N., Evered, C., and Winstanley, D. (2009). Densovirus induces winged morphs in asexual clones of the rosy apple aphid, *Dysaphis plantaginea*. *Proc Natl Acad Sci USA* 106**,** 8465-8470.

Sanchez-Arcos, C., Reichelt, M., Gershenzon, J., and Kunert, G. (2016). Modulation of Legume Defense Signaling Pathways by Native and Non-native Pea Aphid Clones. *Front Plant Sci* 7.

Sandström, J. (1994). High variation in host adaptation among clones of the pea aphid, *Acyrthosiphon pisum* on peas, *Pisum sativum*. *Entomol Exp Appl* 71**,** 245-256.

Sandström, J., and Pettersson, J. (1994). Amino acid composition of phloem sap and the relation to intraspecific variation in pea aphid (*Acyrthosiphon pisum*) performance. *J Insect Physiol* 40**,** 947-955.

Simon, J.C., d'Alencon, E., Guy, E., Jacquin-Joly, E., Jaquiery, J., Nouhaud, P., et al. (2015). Genomics of adaptation to host-plants in herbivorous insects. *Brief Funct Genomics* 14(6)**,** 413-423.

Teixeira, M.A., Sela, N., Atamian, H.S., Bao, E., Chaudhary, R., MacWilliams, J., et al. (2018). Sequence analysis of the potato aphid *Macrosiphum euphorbiae* transcriptome identified two new viruses. *PLoS One* 13(3)**,** e0193239.

Teixeira, M.A., Sela, N., Ng, J., Casteel, C.L., Peng, H.C., Bekal, S., et al. (2016). A novel virus from *Macrosiphum euphorbiae* with similarities to members of the family *Flaviviridae*. *J Gen Virol* 97**,** 1261-1271.

van der Wilk, F., Dullemans, A.M., Verbeek, M., and Van den Heuvel, J.F.J.M. (1997). Nucleotide sequence and genomic organization of *Acyrthosiphon pisum* virus. *Virology* 238**,** 353-362.

Will, T., Tjallingii, W.F., Thonnessen, A., and van Bel, A.J.E. (2007). Molecular sabotage of plant defense by aphid saliva. *Proc Natl Acad Sci USA* 104(25)**,** 10536-10541.

Xu, Y., and Gray, S.M. (2020). Aphids and their transmitted potato viruses: A continuous challenges in potato crops. *J Integrat Agric* 19(2)**,** 367-375.

Table 6.1. List of primers used.

Primer	Sequence	Reference
L27F	CCGAAAAGCTGTCATAATGA	Atamian et al. 2013
L27R	GGTGAAACCTTGTCTACTGTTACATCTT	Atamian et al. 2013
MeV-1F	CATTACCAGCGCTTCTGTCA	This Study
MeV-1R	ATGGCGACTAGCCTACGAGA	This study
MeV-2F	ATTGGGCCGTGAAACATTTG	This study
MeV-2R	AATAGGCGCAGAGATGGACG	Teixeira et al. 2018
MeV-3F	ACATTCTCATCCCCACCAAT	This study
MeV-3R	AGCCAATTTAGTACCATCACTACGT	Teixeira et al. 2018

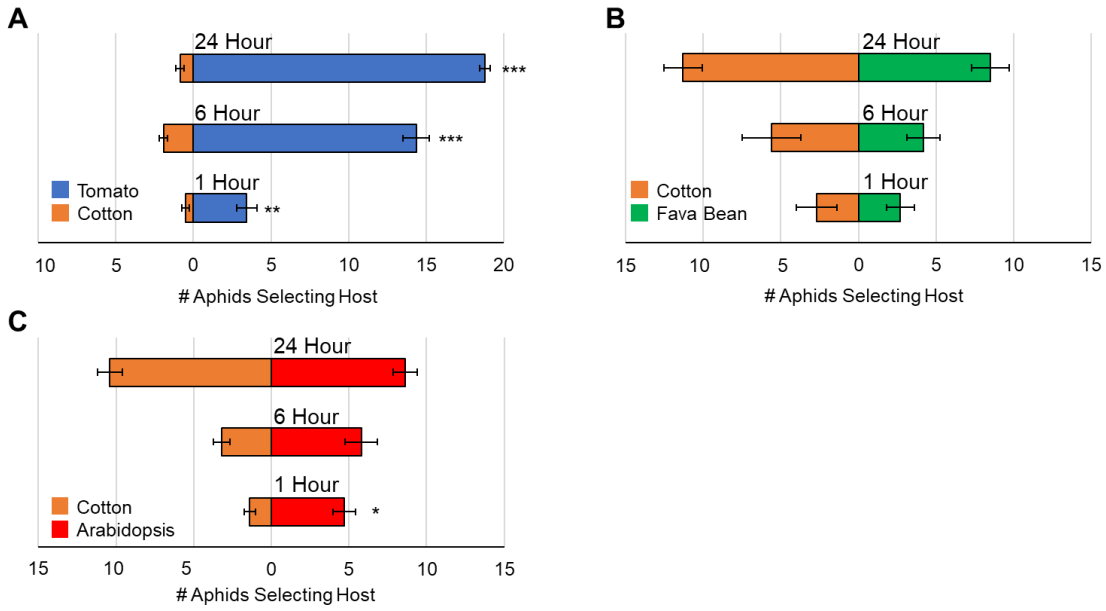

Fig 6.1. Choice assays of potato aphids adapted to cotton. Potato aphids were collected from a cotton adapted aphid colony and introduced into a large petri dish arena to choose between hosts. Twenty potato aphids (4th stage and adults) in each arena. **A)** Aphids chose between a cotton leaf and a tomato leaflet. Fourteen plant pairs were tested in 5 different experiments. **B)** Aphids chose between a cotton leaf and a fava bean leaf. Ten plant pairs were tested in 2 different experiments. **C)** Aphids chose between a cotton leaf and an Arabidopsis leaf. Ten plant pairs were tested in 2 different experiments. Graphs show the mean number of aphids on either host at the designated time points with error bars representing SE of the means. Statistical analysis was done using two-way ANOVA (genotype x time points), followed by Tukey HSD; asterisks indicate significance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

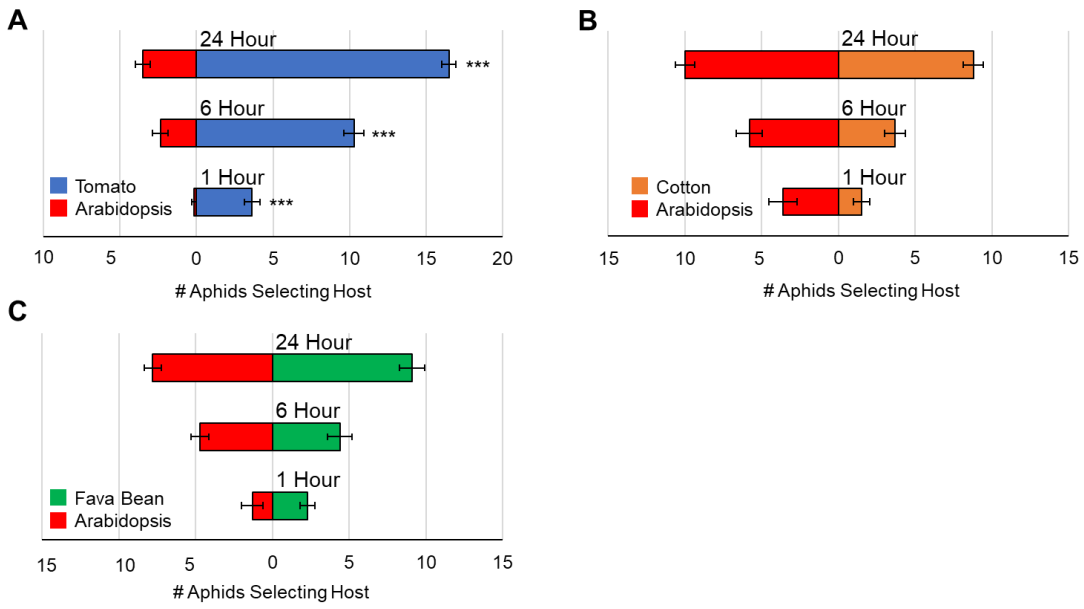

Fig 6.2. Choice assays of potato aphids adapted to Arabidopsis. Potato aphids were collected from an Arabidopsis adapted aphid colony and introduced into a large petri dish arena to choose between hosts. Twenty potato aphids (4th stage and adults) in each arena. **A)** Aphids chose between an Arabidopsis leaf and a tomato leaflet. Fourteen plant pairs were tested in 5 different experiments. **B)** Aphids chose between an Arabidopsis leaf and a cotton leaf. Ten plant pairs were tested in 2 different experiments. **C)** Aphids chose between an Arabidopsis leaf and a fava bean leaf. Ten plant pairs were tested in 2 different experiments. Graphs show the mean number of aphids on either host at the designated time points with error bars representing SE of the means. Statistical analysis was done using two-way ANOVA (genotype x time points), followed by Tukey HSD; asterisks indicate significance *** $p < 0.001$.

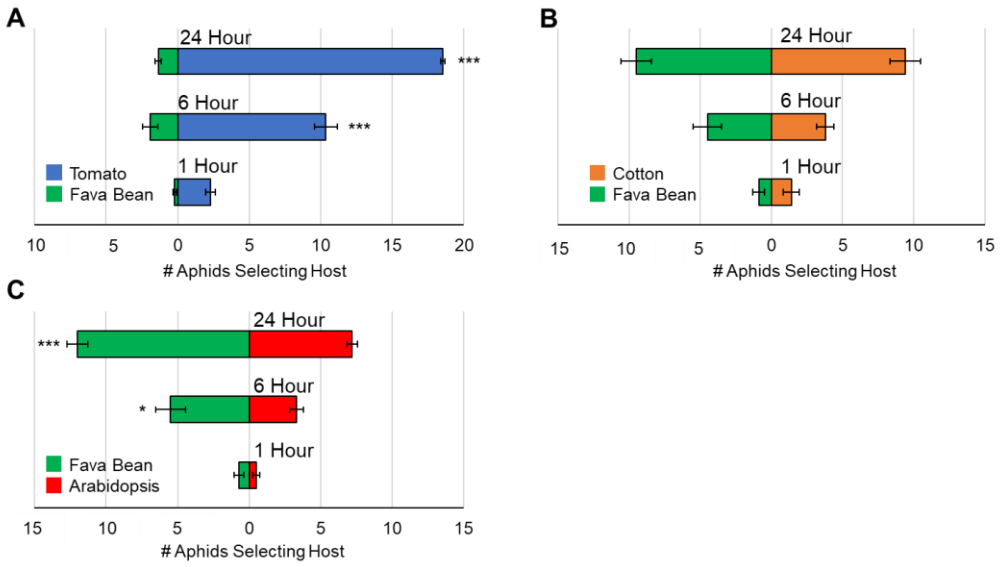

Fig 6.3. Choice assays of potato aphids adapted to fava bean. Potato aphids were collected from a fava bean adapted aphid colony and introduced into a large petri dish arena to choose between hosts. Twenty potato aphids (4th stage and adults) in each arena. **A)** Aphids chose between a fava bean leaf or tomato leaflet. Fourteen plant pairs were tested in 5 different experiments. **B)** Aphids chose between a fava bean leaf or cotton leaf. Ten plant pairs were tested in 2 different experiments. **C)** Aphids chose between a fava bean leaf or Arabidopsis leaf. Ten plant pairs were tested in 2 different experiments. Graphs show the mean number of aphids on either host at the designated time points with error bars representing SE of the means. Statistical analysis was done using two-way ANOVA (genotype x time points), followed by Tukey HSD; asterisks indicate significance *** $p < 0.001$.

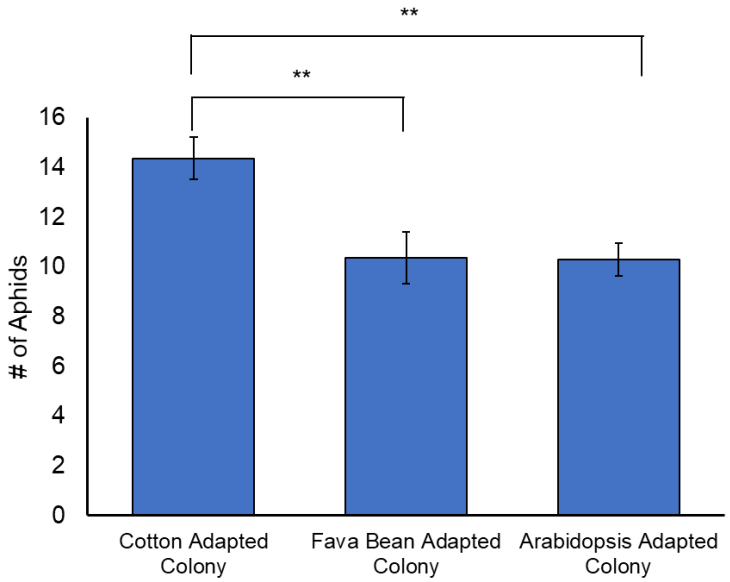

Fig 6.4. Aphids adapted to new hosts overwhelmingly chose their original host tomato. The 6-hour time points of each of the choice experiments with tomato as one of the hosts and the newly adapted host as the other host to choose from. Fourteen plant pairs were tested with 20 potato aphids (4^{th} stage and adults) each in 5 different experiments. Graphs show the mean number of aphids on either host at 6 h with error bars representing SE of the means. Statistical analysis was done using one-way ANOVA, followed by Tukey HSD; asterisks indicate significance ** $p < 0.01$.

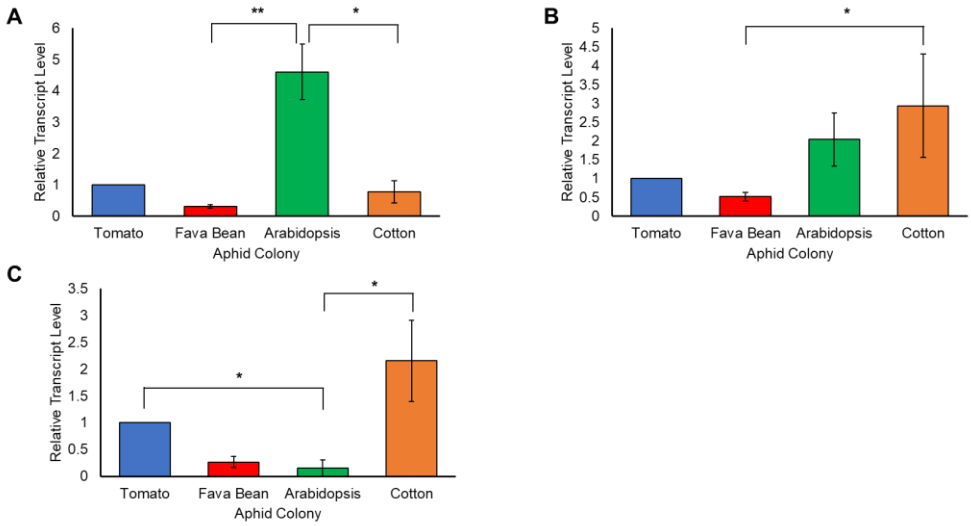

Fig 6.5. Potato aphid virus transcript levels in the potato aphid colonies adapted to different hosts. Ten potato aphids (4th stage or adult) were collected from each colony and the levels of **A)** MeV-1, **B)** MeV-2 and **C)** MeV-3 were quantified using the aphid ribosomal gene L27 and compared to the respective virus levels in aphids on the original host tomato. Error bars represent SE of the mean of three biological replicates with two technical replicates each. Statistical analysis was done with one-way ANOVA, followed by Tukey HSD; asterisks indicate significance * $p < 0.05$, ** $p < 0.01$.