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Game of 'Mones: Comprehending Bemisia tabaci MEAM1 Nymph-Based Resistance and Defense Phytohormone Signaling in Alfalfa

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ABSTRACT OF THE DISSERTATION

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Hemipteran insects are a significant threat to food security in California and worldwide; their piercing-sucking mouthparts make them difficult for host plants to perceive. Of these insect pests, the whitefly (*Bemisia tabaci*) is a cosmopolitan pest which stunts plant growth and development, vectors viruses, and also secretes honeydew which can result in sooty mold growth on host plants. Whiteflies are extant on all continents except Antarctica and climate change increases the propensity superabundant whitefly populations will be more common worldwide over time. Whiteflies are difficult to control as the release of natural enemies in crops has limited effectiveness and whiteflies circumvent the pesticides by rapidly adapting by developing insecticide resistance. For this reason, integrated pest management (IPM) programs centered around host plant resistance (HPR) may be the most effective means of controlling whiteflies. An effective, nymph-based whitefly-resistance mechanism was identified in alfalfa. While this mechanism may greatly inhibit whitefly population over generations, the polyploid nature

and limited genomic resources in alfalfa make elucidating this resistance a challenge. Here, we describe whitefly-resistance found among three alfalfa populations. Upon screening 84 individual lines from the three populations (two resistant and one susceptible), we concluded whitefly resistance was multigenic as a continuous spectrum of phenotypes from highly resistance to highly susceptible in each population. We identified several highly resistant (R1, R2 and R3) and susceptible lines of alfalfa (S1) for further studies. Through a series of experiments exploring *B. tabaci* MEAM1, MED1 and NW1 behaviors, we determined the whitefly-resistance displayed in R1, R2 and R3 lines were distinct and both antibiosis and antixenosis were detected for all three whitefly species. MEAM1 nymph mortality was displayed in all R lines, while the nymph mortality mechanisms did not impact MED whitefly. In addition, differences in host-choice, adult longevity and fecundity on R1, R2, R3 and S1 lines were whitefly-species specific. To gain insights into the mechanisms of resistance deployed in R1 plants vs S1 plants, *B. tabaci* MEAM1 whitefly-infestation time courses in R1 and S1 plants were performed. To understand the phytohormone underpinnings of alfalfa's defense response to whitefly, S1 alfalfa's response to salicylic acid (SA) and jasmonic acid (JA) treatments was also evaluated. *De novo* transcriptomic assembly of these libraries led us to postulate alfalfa's whitefly resistance mechanism is independent of SA, JA and abscisic acid (ABA) signaling and is ET-dependent. In addition, the downregulation of several pattern-triggered immunity receptors, suggests defense signaling in R1 plants is distinct for S1 plants and unique in the resistance responses reported to date in Hemipteran literature. In addition, R1 alfalfa have substantial difference in the expression of cutin, wax and suberin biosynthesis transcripts implicating the role of cuticle/cell wall alterations in R1's whitefly resistance. Analysis of phytohormone-response libraries led us to conclude

alfalfa's SA and JA responses are distinct from Arabidopsis as there was no evidence for reciprocal regulation of SA and JA responses and a substantial number of genes are responsive to both hormones. Finally, unlike the previously characterized basal immunity response Arabidopsis to whiteflies, where JA has an important role in deterring nymph development, and there is little correlation between alfalfa's whitefly response and SA- and/or JA-regulated genes. Collectively, these data provide the first insights into the alfalfa's mechanism of resistance to the global pest *Bemisia tabaci.*

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https://drive.google.com/drive/u/1/folders/0ACVLXbHIWKAyUk9PVA

Introduction

Insect pests have always been a challenge to mitigate in agricultural operations. At the core of any insect control operation is an integrated pest management (IPM) plan centered around host plant resistance (HPR) (Naranjo and Ellsworth 2009; Barzman et al. 2015; Stenberg 2017; Michel and Harris 2021). HPR is foundational to any IPM program, as it is less economically and environmentally taxing to growers (Naranjo and Ellsworth 2009; Barzman et al. 2015). One of the challenges with utilizing resistance (*R*) genes for insect control is the ability for an insect pest to evolve to avoid detection and activation of HPR (Kaloshian and Walling 2016). Therefore, durable resistance is often multigenic; relying on the additive effects of multiple loci, which is harder for a pest or pathogen to evade (Natukunda et al. 2021).

Hemipteran insects, such as whiteflies, aphids, mealybugs, psyllids, planthoppers, and leafhoppers, are sap-feeding insects that greatly diminish agricultural productivity (Kaloshian and Walling 2005). Four Hemipteran insects are rated in the top ten pests currently devastating global agriculture including: whiteflies (*Bemisia tabaci*), the green peach aphid *(Myzus persicae), the cotton aphid (Aphis gossypii), and the brown planthopper (Nilaparvata lugens) (*https://www.kew.org/read-and-watch/insect-pests-biggest-threat-plants). These insects use modified mouth parts called stylets to consume phloem. Depending on their feeding mechanism, Hemipteran stylets can puncture cells indiscriminately, puncture mesophyll cells along their way to phloem, or weave between host plant cells to minimize cellular damage and avoid deployment of host plant defenses. In addition, Hemipteran insects secrete small molecules called effectors in their saliva to suppress host plant defenses to the herbivore's benefit (Kaloshian and Walling 2016; Huang et al. 2021). Integrated pest management is an

environmentally friendly means of managing insect pests and pathogens (Onstad 2019; Stenberg 2017). Host plant resistance is the most sustainable way to manage agricultural pests (Smith and Clement 2012; Walling and Thompson 2013), however, to date there are few Hemipteran resistance genes isolated and characterized at the molecular level (*Mi-1.2*, *Bph2/3/6/9/14/17/18/29/32*, and *Vat*). Recently, a potent source of whitefly resistance was identified in the legume alfalfa (*Medicago sativa*) (Jiang et al. 2003; Teuber et al. 1997; Jiang and Walker 2007).

In this Dissertation, I focus on identifying and characterizing alfalfa's whitefly resistance mechanism. Based on transcriptome analyses of highly resistant and highly susceptible alfalfa lines, my dissertation has revealed that alfalfa's resistance to whiteflies impacts many levels of plant immunity. Comparisons of resistant and susceptible plants show differences in physical barriers (ie., cell walls and the cuticle), differential activation of phytohormone-regulated defense pathways, and distinctions in pattern triggered immunity (PTI) and basal immunity. To provide context, I provide an introduction to *Bemisia tabaci* (the whitefly) and then I overview of plant immunity emphasizing hemipteran insect-plant interactions. I begin with a description of plant physical and chemical defenses, and the roles of the major defense phytohormones associated with immunity – salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), and ethylene (ET). I also review what is known about Hemipteran resistance genes and more specifically host plant resistance to whiteflies. I then introduce the powerful whitefly resistance that has been identified in alfalfa, as well as some of the challenges of working in alfalfa. Finally, I will close with the objectives of my Dissertation.

Bemisia tabaci – a global pest

Whiteflies (Aleyrodidae) are Hemipteran insects that use piercing-sucking mouthparts to consume phloem sap. Whiteflies received their name because the adults are covered in a fine, powdery white wax (Hodges and Evans 2005). There are currently 1500 species of whitefly known and only 150 (10%) are found in the United Stated (Hodges and Evans 2005). Among these species, several are agricultural pests including the greenhouse whitefly (*Trialeurodes vaporariorum*), the citrus whitefly (*Dialeurodes citri*), cloudy-winged whitefly (*Singhiella citrifolii*), woolly whitefly (*Aleurothrixus floccosus*), rugose spiraling whitefly (*Aleurodicus rugioperculatus* Martin) and several species of *Bemisia tabaci* (Hodges and Evans 2005; Stocks and Hodges 2012).

B. tabaci is among the most economically devastating insect pests in modern agriculture and are commonly found in tropic and subtropic regions (Mound and Halsey 1978). The speciation of *Bemisia tabaci* has undergone several changes to nomenclature (De Barro et al. 2011). *Bemisia tabaci* were originally referred to as biotypes, or host-plant-related races (Bird et al. 1957). This concept was originally based on the biological differences of host utilization between sympatric populations (Bird et al. 1957). This definition of *Bemisia tabaci* biotype evolved to include allopatric populations (Bethke et al. 1991). As more biotypes were designated, it was unknown if there were distinguishable traits that could characterize each biotype. Observations of physiological changes to the host plant, dispersal capabilities, and the propensity to develop insecticide resistance were not sufficient in distinguishing between biotypes (Bedford et al. 1994; Brown et al. 1995). Several advances in genetic tools in *Bemisia tabaci* genomics showed genetic differences between biotypes, but mtCO1 sequencing was the

most significant resource developed (Lee et al. 2013). The sequencing and comparison of *Bemisia tabaci* mtCO1 sequences pointed to the species being a cryptic species complex of unique, yet morphologically indistinguishable species (Boykin et al. 2013; De Barro et al. 2011). Today, there are currently 37 identified species which comprise the *Bemisia tabaci* cryptic species complex of unique, yet morphologically indistinguishable species (Wang et al. 2019a).

Whiteflies are a multivoltine fecund insects whose feeding, migratory, and reproductive behaviors enable it to have a significant impact on modern agriculture globally (Stansly 2010; Butler and Henneberry 1989; Legg et al. 2014; Maruthi et al. 2017). Whiteflies share properties with other Hemipteran insects including piercing-sucking mouthparts, reduced hind wings, and incomplete metamorphosis (Walker et al. 2010). Female whiteflies can lay 60 to 300 eggs on the abaxial side of their plant host through their lifetime (30 - 40 days) (Stansly 2010; Naranjo 2004). Eggs are supported on a small pedicel (stalk) that penetrates the epidermal cells of the host (Walker et al. 2010; Buckner et al. 2002). In addition to providing support for the egg, the pedicel also transports water and some solutes to the egg (Walker et al. 2010; Byrne et al. 1990). Within the egg, the developing embryo contains a bacteriome that harbors primary and secondary endosymbionts. The primary endosymbiont is *Candidatus* Portiera aleyrodidarumin (Baumann et al. 2006). The secondary endosymbiont complement within each of the different *B. tabaci* species varies and the most common secondary endosymbionts include: *Hamiltonella, Rickettsia, Wolbachia, Arsenophonus, Cardinium, Fritchea,* and *Hemipteriphilus* (Andreason et al. 2020). The primary endosymbionts are responsible for providing nutrients, vitamins and minerals while the secondary

endosymbionts have roles in host adaptation and predator/pesticide resistance (Hedges et al. 2008; Scarborough et al. 2005; Oliver et al. 2012; Wang et al. 2019a)

The fecund nature of whiteflies is beneficial as insect eggs are prone to predation and dislodgement (Naranjo 2004). The nymphs that emerge undergo four phases of metamorphosis: first (0.3 mm), second (0.4 mm), third (0.5 mm), and fourth (0.7 mm) instars. The first instar is the only mobile nymph form, therefore finding a desirable feeding site for subsequent phases is critical. Often called crawlers, the first-instar nymphs find a location on the abaxial leaf side that is near either a major or minor vein. While nymphs prefer minor veins, they are capable of feeding on other sites as long as the phloem can be reached by their stylets (Walker et al. 2010). The first instar molts into the second instar within two to three days, depending on the temperature (McAuslane 2000). The metamorphosis process continues for the subsequent instars and in largely a temperature-dependent process. In cotton, it takes 17 days for a whitefly to develop from egg to adult (Butler and Henneberry 1989) The final immature stage is the fourth instar. Towards the end of the fourth instars, nymphs develop into pseudo-pupa, which is hallmarked by the development of large red-brown eyes (also known as the “red eye stage”) (Gelman et al. 2002). The fourth instar and egg stage have the highest levels of mortality among all whitefly development stages (Naranjo 2004; Stansly 2010). As adults, *Bemisia tabaci* are haplo-diploid with male offspring being haploid (1N) and female offspring being diploid (2N)(Byrne et al. 1996). Female whiteflies are also larger and live longer than male whiteflies (Gerling et al. 1986). Upon emergence, adults can migrate up to 150 m and begin feeding on their new host and producing progeny to continue infestations(Ludwig et al. 2019) (Byrne 1999).

As mentioned previously, whiteflies use their feeding mouthparts (the stylet bundle) to consume plant sap (Walker et al. 2010). Whiteflies move their stylets using a combination of muscle and head movements (Walker and Perring 1994). Whiteflies secrete two types of saliva to counteract the host plant's ability to perceive whiteflies. These salvias, the sheath and watery saliva, are made by the primary and accessory salivary glands, respectively (Walker and Perring 1994). Whitefly feeding is initiated by the release of sheath saliva on the chosen feeding site and the stylets pushing through the sheath saliva to penetrate the epidermal cells (Walker and Perring 1994; Buckner et al. 2002). Form this point, the stylets weave between cells using an intracellular path that seldom ruptures adjacent cells (Byrne and Bellows 1991). The feeding whitefly continues to release sheath saliva in small increments so the entire length of the stylet is protected by sheath saliva (Walker and Perring 1994). Once the stylet reaches the phloem, watery saliva is quickly released to prevent the sealing response, spread viruses, and for chemosensory evaluation of vascular tissue (Walker and Perring 1994). Whiteflies primarily feed on the phloem but may consume xylem contents in events of dehydration (Stansly 2010).

Like other phloem-feeding insects, the voracious feeding of whiteflies compromises plant growth due to depletion of the phloem and its C and N resources. After feeding, whiteflies use their vasiform orifice to secrete a sugar-rich honeydew secretion that can impair photosynthesis and the aesthetic appeal of crop. In addition, whiteflies pose additional unique challenges to plant hosts (Stansly 2010). Whiteflies can also impair their plant host through virus vectoring. *B. tabaci* species predominately vector begomoviruses to their hosts, although they can also transit crinivriuses, ipomoviruses, torradoviruses, and carlaviruses. (Navas-Castillo et al. 2011; Maruthi et al. 2017; Polston

and Capobianco 2013; Pan et al. 2012; Colvin et al. 2004). One species, *Bemisia tabaci* MEAM1(Byrne et al. 1996), is known to vector hundreds of viruses, a phenomenon not seen in other whiteflies (Navas-Castillo et al. 2011). *B. tabaci* has a host range of over 500 plant species with a particular preference for Malvaceae, Cucurbitaceae, and Euphorbiaceae (Malka et al. 2018). This wide host range is exploited by whiteflies throughout the year as they can move from host to host as seasons progress.

Whiteflies are hard to control. In addition to their ability to move between hosts, whiteflies feed on the abaxial side of the leaf, making it easier to evade pesticides. They additionally have the ability to develop resistance to insecticides (Stansly 2010; Naranjo and Ellsworth 2009). Biocontrol by natural enemies, such as predators and parasitoids, are effective for whitefly control the controlled environment of greenhouses. However, in the field success stories for whitefly management using natural enemies is limited to those crop systems where the principles of IPM are fully embraced (Naranjo and Ellsworth 2009; Wang et al. 2019a). Finally, as advances in the understanding of whitefly endosymbionts contributions to whitefly success and adaptation to environmental stress are rapidly advancing (Milenovic et al. 2022), it has been proposed that engineering endosymbiont genomes may provide new methods for whitefly control. Host plant resistance to whiteflies has been discovered, but limited deployment has occurred to date (see Introduction Section 7).

Among the ~ 37 unique *B. tabaci* species, there are at least two known to be invasive: *Bemisia tabaci* Middle East Asia Minor 1 (MEAM1) and Mediterranean 1 (MED1). *Bemisia tabaci* New World 1 is the native whitefly of the New World and while there is evidence it was a formidable pest, *B. tabaci* MEAM1 had a much greater impact on North American agriculture (Stansly 2010). *Bemisia tabaci* MEAM1 was first identified

on poinsettia crops in Florida and again identified throughout the southwestern US in the 1980s (Stansly 2010; Costa and Brown 1991; Cohen et al. 1992). Agricultural operators noticed a new “silvering” phenomenon on leaves that caused considerable physiological damage to the host not associated with *Bemisia tabaci* NW1 (Costa and Brown 1991; Cohen et al. 1992; Brown et al. 1995). Researchers then began to propose a new whitefly species had established itself in the US. Further genetic analyses confirmed their hypothesis and over time this invasive species overtook the native NW1 species becoming the dominant species in the US (Costa et al. 1993). Several factors make MEAM1 a more challenging pest to manage than NW1 including a larger host range, better migratory abilities the ability to develop insecticide resistance (Prabhaker et al. 1985; Costa et al. 1993; Cahill et al. 1994; Horowitz and Ishaaya 2014). While not currently identified in fields in the US, *Bemisia tabaci* MED1 has been identified in greenhouses and poses a threat to US agriculture (Hodges and McKenzie 2008; Dennehy et al. 2010; McKenzie et al. 2012; Horowitz and Ishaaya 2014). *Bemisia tabaci* MED1, like *Bemisia tabaci* MEAM1, is more likely to develop insecticide resistance and has a larger host range than *Bemisia tabaci* NW1 (McKenzie et al. 2012). As a consequence of their invasiveness, MEAM1 whiteflies are an ever increasing threat to California and US agriculture: *Bemisia tabaci* has been estimated to cause in excess of $1B in damages in the US since 1991 (Paine and Hoddle 2022).

Plant Immunity

All life forms are challenged by a plethora of biotic invaders and abiotic stresses (e.g., temperature, etc.). Some core principles of immunity are shared in plants and animals, while other immunity strategies are unique (Taylor 1998; Haney et al. 2014; Király et al. 2013). The first strategy for evasion of attackers is mobility. Readily realized

in animals who swim, fly, slither, crawl, or walk, plants do not have the capacity to move. For this reason, plants have evolved different mechanisms to combat pathogen and pest attack. The following sections will discuss the arsenal of defense mechanisms available to plants.

Physical, Constitutive and Induced Defenses

Upon arrival of a pest or pathogen on a host plant, the plant relies on robust physical barriers and a finely tuned sensing and signaling machinery to limit pathogen/pest damage. Two layers of defense are encountered: constitutive defenses and induced defenses (Walling 2000) (War et al. 2012). Constitutive defenses are present continuously and serve as a primary defense layer, while induced defenses are those triggered by a pest or pathogen.

Plant constitutive defenses can be classified as either physical or chemical and myriad modalities are available. These defenses can impact movement, feeding, development, and reproduction of herbivore pests. Constitutive physical plant defenses are the first line of defense against phytopathogens. These physical defenses are often manifested as “structural defense” for a plant host and refer to “any morphological or anatomical trait that confers a fitness advantage to the plant by directly deterring herbivores from feeding on it” (Hanley et al. 2007). Because these traits are inherent and don’t require diversion of resources from growth or development to defense, constitutive physical defenses are an invaluable resource to all plant families. A select number of physical defenses that deter herbivores (plant cuticle, cell wall, and trichomes) are highlighted below.

Plant Cuticle

The plant cuticle is a biochemically complex and multifunctional barrier that provides protection from xenobiotics, drought, extreme temperatures, UV radiation, mechanical injuries, pathogen infection, and insect/pathogen attack (Ziv et al. 2018; Serrano et al. 2014; Domínguez et al. 2017). At the epidermal surface of aerial plant organs (leaves, stems, flowers, and fruit), the cuticle is ostensibly the first physical barrier an herbivore encounters (Ziv et al. 2018). The cuticle has two distinct layers, the inner and outer cuticular layers. Both layers contain cutin, which is composed of esterified ω- and mid-chain hydroxy and epoxy C16 and C18 fatty acids (Serrano et al. 2014; Heredia 2003). The inner cuticular layer interacts with the epidermal cell walls and is composed of cutin and polysaccharides. The outermost layer of the cuticle, also called the cuticle proper, is structurally diverse, but is predominately comprised of cutin, as well as intracuticular and epicuticular waxes. A majority of the genes that control the biosynthesis of cutin and its waxes are known (Fich et al. 2016; Lee and Suh 2013; Suh et al. 2005). In addition, advances in understanding the enzymes that modify, proteins that transport, and transcription factors that control these genes are being made (Yeats and Rose 2013). Plant cuticles also store volatile and non-volatile secondary metabolites, including flavonoids and triterpenoids, with known antibiotic and antixenotic roles in defense against pathogens and insects (Zacchino et al. 2017; Arif et al. 2009; Ziv et al. 2018; Powell et al. 1999; Simmonds 2001). As might be anticipated, while the core components are shared, plant cuticles are often plant species specific. In plant-insect interactions the cuticle provides a “slippery” surface, necessitating herbivores to use tarsi to adhere to waxy plant surfaces (Friedemann et al. 2015; Gorb and Gorb 2017; Gaume et al. 2004); these waxes adhere to the tarsal pads of insect feet and the

waxes must be dislodged to regain traction on surfaces. The cuticle's slippery surface also enables pitfall carnivorous plants to capture their prey (Gaume et al. 2004).

Recent studies suggest that plant DAMP (damage-associated molecular pattern) receptors sense plant cuticle damage. Non-intuitively, *Arabidopsis* and maize mutants that increase cuticle permeability display resistance to necrotrophic pathogens either via DAMP recognition or detection of cuticle monomers (Serrano et al. 2014). Increases in reactive oxygen species also occur with increases in cuticle permeability (Ziv et al. 2018; Serrano et al. 2014). This increased cuticle permeability enhances plant defenses; hormone changes linked to increased cuticle permeability/enhanced defenses included increases in nitric oxide and ethylene levels and abscisic acid deficiency (Curvers et al. 2010; Romero and Lafuente 2022; L'Haridon et al. 2011; León et al. 2016). *Arabidopsis* lines that increase the quantities of very long-chain-n alkanes of the cuticle, decrease cuticle permeability and are more resistant to water stress (Bourdenx et al. 2011).

Cell Walls

Plant cell walls are comprised of a network of cellulose microfibrils cross-linked with hemicellulose are the second physical barrier encountered by pests and pathogens (Malinovsky et al. 2014). Plant cell walls have two layers: a primary and a secondary cell wall. The primary cell wall's cellulose network is entwined in a matrix of pectic polysaccharides. In contrast, the secondary cell wall has less pectin and is fortified with lignin. Not all plant cells have secondary cell walls; vascular tissue (phloem and xylem) and specific tissues with roles that might require additional structural fortification typically possess them (Zhong and Ye 2014). While there is diversity in the three-dimensional structure of cell walls, perception of cell wall damage as a initiator of defense cascades

is shared among plants (Popper et al. 2011; Fangel et al. 2012; Hou et al. 2019). Pathogens possess evolved evasive measures against the cell wall, as the breaching of cell wall integrity is vital for phytopathogens to colonize their hosts (Malinovsky et al. 2014; Underwood 2012). Plant hosts have developed a number of responses to counteract this form of pathogenicity.

Best characterized in plant-pathogen interactions, the major cell wall structural components play active roles in defense. Cellulose is a β-1,4-glucose polymer critical for cell structure (Malinovsky et al. 2014). Cellulose polymers assemble into microfribils that are densely-packed to make cellulose more resistant to cell-wall degrading enzymes introduced by pathogens and pests. Examples include *Clostridium thermocellum* and *Fusarium graminearum* that secrete cellulases that are induced upon infection (Van Vu et al. 2012; Zhang et al. 2016; Artzi et al. 2017; Kesten et al. 2017). Some phloem-feeding insects secrete cellulases in their saliva (Adams and Drew 1965) and tissue-damaging herbivores that consume leaves digest plant cell walls by the gut cellulases, often provided by microbes within their guts (Martin 1983).

Cellulose is synthesized by the catalytic subunits of cellulose synthase terminal complexes (CESAs) and mutations in CESAs have been linked to tolerance to abiotic and biotic stress. Defects in CESAs involved in primary (*CESA3*) and secondary (*CESA4/7/8*) cell wall formation result in tolerance to osmotic stress and resistance to powdery mildew and necrotrophs (*Plestosphaerella ccucumerina* and *Ralstonia solanacearum*), respectively (Chen et al. 2005; Hernández-Blanco et al. 2007; Ellis and Turner 2001). Mutations in the CESA mutant *lew2/cesa8* increase ABA and carbohydrate levels (Chen et al. 2005; Hernández-Blanco et al. 2007). Surprisingly, a literature search indicates that this is an unexplored area in plant insect interactions.

Hemicellulose is a major cell wall component known to interact with cellulose in the secondary cell wall. Hemicelluloses are a large class of polysaccharides that have β-(1→4)-linked backbones including xyloglucans, xylans, mannans, and glucomannans (Scheller and Ulvskov 2010). Xylans are the most common hemicellulose class in the secondary cell wall and some pathogenic microbes are capable of degrading hemicellulose with their secreted xylanases (Malinovsky et al. 2014).

Pectin is a major component of the primary cell wall with roles in defense. Pectin is a polysaccharide comprised of structurally distinct domains of either homogalacturonan or rhamnogalacturonan. Some pectin fragments, known as oligologalacturonorides (OGAs), are DAMPs that are perceived by plant hosts to trigger wound signaling and initiate subsequent defense mechanisms (Ridley et al. 2001; Côté and Hahn 1994; De Lorenzo and Ferrari 2002; De Lorenzo et al. 2001). For example, the defenses activated in *Arabidopsis* after the perception of hemicellulose degradation has been elucidated (Claverie et al. 2018; Malinovsky et al. 2014). WAKs are known as a detector of OGAs and longer pectin fragments released during microbe attack (Kohorn et al. 2014), as well as wounding and insect feeding. Upon detection of these pectin fragments, WAKs function as a signaling hub for the OGA-responsive defense pathways (Rui and Dinneny 2020; Yang et al. 2019c; Saintenac et al. 2018; Rosli et al. 2013; Amsbury 2020). Few studies have explored how changes in pectin composition impact feeding by sap-feeding insects. However, aphids are known to feed better on pectin methylesterase mutants of Arabidopsis (Kloth et al. 2019; Silva-Sanzana et al. 2019).

Lignin is a complex polyphenolic polymer that is a component of plant secondary cell walls (Liu et al. 2018). Lignin fortifies the cell walls of cells associated with the phloem and xylem, but not the vascular tissue itself. Lignin monomers are synthesized

from phenylalanine/tyrosine in the cytosol and modified by deamination, hydroxylation, methylation and reduction (Liu et al. 2018). Three types of lignin monomers are transported to the apoplast and to assemble the lignin polymer including: sinapyl alcohol (S unit), coniferyl alcohol (G unit) and p-coumaryl alcohol (H unit). The monolignols are polymerized by peroxidase (POD) and laccase (LAC) in secondary cell wall. Lignin composition and quantity is dynamic and responds to both biotic and abiotic stress. Lignin has a critical role in plant structure, growth and development and is a critically important barrier that protects plant organs from pathogens and pests (Moura et al. 2010).

Lignification physically deters some pathogens from establishing a presence on a host and can also be induced upon Hemipteran infestation of host plants (Bhuiyan et al. 2009; Lee et al. 2019). *PAL, C4H* and *PR9,* which are important in lignin biosynthesis, are induced in rice upon brown planthopper resistance (*Nilaparvata lugens* (Stål)) and *CmMYB15/19*, which are transcription factors important in activation of lignin biosynthesis genes, are induced in aphid-infested chrysanthemum (Duan et al. 2014; Jannoey et al. 2017; An et al. 2019; Wang et al. 2017). Lignin can also be induced by the antimicrobial molecule sclareol and the insect peptide LqhIT2 to enhance root-knot nematode resistance in *Arabidopsis* and to leafroller resistance in rice, respectively (Fujimoto et al. 2015; Tianpei et al. 2015). Lignin is also a major component of other crop defense responses to pathogens including cassava's response to whitefly. Metabolomic analysis of resistant and susceptible cassava shows higher levels of lignin accumulation in resistant varieties suggesting that cell wall fortification is critical in resistance to whiteflies (Perez-Fons et al. 2019; Garceau 2021). In alfalfa, the lignin biosynthetic pathways and biotic/abiotic stress tolerance has been investigated (Gallego-

Giraldo et al. (2011). Downregulation of alfalfa's hydroxycinnamoyl COA: shikimate hydroxycinnamyl transferase reduced lignin levels, but elevated salicylic acid, jasmonic acid, and abscisic acid levels along with enhancing tolerance to abiotic and biotic stress, including fungal infection.

Trichomes

Trichomes are hair-like appendages found on the organs of higher plants (Peter et al. 1995). While not all plants or plant organs produce trichomes, they have important defensive and protective roles when present. There is a wide array of structural and chemical diversity in trichomes. They are important for defense against pathogens and pests and protection from abiotic stresses (UV-radiation, drought, heavy metal accumulation) (Peter et al. 1995; Dalin et al. 2008b; Gao et al. 2021; Galdon-Armero et al. 2018; Skaltsa et al. 1994). Trichomes are either unicellular or multicellular structures and grouped into two major classes: glandular and non-glandular trichomes based on shape and chemical composition. The appearance of trichomes is developmentally programmed in plants and several glabrous genes important for trichome production have been identified in *Arabidopsis* and cucumber (Marks et al. 2009; Cui et al. 2016).

Glandular trichomes produce, store, and secrete large quantities of secondary metabolites (such as terpenoids, methyl-ketones, acyl-sugars, and phenolics), which vary within and between plant genera. These trichomes are generally associated with defense to pests and pathogens (Dalin et al. 2008a). Plant hosts will often respond to herbivory with increased trichome production (Traw and Bergelson 2003; Dalin et al. 2008b). In contrast, non-glandular trichomes lack a secretory mechanism but some are known to store large quantities of specialized metabolites including phenolics

(Karabourniotis et al. 2020). The role of trichomes in the whitefly resistance of tomato will be discussed in a later section.

Secondary Metabolism

Secondary metabolites, also referred to as specialized metabolites, can be classified as any metabolite a plant synthesizes that is non-essential for life; often these chemicals are involved in plant/non-plant communication and stress responses (Moghe and Last 2015). Many of these metabolites are associated with defense to pathogens and pests. Secondary metabolites are broken into two classes: phytoanticipins and phytoalexins (Piasecka et al. 2015). Phytoanticipins are present before pathogen/pest attack. These metabolites are often present within the cuticle, trichomes or vacuoles (Tiku 2018). They are the first-line chemical defense against attackers and are often associated with antixenosis in herbivores (VanEtten and Bateman 1971).

Phytoalexins are secondary metabolites that are induced upon attack (Jeandet 2015). These molecules can function as antimicrobial compounds or can modulate defense signaling pathways (Piasecka et al. 2015). There are numerous secondary metabolites classified as phytoalexins, and often their production is dependent on complex biochemical pathways involving a suite of genes; in some cases, there is functional redundancy and gene families are involved in these pathways to increase the diversity of related bioactive chemicals. Several important secondary metabolites with known roles in plant defense to herbivores will be highlighted below. For comprehensive reviews on this topic, see Moghe and Last (2015), Piasecka et al. (2015), and Tiku (2018).

Cyanogenic Glucosides

Cyanogenic glucosides (CGN) are among the most lethal plant secondary metabolites. These secondary metabolites are found in over 2000 plant species and are often vacuole-localized (Gleadow and Møller 2014). CGNs are catabolized to release hydrogen cyanide (HCN), a universal respiratory poison, upon tissue disruption. β-glucosidases and α-hydroxynitrile lyases hydrolyze CGN. Depending on the plant species, β-glucosidases are stored either in the chloroplast or apoplast (Ketudat Cairns et al. 2015), while α-hydroxynitrile lyases function in protein bodies, respectively (Hickel et al. 1996). Upon cellular damage, CGNs and β-glucosidases or α-hydroxynitrile lyases occupy the same cellular space to release HCN (Zagrobelny et al. 2004). CGNs are a feeding deterrent to insects as HCN inhibits respiration and certain enzymes (Kassim and Rumbold 2014; Morant et al. 2008). There are several factors impacting the effectiveness of CGNs on insect herbivory including the insect's threshold for toxicity, generalist/specialist species status, whether or not the insect's diet dilutes CGN levels, or if the insect pest does minimal damage during feeding (i.e; phloem feeders) (Gleadow and Møller 2014; Zagrobelny et al. 2004).

While CGN and subsequent HCN can be effective insect deterrents, they are also toxic to humans. Cyanide poisoning from cassava remains a public health problem in Africa, hence low CN cassava ("sweet cassava") genotypes have been developed (Alitubeera et al. 2019). HCN is also found in tropical legumes and lima bean. In both crops, proper cooking is an effective way of reducing HCN levels to acceptable levels (Okolie and Ugochukwu 1989; Akpapunam 1985). There is also evidence of alfalfa producing HCN that can be toxic to livestock (Majak et al. 1990).

Glucosinolates

Glucosinolates are a distinct class of secondary metabolites found among the *Brassicaceae* family and a small number of other plants (Barba et al. 2016). Glucosinolates are derived from the amino acids alanine, valine/leucine and isoleucine, methionine, phenylalanine/tyrosine, tryptophan, and possibly glutamate to synthesize aliphatic, indole, and aromatic glucosinolates (Ishida et al. 2014). Each amino acid is decorated with side chains allowing the synthesis of over 120 glucosinolates. Studies in the model plant *Arabidopsis thaliana* have elucidated the mechanisms of glucosinolate biosynthesis, transport and catabolism (Wittstock and Burow 2010; Wittstock and Halkier 2002). Furthermore, use of mutants that fail to produce different classes of glucosinolates have allowed an understanding of their role in defense to pathogens and pests.

Like cyanogenic compounds, the glucosinolates and their thioglucosidases (myrosinases) that hydrolyze glucosinolates to their more active form are stored in different cells. Therefore, glucosinolates are relatively stable in unperturbed plants. After mechanical damage or herbivory, cellular contents mix and the aglycone glucosinolate is released to produce bioactive isothiocyanates (ITC) (Ishida et al. 2014; Rask et al. 2000; Bones and Rossiter 2006; Bones and Rossiter 1996; Baenas et al. 2020). While glucosinolates repel most insects, some insects have adapted to plants with high glucosinolate levels (Hopkins et al. 2008). These specialist insects survive through either enzymatic detoxification, excretion, or sequestration of glucosinolates and/or behavioral adaptations (Mainguet et al. 2000; Hopkins et al. 2008).

Phenolics

Phenolics are among the most common secondary metabolites found in plants and are derived from either the shikimate or the phenylpropanoid pathway (Marchiosi et al. 2020; Dai and Mumper 2010). Phenolic compounds have at least one hydroxyl group attached to an aromatic ring. Phenolics are used as defense mechanisms against herbivores, microorganisms and other competing plant species (War et al. 2012). Phenolic compounds impair herbivore success by physical deterrence (lignin biosynthesis) (Bhonwong et al. 2009; Barakat et al. 2010). In addition to their roles in defense, phenolics contribute to structure and development in their plant hosts as interconnectors of cell wall polysaccharides and lignin anchoring (Marchiosi et al. 2020). Among the most common phenolics with roles in plant defense are lignin, flavonoids, tannins, and phenolic acids.

Flavonoids are among the most abundant secondary metabolites in plants (Panche et al. 2016). Among the over 4000 flavonoid compounds identified, they share the same general structure with a 15-carbon skeleton composed of two aromatic rings connected by a three-carbon bridge (Kulbat 2016; Kumar et al. 2020). Flavonoids can be classified as flavonoids/bioflavonoids, isoflavones, or neoflavonoids based on the degree of saturation and oxidation of the central carbon ring (Gutiérrez-Lomelí et al. 2012). Flavonoids can also be further divided into one of several subclasses: anthoxanthins, anthocyanidins, chalcones, flavanidols, flavans, pyroanthocyanidins, flavones, flavanols, and tannins (Panche et al. 2016). Flavonoids partake in numerous plant functions including floral pigmentation, aiding in symbiotic prokaryotic relationships, antioxidant activity and protection against abiotic and biotic stressors including UV radiation and insect herbivory. Several instances of flavonoids inhibiting plant pathogens have been

identified including groundnut (*Arachis hypogaea*)-derived quecetin contributing to tobacco armyworm (*Spodoptera litura*) larval mortality, maysin in transgenic maize inhibiting corn earworm (*Helicoverpa zea*) larvae, and upregulation of several isoflavone and isoflavanone biosynthesis genes in *Medicago truncatula* in response to *Pseudomonas syringae* (Mallikarjuna et al. 2004; Johnson et al. 2007; Samac and Graham 2007). Flavonoids have also been linked to conferring resistance to multiple species of Hemipteran insects including aphids, red- (*Piezodorus guildinii*) and brown-banded stink (*Euschistus heros*) bug in soybeans (Lattanzio et al. 2000; Bentivenha et al. 2018; Michereff et al. 2019). Flavonoids are also quite abundant among many *Fabaceae* family members, including alfalfa (Wink 2013; Tsai and Phillips 1991).

Tannins and phenolic acids are two of the largest groups of phenolics with significant roles in plant defense. Phenolic acids are among the largest group of polyphenols and are produced via the phenylpropanoid pathway and monolignol pathways. Phenolic acids are derived from benzoic acid or cinnamic acid (Gutiérrez-Lomelí et al. 2012). They have roles in promoting symbiotic relationships with microbes and conferring defense against herbivores (Mandal et al. 2010; Sarma and Singh 2003; Seneviratne and Jayasinghearachchi 2003; Nicholson and Hammerschmidt 1992). Instances of herbivores being inhibited by phenolic acids include *Spodoptera litura F.* feeding in cotton, *Sitodiplosis mosellana* feeding on wheat, and jabuticaba extracts inhibiting *Spodoptera frugiperda* (Rani and Pratyusha 2013; Ding et al. 2000; Usha Rani and Pratyusha 2013). Phenolic acids have also been linked to antimicrobial activity against pathogenic bacteria (Cueva et al. 2010). There are also several documented instances of Hemipteran insects being inhibited by phenolic acids (Chrzanowski and Leszczyński 2008; Kariyat et al. 2019)

Tannins are water-soluble derivates of phenols and can form complexes with polysaccharides, nucleic acids, and other plant-derived compounds. Tannins are classified as hydrolysable or condensed, with condensed tannins being the more common type in plant hosts. Hydrolysable tannins contain a central glucose core or another polyol esterified with gallic acid, while condensed tannins are oligomers or polymers of flavan-3-ol linked through an interflavan carbon bond (Hassanpour et al. 2011). Condensed tannins accumulate in the vacuole, while hydrolysable tannins accumulate in the cell wall (Barbehenn and Peter Constabel 2011). Herbivore feeding is controlled by plant-derived tannins via antioxidant activity, prooxidant activity and as a toxins (Khennouf et al. 2003; Barbehenn et al. 2005b; Barbehenn et al. 2009a). Caterpillar midguts can be oxidized by ellagitannins, a form of hydrolyzed tannins (Barbehenn et al. 2005a; Barbehenn et al. 2005b; Barbehenn et al. 2009a, b). Additionally, tannins have been linked to inhibition of feeding of the Hemipteran cotton aphid (*Aphid gossypii* Glover) and tarnished plant bug (Lygus lineolaris) on cotton (Ma et al. 2019; Cervantes et al. 2017).

Alkaloids

There are currently approximately 12000 known plant-derived alkaloids (Ali et al. 2019). Among the most commonly known alkaloids are caffeine, nicotine, and morphine. Alkaloids are nitrogen-containing, low-molecular weight compounds classified into three major groups: true alkaloids, pseudoalkaloids, and protoalkaloids. True alkaloids are basic, derived from amino acids, and possess a nitrogen atom in a heterocyclic ring. Pseudoalkaloids are basic, but are not derived from amino acids and are common in Solanaceae. Protoalkaloids are derived from amino acids and are basic, however, do not

possess their nitrogen atom in a heterocyclic ring (Dey et al. 2020). Alkaloids are stored throughout the plant, though in uneven amounts and are turned over quickly as they take part in myriad functions (Kurek 2019). While alkaloids are both toxic to both humans and insects, they are found in lower levels in the food humans consume, so they pose less of a threat.

The role of alkaloids in plant defense has been explored extensively and there are strong links between alkaloid production in plant hosts and defense against insects (Yao et al. 2019; Shao et al. 2018; Santos et al. 2018; Kim and Ahn 2017). Several herbivore behaviors are inhibited by alkaloids including *Spodoptera exugia* egg hatching and the heart contractile activity of three beetle species (*Zophobas atratus*, *Tenebrio molitor*, and *Leptinotarsa decemlineata*) (Marciniak et al. 2010; Thawabteh et al. 2019). The specialist insect *Manduca sexta* is capable of suppressing the nicotine biosynthesis in its host *Nicotiana attenuata* and impacting resulting JA/ET crosstalk (Winz and Baldwin 2001). While alkaloid distribution among plant families is uneven, legumes do possess alkaloids with NPAA, pyridine alkaloids, and piperidine alkaloids being present widely across the family (Wink 2013).

Terpenes

Terpenes are the largest and most diverse family of secondary metabolites in plants with over 25,000 currently identified. Terpenoids are the modified form of terpenes derived from isopentyl diphosphate (IPP) and its isomer dimethyl diphosphate (DMAPP). In plants, IPP and DMAPP are synthesized via the cytosolic mevalonic acid (MVA) pathway or the plastidial methylerythritol (MEP) pathway (Oldfield and Lin 2012). It is also noteworthy that in addition to synthesizing IPP, the penultimate metabolite for

the MEP pathway, methylerythritol cyclodiphosphate (MEcPP), has been linked to resistance against aphids (Onkokesung et al. 2019). IPP is a five-carbon molecule (C_5) that is used to synthesize monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), and triterpenes (C_{30}) (Zwenger and Basu 2008). The diversity of the terpene family is rooted in the diverse array of terpene synthases (TPS) used to synthesize terpenoids (Boncan et al. 2020; Singh and Sharma 2015; Cheng et al. 2007). Terpenes are both temporally- and spatially-regulated (Singh and Sharma 2015). Terpenes accumulate in trichomes with the capacity to hold these secondary metabolites (Singh and Sharma 2015). While conifers are well known for their diversity in terpenes production, most other plant families also produce terpenes (Zwenger and Basu 2008). These terpenes have myriad roles in mitigating abiotic and biotic stress and are often induced in response to herbivory, redox stress, thermal stress, and osmotic stress. Both terpenes and terpenoids are important in attracting beneficial insects to herbivore infested plants or interfering with herbivore feeding (Yan and Thompson 1995; Sharma et al. 2017).

Pathogen Recognition and PTI/ETS/ETI

The crux of plant defense is a host's ability to perceive damage, herbivory, pathogens, and non-adapted organisms in an expedient manner. Two related and yet distinct mechanisms are deployed to recognize and respond to attacks by pathogens and pests in plants (Jones and Dangl 2006). Using PRRs (pattern recognition receptors), plants are able to perceive highly conserved molecules from non-host microbes, pathogens and pests called MAMPs or PAMPs (microbe- or pathogen-associated molecular patterns, respectively). The interaction with a PRR and its cognate MAMP induces PAMP/MAMP-Triggered Immunity (PTI) and its associated defense signaling cascade. PTI, also known as basal resistance, prevents non-adapted pathogens from

colonizing the plant. To colonize a host, pathogens and pests secrete effectors into plants to suppress PTI's defense signaling pathways, resulting in disease symptoms or insect colonization; this process is called effector-triggered susceptibility (ETS).

Plants counter effectors using Effector-Triggered Immunity (ETI) (Tsuda and Katagiri 2010; Dodds and Rathjen 2010). This second mechanism of plant defense occurs when a plant has evolved a resistance (R) protein to recognize a pathogen/pest-derived effector. During ETI, a rapid defense response is deployed and this resistance response is often accompanied by hypersensitive defense response. The now classical "zigzag model" explains the dynamics of PTI, ETS and ETI and this model has accelerated our understanding of the overlap and distinctions between PTI and ETI (Jones and Dangl 2006).

PAMP/MAMP-Triggered Immunity (PTI)

PTI is the first level of innate immunity in plants (Zipfel 2009). PTI is initiated upon perception of a MAMP, which is a conserved epitope derived from a pathogen. MAMPs, such as chitin or flagellin, are essential to the pathogen, which limits the chance a mutation in a MAMP will evade PTI. The hallmarks of PTI include reactive oxygen species (ROS) burst, defense gene expression, and a MAPK cascade (Zhang and Zhou 2010). MAMPs are perceived by PRRs, which are cell surface-localized receptors that are either a receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (Zipfel 2014). RLKs possess a ligand-binding ectodomain, a single-pass transmembrane domain, and an intracellular kinase domain. RLPs possess a similar architecture but lack the intracellular kinase domain; RLPs and RLKs work together in complexes (Gust and Felix 2014). These supramolecular protein complexes consist of: a primary ligand-binding

receptor protein, one or two co-receptors, cytoplasmic kinases, and regulatory proteins (Kim and Castroverde 2020). The co-receptors play a critical role in PRR detection of MAMPs and function as either homodimers or heterodimers (Noman et al. 2019)

The most well characterized MAMP-PRR associations were first characterized in bacterial pathogen-plant interactions. These interactions include: the conserved 22-amino acid peptide in bacteria flagellin (flg22) with the RLK Flagellin Insensitive 2 (FLS2); the translation elongation factor thermo unstable (EF-Tu)-derived N-acetylated terminal peptide (elf18) and the elongation factor Tu receptor (EF), and AvrXa21 and the rice resistance gene Xa21 (a RLK) (Chinchilla et al. 2006; Zipfel et al. 2006; Lee et al. 2006). All three PRRs use their LRR domain for ligand binding.

EFR requires the co-receptor BRI1-associated receptor kinase I (BAK1), while FLS2 requires both BAK1 and *BOTRYTIS INDUCED KINASE 1* (BIK1). The phosphorylation of EFR by BAK1 results in control over plant growth, innate immunity, and cell death (Schwessinger et al. 2011). In the case of the FLS2-BAK1-BIK1 complex, BIK1 is phosphorylated upon flagellin detection. BIK1 phosphorylates BAK1 and FLS2 to initiate downstream MAMP signal transduction, a hallmark of PTI, that results in immunity to nonpathogenic bacteria (Lu et al. 2010; Wang et al. 2014b; Chinchilla et al. 2006). XA21 is able to form an immune complex with SERK2; SERK2 is also capable for forming complexes with XA3 and FLS2 in rice (Chen et al. 2014).

In contrast, peptidoglycans (PGNs) derived from cell walls are recognized by a multimer of RLPs including LYM1, LYM3, and CERK1 (Willmann et al. 2011; Lee et al. 2006; Zipfel et al. 2006; Heese et al. 2007; Chinchilla et al. 2006). LYM1 and LYM3 are plasma membrane proteins that contain three lysin-motif domain proteins that are

required for PGN detection. CERK1 possess an ectodomain with three distinct LysM domains with the ability to bind chitin (Petutschnig et al. 2010).

PRRs also recognize fungal-derived MAMPs. Like PGNs, chitin is detected by LysM-domain proteins including CERK1 and the RLPs *LYK4* and *LYM2* (Miya et al. 2007; Gu et al. 2017; Wan et al. 2012). Other PAMP/PRR pairs include: xylanase and tomato's RLP Eix2; the *Cladosporium*-secreted AVR9 detected by Cf-9, and polygalacturonases perceived by RBGP1/RLP42 (Zhang et al. 2014; Romeis et al. 2000; Jehle et al. 2013). The RPL SUPPRESSOR OF BIR1 (SOBIR1) forms a number of complexes: SOBIR1 has roles in growth and development and interacts with BAK1 for immune signaling (Gust and Felix 2014; van der Burgh et al. 2019). Production of reactive oxygen species by Respiratory burst oxidase homolog protein D (RbohD) requires C-terminal phosphorylation from the PBL13 receptor-like cytoplasmic kinase (Lee et al. 2020). Finally, PRRs important in viral immunity have also been recently identified; NIK1 has been implicated in the detection of virus-derived MAMPs (Teixeira et al. 2019).

Tomato's SERK3A/3B and BAK1 have roles in conferring resistance to both root-knot nematodes and the bacteria pathogen *P. syringae* (Peng and Kaloshian 2014). Feeding on tobacco from the specialist insect *Manduca sexta* is inhibited by BR1-dependent JA signaling and accumulation of carbon-rich secondary metabolites (Da-Hai Yang and Wu 2013).

While the phloem feeding of Hemiptera is harder to detect than that of other insect pests due to more limited cellular damage, Hemipteran pests are also capable of inducing PTI (Naalden et al. 2021). Aphids are capable of triggering PTI and PTI is important for basal resistance. For example, the *bak1-5* mutant has enhanced

susceptibility to the generalist pea aphid (Zipfel 2014). The endosymbionts of phloem-feeders can also trigger PTI. For example, GroEL chaperonin of the obligate endosymbiont γ-Protobacterium, triggers PTI in *Arabidopsis* and tomato (Chaudhary et al. 2014; Elzinga et al. 2014). In the case of aphid-induced PTI, The BAK1-TPC1-GLB3.3/3.6 and BAK1-SERK3 complexes contribute to green peach aphid resistance in Arabidopsis (Vincent et al. 2017; Prince et al. 2014).

The roles of PTI in perception and defense signaling in other plant-insect interactions is also emerging. For example, recognition of *Pieris brassicae* eggs bears the hallmarks of PTI, with rises in ROS, SA and cell death (Li et al. 2016a). Eleven *LecRK-I* RNAs (LecRK-I.1-8) increase in response to egg deposition and based on mutant analysis, two of these genes, *LecRK-I.8* and *LecRK-I.1* are responsive to *P. brassicae* egg secretions (Gouhier-Darimont et al. 2019; Yang et al. 2011b; Groux et al. 2020). Phosphatidyl choline (PC), but not phosphatidyl ethanolamine, is the *P. brassicae* egg-derived ligand perceived by plants (Stahl et al. 2020). This suggests that PC, a highly conserved molecule present in insect, pathogens, and plants may be an egg-associated molecular pattern (EAMP), MAMP, or damage-associated molecular pattern (DAMP).

The perception of DAMPs, plant-derived molecules released by cellular damage, by PRRs is established (Gust et al. 2017; Zipfel 2014). DAMPs are generated by wounding, pathogen infection, and herbivore attack. Known DAMP-PRR pairs include PROPEP-derived AtPep1 sensed by PEPR1/2, cell wall molecules (OGAs) sensed by WAK1, extracellular ATP sensed by DORN1, and extracellular self-DNA (exDNA) (Chen et al. 2017; Krol et al. 2010; Yamaguchi et al. 2010; Brutus et al. 2010; Veresoglou et al. 2015; Mazzoleni et al. 2015b; Mazzoleni et al. 2015a; Duran-Flores and Heil 2015). The

role of DAMPs in herbivore-plant interactions is best characterized for tissue-damaging herbivores (Malik et al. (2021).

Effector-Triggered Immunity (ETI)

While PTI provides plants with a robust defense response providing protection to non-adapted pathogens and basal immunity, phytopathogens are successful at evading and intercepting PTI by deploying virulence factors called effectors (Dodds and Rathjen 2010; Jones and Dangl 2006; Huang et al. 2021). The suppression of PTI via pathogen and insect pest effectors results in effector-triggered susceptibility (ETS) (Jones and Dangl 2006). To counter pathogen/pest virulence factors, plants have evolved resistance (*R*) genes, which participate in the cascade of events called effector-triggered immunity (ETI) (Jones and Dangl 2006).

Most *R* genes encode NBS-LRRs (NLRs) that contain both nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains (McHale et al. 2006b). Most NLRs contain four functional domains: (1) a variable amino-terminal domain involved in protein-protein interactions, (2) the NBS domain involved ATP hydrolysis and signal transduction, (3) the LRR domain involved in ligand binding and protein-protein interactions, and (4) a carboxy-terminal domain. While little is known about the carboxy-terminal domain, some nuclear-localized NLRs have WRKY-binding motifs in these domains. NLRs have myriad roles in plant biology detecting ligands to control defense, as well as cell expansion and development, stem-cell maintenance, and stomatal development. NLRs have critical roles in resistance against insects, bacteria, fungi, viruses, and oomycetes. As their extracellular domains facilitate protein-protein interactions, plant LRR-RKs have evolved into four major subnetworks to promote

defense or growth/development and to prevent aberrant associations that would dilute ligand-triggered “messages” (Smakowska-Luzan et al. 2018)

Plant NLRs mediate ETI in three major steps: (1) direct/indirect perception of pathogen/pest effector molecules by its cognate NLR, (2) activation of the NLR through a confirmational change, and (3) downstream signaling to deploy defense-signaling pathways. Upon recognition of its effector, NLRs mediate a rapid and robust activation of a ROS burst, calcium ion flux, a mitogen-associated protein kinase (MAPK) cascade, induction of *PATHOGENESIS-RELATED* (PR) proteins, and often a hypersensitive response (HR) (McHale et al. 2006a). These molecular events overlap with PTI, but are distinct (Yuan et al. 2021b).

Plant NLRs are large proteins (approximately 860 – 1900 amino acids long) and are members of large gene families (McHale et al. 2006a). For example, *Arabidopsis* contains approximately 150 NLRs; but both the total number of *NLR* genes, copy the number of *NLR* gene paralogs, and number of loci where *NLRs* are clustered varies considerably between plant species and plant families (Baggs et al. 2017; Meyers et al. 2003; Monteiro and Nishimura 2018; McHale et al. 2006a).

Plant NLRs are classified into two subfamilies based on their amino-terminal domain: the Toll/interleukin-1 receptor (TIR) or coiled-coil (CC). TIR-NLRs (TNLs) and CC-NLRs (CNLs) are distinct in structure and function (McHale et al. 2006b). TNLs are likely to have larger carboxy-termini (200 – 300 amino acids) compared to CNLs (40 – 80 amino acids). Both NLR subfamilies are present in domesticated plant families with the exception that TNLs are absent from cereals (McHale et al. 2006a). TNLs transduce defense signals through *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*) and CNLs signal through *NON-RACE-SPECIFIC DISEASE RESISTANCE1* (*NDR1*) (Bhattacharjee

et al. 2011; Knepper et al. 2011). There are high levels of sequence variation between NLRs, which may reflect selective pressure imposed in different geographical regions and environmental conditions (Monteiro and Nishimura 2018; Baggs et al. 2017). Activation of ETI by NLR recognition of its effector has a high fitness cost, as carbon and nitrogen resources are diverted from growth and development to a robust defense response (Brown and Rant 2013; Huot et al. 2014). For this reason, NLRs means are under strict transcriptional, post-transcriptional, and post-translational control (Yin et al. 2019; Lai and Eulgem 2018).

Some NLRs interact directly with their effectors consistent with the original gene-for-gene model of Flor (1971). Examples of this relationship include the rice Pi-Ti and its *Magnaporthe oryzae* effector AvrPi-Ta, *Arabidopsis* RPP1 and *Hyaloperonospora arabidopsidis* ATR1 and powdery-mildew resistance loci *mildew loci a* (*mla*) (Saur et al. 2019; Krasileva et al. 2010; Dodds et al. 2006). However, a number of indirect effector/NLR interactions have been characterized including the guarding of RIN4 by multiple R proteins and the RPS4-RRS1 NLR complex (Liu et al. 2009; Huh et al. 2017).

The guard and decoy models build upon the “gene-for-gene” model to accommodate these indirect interactions (van der Hoorn and Kamoun 2008). These models propose that NLRs monitor effector-induced changes to the immune signaling network. For example, an NLR may detect the structural change in a plant protein (a virulence target) that interacts with an effector. Alternatively, an NLR will perceive a structural change in an NLR-mimic protein. Guard and decoy proteins are evolutionarily distinct: decoys arose due to the narrow roles of guard proteins in defense and signaling (Kapos et al. 2019; Lorang et al. 2012). Decoy proteins, however, have roles exclusive to ETI (Zhou and Chai 2008; van der Hoorn and Kamoun 2008). Guardees must also

remain recognizable by the corresponding NLR, while decoys only need for its effector-induced change(s) to be recognized by the NLR (van Wersch et al. 2020). Perception of these structural changes provokes a conformational change in the NLR to initiate ETI. The bait model is a hybrid of the guard and decoy models; in this mode, an accessory protein "baits" the effector and the accessory protein's direct interaction with the NLR initiates downstream defense signaling and ETI (Collier and Moffett 2009). The guard model is best exemplified by Resistance to *P. syringae* pv. Maclicuola protein 1 – interacting protein 4 (RIN4) in ETI, which is triggered by Resistance *to P. syringae* protein 1 (RPM1) and Resistance to *P. syringae* protein 2 (RPS2) after detection of their effectors AvrRpm1 and AvrRpt2, respectively (Mackey et al. 2002; Kunkel et al. 1993). RPM1 detects AvrRpm1-mediated RIN4 phosphorylation via a receptor interacting protein kinase (RIPK) (Liu et al. 2011). In contrast, RPS2 detects the decline in RIN4 abundance that is triggered by AvrRpt2-mediated auxin/indole acetic acid turnover (Cui et al. 2013). An example of the decoy model is the relationship between ZED1 and the NLR ZAR1. ZED1 is a pseudokinase acetylated by the HopZ1a effector and upon the trapping of HopZ1a in a complex with ZAR1 and ZED1, ZAR1-mediated immunity can be activated (Lewis et al. 2013). Amazingly, there are no insect effector-R protein interactions that have been elucidated at the molecular level, although some insect effectors in involved in ETI in Hessian fly-wheat interactions have been identified (Aljbory et al. 2020).

NLRs have also been recently characterized as "sensors and helpers" that can monitor host changes induced by pathogens and cooperate as "helpers" to signal downstream defense responses (Baggs et al. 2017). While the direct interactions of helper NLRs, such as ADR1, NRC1, and NRG1, with other NLRs is not currently present

in the literature, they are known to play a role in the regulation of the ETI's defense-signaling pathways (Collier et al. 2011; Wu et al. 2016). Sensor NLRs, on the other hand, can form complexes with co-regulated partner NLRs to execute downstream signaling (Jubic et al. 2019).

While ETI provides a robust defense response to the effectors that help pathogens/pests evade PTI, the evolutionary arms race between attacker and plants continues. Some effectors evolve to evade and suppress ETI (Jones and Dangl 2006). The expansive diversity of plant NLR families is essential to counter and evade the impacts of pathogens/pests that have adapted to a formerly "resistant" plant host.

Host plant resistance (HPR) to pathogens and pests can be classified as quantitative or qualitative resistance. Qualitative resistance causes discrete resistance phenotypes and are driven by few genes that dominate and determine the resistance phenotype (Corwin and Kliebenstein 2017). Quantitative resistance, however, results in a spectrum of phenotypes that is driven by many genes with low or moderate effect on resistance by themselves. However, there are instances of individual loci greatly impacting resistance as with *rx1, rx2* and *rx3* impacting *Xanthomonas campetstris* resistance in tomato (Stall et al. 2009) (Pilet-Nayel et al. 2017b, a). Holistic breeding programs utilizing the best practices of integrated pest management (IPM) breeders identify and deploy multiple *R* genes simultaneously (referred to as "pyramiding") in a cultivar; this is means for more durable resistance that is less likely to be evaded by pathogen or pest adaptations (Grafius and Douches 2008; Pilet-Nayel et al. 2017a; Mundt 2018; MacIntosh 2019).

Recently, the active oligomeric state, or "resistosome" status of NLRs has been elucidated (Burdett et al. 2019). A resistosome consists of a NLR, a decoy kinase and a

pseudokinase (Liang and Zhou 2018; Burdett et al. 2019). These resistosomes function as sensors and executors of programmed cell death (Ullrich 2021).The first resistosome complex identified included the CNL ZAR1, the RLK RESISTANCE RELATED KINASE 1 (RKS1), and protein kinase AVRPPHB SUSCEPTIBLE 1 LIKE 2 (PBL2) (Wang et al. 2019b). In this system, the effector AvrC uridiylates the decoy pseudokinase PBL2. Upon uridylation, PBL2 interacts with the ZAR1 complex and the interaction with the preformed RKS1 and ZAR1 complex results in the hydrolysis of an ATP from ZAR1. This results in a subsequent conformational change and activation. In its active form, ZAR1 can form a homo-pentamer to form a pore in the plasma membrane through which calcium ions can enter to trigger defense-signaling pathways (Wang et al. 2019c). Additional plant resistosomes have been identified and are further described in (Ullrich 2021).

Host plant resistance to Hemipteran insects

While *R* genes conferring resistance to insects have been identified (Walling and Thompson 2013; Smith and Clement 2012), a handful have been characterized at the molecular level. For the Hemiptera, resistance genes to the brown planthopper (*Nilaparvata lugens*) in rice, cotton melon aphid (*Aphis gossypii*) and pink potato aphid (*Macrosiphum euphorbiae*) in rice, melon and tomato have been characterized. The tomato *Mi-1.2* gene will be discussed in the whitefly resistance gene section as *Mi-1.2* confers resistance to multiple animal species including two *B. tabaci* species (Zhao et al. 2016; Ji et al. 2016; Du et al. 2009a; Jairin et al. 2007; Casteel et al. 2006; Nombela et al. 2003).

Brown planthopper (BPH) is among the most devastating pests to rice, which is a staple crop throughout the developing world. The introduction of *Bph* resistance genes into rice varieties has become an economically and ecologically sustainable means of controlling for BPH. The necessity for identifying more *BROWN PLANTHOPPER* (*Bph*) genes has increased recently considering the advantages of stacking resistance genes and the fact that BPH can evolve into biotypes that overcome most *Bph R* genes. There are over 37 identified genes and nine successfully cloned genes in rice that confer resistance to BPH (Cheng et al. 2013; Sani Haliru et al. 2020). Each of these cloned resistance loci have unique characteristics but share some features. Four *Bph* genes encode for a coiled-coil (CC) nucleotide-binding site (NBS) leucine-rich repeat (LRR) protein: *BROWN PLANTHOPPER (Bph) 2/26, 9, 14,* and *18*. Three CNLs (*Bph2/26*, *14,* and *18)* have roles inhibiting phloem feeding and inducing callose deposition. In contrast, BPH-feeding induces cell death in *Bph9* plants along with both salicylic acid (SA)- and jasmonic acid (JA)-dependent defenses (Zhao et al. 2016; Ji et al. 2016; Tamura et al. 2014; Du et al. 2009b). Two loci (*Bph3* and *Bph17*) encode for lectin-domain receptor kinases (RK) that localize to the plasma membrane to mediate a potent durable resistance (Jairin et al. 2007; Liu et al. 2015). Three loci encode for proteins that are neither NLRs or RKs: *Bph6* is a exocyst-localized protein that also contributes to cell wall maintenance (Guo et al. 2018). While *Bph29* is a B3 DNA-binding domain that induces SA-dependent defense and callose deposition (Wang et al. 2015). Finally, *BROWN PLANTHOPPER 32* is a short-consensus repeat protein localized in the plasma membrane of leaf sheaths which inhibits insect feeding (Ren et al. 2016). Most cloned *Bph* genes are either exclusively antibiotic (*Bph2/26, 3, 6, 9, and 32)* or antixenotic (*Bph29)* resistance genes, though *Bph29* displays both antibiosis and antixenosis. Most

Bph genes are also dominant alleles, with the exception of *Bph29* and *Bph2/2,6* which are recessive alleles.

Cotton-melon aphid (CMA) resistance was identified in multiple melon germplasm sources. This resistance was mapped to a locus responsible for conferring CMA-directed antibiosis and antixenosis. Two melon genotypes were used to identify the loci conferring cotton-melon aphid resistance, which was determined to be a dominant allele encoding for a CNL (*Vat*). *Vat* is a phloem-mediated resistance gene containing a soluble component that inhibits imbibition and also inhibits cotton-melon aphid mediated virus transmission. Global deployment of *Vat* In melon production systems has been a durable and effective means of CMA control (Dogimont et al. 2014).

The Convergence of PTI and ETI

Immune responses mediated by PTI and ETI have different triggers, but both result in two related defense responses (Chang et al. 2022; Yuan et al. 2021b). Both PTI and ETI elicit Ca^{2+} fluxes, reactive oxygen species (ROS) bursts, mitogen-activated protein kinase (MAPK) cascade activation, transcriptional reprogramming, and localized callose deposition (Chang et al. 2022; Noman et al. 2019; Thomma et al. 2011; Yuan et al. 2021b; Tsuda and Katagiri 2010). While ETI was originally perceived to be stronger than PTI, recent experiments have shown both PTI and ETI can range in strength of response (Dodds and Rathjen 2010; Thomma et al. 2011; Wirthmueller et al. 2007; Ritter and Dangl 1996; Tao et al. 2003; Hofius et al. 2009).

While largely overlapping, there are differences between both defense responses. For example, ETI has a stronger ROS response than PTI. PTI's ROS burst is

monophasic, rapid and occurs immediately after pathogen detection, while ETI has a longer lasting biphasic ROS response (Torres et al. 2006; Zhang et al. 2007). MAPK signaling in both pathways is also different. More MPKs (MPK1/3/4/6/11/13) have been linked to PTI than ETI (MPK3/6) and the MAPK signaling in ETI is more persistent than PTI (Peng et al. 2018; Tsuda et al. 2013; Asai et al. 2002; Teige et al. 2004; Nuhse et al. 2000; Nitta et al. 2014; Droillard et al. 2004; Bethke et al. 2012). Ligand specificity is also a significant differentiator of PTI and ETI (Bent and Mackey 2007; Macho and Zipfel 2014). As described in the section above, PRRs bind directly to highly conserved ligands from pathogens, pests or plant-derived molecules released after damage. In contrast, NLRs may or may not bind their ligand directly. Often helper NLRs (hNLRs) and sensor NLRs (sNLRs) bridge this gap (Jubic et al. 2019; Baggs et al. 2017). There are also instances of NLRs detecting multiple ligands and single ligands perceived by multiple NLR receptors (Ngou et al. 2021b). For example, the *Arabidopsis* NLRs WRR4A/B detect multiple CX_2CX_5G effectors of the oomycete *Albugo cadida* and the NLRs *RRS1/RPS4* and *RRS1B/RPS4B* both detect the T3S *AvrRps4* effector from *P. syringae* (Saucet et al. 2015; Redkar et al. 2021; Huh et al. 2017). ETI-dependent hormonal responses are also redundant and typically one hormone predominates the defense cascade; therefore, it is more difficult for a pest or pathogen to perturb the defense response (Dodds and Rathjen 2010; Tsuda and Katagiri 2010).

Both immune responses share properties and function interdependently to contribute to maintaining active defenses against pathogens/pests (Chang et al. 2022). While the ligands differ between PRRs and NLRs, both use co-receptors or resistance proteins working in tandem to synergistically control PTI and ETI (Ngou et al. 2021b). Recently, Ngou et al. (2021a) showed that PTI and ETI components potentiate each

other to confer resistance to *P. syringae.* ROS burst is a product of both ETI- and PTI-mediated defenses. Yuan et al. (2021a) discovered that BIK1 is essential for activation of RBOHD and subsequent ROS signaling. Further evidence of distinct components of these immune systems working synergistically is the requirement of ETI components (EDS1, PAD4, and the helper NLR ADR1) for RLP23-mediated PTI and the fact that TNL signaling enhances detection of the PAMPs flg22 and nlp20 (Pruitt et al. 2021; Tian et al. 2021).

Finally, recent studies have shown that NLR activation contributes to the maintenance and priming of PRRs supporting the hypothesis PRR-NLR crosstalk can happen in a synergistic manner. This priming enables a plant to enter a physiological state more ready to deploy defense responses (Conrath et al. 2002). ETI and PTI can synergistically enhance host HR, ROS production, defense transcriptome expression, and physiological changes associated with defense (Ngou et al. 2021b). Finally, both PTI and ETI activate systemic acquired resistance (SAR), which is a long-lasting immune response that occurs in infected/infested tissues and is propagated to distal parts of the plant (Klessig et al. 2018). SAR and SAR signals are describe in the section on SA signaling.

Phytohormone-Mediated Defenses and Crosstalk

In the previous sections, the signaling machinery that perceives plant attackers was outlined. The plant immunity triggered by PTI and ETI is deployed by phytohormones and reactive oxygen species (Tsuda and Katagiri 2010). Although virtually, all phytohormones have some role in plant defense (Checker et al. 2018), four phytohormones (SA, JA, ABA, ET) are at the core of these responses. These pathways

can act additively, synergistically or antagonistically to orchestrate the “appropriate” defense responses to a particular pathogen or pest; the communication between phytohormone pathways is often called crosstalk (Pieterse et al. 2009; Grant and Jones 2009a). In addition, defense phytohormone pathways must be balanced with host plant growth, development, and reproduction (Huot et al. 2014). This section will briefly discuss phytohormone biosynthesis, signaling, and crosstalk and how plant pathogens can manipulate crosstalk for their benefit.

SA Biosynthesis, Perception, and Signaling

SA has roles inhibiting growth, inducing flowering, inducing senescence, and is essential for several components of plant immunity including PTI, ETI, Systemic Acquired Resistance (SAR), N-hydroxy-pipecolic acid (NHP) biosynthesis, NHP-mediated immunity, and defense against biotrophic pathogens (Huang et al. 2020b; Peng et al. 2021; Lefevere et al. 2020; Zhang and Li 2019a). SA’s important role in PTI and ETI is indicated by the numbers of pathogen/pest effectors targeting this pathway as reviewed by An and Mou (2011), Pajerowska-Mukhtar et al. (2013), Kazan and Lyons (2014),and Zhang and Li (2019a). Plants must maintain tight regulatory control of the SA-defense signaling pathway and SAR due to their high fitness cost.

SA biosynthesis and transport

The importance of SA (2-hydroxybenzoic acid) in SAR was established over 25 years ago. SA accumulates in local and systemic tissue after pathogen/pest attack (Durrant and Dong 2004; Ryals et al. 1996; Ross 1961; Cui et al. 2019). In plants, SA is synthesized via the isochorismate synthase (ICS) and the phenylalanine ammonia-lyase (PAL) pathways, which both initiate with chorismate (Lefevere et al. 2020). Two ICS

pathway genes (*ICS1/SID2* and *ICS2*) in Arabidopsis control SA biosynthesis in the plastid (Dempsey et al. 2011). In contrast, PAL uses phenylalanine to synthesize SA in the cytosol. Phe is synthesized in both the plastid and cytosol. *EDS5* is an chloroplast envelope SA transporter that moves SA to the cytosol (Serrano et al. 2013). Cytosolic SA is transported to the cuticle/apoplast in a proton-dependent manner and can also be delivered to the nucleus via stromules (Gu and Dong 2015; Caplan et al. 2015).

The contributions of the PAL and ICS pathways to SA accumulation during SAR is plant-species dependent. For example, in *Arabidopsis* the ICS pathway primarily contributes to local and systemic SA synthesis after pathogen/pest attack (Wildermuth et al. 2001; Chen et al. 2009); while the PAL pathway is a minor contributor to pathogen/pest SA biosynthesis. In contrast, in soybean, the PAL and ICS pathways contribute equally to SA production (Shine et al. 2016). Tobacco, on the other hand, primarily utilizes the PAL pathway in response to pathogens (Ogawa et al. 2006).

SA can be toxic to a plant host when accumulated at high levels. Therefore, SA is modified into derivative forms by glucosylation, methylation, sulphonation, and amino acid conjugation (Dempsey et al. 2011). While there is not a comprehensive knowledge of SA-amino acid conjugates, they are believed to have a role in SA catabolism (Dempsey et al. 2011; Klessig et al. 2018) The inactive derivative of SA (glucosylated SA, SAG) is a storage form; while methyl salicylate (MeSA) is a volatile form of SA. This volatile form releases SA from cells and prevents high levels of SA accumulating *in planta*, which can be toxic. (Kumar 2014; Lee and Raskin 1998; Chini et al. 2004). While these forms of SA may not be active, there is evidence SAG plays a role in plant defense by modulating MeSA/SA homeostasis (Ninkovic et al. 2021; Chen et al. 2019; Ratzinger et al. 2009).

SA signaling

SA signaling is regulated vis a *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES* 1 (*NPR1*)-dependent or -independent pathway (Uquillas et al. 2004; Spoel et al. 2003). The NPR1-independent pathways are less well studied but one *NPR1*-independent pathway relies on the *WHIRLY1* transcription factor (Durrant and Dong 2004). Here, I will primarily focus on *NPR1*-dependent immunity, which is largely gleaned from studies in Arabidopsis. In Arabidopsis ~90% of the SA-dependent defense response is NPR1 dependent (Sun et al. 2018b). NPR1 and NPR3 and NPR4 are SA-binding proteins that fine tune SA-regulated defense responses and systemic acquired resistance (SAR) depending on plant cell SA content (Zhang and Li 2019b; Peng et al. 2021). NPR1 is a redox-sensitive, positive regulator of SA-dependent signaling that binds to TGA transcription factors to active SA-dependent defenses (Mou et al. 2003). While NPR3 and NPR4, were initially proposed to be E3 ubiquitin ligases that turnover NPR1 after SA binding (Fu et al. 2012), these proteins are now thought to negatively regulate SA signaling by suppressing TGA-dependent gene expression (Ding et al. 2018).

Three upstream SA-signaling components that positively regulate SA signaling are *EDS1, PAD4,* and *SAG101*. EDS1, PAD4, and SAG101 are members of the EDS1 family and are pseudoenzymes characterized by their N-terminal lipase-like domain (LLD) and a unique C-terminal α-helical bundle (the EP domain) (Dongus and Parker 2021). The putative lipase *EDS1* interacts with *PAD4* or *SAG101* to influence SA biosynthesis and SA-mediated defense signaling (Dongus and Parker 2021; Cui et al. 2017; Wiermer et al. 2005). While PAD4 and SAG101 are not found in the same EDS1 complex, *PAD4* and *SAG101* expression are both *EDS1*-dependent (Wiermer et al.

2005; Cui et al. 2017; Zhu et al. 2011a). Both PAD4 and EDS1 are induced by SA (Jirage et al. 1999; Falk et al. 1999). The EDS1-SAG101 heterodimer functions with TNLs in programmed cell death and pathogen resistance; while the EDS1-PAD4 heterodimer has a role in basal immunity and is not exclusive to TNL-mediated ETI. Helper NLRs (hNLRs) (such as N REQUIRED GENE 1 (NRG1) and ACTIVATED DISEASE RESISTANCE 1 (ADR1)) also positively influence these interactions. For example, NRG1 and other RNLs interact with the EDS1-SAG101 heterodimer and this complex has a role in ETI signaling (Lapin et al. 2019). *NDR1* also functions upstream of SA biosynthesis, but in a *EDS1*-independent manner (Aarts et al. 1998). In contrast, ADR1 interacts with the EDS1-PAD4 heterodimer and this complex has a role in the rapid induction of localized and systemic SA-mediated responses along with the repression of JA-mediated responses. (Dongus and Parker 2021).

NPR1, and the related NPR3 and NPR4, are a key transcriptional co-regulators of SA-mediated defenses. Until recently, NPR3 and NPR4 were thought to be E3 ligases with a role in controlling NPR1 levels (Fu et al. 2012). More recently, Ding et al (2018) showed that all three proteins are bone fide SA receptors and also bind TGA transcription factors to execute their regulatory activities. NPR1 and NPR3/4 execute opposite roles in regulating transcriptional response to SA (Ding et al. 2018). While NPR1 is a transcriptional co-activator, NPR3 and NPR4 are transcriptional co-repressors; all three proteins execute their regulatory roles by binding TGA transcription factors. TGAs are transcription factors that bind to the TGACG recognition sequence also known as the *as-1* motif (Gatz 2013). There are ten TGAs identified in *Arabidopsis* and all TGAs interact with NPR1 constitutively with the exceptions of TGA1/4, which only interact with monomerized NPR1 (Johnson et al. 2003; Rochon et al. 2006; Després et

al. 2000; Zhou et al. 2000a). NPR3/4, on the other hand, only bind with TGA2/5/6. The interaction of TGAs with NPR1 activates transcription of *PATHOGENESIS-RELATED* proteins and other SA-responsive genes when there are sufficient levels of SA (Kesarwani et al. 2007; Zhou et al. 2000a). Post-translational modification of NPR1 is critical for modulating NPR1 activity. Phosphorylation is critical for NPR1 activation of SA-mediated defenses (Kumar 2014). In addition, NPR1 is also modified via S-nitrosylation, sumoylation, and ubiquitination, which influences NPR1's ability to bind SA and function as a transcriptional co-activator (Peng et al. 2021).

The current model for NPR1/3/4 and SAR is briefly described below. In healthy, non-stressed plants, SA levels are low and the NPRs are not bound to SA. Under these conditions, NPR3 and NPR4 bind to TGAs and NPR1 is primarily an inactive oligomer localized in the cytosol, which prevents unnecessary triggering of SA-mediated defenses (Kinkema et al. 2000; Ding et al. 2018). The small quantities of NPR1 that are in the nucleus are sequestered in an oligomeric complex with NON INDUCIBLE IMMUNITY PROTEIN 1 INTERACTING proteins (NIMINs) (Hermann et al. 2013). Upon activation pathogen attack, SA accumulates and there is an accompanying change in the redox status of plant cells. NPR1 is phosphorylated and undergoes a change from an oligomer into an active monomer and the activated SA-bound monomer is transported to the nucleus (Mou et al. 2003; Kinkema et al. 2000), where is associates with TGAs to activate SA-dependent gene expression (Després et al. 2000; Eckardt 2003; Fan and Dong 2002; Zhou et al. 2000b). In addition, NPR3 and NPR4 bind SA and this blocks their ability to interact with TGAs, releasing these TGAs and allowing their interactions with NPR1 to activate SA-mediated defenses.

Other components of SA signaling pathway influence SA in a positive or negative manner. For example, the transcription factors SARD1 and CBP60g are broad regulators of plant immunity (Sun et al. 2015). SARD1 and CBP60g are recruited to the *ICS1* promoter and enhance SA production and are also key transcriptional regulators of N-hydroxy pipecolic acid production (Wang et al. 2011). There are also many other positive (NTL9, WRKY28/46/48/75, TCP8/9, ANAC019/55/72, PCRK1/2, CDK8, TGA1/4, and GTL1) and negative (WRKY18/40/70, CAMTA1/2/3, NPR3/4, and NIMIN1) influencers of SA-regulated defenses that have been characterized (Zhang and Li 2019a). In addition, proteins from the mediator complex, responsible for RNA polymerase II and transcription factor binding, have variable relationships to SA responses. *MED21/25* have all been linked to negative regulation of SA-mediated responses, while *MED15/16* have been linked to positive regulation of SA responses (Zhang et al. 2012; Canet et al. 2012; Dhawan et al. 2009; Kidd et al. 2009).

Systemic Acquired Resistance and the mobile signals

Induced by both PTI and ETI, chemical communication between infested/infected leaves and distant leaves (systemic leaves) activates SAR (Spoel and Dong 2012; Vlot et al. 2009). SAR is associated with rapid and enhanced immunity that occurs when a plant is subsequently challenged with a different pathogen/pest. The “ready for response” status of SAR-induced leaves is also called defense priming. Several molecules are associated with SAR activation: salicylic acid (SA), N-hydroxypipecolic acid (NHP), the NHP precursor pipecolic acid (Pip), azaleic acid, glycerol-3-phosphate, DEFECTIVE IN INDUCED RESISTANCE 1 (DIR1), DIR1-LIKE, dihydroabientinal, and α- and β-pinene (Hartmann and Zeier 2019; Riedlmeier et al. 2017; Chaturvedi et al.

2012; Chanda et al. 2011; Champigny et al. 2013; Wang et al. 2014a; Wang et al. 2018a; Park et al. 2007; Wenig et al. 2019).

Pip (and its derivative NHP) synthesis from L-lysine is controlled by *ALD1, SARD4*, and *FMO1* (Mishina and Zeier 2006; Ding et al. 2016b; Hartmann and Zeier 2019). These loci are positively regulated by the SA-signaling components EDS1 and PAD4, indicating synergy between SA and Pip biosynthesis (Joglekar et al. 2018). While both compounds positively contribute to SAR, NHP is transported systemically and Pip is not transported through the plant and is associated with a more localized response (Hartmann et al. 2017; Ding et al. 2016a; Chen et al. 2018b).

Other SA signaling components influence NHP and Pip biosynthesis. WRKY33 binds to ALD and positively regulates Pip biosynthesis and a strong, sustained MAPK activity via MPK3/6 results in increased levels of Pip (Wang et al. 2018b; Mao et al. 2011). Treatment of *Arabidopsis* with NHP leads to enhanced SA production, an elevated HR, and enhanced camalexin production. Camalexin and NHP production are both *FMO1*-dependent (Návarová et al. 2012). Pip treatment induced SAR and leads to *FMO1* upregulation (Bernsdorff et al. 2016; Chen et al. 2018b).

Because SAR lasts considerably longer (up to weeks or months) than PTI and ETI, this defense mechanism is under tight regulation. While SAR provides robust defense against secondary infection, the costs to plant growth and development are significant. SA has long been linked to being detrimental to plant fitness, so SAR's deleterious consequences are consistent with the function of its major hormonal component. Surprisingly, however, there are also deleterious consequences to plants unresponsive to SAR (Durrant and Dong 2004; Fu and Dong 2013).

SA crosstalk with other phytohormone pathways

There are multiple mechanisms that prioritize, coordinate and fine-tune SA pathway deployment in response to pathogens, pests, and abiotic stress (Yang et al. 2015; Thaler et al. 2012; Yang et al. 2019b). Best studied in Arabidopsis, biotrophs and necrotrophs elicit different hormonal responses and defense signaling cascades. These responses are mediated by SA and JA, respectively. Therefore, the SA and JA-mediated defenses are often antagonistic to each other (Fu et al. 2012; Glazebrook 2005; Mur et al. 2006; Yang et al. 2019b; Yang et al. 2015). Although additive and synergistic interactions of SA and JA are also known (Mur et al. 2006). This topic is well reviewed in the literature and continues to be elucidated and integrated with interactions with other phytohormones, as discussed above (Yang et al. 2019a; Yang et al. 2015). Four *Arabidopsis* genes are significant players in SA-JA cross-talk: *NPR1*, *WRKY70, MPK4,* and MYC2.

NPR1, and the related *NPR3* and *NPR4*, are SA receptors and their roles as transcriptional co-activators and co-repressors as was described above. It is noteworthy that NPR1 is also a negative regulator of JA-mediated defenses. The SA-deficient mutant *npr-1* displays enhances susceptibility to biotrophs and has elevated levels of JA upon *P. syringae* infection compared to Col-0 (Spoel et al. 2003; Rayapuram and Baldwin 2007b). The mechanism of NPR1's influence on JA signaling is cytosol localized and this mechanism of resistance is not completely understood to date. However, NPR1's role in controlling downstream transcription factor expression to mediate SA-JA crosstalk is understood.

The transcription factor *WRKY70* has a role in JA-SA crosstalk. The expression of *WRKY70* is controlled by NPR1-dependent and -independent mechanisms (Li et al.

2004). *WRKY70* is a positive regulator for SA-responsive genes and a negative regulator of JA signaling in an NPR1-dependent manner. After infection or SA treatments, WRKY70 upregulates a wide variety of genes including pathogenesis-related protein genes (*PR1, PR2* and *PR5*) and the master defense regulator *SARD1 (SYSTEMIC ACQUIRED RESISTANCE DEFICIENT1),* which provides protection against abiotrophic pathogen and down-regulates JA-responsive genes (Li et al. 2006; Li et al. 2017a; Li et al. 2004). It was recently shown that *WRKY70* is phosphorylated after infection and WRKY70-P activates *SARD1* expression; WRKY70-P activity is transient, as it is turned over by the 26S proteosome (Liu et al. 2021). Furthermore, in healthy leaves, the non-phosphorylated *WRKY70* represses *SARD1* indicating that *WRKY70* serves as both a positive and negative regulator of SA-responsive genes (Ren et al. 2008). In addition, analysis of *wrky70* mutants clearly indicates that WRKY70-mediated crosstalk is only one of several mechanisms to prioritize deployment of the SA- or JA-signaling pathways (Ren et al. 2008).

The mitogen-activated protein kinase *MPK4* is a negative and positive regulator of SA and JA signaling, respectively, and this regulation is mediated by *PAD4* and *EDS1* (Brodersen et al. 2006). *MPK4* is guarded by SUMM2 and disruption of the MEKK1-MKK1/2-MPK4 cascade triggers MEKK2, which positively regulates SUMM2 (Zhang et al. 2017c). Experiments with *mpk4* mutants show that *MPK4* functions as a negative regulator of SA signaling and MEKK1 and MKK1/2 work with MPK4 to suppress PTI. MPK4 interferes with EDS1/PAD4-mediated SA signaling and promotes JA signaling. (Gao et al. 2008; Petersen et al. 2000). There are other branches of the SA signaling pathway that are regulated by phytohormone crosstalk. SA signaling can also be disrupted by modulation of NAC TFs (*ANAC019*, *ANAC055*, and *ANAC072)* via MYC2

(Zheng et al. 2012). MYC2 is a transcription factor that coordinates the JA pathway activation by coordinating crosstalk between two of the JA branches of defense signaling (See JA section). The modulation of ANACs by MYC2 inhibits induction of *ICS1* and promotes the SA methylation gene *BSMT1* (Zheng et al. 2012).

While orthologs for major components of crosstalk between the JA and SA signaling pathways are found in most plant species, these proteins do not always have the same function (Thaler et al. 2012; Rayapuram and Baldwin 2007a). *NPR1* in tobacco is a negative regulator of SA and an NPR1-like gene in strawberry (FvNPRL-1) functions more like *Arabidopsis* NPR3/4 (Shu et al. 2018; Rayapuram and Baldwin 2007a).

Temporal deployment of the JA and SA pathways provides numerous opportunities to prioritize and attenuate signaling pathways and, not surprisingly, is a component of JA-SA crosstalk. JA and SA can both be deployed to additively or synergistically impact defense responses, however prolonged or higher accumulation of these phytohormones can result in antagonistic actions (Mur et al. 2006; Spoel et al. 2007; Thaler et al. 2012). Plants generally prioritize SA- over JA-mediated response because SA-mediated SAR provides a more robust defense against a wider array of nonhost pathogens (Klessig et al. 2018). As JA and ET collaborate to express many defense genes, SA is most often antagonistic to ET-mediated signaling (Pieterse et al. 2012; Leon-Reyes et al. 2009; Leon-Reyes et al. 2010).

In many cases, SA has an antagonistic relationship with ABA signaling pathways (Mauch-Mani and Mauch 2005; Asselbergh et al. 2008b). Evidence for ABA's antagonistic role in SA-regulated immunity is derived from the facts that: (1) ABA and ABA-responsive genes are induced after pathogen infection, which suppresses SA biosynthesis and SA-mediated responses (Whenham et al. 1986; de Torres-Zabala et al.

2007); (2) ABA treatments inhibit SAR elicited by *P. syringae* and other biotrophs (Mohr and Cahill 2001; Yasuda et al. 2008; Jiang et al. 2010); (3) SA treatments suppress ABA signaling in both an NPR1-dependent and -independent manner (Cao et al. 2011; Yasuda et al. 2008); (4) ABA insensitive mutants have increased resistance to biotrophic pathogens (Audenaert et al. 2002); and (5) pathogens can modulate SA signaling to induce ABA signaling and enhance host plant susceptibility (de Torres Zabala et al. 2009). There is however some evidence of SA-ABA synergism (Cao et al. 2011). Additionally, the transcription factor MYB96 positively regulates both SA- and ABA-regulated genes and early stomatal immunity, which limits the movement of bacterial pathogens into the leaf and is associated with PTI (Seo and Park 2010).

Jasmonic acid

Jasmonic acid (JA) has roles in both growth, development and defense; it plays a prominent role in flowering and in defense against insects and necrotrophic pathogens (Thomma et al. 2001; McDowell and Dangl 2000). Jasmonic acid also has a prominent role in wounding responses in plants (Wang et al. 2000). There are two defense signaling branches regulated by JA. One branch is controlled by MYC transcription factors (the MYC branch) and the second is controlled by both JA and ET (the ERF branch) (Broekgaarden et al. 2015b). There is extensive cross-talk between the two JA regulated defense signaling pathways. JA also communicates with other phytohormone pathways including SA (as described above), ET, GA, and ABA.

Increased levels of JA-Ile increases resistance to insects and necrotrophs (Gui et al. 2004) via induction of volatiles and secondary metabolites that directly deter feeding and recruit natural predators (Bruinsma et al. 2009), as well as antinutritive proteins that

deter the ability of an insect to digest proteins in the diet (Liu et al. 2005). Whereas in collaboration JA and ET, defend against necrotrophs. Some insect pests, such as *Spodoptera exigua*, *Leptinotarsa decemlineata* and *Bemisia tabaci,* are capable of manipulating JA signaling either through induction of SA signaling or directly targeting JA signaling components (Bede et al. 2006; Li et al. 2014a; Bruessow et al. 2010b; Zhang et al. 2017b).

JA Biosynthesis

The JA biosynthetic pathway is well characterized in *Arabidopsis*, tomato and rice (Wasternack and Hause 2013). JA synthesis is initiated within the chloroplast upon release α-linolenic acid (18:3) by phospholipases A1 *DAD1* or *PLD*. In *Arabidopsis*, AtDAD1 is associated with JA biosynthesis during development, while PLDs are responsible for wound-induced JA biosynthesis (Ishiguro et al. 2001; Wang et al. 2000). α-Linolenic acid is then converted to 12-oxo-phytodienoic acid (OPDA) by sequential action of lipoxygenases (LOXs), allene oxide synthase (AOS), and allene oxide cyclase (AOC). LOXs catalyzes the oxygenation of fatty acids to hydroperoxyl derivative, then AOS catalyzes the dehydration of 13-hydroperoxy-octa-decatrienoic acid to an unstable epoxide. This unstable epoxide is then converted into OPDA via AOC (Stenzel et al. 2012; Schaller 2001; Farmer and Goossens 2019; Turner et al. 2002). All three metabolic steps are localized to the chloroplast. While there is only one AOS in *Arabidopsis*, there are multiple AOCs and LOXs with roles in JA biosynthesis in *Arabidopsis*. There are six lipoxygenases in *Arabidopsis*: four 13S-lipoxygenases (LOX2/3/4/6) and two 9S-lipoxygenases (LOX1/5) but only LOX2/3/4/6 actively participate in JA biosynthesis (Nalam et al. 2015; Chauvin et al. 2012). LOX6 is

noteworthy as it is important is systemic accumulation of JA (Chauvin et al. 2012). Finally, the four AOCs of *Arabidopsis* are functionally redundant (Stenzel et al. 2012).

OPDA is then transported to the peroxisome via 12-oxophytodienoate reductases (OPRs). Of the three OPDA reductases (*OPR1/2/3*) in *Arabidopsis*, only OPR3 converts OPDA into 3-oxo-2-(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid (OPC 8:0) (Stintzi and Browse 2000; Schaller 2001). OPC-8.0 then undergoes three rounds of β-oxidation to form JA. JA is exported to the cytoplasm by jasmonate transporter 1 (JAT1), where JA is chemically modified for its different functions (Li et al. 2017b).

There a many cellular forms of JA and these have been extensively reviewed (Wasternack and Song (2016); (Wasternack and Strnad 2018)). A few important JA forms are highlighted here. JA conjugated to isoleucine by the jasmonate-amino acid synthetase JASMONATE-RESISTANT1 (JAR1) (Staswick and Tiryaki 2004). JA-Ile is a bioactive form with a role in signaling and activation of JA-mediated defenses. Additional forms of conjugated JA exist including JA-Ala, JA-Val, JA-Leu, and JA-Met; these conjugated forms also interact with the COI1-JAZ complex pointing to an essential role in JA signaling but JA-Ile is the most bioactive form (Yan et al. 2016). JA has a positive effect on JA biosynthesis. Exogenous applications of JA and MeJA lead to induction of the octadecanoid pathway and increased resistance to insect pests and necrotrophic pathogens

There are also two volatile forms of JA: JA can be modified into *cis*-jasmone (*cis*-JA) or methyl jasmonate (MeJA) (Wasternack and Song 2016). Inactive forms of JA also exist including 12-hydroxy-JA, JA methyl ester, 12-hydroxy-JA-Ile, and 12-carboxy-JA-Ile. After synthesis of JA, the carboxyl methyltransferase (JMT) converts JA to MeJA; MeJA can also be converted back to JA via a methyl esterase (JME). Inactive MeJA is

converted to JA-Ile by JAR1 (Staswick and Tiryaki 2004).The volatile MeJA has a role in intra- and interplant communication in defense against plant pathogens (Seo et al. 2001; Thomma et al. 1998; Wu et al. 2008; Turner et al. 2002). MeJA has roles in both pollination and JA signaling, while *cis*-JA has also been linked to pollination and interactions between aphids and parasitoids (Bruce et al. 2008; Wasternack and Song 2016).

JA perception and signaling: the MYC dependent branch of JA signaling

MYC2, 3 and 4 regulate the MYC-dependent branch of JA signaling (Kazan and Manners 2013). In addition, MYC2 is a transcriptional activator of some ABA-responsive genes (Abe et al. 2003). As with SA-induced defenses, plant cells have evolved stringent but flexible mechanisms to control activation and inactivation of JA-dependent defense responses (Ruan et al. 2019). In *Arabidopsis*, the F-box protein *CORONATINE INSENSITIVE 1* (COI1) serves as the central hub of JA signaling (Chini et al. 2007; Devoto et al. 2005; Ren et al. 2005; Katsir et al. 2008). COI1 is the JA-Ile receptor. In a nutshell, COI1 is part of a Skp1, Cullin, F-box containing (SCF) complex that is activated after JA-Ile is bound by COI1; the COI1-SCF complex degrades critical transcription factors that repress JA-responsive genes. Critical for the downstream of the COI1-SCF complex is the transcription factor *MYC2*, which is in a complex with negative regulators of JA signaling - the *JASMONATE ZIM-DOMAIN* (JAZs) proteins (Lorenzo et al. 2004b; Cheng et al. 2011; Chini et al. 2007). The MYC2-JAZ also interacts with transcriptional repressor *NOVEL INTERACTOR OF JAZ* (NINJA) and the transcriptional corepressor *TOPLESS* (*TPL*) (Pauwels et al. 2010; Cheng et al. 2011). In an unperturbed state, low levels of JA-Ile are synthesized and are incapable of releasing MYC2 from the JAZ-

NINJA-TPL complex, thereby inhibiting MYC2 -mediated activation of JA-responsive genes.

With JA accumulation, COI1 binds JA-Ile and JAZs are recruited to the COI-SCF complex for degradation by 26S ubiquitination (Xu et al. 2002; Chini et al. 2007; Devoto et al. 2005). This liberates MYC2 from its interactions with NINJA and TPL and MYC2 now binds to the G-box motif to induce expression of genes such as: *VEGETABLE STORAGE PROEIN 2 (VSP2), MYC2/3/4, MYB21/24, ETHYLENE RESPONSIVE FACTORs ½/4 (ERF1/2/4),* and OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF59 (*ORA59*) (McGrath et al. 2005; Lorenzo et al. 2003; Mandaokar et al. 2006; Pré et al. 2008; Kazan and Manners 2013; Boter et al. 2004). Several of these genes (*ERF1/2/4*, *MYC2/3/4*, *ORA59*) are transcription factors that positively influence JA signaling, while *VSP2* is an antinutritive protein that deters insect herbivory.

The JA and ET dependent signaling pathway: The ERF pathway

JA works with ET to induce a suite of genes active against necrotrophic pathogens. These genes include the well-studied *THIONIN-2.1* (*THI2.1*) and *PLANT DEFENSIN 1.2* (*PDF1.2*) (Brown et al. 2003; Vignutelli et al. 1998); which are considered sentinels for the JA/ET-pathway, which is designated as the ERF branch of JA signaling (Lorenzo et al. 2004a). Not unexpectedly, JA- and ET-signaling pathways can act independently, synergistically or antagonistically (Broekgaarden et al. 2015b).

The ERF1 pathway is a signaling pathway that can be utilized for JA signaling or ET signaling. MYC2 and EIN3/EIL1 are the determinants of JA-ET crosstalk. ETHYLENE INSENSITIVE 3 (EIN3) and EIN3 LIKE 1A (EIL1) are transcription factors involved in ET signaling (See ET signaling section). EIN3 and EIL1 integrate ET and JA

signaling to modulate gene expression, root development, and necrotrophic pathogen defense. As mentioned above, JAZ proteins bind MYC2 preventing JA-regulated gene expression (Kazan and Manners 2013). In addition, JAZ proteins with the help of HISTONE DEACETYLASE 6 (HDA6) repress EIN3/EIL1-regulated ET-responsive genes (Zhu et al. 2011b). A rise in JA results in the turnover of JAZ proteins freeing MYC2 and EIN3/EIL1 to activate JA- and ET-response genes (Chini et al. 2007; Zhu et al. 2011b). The de-repression of EIN3 allows ERF1 and ORA59 to be activated, which induces downstream defense genes, such as *PDF1.2* (Liu and Timko 2021). EIN3 and MYC2 directly interact with each other to modulate each other's responses, which results in JA-ET crosstalk (Song et al (2014). Additionally, JAZ proteins are capable of interacting with MYC2, EIN3, and EII1: this interaction causes a repression of EIN3 and EIL1 which causes a reduction in ET-mediated responses (such as formation of the apical hook after ET treatment) and a repression of MYC2 resulting in a reduction of the expression of MYC2-dependent, JA-dependent defense genes (Song et al. 2014; Lorenzo et al. 2004b).

The relationship the ABA and ERF1 branch of the JA-signaling is antagonistic, while the role of the MYC2 branch is more inconclusive (Kazan and Manners 2013). Exogenous ABA or ABA deficiencies suppress or induce JA-responsive genes, respectively (Adie et al. 2007; Asselbergh et al. 2008a). Several components of the ABA- and JA-signaling pathways interact with each other. For example, the ABA receptor *PYL* interacts with JAZ repressors and in turn cause a reduction in anthocyanin production in *Arabidopsis* (Lackman et al. 2011) .

ABA biosynthesis and signaling

Abscisic acid (ABA) is 15-carbon sesquiterpenoid that is well known for its role in regulating seed dormancy and responses to abiotic stresses such as water-deficit (drought), cold and osmotic stress (Vishwakarma et al. 2017; Tuteja 2007; Kuromori et al. 2018). There is growing evidence for ABA's role in biotic stress (Bharath et al. 2021; Cao et al. 2011; Ton et al. 2009). ABA mediates (a)biotic stress-induced closure of stomates to limit evapotranspiration and pathogen entry (Mauch-Mani and Mauch 2005; Munemasa et al. 2015; Bharath et al. 2021). ABA also negatively modulates the ERF signaling pathway that requires JA and/or ET for activation.

As an isoprenoid, ABA's precursor is the five-carbon IPP that is synthesized via the plastidal isoprenoid pathway (the MEP pathway). Numerous recent reviews have described the enzymes associated with this pathway and its complex regulation (Banerjee and Sharkey 2014; Rodríguez-Concepción and Boronat 2015). IPP undergoes several condensation reactions to generate the isoprenoids geranyl diphosphate (10C), farnesyl diphosphate (15C), and geranyl geranyl diphosphate (GGPP; 20C). Two GGPP units are then condensed by phytoene synthase (*PSY*) to create a C40 phytoene (Ruiz-Sola and Rodríguez-Concepción 2012; Kirby and Keasling 2009). Four subsequent dehydrogenation reactions using the enzymes phytoene desaturase (*PDS*), zeta-carotene desaturase (*ZDS*), and carotenoid isomerase (CRTISO) convert phytoene to lycopene (Avendaño-Vázquez et al. 2014; Bartley et al. 1999; Park et al. 2002).

Lycopene serves as a branch point for ABA biosynthesis; it is catabolized to α-carotene or β-carotene, with the latter being a precursor of ABA. β-carotene is metabolized into zeaxanthin by beta-carotene 3-hydroxylase 1 and 2 (*BCH1/2*) in a tightly regulated manner due to limited storage reservoirs for zeaxthanin in plants

(Finkelstein 2013; Sun et al. 1996). The first committed step of ABA biosynthesis is the conversion of zeaxanthin to all-*trans*-*violaxanthin* via two-step epoxidation. Zeaxanthin epoxidase (*ZEP/ABA1*) converts zeaxthanin to violaxanthin and this biochemical reaction can be reversed by violaxanthin de-epoxidase (*VDE*) (Xiong et al. 2002; Havaux et al. 2000). While *ABA1* is the only known *ZEP* in *Arabidopsis*, experiments with the ABA-deficient mutant *aba1* mutant indicate there is a minor ABA-biosynthesis pathway independent of ZEP/ABA1 (Barrero et al. 2005). In this case, violaxanthin is converted to either 9-cis neoxanthin by ABA4 or 9-cis-violaxanthin via an unknown enzyme (North et al. 2007; Finkelstein 2013; Dejonghe et al. 2018). The rate-limiting step for ABA biosynthesis is mediated *9-CIS-EPOXYCAROTENOID DIOXYGENASE* (*NCED*) to convert violaxanthin to xanthonin (Xiong and Zhu 2003; Tan et al. 2003). Xanthonin then undergoes several oxidation steps and is then converted to abscisic aldehyde by *ABA2*, then finally to abscisic acid via *AAO3/ABA3* (Léon-Kloosterziel et al. 1996; Schwartz et al. 1997; Seo et al. 2000). After synthesis, ABA can be glycosylated to an inactive storage form (ABA-GE), transported and stored in the vacuole for ready deployment in times of (a)biotic stress. ABA signaling is also dependent on ABA transport to guard cells, which is fulfilled by ATP-binding cassette (ABC) transporters in a manner yet to be elucidated (Ng et al. 2014).

Our current understanding of the mechanisms of ABA perception and signaling has been mainly elucidated in *Arabidopsis* and is dependent on three major components: (1) the ABA receptors, which include *PYRABACTIN RESISTANCE 1* (*PYR1*) and 13 *PYR1-like* (*PYL*) proteins, (2) group-A protein phosphatases type 2C (PP2Cs), and (3) SNF1-related kinase 2s (SnRK2s) (Kulik et al. 2011; Dittrich et al. 2019; Hirayama and Umezawa 2010). Under non-stress conditions, plants produce low

levels of ABA and SnRK2s are associated with PP2Cs. SnRK2s are unable to phosphorylate downstream ABA-responsive targets. When ABA levels rise during (a)biotic stress, PYR/PYL receptors bind ABA and release SnRK2s. SnRK2s are now able to phosphorylate downstream substrates to activate ABA-dependent gene expression and cellular events that mediate stomatal closure (Komatsu et al. 2013; Kobayashi et al. 2005; Soon et al. 2012; Kulik et al. 2011; Park et al. 2009; Umezawa et al. 2009; Hirayama and Umezawa 2010).

In addition to SnRK2 activity, ABA signaling is heavily reliant on additional phosphorylation and dephosphorylation of proteins in the ABA-signal transduction chain. For example, the phosphorylation status of *PYLs* is tightly regulated by multiple protein kinases. Phosphorylation of PYLs by Early flowering 1 (EL1)-like casein kinase (AEL) and C-terminally encoded peptide receptor 2 (CEPR2) target PYLs for turnover by the proteasome to negatively regulate ABA signaling. *TARGET OF RAPAMYCIN* (*TOR*) phosphorylation of PYLs disrupts the ability of PYLs to bind ABA; under these conditions, PP2C binds SnRKs to prevent activation of ABA signaling in unstressed plants and prioritize growth (Kravchenko et al. 2015; Yu et al. 2019; Zhang et al. 2018b; Chen et al. 2018a). Finally, the *Arabidopsis* and cytosolic ABA receptor kinase 1 (CARK1) phosphorylation of PYL positively impacts ABA signaling (Zhang et al. 2018b). In addition, several class A PP2Cs serve as negative regulators the ABA response by inhibiting SnRK2s including: ABI1, ABI2, HYPERSENSITIVE TO ABA1 (HAB1), HAB2, ABA-HYPERSENSITIVE GERMINATION1 (AHG1), AHG3/PP2CA, and HIGHLY ABA-INDUCED1 (HAI1), HAI2 and HAI3 (Yoshida et al. 2005b; Kim et al. 2013; Antoni et al. 2011; Kuhn et al. 2005; Nishimura et al. 2007; Merlot et al. 2001; Kobayashi et al. 2005; Chen et al. 2018a).

Finally, two transcription factor families with positive roles in ABA regulation have been identified. The ABI5 family is associated with germination and seed development and the AREB/ABF family is associated with activation of abiotic stress responses (ABF1, AREB1/ABF2, AREB2/ABF4, and ABF3) (Kang et al. 2002; Uno et al. 2000; Choi et al. 2000; Kim et al. 2002; Bensmihen et al. 2005). Recently, several of these transcription factors have been linked to cold stress (ABF1, SNAC1/2), drought (ABF4, ATAF1, SNAC1/2, MYC2, DREB2) , and salt stress (ABF2/3/4, MYB2, MYC2, CBF4) (Agarwal and Jha 2010). Additional transcription factors are also involved in ABA-regulated responses including well characterized MYCs and MYBs; the diversity of factors utilized in the ABA-signaling network was recently reviewed by Kuromori et al. (2018) and Chen et al. (2020).

ABA and ethylene are known to be antagonistic to each other (Ton et al. 2009) The ABA mutants *aba2* were found to have elevated levels of ET, while the ET mutants *etr1* and *ein2* were found to have elevated levels of ABA (Chiwocha et al. 2005; LeNoble et al. 2004; Ghassemian et al. 2000). For example, ABA inhibits ET signaling via the transcription factor HY5. HY5 binds to the ethylene-responsive transcription factor ERF11 and HY5 can inhibit ET biosynthesis by binding directly to ACS genes responsible for ET biosynthesis (Li et al. 2011). There is also evidence for ABA-ET synergism. ABA-deficient mutant *aba3-1* was found to have increased susceptibility to the necrotroph *Alternaria brassicola*, pointing to some synergism between the pathways (Fan et al. 2009). The relationship between JA and DELLAs will be discussed later.

Ethylene: Biosynthesis and roles in defense

The hydrocarbon ethylene (ET) is associated with plant development, fruit ripening, senescence and defense to pathogens and pests (Broekgaarden et al. 2015b). In defense, as mentioned above ET is a key regulator of the ERF1 pathway that is co-regulated by JA and ET. ET perception is known to be important in symptom development in tomato infected with *Xanthomonas campestris* pc *vesticatoria*, *P. syringae* pv *tomato* and *Fusarium oxysporum* f sp *lycopersici* (Lund et al. 1998). ET can also promote interactions with beneficial, mutualistic fungi to plant hosts (Khatabi et al. 2012; Khatabi and Schäfer 2012). Finally, the roles of ET in defense to herbivores varies significantly and is often host plant species or cultivar dependent. These varying roles for ET in plants requires tight regulation of ET biosynthesis and signaling.

Ethylene biosynthesis

ET biosynthesis is initiated with the amino acid methionine (Met) (Wang et al. 2002). Approximately 80% of Met produced in plant hosts in converted by SAM synthetase into the major electron donor *S*-AdoMet that is used in myriad biosynthetic processes (Ravanel et al. 1998). For ET biosynthesis, ACC synthase (ACS) uses *S*-AdoMet to synthesize 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is used as a substrate to produce into ET by ACC OXIDASE (ACO); alternatively, ACC can be conjugated to malonate or glutathione to form MACC and GACC, respectively; MACC and GACC are thought to be inactive ACC storage forms (Kionka and Amrhein 1984; John et al. 1999).

Both *ACS* and *ACO* are members of multigenic families in Arabidopsis and other plants (Babula et al. 2006). In most conditions, *ACS* in the rate limiting step of ET

biosynthesis (Wang et al. 2002). In Arabidopsis, ten of the twelve *ACS* genes are enzymatically active and convert *S*-AdoMet to ACC. Only two do not have roles in ET biosynthesis; *ACS1* is catalytically inactive and *ACS3* is a pseudogene (Liang et al. 1995; Liang et al. 1992). More recently, it was revealed that under selected conditions, *ACO* can be a rate limiting step for ET biosynthesis (Houben and Van de Poel 2019).The Arabidopsis *ACO* gene family has five members and substantial expansions of the *ACO* gene family has occurred in other plant species (Wang et al. 2016b; Terol et al. 2010).

ET biosynthesis is tightly regulated by ACS activity and protein levels. ETO1 (ETHYLENE OVERPRODUCER 1) binds ACS to inhibit its catalytic activity and to promote its turnover by the 26S proteasome; interestingly, cytokinin prevents ACS turnover using a yet to be identified mechanism (Christians et al. 2009; Yoshida et al. 2005a; Wang et al. 2004). To counter ACS instability and promote ET synthesis, ACS can be stabilized phosphorylation (Wang et al. 2002; Kende 1993). ACS can be phosphorylated by calcium-dependent protein kinase (CDPK) or by both CDPK and mitogen-activated protein kinase 6 (MPK6) (Argueso et al. 2007).

Ethylene signaling

ET is perceived by five ethylene receptors (ETR1, ETR2, EIN4, ERS1, and ERS2) located on the endoplasmic reticulum membranes (Sakai et al. 1998; Hua and Meyerowitz 1998; Hua et al. 1995; Hua et al. 1998; O'Malley et al. 2005; Qu and Schaller 2004). The ET receptors also requires a copper co-factor, which is donated by the copper exporter RAN1 (RESPONSIVE-TO-ANTAGONIST1) (Binder et al. 2010). CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1), EBF1/2 (ETHYLENE BINDING FACTORS 1 and 2), and EIN2 targeting protein 1 and 2 (ETP1/2) are negative

regulators and prevent inadvertent triggering of the ET-response cascade in the absence of ET (Qiao et al. 2009). In the absence of ET, the ET receptors bind and activate the Raf protein kinase CTR1 (Kieber et al. 1993). Both CTR1 and its target EIN2 bind to the ET receptors. CTR1 phosphorylates and inactivates the ER membrane-localized and central integrator of ethylene responses EIN2 (ethylene insensitive protein 2) (Ju et al. 2012a). Furthermore, ETP1/2 promote the turnover of EIN2 by the 26S proteosome, to keep this positive regulator at low levels. Finally, EBF1/2 bind to the nuclear-localized transcription factors EIN3 and EIN3-like 1/2(EIL1/2) to target these proteins for degradation by the 26S proteasome (Dolgikh et al. 2019).

When ET levels rise, the ET receptors bind ET and dissociates from -CTR1, which inhibits CTR1 kinase activity (Kieber et al. 1993; Ju et al. 2012a). The non-phosphorylated EIN2 is cleaved to release its active C-terminal domain (EIN2-CEND), which has two modes of action. With EIN5, 5’ – 3’ exoribonuclease (XRN4) and LARP1A, EIN2 binds to the EBF1/2 RNAs and sequesters them in P bodies in the cytosol, to limit EBF1/2 protein accumulation and action (Olmedo et al. 2006; Merret et al. 2013; Li et al. 2015b). EIN2-CEND also migrates to the nucleus activate the central ET regulators EIN3 and EIL1/2 (Ju et al. 2012b; Dolgikh et al. 2019; Chao et al. 1997). These ET-responsive transcription factors activate transcription of a battery of ET-response genes such as ERFs (ETHYLENE RESPONSE FACTORs) and other ethylene-responsive element binding proteins (EREBPs) to deploy ET-mediated responses (Riechmann and Meyerowitz 1998; Chen et al. 2016; Dietz et al. 2010).

EIN2 interacts with a number of proteins to modulate ethylene signaling. EIN2-CEND interacts with a novel proteasome subunit EER5 that mediates Ein2-CEND turnover to enable the resetting of ethylene signaling (Christians et al. 2008). EIN2-

CEND action is also modulated by ECIP1 (EIN2 C-TERMINUS INTERACTING PROTEIN 1) to influence salt tolerance (Lei et al. 2011). Finally, histone acetylation and deacetylation are also involved in transcriptional regulation of ethylene signaling. Interestingly, upon binding of EIN2, the chromatin-associated ENAP1 (ETHYLENE NUCLEAR ASOCCIATED PROTEIN1) promotes opening of chromatin surrounding ET-response genes to provide better access to the EIN3/EIL transcription factors (Zhang et al. 2017a). In addition, ENAP1 and EIN2 participate in the suppressing ET-downregulated genes by their interactions with the histone deacetylases SIRTUIN1/2 (Zhang et al. 2018a).

As with other phytohormone-signaling pathways, the ET-signaling pathway deploys transcription factors that can enhance or repress ET signaling. The ethylene-responsive factors (ERFs) are well known for their responses to ET having myriad roles in growth, development, and defense (Licausi et al. 2013; Thirugnanasambantham et al. 2015; Heyman et al. 2018). ERFs are a part of the APETALA2/ERF superfamily of transcription factors that have one or more AP2 DNA-binding domains and are classified into four subfamilies: the ERF, AP2, and RAV (RELATED TO ABI3/VP) proteins) and the small Soloist group with highly divergent structures. The size of this superfamily varies in plants from as few as 131 (cucumber) to 200 in poplar (Thirugnanasambantham et al. 2015). In addition, the members of the ERF family have been classified several times. Sakuma et al. (2002) divided the Arabidopsis ERFs into 6 DREB (DEHYDRATION RESPONSIVE ELEMENT BINDING) groups (A1-A6) and six ERF groups (B1-B6). In contrast, Nakano et al (2016) divided the Arabidopsis and rice ERF subfamily into ten groups with I-IV corresponding proteins in the A1-A6 class and V-X corresponding the

B1-B6 proteins (Sakuma et al. 2002). Unfortunately, there is not a one-to-one correspondence of the groups (Licausi et al. 2013).

The ERF family is further classified into groups and subfamilies (Licausi et al. 2010; Nakano et al. 2006). AP2/ERF superfamily members can be transcriptional activators, passive transcriptional repressors, or active transcriptional repressors (Licausi et al. 2013). The diversity of the ERF TF superfamily means superfamily members can play many roles in (a)biotic stress and phytohormone response (Libault et al. 2007; Yang et al. 2005; Yang et al. 2011a; Ogawa et al. 2005; Sakuma et al. 2006; Warmerdam et al. 2019).

ET's role in insect defense

As a volatile, ET modulates volatile organic compound levels in conjunction with JA in intra- and interplant signaling (Schmelz et al. 2003). JA and ET act together to induce mediate basal resistance against necrotrophic pathogens (Huffaker et al. 2013; Holopainen and Blande 2012; Han et al. 2010). The simultaneous activation of JA and ET results in the transcriptional activation of ERF1 (Lorenzo et al. 2003) and ERF1 activates the expression of a battery of genes called JA/ET-responsive genes. ET also plays a role in PTI and ETI. ETI induces rapid increases in ET and PTI displays a biphasic accumulation of ET with an early and later burst (Mur et al. 2009; Broekgaarden et al. 2015b; Boller and Felix 2009).

ET has multiple impacts on insect herbivory. Ethylene's impact on herbivory was first documented in rose infested with red spider mites where infested tissue released more ET than uninfested tissue (Williamson 1950). ET production was subsequently linked to insect feeding by Duffey and Powell (1979) and Rieske and Raffa (1995). Since

these first experiments exploring ET's relationship to herbivory, ET's role in herbivory responses has been further explored. Insect herbivory has been linked to ET induction by fall armyworm in corn (Harfouche et al. 2006) and by *Tetranychus urticae* in Lima bean (Arimura et al. 2002). ET induction is also detected in response to multiple phloem-feeding insects including brown planthopper (Lu et al. 2011) and multiple species of aphids (Anderson and Peters 1994; Arga**n**dona et al. 2001; Botha et al. 2014; Hu et al. 2011; Li et al. 2013a; Zhang et al. 2019). Additionally, ET was found to induce isoflavonoid levels in soybean (Dillon et al. 2020). Finally, ET is linked to the volatiles associated in tritrophic interactions as silencing of ET biosynthesis genes reduces attraction of carnivorous mites and decreases resistance to *Chilo suppressalis* (Lu et al. 2014; Broekgaarden et al. 2015b).

ET is important is for basal resistance responses to several insects. Several ET-response genes are induced by insect feeding including several ERFs in chickpea (Pandey et al. 2017) and barley (Leybourne et al. 2019). Several ET-associated genes have also been linked to Hemipteran resistance including *Pti5* conferring resistance to potato aphid in tomato, *MYB44* and *EIN2* conferring resistance to green peach aphid and diamondback moth, and ERF113 conferring resistance to spotted alfalfa aphid in *Medicago truncatula* (Lü et al. 2013; Li et al. 2015a; Jacques et al. 2020). ET was also implicated as a phytohormone involved in *Vat*-mediated resistance to aphid in melon (Anstead et al. 2010). Another ET-mediated resistance mechanism against aphids was also found in corn as *mir1* is responsible for deterring aphid settling and aphid populations (Louis et al. 2015). Conversely, ET has been linked to negative responses to herbivores (Tian et al. 2014; Stotz et al. 2000), with several instances being linked to Hemipteran in particular (Mantelin et al. 2009; Ye et al. 2020; Hu et al. 2016)

Balancing growth and defense: Roles of Brassinosteroids, Cytokinins, Auxin, and Gibberellic Acid

Deployment of the plant defense machinery is a spendthrift function that compromises plant growth and reproduction. Therefore, plants need to balance the trade-offs between utilizing resources for growth/development/reproduction versus defense against phytopathogens and pests (Huot et al. 2014). As JA, SA, ET, and ABA are the major phytohormones critical in mounting effective defenses, communication between these defense-signaling pathways is critical to mounting an effective defense against an invader and to limit the toll on plant health, growth and reproduction. The lifestyle strategy of phytopathogens, as biotrophs, hemi-biotrophs, or necrotrophs, trigger different defense signaling pathways and responses to biotrophs and necrotrophs are often antagonistic (Grant and Jones 2009b; Huot et al. 2014; Checker et al. 2018). However, there is now an emerging picture of synergistic communication between different phytohormones (Checker et al. 2018). This section will explore the synergistic and antagonistic plant hormone relationships and how they impact plant defense outcomes. Of particular interest are the interactions with brassinosteroids (BRs), cytokinins, auxin, and gibberellic acid.

Brassinosteroids

Brassinosteroids (BRs) regulate plant growth. BRs are a class of nearly 70 polyhydroxylated sterol-derived steroids found widely among the plant kingdom and among plant steroids, BRs are most closely related to animal steroid hormones (Kutschera and Wang 2012; Clouse 2011). BRs can be found in myriad tissue types throughout individual plants, thought they are most commonly found in reproductive tissues and at lower levels in leaves/shoots (Takatsuto 1994). BRs mostly function in

growth and reproduction processes including cell expansion, cell elongation, cell differentiation, and etiolation (Nolan et al. 2019).

The role of BRs in plant defense is not well studied. BRs been linked to positive and negative roles in plant defense. For example, endogenous application of BRs to rice and barley contributed to increased tolerance to leaf pathogens and increased *Fusarium* resistance in barley (Nakashita et al. 2003; Ali et al. 2013). However, BRs did not improve *Arabidopsis* or rice resistance to *P. syringae* pv. tomato (Pto) or *Pythium garminicola*, respectively (De Vleesschauwer et al. 2012; Albrecht et al. 2012). The interactions of BRs some defense phytohormones (ET and ABA) is not yet resolved (Wasternack 2014; Huang et al. 2010; Peng et al. 2011). However, there is evidence BRs might be antagonistic to SA and JA: BRs may suppress SA-signaling and BRs are also linked to the suppression of JA-mediated anthocyanin accumulation and root inhibition (Huang et al. 2010; Peng et al. 2011).

Cytokinins

Like BRs, the role of cytokinin (CKs) in defense is poorly understood. CKs are N^6-substituted adenine derivatives generally involved in root and shoot growth and development and are negative regulators of senescence (Kieber and Schaller 2014). A few studies suggest that CKs can act synergistically with SA to induce phytoalexins and increase resistance to rice blast fungus (O'Brien and Benková 2013; Jiang et al. 2013; Checker et al. 2018). In addition, two proteins of the CK histidine phosphotransfer signaling machinery (i.e., AHP and ARR) may provide an alternate mechanisms of ET perception that is EIN3-independent (Binder 2020).

Auxins

Auxins are low molecular weight, weak organic acids that primarily found in roots, shoots, and younger leaves and are primarily involved in development and cell elongation. Auxin is a negative regulator in plant stress responses and is often antagonistic to the action of other defense hormones (Fahad et al. 2015). SA and SA-induced SAR suppresses auxin-responsive genes, which results in stabilization of auxin repressors and the repression of auxin-responsive genes (Wang et al. 2007). One reason for suppressing auxin responses is that auxins loosen the plant cell wall, which plant hosts more susceptible to pathogens (Cosgrove 2005; Ding et al. 2008; Wang et al. 2007; Robert-Seilaniantz et al. 2011; Checker et al. 2018).

Gibberellins

Gibberellins (GAs) are growth-promoting hormones that impact germination, seed development, flowering, stem elongation, and leaf expansion. GAs have both synergistic and antagonistic relationships with defense hormones JA and SA, respectively. The JA and GA signaling pathways intersect due DELLA protein-JAZ protein interactions. DELLA proteins are part of the GA-signaling pathway, where they serve as negative regulators by binding growth-promoting transcription factors such as PIFs (PHYTOCHROME-INTERACTING FACTORs); DELLAs are turned over by the 26S proteasome and this is correlated with activation of GA signaling (Silverstone et al. 2001).

In defense, DELLAs appear to fine tune JA signaling (Bao et al. 2020) As noted earlier in a resting state, MYC2 is complexed with JAZ proteins and cannot activate JA-responses. When JA levels rise, JAZ degradation occurs and MYC2 can now activate

JA-response genes. DELLAs also compete with JAZs to bind to MYC2. At low GA levels, DELLAs interact with JAZ1 and MYC2 is liberated and JA-mediated responses are deployed (Hou et al. 2010). However, as GA increases, DELLAs are turned over and MYC2 and JAZ1 interact, which represses JA-mediated responses (Bao et al. 2020). In this manner, GA and JA act antagonistically to modulate the levels of free MYC2 proteins to regulate the robustness of the JA-defense response. Reciprocally, JA antagonizes GA-mediated growth by increasing the levels of DELLAs (Yang et al. 2012). In addition, RGA interacts with the transcription factor MYC2 to repress JA-defense responses (Hong et al. 2012). This complex "give and take" between the JA- and GA-signaling pathways provides the fine tuning needed to coordinate JA-mediated defense and GA-mediated growth.

Finally, GAs also promote JA-regulated gene expression. In flowers, GA enhances JA-signaling by increasing JA biosynthesis, inducing *MYB21*, *MYB24* and *MYB57* expression to stimulate stamen development (Cheng et al. 2009). In addition, GA stimulates JA-regulated terpene synthesis (Hong et al. 2012). While much less is known about SA's relationship with GAs, early evidence points to some synergism between the two hormones (Emamverdian et al. 2020).

Pathogen Manipulation of Plant Immunity

Since both pathogens/pests and plants deploy an arsenal of mechanisms to induce or deter infection/infestation, respectively, resulting a perpetual evolutionary arms race exists between them (Martel et al. 2021; Jones and Dangl 2006). Many plant pathogens/pests have evolved mechanisms to manipulate phytohormone crosstalk to benefit their subsistence on their host (Grant and Jones 2009a; Kaloshian and Walling 2016). Pathogen suppression of phytohormone defense pathways confirms the

importance of PTI, ETI and phytohormone signaling pathways importance in immunity; for this reason, some effectors are considered virulence factors. Other effectors trigger ETI by their direct or indirect recognition by NLRs. While a few effectors that trigger ETI are known from Hessian flies and the NLR loci that are important for “gene-for-gene” resistance are known, the molecular interactions between these molecules have yet to be identified (Aggarwal et al. 2014). Effectors in pathogens and pests have been extensively reviewed and are described further in Kazan and Lyons (2014), Kaloshian and Walling (2016), Basu et al. (2018), Naalden et al. (2021), and (Huang et al. 2020a) . Below I will discuss a select number of Hemipterans, including whiteflies, with effectors known to impact herbivore success.

Hemipteran insects introduce effectors to suppress host plant defenses and to trigger host plant resistance. Whiteflies are phloem-feeders and obligate biotrophs. The first indirect evidence for whitefly effectors that suppress host-plant defenses were first gleaned from studies in Arabidopsis. In these interactions, *B tabaci* growth is inhibited by JA mediated defenses (Zarate et al. 2007; Kempema et al. 2007), as whitefly nymph development was accelerated on JA-deficient/SA-overexpressing mutants and inhibited on JA-deficient/SA-overexpressing mutants. *B. tabaci* activates SA-mediated defenses to suppress JA-mediated defenses, thereby making Arabidopsis a better host for nymph development (Kempema et al. 2007; Zarate et al. 2007). Zhang et al. (2013c) repeated and extended these studies an ethylene mutant (*ein2-1) and* replicated these studies in semi-field conditions. Whitefly nymph development on *ein2-1* mutants was accelerated; similar to JA biosynthesis and perception mutants (Zhang et al. 2013b). Interesting, the elevated SA produced during whitefly infestation of Arabidopsis is also detrimental to this insect (Zhang et al. 2013c). Whitefly infestation of Arabidopsis induces a

ocimene/myrcene synthase, which causes infested plants to emit a volatile blend that is more attractive to its parasitoids, thereby enhancing biocontrol (Zhang et al. 2013a). Finally, in Lima bean whiteflies impact ocimene in a different manner (Zhang et al. 2009). In Lima bean, whiteflies suppress the ocimene is usually associated with spider mite infestation.

As stated earlier, Hemipteran species have effectors capable of interfering with defense signaling. Hemipteran effectors have been explored more comprehensively in aphids and are reviewed in Van Bel and Will (2016) and Huang et al. (2019). Knowledge of whitefly effectors, however, has been recently expanded. The *B. tabaci* salivary effector *Bsp9* interferes with defense signaling through WRKY33 and the MAPK3/6 signaling cascade (Wang et al. 2019d). Whereas, *Bt56* induces SA signaling and *BtFer1* reduces H_2O_2, callose deposition, and JA in host plants to promote whitefly performance (Su et al. 2019; Xu et al. 2019). Additionally, several *Bemisia tabaci* specific salivary proteins were identified including hydrolases, oxioreductases, apolpophorins, and vitellogenins (Huang et al. 2021).

Mealybugs (*Phenacoccus solenopsis*) are also capable of commandeering host plant defenses to their own advantage. Tomato's JA-regulated defenses interfere with mealybug feeding and nymph development (Zhang et al. 2015). Mealybugs manipulate the tomato host to suppress JA production and enhance SA levels and SA signaling (Zhang et al. 2015). There are instances of the necrotrophic pathogen *Alternaria solani* and several Lepidopteran species capable of manipulating SA-JA crosstalk to their benefit (Bruessow et al. 2010a; Diezel et al. 2009; Rahman et al. 2012; Zhang et al. 2015). There are similar bouts of crosstalk manipulation observed among *P. syringae*,

aphids, and psyllids (Laurie-Berry et al. 2006; Fernández-Calvo et al. 2011; Morkunas et al. 2011).

Host Plant Resistance

Host plant resistance (HPR) is a critical part of an IPM program (Onstad 2019; Lefebvre et al. 2020; Stout and Davis 2009). IPM programs centered around HPR are more sustainable, economically viable, and more durable. HPR centered around multigene resistance is particularly ideal because it is more difficult for pests and pathogens to break multi-component resistance mechanisms. When thinking about phloem-feeding hemipteran pests, many R mechanisms are phloem-mediated (Walling 2000). Many HPR mechanisms against phloem-feeding insects have been identified and planthopper resistance in rice and aphid resistance has been identified in wheat, corn, tomato, *Medicago truncatula,* and soybeans (Gururani et al. 2012). However few have been studied at the mechanistic level. A discussion of the *R* genes to brown planthopper and aphids appears in the section entitled Host plant resistance to Hemipteran insects (Introduction Section 4.3) . HPR to whiteflies has been found in tomato, cassava, Brassica, melon, soybean, cowpea, common bean, and cotton (Teuber et al. 1997; Silva et al. 2019; Gulluoglu et al. 2010; Cruz et al. 2014b; Simmons et al. 2019; Simmons and Levi 2002; Broekgaarden et al. 2012; Carabalí et al. 2010; Nombela et al. 2003). Many of these resistance mechanisms are multigenic. This section will focus on further describing these whitefly resistance mechanisms.

Tomato

Tomato (*Solanum lycopersicum*) is a perennial that is grown as an annual in non-tropical regions and is crop with significant agricultural value to the United States and the world. With domestication, many of the potent resistance mechanisms to Hemipteran pests and other herbivores have been lost (Ferrero et al. 2020). For this reason, resistance to whiteflies in wild tomato species has been intensively investigated. Whitefly resistance was identified in numerous wild tomato species including: *Solanum pennellii*, *S. habrochaites*, *S. habrochaites* f. *glabratum*, *S. pimpinellifolium*, S, *galapagense*, and *S. chilense* (Firdaus et al. 2012). Tomato's resistance influences multiple whitefly behaviors with both antibiotic or antixenotic resistance being displayed (Vosman et al. 2018).

Tomato's whitefly resistance mechanism is largely trichome-dependent. This trichome-mediated resistance is broad based and effective against a large spectrum of herbivores (Alba et al. 2009; Firdaus et al. 2012; Silva et al. 2014). While multiple types of trichomes are present on resistant wild tomato species, type IV trichomes are largely responsible for whitefly-resistant allelochemical production (Silva et al. 2014). Depending on the wild tomato species, trichomes produce a suite of compounds inhibiting insect growth and development including acylsugars, methyl ketones, and sesquiterpenoids (Yao et al. 2019; Firdaus et al. 2013; Mutschler et al. 1996; Liedl et al. 1995; Leckie et al. 2012; Frelichowski and Juvik 2005; Firdaus et al. 2012; Escobar-Bravo et al. 2016). Flavonoids have also been identified as repellant to whiteflies in certain tomato species (Yao et al. 2019). The synthesis of each of these secondary metabolites is controlled multiple genes, which makes breeding and developing allelochemical-producing commercial varieties difficult (Firdaus et al. 2012; Firdaus et al. 2013; Leckie et al. 2012).

Broad spectrum resistance to herbivores was discovered in *Solanum pimpinellifolium* L. spp. including aphids, two-spotted spider mites (*Tetranychus urticae* Koch), tomato leafminer (*Tuta absoluta*), thrips, and two species of whiteflies (*Bemisia tabaci* and *Trialeurodes vaporariorum*) (Alba et al. 2009; Vosman et al. 2018; Rodriguez-Lopez et al. 2011; Rakha et al. 2017; McDaniel et al. 2016). *S. pimpinellifolium* accessions produce and store acyl sugars in type IV trichomes (Rodriguez-Lopez et al. 2011; Fernandez-Munoz et al. 2003; Silva et al. 2014). Acyl sugars are known to repel and irritate whiteflies, constrain oviposition, induce adult mortality, and delayed nymph development in whiteflies (Fernández-Muñoz et al. 2000; Alba et al. 2009; Escobar et al. 2010; Rodriguez-Lopez et al. 2011; Silva et al. 2014). EPG studies also show that *T. vaporariorum* (the greenhouse whitefly) has difficulty feeding on *S. pimpinellifolium* (McDaniel et al. 2016). This multigenic resistance mechanism has been successfully moved from S. *pimpinellifolium* to cultivated tomato (Rodriguez-Lopez et al. 2011; McDaniel et al. 2016), where it confers antixenotic resistance to *B. tabaci* MEAM1 and *T. vaporariorum* (Muigai et al. 2002).

Solanum habrochaites f. *glabratum* (formerly known as *S. hirsutum f. glabratum*) has type IV trichomes that are rich in methyl ketones which have a strong antibiotic effect on herbivores including spider mites, aphids, and whiteflies (Chatzivasileiadis and Sabelis 1997; Williams et al. 1980). Methyl ketones reduce the oviposition rate, as well as nymph and adult survival rates of *T. vaporariorum* (Bas et al. 1992; Romanow et al. 1991). This resistance is plant age-dependent; as the secondary metabolites in trichomes of older plants were more likely to confer resistance than younger plants (Bas et al. 1992). Several QTLs across multiple chromosomes are linked to methyl ketone production (Erb et al. 1994; Firdaus et al. 2012; Firdaus et al. 2013). Sesquiterpenoids

from type VI trichomes of *Solanum habrochaites* are also an effective means of whitefly control. The introduction of sesquiterpene biosynthesis genes into glandular trichomes made greenhouse whiteflies less fecund (Bleeker et al. 2012).

Tomato's Mi1.2 is one of the few gene-for-gene resistance genes that confer resistance to phloem-feeding pests have been characterized at the molecular level. Tomato's NBS-LRR gene. *Mi-1.2* was first identified as conferring resistance to several root-knot nematode species (*Meloidogyne spp*) and was later shown to confer resistance to muliple insects including: whitefly (*B. tabaci* MEAM1 and MED, the pink potato aphid (*Myzus persicae*), and a psyllid (*Bactericera cockerelli*) (Nombela et al. 2003; Nombela et al. 2001; Goggin et al. 2006; de Ilarduya and Kaloshian 2001; Casteel et al. 2006). *Mi1.2* resistance, however, has several limiting factors. First, *Mi1.2* resistance to whitefly is temperature- and age-dependent (Nombela et al. 2003; de Ilarduya and Kaloshian 2001). Second, while aphid resistance is phloem mediated, whitefly resistance factors for other phyla are not. Mi-1.2's whitefly resistance is apoplast-mediated. Jiang and Walker (2007) conducted studies comparing phloem feeding of whiteflies on resistant and susceptible alfalfa. They found while whiteflies were able to reach the phloem on resistant alfalfa, their feeding time was short: they postulated that feeding was plausibly inhibited by a toxin or a p-protein. Third, the resistance conferred to psyllids is unique as it influences adult choice and development from egg to adult, but not oviposition rates or development time (Casteel et al. 2006). Finally, transferal of *Mi1.2* to eggplant and tomato did not confer resistance to aphids or whiteflies, respectively (Nombela et al. 2003; Goggin et al. 2006), suggesting the *Mi1.2* resistance requires additional genes for deployment. A microarray study was also conducted with a resistant and a susceptible tomato variety to identify genes involved in whitefly resistance and among them an

ortholog to the pathogenic bacteria resistance gene AIG and a gene encoding diaminopimelate epimerase were upregulated (Rodríguez-Alvarez et al. 2019).

Whitefly resistance in Cassava

Cassava (*Manihot esculenta*) is a perennial shrub grown in the tropic and subtropic regions. While indigenous to the neotropics, cassava was introduced to Africa in the 16th century, followed by Asia in the late 18th and 19th centuries (Bellotti and Arias 2001; Hershey et al. 2001). It is largely viewed as a resilient crop for farmers under socioeconomic limitations (Bellotti and Arias 2001; IITA 2020). Most of its nutritional and economic value lies in its edible roots containing over 80% starch in dry matter, however its leaves have some value as a food source due to relatively high protein levels (El-Sharkawy 2004). As mentioned earlier in the *Introduction*, cassava roots contain metabolites (such as linamarin) that release hydrocyanic acid (HCN) upon cellular damage (Hillocks et al. 2002). This poses a threat to humans and animals, but the deployment of low HCN cassava ("sweet" cassava) and post-harvest processing methods such as grating, fermentation, or dehydration are effective means in mitigating HCN content in cassava (IITA 2020). High HCN producing cassava deter generalist insect pests, but not specialist insects, from feeding on cassava in the field (Bellotti et al. 1999; Bellotti et al. 1994). Most recently, it was shown that despite the limited tissue-damage that occurs during whitefly feeding, limamarin and HCN are induced by *B. tabaci* Sub-Saharan African 1 (SSA1) after feeding on cassava (Easson et al. 2021). *BtSSA1* can adapt to cassava by glycosylating the cyanogenic glucoside linamarin several times and also by phosphorylating linamarin and its derivatives into an inert form.

Several genera of whiteflies are severe pests of cassava including: *Aleurothrixus aepim, Aleurotrachelus socialis,* and a number of *Bemisia tabaci* species from SubSaharan Africa (Bellotti et al. 2012; Macfadyen et al. 2018). *B. tabaci* causes both direct and indirect impacts on the value of the cassava crop. The superabundant *B. tabaci* populations cause direct damage including plant stunting and reducing root yields by more than 45% (Legg et al. 2014; Thresh et al. 1997). In addition, virus-transmission by *B. tabaci* spp poses a severe threat to cassava production in the field. These viruses included the African Cassava Mosaic Virus (ACMV), East African Cassava Mosaic Virus (EACMV), Cassava Brown Streak Virus (CBSV), and Ugandan Cassava Brown Streak Virus (UCBSV) (Colvin et al. 2004; Maruthi et al. 2017). While these diseases are most common to Africa, they are beginning to emerge in Asia (Minato et al. 2019; Malik et al. 2020; McCallum et al. 2017). Viruses also impact root size and quality causing significant economic losses with up to 50% yield loss upon infection (Legg et al. 2004; Munthali 1992; Bellotti and Arias 2001; Bellotti et al. 1999). Finally, whitefly honeydew supports the growth of the black sooty mold, which are so common that farmers coined the common name “black mosaic” (Omongo et al. 2012). Sooty mold impairs photosynthesis to indirectly impact cassava growth.

In the early 2000s, *B. tabaci* populations rose to superabundant levels and drove a severe CMD and CBSD pandemic in Sub-Saharan Africa (Legg et al. 2011; Legg et al. 2014) and methods to enhance vector control are acutely needed. Due to the fact that CBSD resistant genotypes were not available to be deployed and despite the fact that some CMV resistant genotypes were being planted, high whitefly populations rose dramatically and both diseases spread rapidly across Africa (Legg et al. 2011; Legg et al. 2014; Macfadyen et al. 2018). While *B. tabaci* populations can be temporarily

diminished using insecticides, these practices are not economically sustainable for the small shareholder farmers in Africa (Legg et al. 2014; Legg et al. 2011);furthermore, whiteflies develop insecticidal resistance rapidly (Prabhaker et al. 1985).

For this reason, there has been an intensive focus on deploying existing mechanisms of whitefly resistance and identifying new sources for whitefly resistance (Legg et al. 2014; Bellotti and Arias 2001; Bellotti et al. 1999). In the 1980s, CIAT (The International Center for Tropical Agriculture) identified cassava varieties resistant to whiteflies (Bellotti and Arias 2001; Carabalí et al. 2010; Parsa et al. 2015). After a large scale screen for whitefly resistance, a highly whitefly-resistant line Ecuadorian 72 (ECU72) was identified. ECU72 delays nymph development, lowers adult survival rates, delays nymph development, and decreases fecundity (Bellotti and Arias 2001) resulting in longer population doubling times in ECU72 vs susceptible *varieties (Carabalí et al. 2010).* In 2015, the African Cassava Whitefly Project was launched to better understand *B. tabaci* species diversity in Africa, *B. tabaci's* natural enemies and genes underlying whitefly resistance (Summers 2015).

To identify the loci that are associated with whitefly resistance in ECU72, a mapping population was developed from the whitefly-susceptible (COL2246) and whitefly-resistant (ECU72) cross (Bellotti and Arias 2001). The most highly resistance individuals from this F1 cross of ECU72 X COL2246 were used to develop F2 and F3 populations (Becerra Lopez-Lavalle et al, unpublished results); some individuals from the F2 population are virtually immune to the Latin American whitefly *A. socialis* (Barilli et al. 2019). Unpublished results from CIAT and the National Resource Institute (UK) indicate the ECU72's resistance is multigenic and negatively impacts five different whitefly species from three different genera including: *Aleurotrachelus socialis,*

Trialeurodes variabilis, *B. tabaci* SSA1, SSA2 and SSA3, and *Bemisia tuberculata* (Barilli et al. 2019; Bellotti and Arias 2001). Genomics, transcriptomics, and metabolomics are being used to better ECU72's defense mechanisms and cassava's overall defense response to whiteflies. Perez-Fonz et al (2019) found higher lignin levels and elevated ferulic acid and *p*-coumaric acid levels in ECU72 relative to COL2246. They postulate cassava contains an antixenotic resistance mechanism towards whiteflies where the cell wall is strengthened. Whitefly-infested ECU72 benefits from reinforced, lignified, vascular tissue, which might prime plant defenses for protection against future whitefly attacks (Perez-Fons et al. 2019). This has been supported by recent transcriptomics analyses (Garceau 2021).

In addition, an understanding of cassava responses to whitefly infestation is also emerging. Irigoyen et al. (2020) discovered that a significant number of cassava *PR* genes are down-regulated in susceptible cassava as a response to whitefly feeding possibly due to whitefly effectors ostensibly muting plant defenses (Irigoyen et al. 2020). This contrasts markedly with the dogma that *PR* genes are induced by biotrophic pathogens and pests. Two other studies have examined gene expression in cassava after whitefly infestations (Antony and Palaniswami 2006; Mwila et al. 2017).

While genetic analysis of cassava's whitefly resistance is still emerging, the impact of cassava resistance is similar to the resistance displayed in alfalfa, the subject of my dissertation. Whitefly-resistant cassava and alfalfa both display severe delays in nymph development and repellence. The overlap and distinctions resistance mechanisms in these species will be interesting to compare in the near future.

Brassica's resistance to whitefly

The *Brassica* family of plants is perhaps one of the more ubiquitous genera used by cultures and societies worldwide used for food, feed, industry, and research (Dixon and Dickson 2006). *Brassica spp.* have a large array of generalist and specialist species that pose a threat to plant survival including some more common agricultural pests such as the diamondback moth (*Plutella xylostella*), cabbage moth (*Mamestra brassicae*), green peach aphid (*Myzus persicae*), and multiple species of whitefly (*Aleyrodes proletella* and *Bemisia tabaci* MEAM1). *Brassicaceae* are well known for glucosinolate biosynthesis, which deter generalist and attract specialist insects (Ahuja et al. 2010). Glucosinolates are described in Section 3.2 of the Introduction.

In addition to glucosinolates, *Arabidopsis* and other *Brassica spp.* synthesize indolic alkaloids (eg., camalexin), which are derived from the indolic precursors that are used for glucosinolate biosynthesis (Frerigmann et al. 2012; Sun et al. 2018a; Gaur et al. 2018). Camalexins are well known for their antimicrobial properties (Ahuja et al. 2010) and are linked to defense against a diverse array of pathogens (Stotz et al. 2011; Lemarié et al. 2015; Stahl et al. 2018).

Brassica spp. and the model plant *Arabidopsis* are excellent whitefly hosts (Trdan et al. 2003; Kempema et al. 2007; Zarate et al. 2007). In the field, *B. tabaci* MEAM1 can discriminate between different *Brassica* species (Farnham and Elsey 1995). *B. tabaci* prefers Brussel sprouts, collard greens (*B. oleracea* var. *viridis*) and kale over broccoli and cauliflower. There was a positive correlation between nymph and adult populations with a preference for non-glossy leaves, which have substantial wax deposits on their leaf surface. The preference for non-glossy *Brassica* leaves was also observed in lepidopterous caterpillars (Eigenbrode et al. 1990). In addition, certain

Brassica species such as *B. cretica* and B. *insularis* have thicker leaves while *B. fruticolosa* and *spinescens* might have higher levels of lectin (Ramsey and Ellis 1996). It should be noted that *B. fruticolosa* and *spinescens* are also believed to be resistant to cabbage aphid (*Brevicoryne brassicae*) due to the presence of a brassica lectin (Ramsey and Ellis 1996).

The glucosinolates and indole alkaloids of *Brassica* spp. and Arabidopsis are not sufficient to confer resistance to whiteflies. As mentioned earlier, some specialist insects have adapted to the glucosinolates of brassicas (Ali and Agrawal 2012). Recently, Brassica species displaying resistance to the cabbage whitefly *Aleyrodes proletella* L. was reported. *A. proletella* has a moderate host range (including alfalfa and other legumes) and is becoming a global pest (Collins 2016). A screen of four broccoli and four cauliflower genotypes after *A. proletella* infestation identified cultivars with whitefly resistance based on differences in oviposition rates, nymph development time, and host preference (Nebreda et al. 2005). Nebreda et al. (2005) saw lower oviposition rates, fewer adults, longer nymph development, and less adult emergence on cabbage than wild broccoli or cauliflower. Using controlled greenhouse studies, Broekgaarden et al. (2009) characterized a whitefly-resistant *B. oleraceae* variety Rivera and a susceptible variety (Christmas Drumhead). Rivera had fewer whiteflies of all developmental stages and whiteflies fed less frequently and for shorter periods of time compared to Christmas Drumhead. In addition, whitefly-resistant *Brassica* had lower densities of other specialist insects, suggesting this mechanism maybe be broad spectrum (Broekgaarden et al. 2009). Furthermore, this whitefly resistance was developmentally regulated (Broekgaarden et al. 2012; Broekgaarden et al. 2018), as adults did not discriminate between resistant and susceptible plants for oviposition until after 11 weeks of growth.

Transcriptomic analysis of whitefly-resistant and -susceptible *Brassica* infested with whiteflies show there is a shift from development-responsive genes to prioritizing defense-responsive genes later in plant development, which also correlated with when *Brassica* plants developed whitefly resistance (Broekgaarden et al. 2018). Several defense-associated genes in the JA-signaling pathway were up-regulated in the whitefly-resistant Rivera plants including *LOX2*, *MYC2, MYC3, GSTU4*, and the anti-nutritional protein genes *trypsin protease inhibitor (TPI)* and *lectin* (Peumans and Van Damme 1995; Dunaevsky et al. 2005). Broekgaarden et. al (2018) observed abscisic acid (ABA) was induced in older plants in response to whitefly feeding and also observed eggs did hatch. They concluded that Rivera's phloem-based resistance mechanism may not be the only resistance mechanism in play (Broekgaarden et al. 2012; Broekgaarden et al. 2018; Lucatti et al. 2014; Broekgaarden et al. 2015a).

Cotton

Cotton species (*Gossypium spp*) are members of the *Mavaceae* family and four species are grown commercially across the world including: *Gossypium hirsutum*, *G. barbadense*, *G.arboreum*, and *G. herbaceum*. Cotton plant leaf morphology impacts whitefly host suitability. Cotton with a thinner leaf lamina, fewer trichome hairs and fewer gossypol glands were identified as less preferred hosts for whiteflies (Butter and Vir 1989). Acharya and Singh (2008) sampled several cotton genotypes and found no correlation between tannins, gossypol, or phenol on whitefly population density. Walker and Natwick (2006) further explored morphological traits associated with whitefly resistance in a wild cotton *G. thurberi* Todaro. They showed resistance was correlated

with smooth and narrow-lobed leaves. However, those results could not be replicated under controlled environmental settings.

Low trichome density was also linked to lower levels of oviposition and prolonged nymph development among *G. barbadense*, *G. arborerum*, and *G. hirsutum* (da Silva Oliveira et al. 2020). In a screen of over 500 cotton species, glabrous leaves and high hair density were also associated with reduced adult choice and oviposition (Jin et al. 2018). Other whitefly resistance mechanisms have been found in cotton. There was a direct correlation between the number of epicuticular waxes, which could interact with chitin, and the number of whiteflies that chose a host among the cotton species *G. abororeum, G. hirsutum*, and *G. harknessi* (Ali et al. 2021). In the same study, the upregulation of *ECIFERUM 3* (*CER3*), a gene that controls the conversion of very long-chain fatty acids to cuticular alkanes, was linked to increased susceptibility to whitefly.

More recently, transcriptome analysis of a susceptible (ZS) and a resistant (HR) cotton cultivar (*Gossypium hirsutum*) infested with whiteflies (*B. tabaci* MEAM1) was performed (Li et al. 2016b). Analysis of both transcriptomes identified several key tenants: Gene expression profiles between HR and ZS cotton are dissimilar throughout the time course, more genotype DEGs (DEGs different across genotypes at the same time point) were identified in the HR genotype compared to the ZS genotype, and the number of temporal DEGs (DEGs different within a genotype at different time points) identified in the HR genotype increased over time. KEGG pathway analyses indicated that flavonoid biosynthesis, chitin degradation, and starch and sucrose metabolism terms were over-represented in the HR genotype. *WRKY40* was also identified as a component of cotton's response to whitefly as it was more highly induced in the HR genotype than the ZS genotype (Li et al. 2016b). *MPK3* was also identified as an

upstream component of cotton's whitefly resistance. The VIGS-mediated silencing of *MPK3* in HR cotton made silenced plants preferred based on whitefly choice and oviposition assays relative to non-silenced HR cotton (Li et al. 2016b).

Whitefly Host Plant Resistance in Other Plant Species

Whitefly-resistant varieties were identified by field screens of melon (*Cucumis melo*), watermelon, cowpeas, soybeans, *Citrullus colocynthis*, and common bean germplasm (dos Santos et al. 2021; Almeida et al. 2021; Simmons et al. 2019; Sari and Sulistyo 2018; Cruz et al. 2014b; Simmons and Levi 2002). To date, most these resistance mechanisms have not been genetically characterized and therefore it is unknown if whitefly resistance is conferred by a single or multiple genes. Furthermore, in most of these crops, resistance/susceptibility experiments have not been performed in controlled environments to evaluate differences in metabolites, proteins or transcripts that are correlated with their whitefly resistance mechanism(s).

Whitefly resistance in melon was first documented in the *Cucumis melo* TGR-1551 accession from Zimbabwe, which has known resistance to cucurbit yellowing stunting disorder virus (Soria et al. 1999). No differences in adult survival, adult longevity, and nymph development time were detected between the TGR-1551 and susceptible melons. However, both free and no-choice experiments show the resistant TGR-1551 is a less desirable host for *Bemisia tabaci* than susceptible genotypes suggesting that resistance may be antixenotic. While the mechanism of resistance is not known, TGR-1551 has potential for use in melon breeding programs (Soria et al. 1999).

B. tabaci MEAM1 resistance has also been identified amongst 42 watermelon (*Citrullus* ssp.) germplasm accessions (Simmons and Levi 2002). Based on choice and

no-choice experiments *Citrullus colocynthis* accessions displayed resistance based on adult settling, female feeding and egg deposition, and survival (egg to adult). The mechanism of resistance has not yet been explored. Resistance to *B. tabaci* was also identified in *C. ecirrhosus,* a wild species native to deserts of southern Africa (Simmons et al. 2019). This resistance was displayed as a reduction of egg to adult survival, reduced oviposition, reduced female size, and avoidance of the resistant genotype. Simmons et al (2019) developed viable F_1 and F_2 progeny from a *C. ecirrhosus* and commercial sweet watermelon *C. lanatus* cross, progeny indicated the potential for development of improved commercial varieties of watermelon.

There is substantial evidence for the existence of whitefly resistance in legumes including common bean, cowpea and soybean. *Bemisia tabaci*-resistant cultivars were first identified in field studies in Brazil. Individual lines (IPR-Eldorado, IAPAR-81 and IPR-Siriri) inhibited oviposition, while IAC-Harmonia inhibited both oviposition and nymph development (Da Silva et al. 2014). While breeding programs continue to identify plants with high levels of antibiosis and antixenosis (dos Santos et al. 2021; Silva et al. 2019), the numbers of genes, biochemical mechanisms or assessment of transcriptomes have not yet been investigated.

Whitefly resistance was also identified in cowpea (*Vigna unguiculata* L. Walp.) . Among 14 genotypes, MNC 99-541 F21 caused prolonged nymph development (Cruz et al. 2014a). Additional advances in screening for whitefly resistance in cowpea have been recently reviewed (Togola et al. 2017). A field screen of 72 cultivars of soybean (*Glycine max*) for whitefly egg, larval, and adult populations led to the discovery of several resistant genotypes of soybean (Gulluoglu et al. 2010). Soybean resistance was also explored by (Sari and Sulistyo 2018), where they identified antixenosis was associated

with trichome density. QTL mapping for whitefly resistance was also performed in two F_2 soybean populations made from two resistant genotypes (Corsoy 79 and Cajeme) and a susceptible genotype (Williams 79) with loci linked to resistance identified on chromosomes 12, 18, and 19 (Perez-Sackett et al. 2011).Finally, Almeida et al. (2021) showed that soybeans expressing the Bt toxin Cry1A and glyphosate resistance transgenes were more susceptible to whiteflies than non-GE soybeans.

Alfalfa – Breeding and Molecular tools

Alfalfa (*Medicago sativa*), also known as lucerne, has a potent whitefly-resistance mechanism that is better studied than the resistance mechanisms in other legumes and is the focus of my Dissertation (see section below). Here, I provide information about alfalfa importance in agriculture, its breeding strategies, and recent advances in molecular tools and genome sequences.

Alfalfa is a perennial legume that was first cultivated approximately 9000 years ago in Eastern and Central Asia. While most commonly utilized as a hay crop for livestock, alfalfa's perennial growth habit and deep root system preserves surface soil and groundwater protection and phytoremediation (Russelle 2001). It is able to fix nitrogen due to its relationship with symbiotic nitrogen-fixing microbe (ie., *Sinorhizobium meliloti*) and therefore is an excellent forage and rotation crop. Rich in N, alfalfa is also rich in antioxidants and several vitamins (A, E, K, and C). Alfalfa is also being used as a system to produce pharmaceutical enzymes (Kumar 2011). California was among the top ten producers of alfalfa in 2021 by acreage. While California remains a significant producer of alfalfa seed due to its high value, although, recent production has declined partly due to continuing drought (Mueller 2007). Alfalfa is also a highly valuable crop to

US agriculture with over $9 billion produced annually and is primarily grown in the northwestern United States and is the most commonly grown crop in the world. (NAAIC 2022).

The genus *Medicag*o contains two subspecies: *Medicago falcata* (yellow flowers; yellow alfalfa) and *Medicago sativa* (purple flowers; alfalfa). Alfalfa is a highly heterozygous outcrossing plant with a relatively large genome (800 - 1000 Mbps) and is found as both a diploid and tetraploid plants; tetraploid alfalfa contains eight chromosomes (2n = 4*x* = 32) (Kumar 2011; Li and Brummer 2012).

Alfalfa is an obligate outcrosser (Hawkins and Yu 2018). Due to the need for outcrossing, alfalfa “cultivars” are actually populations of highly heterozygous individuals with up to 99% variation within a population (Li and Brummer 2012). The genetic diversity within and between alfalfa populations is greater that most inbred crops and, not surprisingly, the strategies deployed in alfalfa breeding are distinct from conventional crops. Alfalfa breeders seek to maintain as much genetic diversity as possible in each cultivar. Select alfalfa individuals expressing a desirable traits are placed in pollinator boxes and random crosses are performed either manually or by pollinating insects. The resulting progeny (a cultivar or line) are a collection of genetically unique individuals distinct from both parents and their siblings. In most breeding strategies, 60-70% of the individuals in a population will express the trait of interest. This is obviously more complex when multigenic traits are being assessed.

While the strategies for alfalfa breeding are distinct, there have been significant advances in alfalfa crop improvement. The earliest efforts to improve alfalfa were driven by a USDA scientist Neils Hansen who identified alfalfa populations with winterhardiness, drought tolerance and resistance to pathogens and pests in European

germplasm. Incorporation of these traits enabled the expansion of US alfalfa production from 2 M acres in 1899 to 30 M acres in 1950 (Russelle 2001). To date, most alfalfa improvement has been primarily driven using classical “alfalfa” breeding strategies by solely evaluating phenotypes, as the deployment of molecular genetic tools for marker-assisted selection (MAS) has been relatively slow (Hawkins and Yu 2018). The development of modern breeding tools and alfalfa genomics has begun in the last decade. While these resources remain limited in alfalfa, significant advances in QTL mapping, association mapping, and SNP discovery have been made (Han et al. 2011; Kumar 2011; Li and Brummer 2012; Hawkins and Yu 2018; Yu and Kole 2021). These advances have assisted breeders and researchers in identifying genes associated with abiotic and biotic stress responses including: freezing tolerance, salinity, heat, bacterial stem blight, aphid resistance, thrip resistance, and livestock grazing (Shu et al. 2017; Lei et al. 2018; Li et al. 2013b; Nemchinov et al. 2017; Tu et al. 2018b; Tu et al. 2018a; Wang et al. 2016a).

Until recently, insights into the alfalfa genome were inferred from the model legume *Medicago truncatula* genome, due to its high sequence conservation and chromosome synteny with *M. sativa* (Tang et al. 2014; Li et al. 2014b). However, in 2020, the first two alfalfa genome assemblies were published. These chromosome level assemblies included the 802-Mb genome of *M. sativa spp. caerulea* (voucher PI464715), which is a progenitor of tetraploid alfalfa, and the 2.7-Gb genome of a tetraploid *M. sativa* (Zhongmu No. 1) (Shen et al. 2020; Li et al. 2020). The chromosome assembly of the autotetraploid alfalfa genome has led to several discoveries: the assembly is nearly complete with over 400 Mb of contigs not aligning to a chromosome during assembly. Also, analysis of the autotetraploid alfalfa transcriptome indicated that the tetraploid

genome is a stable genome. This is unlike most crops that underwent an ancient whole genome duplication to the tetraploid state followed by a return to a diploid state. This transition back to diploidy is associated with massive gene loss (Julier et al. 2003).

There are also a number of resources available in the Alfalfa Breeder's Toolbox developed by the Noble Foundation that include gene expression data, BLAST resources, a depository of MAS traits and QTLs tested in alfalfa along with literature associated with advances in alfalfa genomics (https://alfalfatoolbox.org/resources). There have also been advances in the development of several types of molecular markers to accelerate breeding and identification of genes that underly important traits. This includes: sequence-related amplified polymorphisms which target exon regions, amplified fragment-length polymorphisms that use primers designed to amplify restriction fragments, and start-codon targeted polymorphisms that identify polymorphisms that may impact protein synthesis (Hawkins and Yu 2018).

While there has been a considerable advance in resources available to alfalfa breeders and researchers, there is still a dearth of resources available compared to model organism as neither the *Phytozome* (https://phytozome-next.jgi.doe.gov/) nor NCBI databases currently have any information about alfalfa genome sequences.

Alfalfa's HPR to *Bemisia tabaci* MEAM1

Alfalfa has a non-trichome based nymph-mediated resistance mechanism effective against *Bemisia tabaci* MEAM1. This resistance mechanism was first identified in the field at the UC Desert Research Extension Center (UC DREC) by Teuber et al. (1997) when he screened over 10,000 alfalfa lines with varying levels of resistance by checking for whitefly adult density and leaf stickiness. Stickier leaves were correlated

with higher levels of feeding and increased whitefly susceptibility. After identifying resistant lines in the field, a whitefly-resistant germplasm (UC-356) was developed. From this germplasm, a resistant population was made with four cycles of positive selection for whitefly resistance (UC2458). From this population, Jiang et al. (2003) screened an subset of half-sibs and tracked nymph development on these lines daily. They identified resistant lines where nymphs were unable to develop past the first instar stage. They then conducted EPG studies on nymphs feeding on resistant and susceptible alfalfa and found while whiteflies were able to reach the phloem on resistant lines, their feeding was short and possibly perturbed by a toxin or P-protein (Jiang and Walker 2007). Since that time, little progress has been made charactering alfala's potent resistance to whiteflies. This is where my dissertation project begins.

Objectives

The cosmopolitan hemipteran whitefly *Bemisia tabaci* MEAM1 compromises agricultural operations for hundreds of crops (Blackmer et al. 1995; Bellows et al. 1994; Cohen et al. 1992). Their wide host range, ability to vector viruses, and relatively quick life cycles make them a difficult pest to manage. Coupled with the fact natural enemies and parasitoid wasps are difficult to deploy in large-scale agricultural settings and the ability of whiteflies to develop insecticidal resistance, there is a great need for host plant resistance (HPR) effective against whiteflies (Lee et al. 2011). While a whitefly-resistant germplasm was developed in the legume *Medicago sativa*, there is relatively little known about alfalfa's whitefly resistance mechanism or phytohormone-associated defense responses. Therefore, this Dissertation aims to identify whitefly resistance in alfalfa and characterize this mechanism along with phytohormone responses in alfalfa.

Chapter 1: Screening of alfalfa whitefly-resistant populations and characterization of the resistance against *B. Tabaci* MEAM1, MED1 and NW1.

I began this project by screening three populations of alfalfa: two resistant (UC-2933 and UC-2845) and one susceptible (UC-1872). Because alfalfa is a highly heterozygous tetraploid, each individual in a population has a unique genotype, therefore individuals in the population will have distinct genotypes. Additionally, the whitefly-resistant and -susceptible lines identified and characterized by Jiang et al. (2003); (2007) were lost over time. Therefore, to identify resistant and susceptible individuals in our populations, I conducted a large-scale whitefly-resistance screen with B. tabaci MEAM1 identifying resistant lines where nymphs did not develop past the first-instar stage; lines were placed in one of five phenotypic classes: highly susceptible, susceptible, moderately susceptible, moderately resistant, and highly resistant.

Three highly resistant (UC2845-092 – R1; UC2845-100 – R2; UC2933 – 022 – R3) and a highly susceptible (UC2845-043) individual were assessed for four different behaviors (oviposition, nymph development, adult choice, and adult longevity) with *B. tabaci* MEAM1, a native species of whitefly (*B. tabaci* NW1), and another invasive species of whitefly (*B. tabaci* MED1). Through these experiments, we show alfalfa has a species-specific whitefly-resistance mechanism that impacts all four behaviors, albeit in different manners with the different whitefly species.

Chapter 2: Comparative transcriptomics of whitefly-resistant and -susceptible alfalfa upon *Bemisia tabaci* MEAM1 infestation.

In Chapter 2, we selected two individuals from the UC-2845 population for a comparative transcriptomic analysis: one highly-resistant (UC-2845 092, R1) and one highly-susceptible (UC2854-043, S1) to MEAM1 whiteflies. We infested R1 and S1 with *B. tabaci* MEAM1 adults and collected samples at time points correlated with whitefly

development: 0, 1, 7, 14, and 22 days post-infestation (dpi). After constructing RNA-seq libraries, we sequenced and *de novo* assembled libraries. Differentially-expressed genes (DEGs) were identified and placed into three classes: genotype (gDEGs), temporal (tDEGs), and interaction (iDEGs) DEGs. Analyses indicated that gDEGs and iDEGs being the best determinants of whitefly-resistance in alfalfa. iDEGs, which use a model to account for development, allow for a more scrupulous analysis of the data.

The more rigorous iDEGs allowed us to focus on a smaller set of DEGs and identified key trends in the data sets. From these analyses, we conclude that there is transcriptome reprogramming in the alfalfa R1 versus R2. Three major conclusions were made. First, epicuticular wax and suberin biosynthesis were constitutively expressed in R1 but not S1 indicating that these physical barriers and signals derived from these structures may be distinct. Second, defense phytohormone signaling is distinct in R1 plants. There is a suppression of SA, JA, and ABA signaling, while ET signaling is enhanced in R1 plants prior to infestation. Third, while there is a suppression of many PTI-associated genes, chitin-responsive genes are induced in R1 relative to S1 indicating that early perception events associated with whitefly infestation are different in the resistant genotype.

Chapter 3: Transcriptomic analysis of SA/JA signaling in alfalfa and their correlation to it's whitefly response

In Chapter 3, we established the SA- and JA- dependent transcriptome of alfalfa to provide the first insights into phytohormone-signaling pathways in this tetraploid crop. Alfalfa plants were treated with salicylic acid (SA) and methyl jasmonate (MeJA) to identify the genes that were differential gene expressed in response to these phytohormones; we used whitefly-susceptible S1 plants for these studies. We collected

alfalfa leaves at several times (0, 0.5, 1, 2, 4, 8, 12, 24 hours post-treatment, hpt) for both treatments and performed RT-PCR using sentinel genes for SA signaling (*PAL1*, *PR2*) and JA signaling (*ARAGH2*, *LOX3*) to determine time points for transcriptomic libraries construction. Based on these data, we selected 0, 1, and 8 hpt sample to construct RNA-seq libraries. Upon *de novo* assembly of the SA and JA transcriptomes, we identified SA- and JA-responsive DEGs. These studies revealed the classes of genes induced in the early 1-h leaves were distinctly different than late 8-h leaves. Furthermore, unlike the model plant Arabidopsis, there was no evidence for SA-JA crosstalk and there were a substantial number of genes that were capable of responding to both phytohormones.

With this knowledge of alfalfa's response to SA and MeJA, we correlated these responses to alfalfa's whitefly responses documented in Chapter 2. Surprising, few whitefly-regulated DEGs were also SA and/or JA responsive, suggesting that these phytohormones have a minor role in alfalfa's resistance to *B. tabaci*. However, we did identify some phytohormone responsive gDEGs and tDEGs and can make the following conclusions: first, there are more MeJA-responsive gDEGs than SA-responsive gDEGs. Second, there are more phytohormone-responsive tDEGs in S1 than in R1. Third, more of these tDEGs in S1 are responsive to MeJA at later time points than SA, and finally, there are few, weak correlations between hormone response and whitefly response in alfalfa.

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Chapter 1 Screening of alfalfa whitefly-resistant populations and characterization of the resistance against *B. Tabaci* MEAM1, MED1 and NW1.

Abstract

The whitefly (*Bemisia tabaci*) is a polyphagous, obligate and voracious phloem-feeder that impacts plant growth, vectors plant viruses and causes sooty mold infections due to their honeydew secretions. Host plant resistance is the most effective means of whitefly control, as whiteflies have a high propensity for developing insecticide resistance. Here, we report the mechanisms that underly *B. tabaci* MEAM1(Middle Eastern Asia Minor 1) resistance in alfalfa (*Medicago sativa*). Whitefly-resistant alfalfa inhibit MEAM1 first-instar nymphs from developing into their later-instar stages. High-throughput resistance screens were used to phenotype 84 alfalfa individuals from three germplasm populations developed from MEAM1-resistant parents. From this screen, three whitefly-resistant (R1, R2, and R3) and one whitefly-susceptible (S1) alfalfa were chosen to for further study. Life history parameters for MEAM1 and two other *B. tabaci* species - MED (Mediterranean) and NW1 (New World-1) were examined. These experiments revealed that while 94-99% of MEAM1 nymphs do not develop beyond their first instar on all three whitefly-resistant plants, MED nymphs developed at the same rate on resistant (R1, R2, and R3) and susceptible (S1) genotypes and NW1 did not develop on either past early-instar stages. MEAM1, MED and NW1 adults had different behaviors (egg deposition, host choice, and longevity) on R and S plants. No significant difference in oviposition was seen between the R and S plants for the three *B. tabaci* species. However, NW1 and MED oviposition differed between the R genotypes, while MEAM1 oviposition was similar on all three R and S1 plants. In host choice experiments, MEAM1 and MED selected S over R plants in choice assays and NW1 preferred not to

settle on either S or R alfalfa. Finally, MED and MEAM1 adult had shorter life spans on R2 and R3, respectively, compared to S1 which may point to MED incompatibility with alfalfa. There were also differences in MEAM1 and MED longevity between the different R genotypes. Collectively, these data indicate that the resistance mechanisms in R1, R2 and R3 plants is multigenic, multi-faceted and whitefly species-specific.

Introduction

Hemipteran insects are among the most economically devastating plant pests in agriculture; two-thirds of sequenced Hemipterans have been classified as “high-status” pests (Panfilio and Angelini 2018). Among Hemipteran insects, whiteflies of the *Bemisia tabaci* cryptic complex are among the most omnipresent and invasive worldwide with at least one species from the complex extant on every continent except Antarctica (Perring 2001; Wang et al. 2019; De Barro and Ahmed 2011). *B. tabaci’s* voracious feeding inhibits growth and development due to depletion of resources, enables virus acquisition and transmission, and produces honeydew secretions that are a rich medium for sooty mold growth on plant surfaces. In addition to their geographic ubiquity and broad host range, whitefly control is difficult due to abaxial oviposition, ability to rapidly develop insecticide resistance and limited success in deploying natural enemies in the field (Naranjo and Ellsworth 2009; Inbar et al. 2001).

Bemisia tabaci MEAM1 (also known as *B. tabaci* B, biotype B, or *Bemisia argentifolii*) is recognized for its global pest status (Willis 2017). MEAM1 is invasive and replaced the non-invasive NW1 (*Bemisia tabaci* NW1) population in North America (Perring et al. 1993; Perring et al. 1991; Barinaga 1993). MEAM1 causes physiological disorders in their hosts, such as leaf silvering and irregular ripening of fruit, which gave

rise to its common name - the silverleaf whitefly (Perring 2001; Bellows et al. 1994). MEAM1 also has the ability to vector hundreds of *Begomoviruses* of economic impact (Inbar et al. 2001). Coupled with a larger host range and greater fecundity compared to NW1, the invasive MEAM1 is a greater threat to agricultural systems than its native counterpart (Bird et al. 1957; Brown et al. 1995; De Barro and Ahmed 2011). Recently *Bemisia tabaci* MED (Mediterranean), another invasive species has been detected in North American greenhouses and fields in Florida (Hodges and McKenzie 2008; Hu et al. 2011; Horowitz and Ishaaya 2014; Smith et al. 2020). While both MEAM1 and MED displaced native *B. tabaci* species in China, MED's propensity for developing insecticide resistance has lead to its growing impact in China (Pan et al. 2012; Yao et al. 2017). The prevalence of whiteflies and their widespread damage globally have heightened the urgency for alternative control methods.

Host-plant resistance is foundational for integrated pest management programs to hemipteran insects (Naranjo and Ellsworth 2009). However, to date, relatively few genes that confer resistance to hemipterans have been successfully cloned. Nine rice genes that confer resistance to brown planthopper (*Nilaparvata lugens*) (*Bph2/26, 3, 6, 9, 14, 17, 18, 29, 32*), a tomato's *Mi-1.2* confers resistance to nematodes and three hemipteran pests, and a melon gene (*Vat*) confers resistance to cotton-melon aphid (*Aphis gossyppi*) have been cloned and characterized (Klingler et al. 2001; Vos et al. 1998; Rossi et al. 1998; Tamura et al. 2014; Jairin et al. 2007; Du et al. 2009; Guo et al. 2018; Ji et al. 2016; Ren et al. 2016; Sani Haliru et al. 2020; Wang et al. 2015; Zhao et al. 2016). With one exception, the Hemipteran resistance genes are coil-coiled nucleotide-binding leucine-rich repeat receptors (CC-NLRs). The exception is *Bph3,* which encodes for a cluster of three of receptor kinases (Jairin et al. 2007). *Mi-1.2* in

unique as it confers resistance to four genera of agricultural pests including root-knot nematodes (*Meloidogyne spp.*), potato aphids (*Macrosiphum euphorbiae*), whiteflies (*B. tabaci* MEAM1 and MED) and psyllids (*Bactericerca cockerelli* (Sulc)) (Nombela et al. 2003; Nombela et al. 2000, 2001; Roberts and Thomason 1986; Casteel et al. 2006; Rossi et al. 1998; Milligan et al. 1998; Vos et al. 1998; Kaloshian and Walling 2016). While resistance to aphids is phloem mediated, the resistance to whiteflies is apoplastic (Jiang et al. 2001). In addition, broad-spectrum resistance to insects, including whiteflies, is present in wild tomato species (*Solanum pennellii, S. habrochaites, S. habrochaites f. glabra- tum, S. pimpinellifolium, S. chilense*) that is dependent on glandular trichomes (Rakha et al. 2017; Firdaus et al. 2012; Dalin et al. 2008; Vosman et al. 2018).

Resistance to two other whitefly genera has also been characterized in cabbage (*Brassica oleraceae)* and cassava (*Manihot esculenta).* Broekgaarden et. al (2009; 2012) found a phloem-mediated resistance to the *Aleyrodes proletella* (the cabbage whitefly) in *B. oleracea* Rivera and this resistance is associated with the phytohormone abscisic acid (Broekgaarden et al. 2018) . Cassava's resistance to the Latin American whitefly *Aleurotrachelus socialis* is associated with the lignification of resistant plants to reduce oviposition, prolong nymph development and increase nymph mortality (Bellotti and Arias 2001; Perez-Fons et al. 2019). Whitefly resistance has also been identified but not extensively characterized in wild cotton (*Gossypium thurberi*) and a number of legumes (e.g., soybean, common bean, and cowpea) (Walker and Natwick 2006; Sulistyo and Inayati 2016; Lambert et al. 1995; Cruz et al. 2014; dos Santos et al. 2021; Silva et al. 2019). Recently, the transcriptional reprogramming that occurs during B. tabaci infestation of a whitefly-resistant tetraploid cotton line Mac7 was reported (Aslam et al. 2022). Considering the limited whitefly HPR mechanisms and the prevalence of

whiteflies as pests, identifying effective whitefly HPR for use in integrated pest management programs is a priority (Teuber et al. 1997; Stenberg 2017; Naranjo and Ellsworth 2009; Lefebvre et al. 2020).

Among whitefly hosts, alfalfa (*Medicago sativa*) has a novel, trichome-independent whitefly resistance mechanism (Walker and Jiang 2005; Jiang et al. 2003; Jiang and Walker 2007; Teuber et al. 1997). Alfalfa is a highly heterozygous, obligate outcrossing tetraploid legume used for food, animal feed, phytoremediation, and bioenergy (Kumar 2011). Alfalfa is a high-value seed crop that is often intercropped between other high-value crops (e.g., cotton), making it a potential reservoir for whitefly expansions in agricultural operations (Naranjo and Ellsworth 2009). When *B. tabaci* MEAM1 populations rose to the superabundant level in California in the 1990's, a field screen of alfalfa identified germplasm resistant to MEAM1 (Teuber et al. 1997). Seventy-three lines with low whitefly adult densities and limited honeydew deposition were identified and used to create a whitefly-resistant germplasm (UC-356). The analysis of individuals from this population showed that alfalfa's whitefly resistance influenced first-instar survival, which was < 10% on highly resistant lines and > 50% on susceptible lines, as well as adult fecundity (Jiang et al. 2003). Using electropenetration graphs to monitor whitefly feeding behaviors, Jiang and Walker (2007) showed that whiteflies reached the phloem of both resistant or susceptible lines, but feeding is deterred in resistant lines. Jiang and Walker postulated either a p-protein or toxin caused nymph death on resistant alfalfa.

Despite its promise for whitefly control, alfalfa's complex genetic composition and unique breeding strategies makes genomic analyses challenging, which has resulted in little progress in elucidating the comprehensive function and identity of this resistance

mechanism (Kumar 2011; Li and Brummer 2012; Zhu et al. 2005). Here, we provide a foundation for better understanding alfalfa's resistance to whiteflies. A screen of 84 alfalfa lines for delayed MEAM1 nymph development identified individuals with a varying levels of whitefly resistance. Three resistant and one susceptible line were used to explore whitefly life history behaviors (oviposition, nymph development, adult longevity, and adult preference) for three whitefly species (*B. tabaci* MEAM1*,* NW1 and MED). These whitefly species had distinct behaviors on our resistant lines: nymph development in MEAM1, adult choice in MEAM1, MED1 and NW1, and adult longevity in MEAM1 and MED1 were all impacted by whitefly resistance in alfalfa.

Methods

Host plants and *B. tabaci* colony maintenance

The *Bemisia tabaci* MEAM1 colony was maintained on *Brassica napus* var 'Florida Broad Leaf' (W. Atlee Burpee & Co.) grown in UC Soil mix 3 at 27°C, 55% relative humidity and 16-h light:8-h dark (300 µEin). The *B. tabaci* MED colony was initiated with ~150 adults from a colony maintained by Jesús Navas-Castillo (University of Málaga). The colony was grown in a separate room under the same growth conditions with quarantine protocols in UC Riverside's Insectary and Quarantine facility (IQF). The *B. tabaci* NW1 colony was initiated with whiteflies from a colony maintained by James Ng (UCR). The NW1 colony was maintained on *Phaseolus vulgaris* var 'Fordhook' (W. Atlee Burpee & Co.) in a IQF separate room under the same conditions described above. *P. vulgaris* plants were introduced to the colony once the first leaves emerged. At the time of adult emergence, infested leaves were detached and placed in a 20-L food storage container with a clear lid and cloth sleeve surrounding a 16-in^2 square hole (Cambro Manufacturing). NW1 whiteflies were collected by aspiration.

Generation and propagation of whitefly-resistant and –susceptible lines

UC-356 germplasm pool that was established with 73 whitefly-resistant alfalfa lines was used to create three populations of alfalfa with varying levels of whitefly resistance/susceptibility (Teuber et al. 1997). The UC-1872 population was developed after one cycle of selection for increased whitefly-susceptibility (WF^S). The UC-356 germplasm was also subjected to four cycles of selection for whitefly resistance (WF^R) to create the WF^R UC-2458 germplasm, which served as the progenitor of both resistant populations (UC-2845 and UC-2933) used in this study. UC-2458 germplasm was subjected to three additional selection cycles of selection for WF^R and additional pest/pathogen resistance genes were incorporated during these selection cycles. The resulting UC-2845 population had whitefly resistance as well as resistance to the spotted alfalfa aphid (*Therioaphis maculata*), pea aphid (*Acyrthosiphon pisum)*, and bluegreen aphid (*Acyrthosiphon kondoi*), Phytophthora root rot (*Phytophthora megasperma* f. *medicaginis) ,* fusarium wilt (*Fusarium oxysporum* f. sp. *medicaginis*), northern and southern root-knot nematodes (*Melodigyne* spp.), andanthracnose *(Colletotrichum trifolii)*.

A different strategy was used to develop the UC-2933 population. Four highly resistant individuals from the UC-2458 germplasm were identified by Jiang and Walker (2003). These individuals (clone 3, 10, 27 and 37) were used to create ½ sib families (UC-2527-26, UC-2458-34, UC-2527-60, and UC-2458-177, respectively). These ½ sib families were used to create the highly resistant UC-2933 germplasm.

Cuttings from 84 individuals from the UC-1872, UC-2845 or UC-2933 populations wer collected from field-grown alfalfa from El Centro, CA or from clones provided by UC Davis to establish parent plants. Stem segments (6-cm in length) were clonally

propagated in UC soil mix 3 by dipping the distal end of the cutting into Clonex gel rooting media (Growth Technology Ltd) and in Spinosad insecticide (Tractor Supply Co., Brentwood, TN) to eliminate herbivores accidently brought in from the field. Stem cuttings were placed in soil in a 72-well inserts, with three stem segments per well. Stem segments were housed under a humidity dome (Hydrofarm; Petaluma, CA) and misted daily. Dome vents were opened after clones established roots (approximately 10 – 14 d). Domes were removed after 21 d. Stem segments with established root systems were transferred to 5“-tall rectangular pots and were grown in a growth room at 27°C, 35-50% relative humidity with a 12-h day/12-h night cycle (200 – 300 μmol). Established plants were transferred to 1-gallon pots and parent plants of each genotype were maintained in a greenhouse or growth room with monthly fertilization. For phenotypic screens, clones from each genotype were made as described above. For each phenotypic screen, one whitefly-susceptible genotype and four randomly chosen genotypes were chosen.

Whitefly resistance/susceptibility bioassays

Alfalfa genotypes (lines) were screened for whitefly resistance/susceptibility using a method adapted from Jiang et al. (2003). A total of 29 lines from UC-1872, 25 lines from UC-2845, and 28 lines from UC-2933 were screened. For each bioassay, a known *B. tabaci* MEAM1-susceptible line served as a positive control; in early phenotypic screens CUF101 was used and in later screens UC-2845-043 was used. Four to nine alfalfa lines with unknown whitefly resistance/susceptible phenotypes were evaluated in each screen. Early screens were performed with ten clonally propagated plants per line (four lines per screen). Statistical evaluation of the data indicated that phenotypes could be

accurately called with five plants per line. Later screens assessed nine lines with five replicate plants.

This experimental design required approximately 600 male and 600 female whiteflies per experiment. To facilitate infestations, we established *Brassica* sex-specific holding plants that harbored only male or female whiteflies. To this end, individual male and female whiteflies were collected in 50-mm test tubes from *B. tabaci* MEAM1 colonies. Tubes were immediately capped with corks and the sex of each whitefly was verified under a dissecting microscope. Male- and female-holding plants were established in separate Bugdorms (MegaView Science Company) in the greenhouse used for phenotype bioassay experiments; insects on holding plants were used one to two days after establishment.

Phenotypic screens used plants with at least five trifoliate leaves. Plants were moved into bug dorms in a greenhouse with day-time temperatures ~23^{o}C and natural light; screens were performed from March to October. On the day of an infestation, small plastic cages were place on two young alfalfa trifoliate leaves per plant. Cages were adapted from a design described by Jiang et al. (2003). Infestations were initiated by collecting six male and six female whiteflies by aspiration from each sex-specific holding plant and delivering them to each insect cage. After 48 h, cages were removed from leaves and the number of viable adults/per cage was recorded. Infested leaves were tagged with a jewelry tag and alfalfa plants were returned to the bug dorm. A random block design was used for all infestations. Infestations were terminated when fourth-instar nymphs, an adult, or its exuvium was observed on the susceptible line (positive control). At this time, infested leaves were excised and placed into plastic bags prelabeled with the pot number and the leaf number and stored at 4° C until imaging.

The abaxial and adaxial side of each leaflet was photographed using the Nikon D5000 at UCR's Center for Plant Cell Biology Microscopy and Imaging Core. The number of first, second, third, and fourth instars and exuvia were counted for each image. The percentage of insects in each developmental stage was determined by the number of insects in each instar divided by the total number of instars/exuvia.

The percentage of insects in their first instar and later instars (second-, third- and fourth-instar nymphs) were used to define five classes of resistance/susceptible. Resistance classes were defined based on the percentage of nymphs in their first instar at the end of the phenotypic screen. From these lines, the highly susceptible genotype 2845-043 was selected as a the susceptible control (S1) and three highly resistant genotypes 2845-092 (R1), 2845-100 (R2) and 2933-022 (R3) were selected for further analysis.

The significance of mean proportion of insects in their first instar for each line (N=5-10) was assessed using a Kruskal-Wallis One-Way ANOVA. Data were arcsin transformed. Experiments with a $p \leq 0.05$ indicated at least one line in the screen displayed a resistant phenotype. Resistant lines were confirmed with Dunn's multiple comparison tests against the known susceptible line.

Oviposition Assays

For each alfalfa line (S1, R1, R2, and R3), two young trifoliate leaves from five 6-in tall plants (N=5) were enclosed in cages and infested with five male and five female *B. tabaci* (MEAM1, MED or NW1). Each line was screened twice resulting in 20 biological replications. After 48 h, adult viability was determined, leaves were excised and the number of eggs on the abaxial and adaxial side of each infested leaf were counted. The eggs from each replicate of a line were summed then divided by the sample size to

determine the average oviposition rate on an alfalfa line. Egg oviposition was analyzed using a Kruskal-Wallis H-test. Significantly different samples were determined with a Dunn's multiple comparison test.

Adult-choice experiments

Choice "cages" were created using hinged plastic boxes (140 mm x 168 mm x 76 mm) (mDesign, Amazon.com) (Fig. 7). Each box had two 2.5-cm diameter holes drilled at the base of the cage to allow insertion of alfalfa plants and a 7.5-cm x 5-cm opening at the back of the box was covered in thrips-proof mesh to allow for air flow. The boxes had a central hole (2.5-cm diameter) to which the cap of a 50-ml tube with a central 2.5-cm hole was glued. The cap allowed attachment of the whitefly collection tube, which was a 50-ml centrifuge tube that was truncated at the 40-ml line and sealed with thrips-proof mesh. The collection tube was screwed into the cap to initiate the choice experiment. Cages were mounted on a ring stand using a clamp 30 cm from the tabletop.

Each experiment used a susceptible line (S1) and one of the three whitefly-resistant lines (R1, R2, or R3). Each line was assessed in five biological replicate experiments. On the day of experiment, plants were introduced to the cage by inserting a stem with three trifoliate leaves into the cage and sealing the hole with 3.2-cm^3 of insulation foam. Plants used for the choice studies had a total of 5 – 8 leaves. Prior to the addition of whiteflies, the inside of cages were wiped with a water-dampened Kimwipe to minimize the static electricity that negatively impacts whiteflies. Thirty whiteflies were collected by aspiration into the collection tube from the *B. tabaci* MEAM1, MED or NW1 colonies. Upon capture, whiteflies were held for 15 min at room temperature or 4°C to ensure they were at the bottom of the collection tube. Whiteflies were then introduced to the cages

by screwing the collection tube to the cage. The tube was gently tapped to ensure all whiteflies were released. Upon release of whiteflies, cages were surrounded with white cardstock to minimize external stimuli. Choice cages were left undisturbed except for daily watering and data collection.

At 8, 24, 48, and 72 hpi (hours post-infestation), the number of whiteflies residing on the adaxial and abaxial side of leaves was determined. A flashlight was used to illuminate the leaf from below and the shadows of the whiteflies residing on each leaflet were counted when whiteflies weren't directly visible. Whiteflies that died or were not found on a plant were called as no choice decisions. The number of whiteflies on each line or making no choice were divided by the total number of whiteflies in the cage to determine the proportion of whiteflies choosing the S or R plants. Adult-choice experiments were analyzed using a two-way RM ANOVA with a Geisser-Greenhouse correction on arcsin-transformed proportions at each time point. Significantly different samples were determined using a Tukey's multiple correction test with individual variances calculated for each comparison. Each experiment was conducted at 26°C and 200 – 300 μmol light with a 12-hour day.

Longevity Studies

Whitefly cages were created using 236-ml plastic containers with a 2.5-cm hole cut in the bottom of the container, two 3-cm holes on opposite sides, and a 0.5-cm hole to deliver whiteflies. Cages were mounted on sticks using heavy wire to prevent bending or damage to the leaf petiole. A trifoliate leaf from each plant (with 8 – 10 leaves) was caged and sealed with 3.2-cm^3 of insulation foam. Five pairs of newly emerged whitefly adults (1:1 sex ratio) were added to each cage via aspiration. The number of alive and

dead whiteflies per cages was determined in 24-hr intervals each day for 24 d. Whiteflies were transferred to a clean leaf on the same plant approximately every 7 d or when the leaf was showing signs of damage. A total of five replicates (N=5) were completed for each line. Longevity experiment survival curves were compared using a Mantel-Cox test at the 0.05 interval. Significantly different samples were determined by comparing survival curves between two genotypes in an experiment.

Results

Identification of MEAM1-resistant alfalfa

The UC-356 germplasm was used to develop three alfalfa lines that were screened for whitefly resistance/susceptibility (Figure 1.1) (Teuber et al. 1997; Jiang et al. 2003). UC-1872 was selected for whitefly susceptibility. While the UC-2933 and UC-2845 populations were selected for whitefly resistance using two distinct strategies as described in *Materials and Methods*. A total of 84 lines from UC-1872, UC-2933 and UC-2845 were screened for resistance/ susceptiblity to *B. tabaci* MEAM1 (Figure 1.2). Delayed nymph development was the scoring metric in this resistance/susceptibility bioassay; the proportion of insects that did not progress beyond their first-instar at the end of an infestation experiment reflected either nymph mortality or a developmental delay (Figure 1.3). Resistant plants had fewer insects that developed beyond their first instar. After comparing proportion of first-instars amongst all lines, plants were assigned to one of five phenotypic classes associated with WF^R: highly resistant (> 90% first-instars), moderately resistant (>70–90%), moderately susceptible (>50 – 70%), susceptible (>20 – 50%), and highly susceptible (0-20%). Consistent with the methods used for alfalfa cultivar breeding, each population had a spectrum of plants ranging from highly susceptible to highly resistant (Figure 1.3) (Teuber et al. 1997). On CUF-101, the

susceptible control (Jiang et al. 2003), 78% of the nymphs progressed beyond the first instar. The UC-1872 population was selected once for whitefly susceptibility (Figure 1.1). Of the 29 UC-1872 plants that were phenotyped, 28 were designated as moderately susceptible, susceptible, or highly susceptible. Only one plant (UC-1872-137) was identified as highly resistant (Figure 1.3A).

The two resistant populations (UC-2845 and UC-2933) had significantly more lines exhibiting resistance. Of the 25 lines from the UC-2845 population (Figure 1.3B) that were phenotyped, 11 were designated as either moderately resistant or highly resistant; while four were moderately susceptible and ten were either susceptible or highly susceptible. Of the 28 lines phenotyped from the UC-2933 germplasm (Figure 1.3C), 12 plants were moderately resistant or highly resistant. Three were moderately susceptible and 14 were either susceptible or highly susceptible. As the whitefly-resistant populations (UC2845 and UC2933) were created using different breeding strategies, we determined if there was significant difference between the proportion of resistant and highly resistant genotypes in these populations. There was no significant difference in the number of either moderately and highly resistant plants ($p > 0.99$) or highly resistant plants ($p > 0.99$) in the two populations.

To determine if the proportion of first-instar nymphs found on moderately or highly resistant lines was significantly different from the proportions detected on the plants in susceptibility classes, we statistically analyzed each screen using a Kruskal-Wallis One-Way ANOVA and subsequent Dunn's multiple comparison tests. One representative screen that identified a highly resistant line is shown in Figure 4. Lines classified as moderately (UC-2845-015 and -082) or highly (UC-2845-100) resistant had significantly

more first-instar nymphs than moderately susceptible (UC2845-010) or susceptible (CUF101 and UC2845-010) plants ($p \leq 0.01$).

Three highly resistant lines (UC2845-092, UC2845-100, and UC2933-022) and one highly susceptible line (UC2845-043) were chosen for further evaluation (Figure 1.3). Leaf and stem morphology for all four lines was the same, with one exception. The leaves of UC-2845-092 had more narrow leaves compared to S1, R2, and R3 (Figure 5). Line UC2845-092 (R1) was the best performing line among the 84 plants phenotyped. R1 plants had 0.99 of the nymphs remaining in their first instar. Line UC2845-100 (R2) performed similarly with the proportion of nymphs in their first instar at 0.96. Line UC2933-022 (R3) had a different parentage (Figure 1.1) and was the second-most resistant line in its population (0.94 of first instar nymphs).

The developmental delays caused by the R1, R2 and R3 lines is whitefly-species specific.

The WF^R lines R1, R2 and R3 were selected due to their strong blocks in MEAM1 nymph development (Fig. 1.3B-C). However, it is not clear if the mechanisms of resistance in the three resistant lines were the same or different and whether or not the development of other *B. tabaci* species would be impacted in these genotypes. Therefore, we assessed MEAM1, MED and NW1 nymph development on R1, R2 and R3 lines. Leaves were infested with twelve whiteflies (1:1 sex ratio), the experiment was terminated when late-fourth instars were detected on S1 plants, and numbers of nymphs in each developmental stage was determined.

As demonstrated in the phenotypic screens, MEAM1 nymph development was significantly delayed on all three resistant genotypes relative to S1 with >1 %, 4 % and 6 % of the nymphs progressing beyond their first instar in R1, R2 and R3, respectively

(Figure 1.6A). In contrast, MED nymphs were able to develop at similar rates on S1, R1, R2 and R3 ($p = 0.40$, $N \geq 12$) (Figure 1.6B). MED nymphs developed more slowly on all four alfalfa genotypes as ~ 40-50% of the nymph were in their first instar at the time of emergence of the first adults. while each of our resistant lines had increased susceptibility. Similar experiments with NW1 indicated that alfalfa may be an incompatible host. NW1 nymphs were unable develop on S1 and the three whitefly-resistant alfalfa (Table 1.1).

Alfalfa's whitefly-resistance mechanisms impact host choice in a whitefly species-specific manner.

To assess if R1, R2, or R3 alfalfa also had active mechanisms to deter whitefly settling, two- host choice assays were performed. Thirty MEAM1, NW1, or MED adults were released into a cage with one susceptible S1 and one resistant (R1, R2 or R3) leaf (Figure 1.7). The number of whiteflies that chose S1 versus a resistant plant or did not make a choice was monitored at four times over a 72-h interval (Figures 1.8-1.9).

For MEAM1, adults preferentially choose S1 over R1 plants. While MEAM1 did not make a host choice by 8 hpi, there was a strong preference for S1 over R1 at the 24, 48, and 72 hpi time intervals based on a RM two-way ANOVA ($p_{line} < 0.01$; $p_{time} = 0.95$; $p_{replicates} = 0.07$) (Figure 1.8A). In contrast, MEAM1 did not discriminate between S1 and R2 ($p_{line} = 0.24$; $p_{time} = 0.99$; $p_{replicates} = 0.27$) or S1 and R3 ($p_{line} = 0.23$; $p_{time} = 0.91$; $p_{replicates} = 0.26$) in these choice assays (Figures 1.8B and 1.8C). However, for both R2 and R3, there are trends that suggest S1 was preferred over the resistant genotypes at later times.

In contrast, the host choice behaviors of MED and NW1 was distinct from MEAM1 (Figure 1.9). For MED, there was a slight preference for S1 over R1 at all time points,

although this was not statistically significant (p_{line} = 0.23; p_{time} = 0.96; $p_{replicates}$ < 0.01) (Figure 1.9A). Unlike MEAM1, statistically significant differences in MED adult choice was observed in both the S1/R2 and S1/R3 free-choice experiments. MED prefered S1 over R2 plants (p_{line} = 0.04; p_{time} = 0.92; $p_{replicates}$ < 0.01) (Figure 1.9B), particularly at 48 and 72 hpi (p_{48hpi} = 0.02; p_{72hpi} = 0.02). For S1/R3 choice experiments, S1 was the preferred host at 24 h and similar trends were seen at all other timepoints (p_{line} < 0.01; p_{time} = 0.89; $p_{replicates}$ < 0.06) (Figure 1.9C). Unlike its interactions with R1 and R2, MED interactions in the S1/R3 choice assay were distinct. Relative to R1, there were more MED whiteflies that did not make a choice at 8, 24 and 48 dpi (p_{8hpi} = 0.04, p_{24hpi} < 0.01; p_{48hpi} = 0.02).

Compared to MEAM1 and MED, NW1 displayed a different interaction with S1 and the three resistant hosts. Few NW1 adults chose either S1 or a resistant plant in the two-choice assays (Figure 1.9D-F). Furthermore, NW1 did not discriminate between the susceptible and resistant genotypes in the S1/R1 (p_{line} < 0.01; p_{time} = 0.93; $p_{replicates}$ < 0.01), S1/R2 (p_{line} < 0.01; p_{time} = 0.83; $p_{replicates}$ = <0.01), or S1/R3 (p_{line} < 0.01; p_{time} = 0.97; $p_{replicates}$ = 0.43) two-choice assays. We noticed all NW1 whiteflies died at the conclusion of each experiment. Therefore, based on statistically significant data and strong trends in other datasets, we can conclude that there is a preference for MEAM1 and MED whiteflies to populate S1 plants over any of the resistant plants. Furthermore, all four genotypes (S1, R1, R2 and R3) repel NW1 with high levels of mortality.

Alfalfa's whitefly resistance mechanisms impact adult longevity

As changes in nymph development time and host choice differed between MEAM1 and MED, we determined if the life span of the different whitefly species was influenced while feeding on S1, R1, R2, and R3 (Figure 1.10). Ten newly emerged whiteflies were

added to a caged alfalfa leaf. Adult viability was checked daily until all whiteflies in a cage had expired. Relative to adult longevity on S1 plants, the three whitefly-resistant genotypes significantly influenced both MEAM1 ($p = 0.04$) and MED ($p < 0.01$) longevity, but in whitefly species-specific ways. For MEAM1, the adult lifespan was >2-fold longer on S1 (22 d) than on R3 (8 d) ($p = 0.03$) (Figure 1.10A; C)(Table 1.2). In addition, MEAM1 adults on R3 plants had a shorter lifespan than R1 plants (14 d) ($p = 0.03$).

When comparing MED adult longevity on the four genotypes, we found a significant difference in the lifespan of S1 (15 d) and R2 (6 d) ($p = 0.02$) (Figure 1.10B; D)(Table 1.3). Surprisingly, there was also a compelling trend for enhanced MED survival on R1 vs S1. When comparing the lifespans of MED on the resistant lines, we found significant differences between R1 (19 d) and R2 (6 d) ($p = 0.04$) and between R1 and R3 (10 d) ($p = 0.03$). Collectively these data indicate that alfalfa's whitefly-resistance mechanisms influenced adult longevity and is whitefly species-dependent significant.

MED and NW1 oviposition is influenced by alfalfa's resistance mechanisms.

To assess if the R1-, R2- or R3-mediated resistance influenced oviposition for the three different *B. tabaci* species, trifoliate leaves were caged with five pairs of either MEAM1, MED, or NW1 whiteflies and the number of eggs deposited within a 48-h period (Figures 1.11 A-C). For MEAM1, MED and NW1, there was no significant difference in the number of eggs deposited on S1 vs resistant genotypes. However, a significant difference in oviposition rates for MED and NW1 was observed when different resistant genotypes were compared. For example, MED laid >2-fold more eggs on R2 (32.3 eggs) than either R1 (12.1 eggs) or R3 (14.9 eggs) ($p = 0.01$ and $p = 0.02$ respectively). The impact of the R genotypes on NW1 oviposition was different with >2-fold NW1 eggs laid on R1 (10.6 eggs) than on R2 (4.7 eggs) plants ($p = 0.03$). These data indicated that

while there was no difference in oviposition between resistant and susceptible lines, there were significant differences between resistant lines and some whitefly species

Discussion

As a perennial crop, assuring that the alfalfa lines can meet the current and future challenges of abiotic and biotic stresses delivered by environmental fluxes is essential. With global climate change and warming temperatures, *B. tabaci* MEAM1 habitats are likely to expand (Ramos et al. 2018). Given the voracious feeding habits, ability to vector 100s of devasting viruses, and wide plant host range (Inbar et al. 2001), whiteflies are likely to exert substantial pressure on global agriculture in the future (Curnutte et al. 2014). In the early 1990's, California experienced superabundant whitefly populations with the invasion and establishment of MEAM1 on numerous crops including tomatoes, lettuce, cauliflower, cantaloupe, and cotton causing reduced yields and crop quality. While both NW1 and MEAM1 are capable of colonizing alfalfa (Toscano et al. 1994; Yee and Toscano 1996), MEAM1 nymphs are better adapted to alfalfa (Palumbo et al. 2000). At high whitefly densities, the value and quality of the alfalfa crop significantly declined due to decreases in plant growth, stem lengths, forage yields, dry matter production, and protein content (Palumbo et al. 2000). Chemical intervention can limit whitefly-associated losses; however, with the fluctuating value of alfalfa hay and high cost of insecticides, chemical treatments are not always economically viable solutions for alfalfa (Naranjo and Ellsworth 2009). Additionally, MED whiteflies are starting to establish themselves in North American greenhouses and fields in Florida and are dominant in other regions worldwide that grow alfalfa (McKenzie and Osborne 2017; Hodges and McKenzie 2008). Therefore, new methods of *B. tabaci* control are needed.

Host-plant resistance, which is at the foundation of all integrated pest management strategies, is an environmentally friendly alternative mechanism to control whitefly population expansion and its ensuing damage.

Whitefly-resistant alfalfa germplasm was first reported by Teuber (1997). Previous studies characterized a small number of individuals from the UC-356 germplasm pool and showed that whitefly resistance was phloem-mediated, reduced phloem consumption by nymphs, and caused nymph mortality (Jiang and Walker 2007; Jiang et al. 2003). Their labor-intensive, stage-specific nymph-mortality screen monitored insect development every three days until the completion of adult emergence reporting egg to adult survival; they also reported dead nymphs that were unable to complete emergence from eggs. To enable the screening of the large numbers of alfalfa lines for our study, we streamlined their screen and obtained a snapshot of whitefly nymph developmental progression at the time of emergence of the first adult. Similar to Jiang et al (2003), we identified highly resistant plants that blocked nymph development; since our whitefly lines and those used by Jiang et al (2003) have the same heritage, the block in nymph development is likely to reflect insect mortality.

With our streamlined bioassay, we phenotyped 84 individuals from three UC-356-derived populations including two whitefly-resistant (UC2845 and UC2933) and a whitefly-susceptible (UC1872) population. We expected that there would be an array of resistance/susceptibility phenotypes in all three of alfalfa populations created for this study, because alfalfa breeding focuses on preserving and promoting genetic diversity in breeding populations. Multiple parents are involved in crosses and the resulting germplasm is a population of plants with genetically unique individuals (Teuber et al. 1997). For this reason, a proportion of the plants in each population (50-70%) will

express any desired trait. By assessing MEAM1 nymph development, we showed that the 97% of the individuals characterized from the UC-1872, which was selected for hyper-susceptibility were classified as highly susceptible, susceptible and moderately susceptible genotypes. Consistent with the UC-356 origins of UC1872, one highly resistant genotype was also identified (UC1872-137).

Similarly, the two populations selected for whitefly resistance (UC2933 and UC2845) consisted of both ~43% resistant and ~56% susceptible individuals. Within both R populations, a spectrum of MEAM1-resistance phenotypes was observed consistent with whitefly resistance being a multigenic trait. Significantly fewer highly susceptible and susceptible plants were identified in UC2933 and UC2845 (46% and 40%, respectively) than in our susceptible population (87%). The highly susceptible line S1 (UC-2845-043) was more susceptible than the known whitefly-susceptible CUF101 (Jiang et al. 2003); while S1 plants had less than 14.2% of insects in their first instar, CUF101 had 21%.

Modalities of plant resistance to pathogens/pests fall into three classes: antixenosis (the non-preference of a host), antibiosis (the inhibition of development or survival of a pathogen/pest on a host), or tolerance (the ability of a host to limit symptoms of damage despite an active infection/infestation) (Radcliffe and Hutchison 1999; Smith and Clement 2012). Collectively, our assessment of nymph development, host choice, rate of oviposition, and adult longevity suggest that we have both antibiotic and antixenotic mechanisms active in MEAM1-resistant alfalfa. Quite surprisingly, the resistance mechanisms deployed in R1, R2 and R3 impact all three whitefly species but in very different ways, supporting the premise that MEAM1, MED and NW1 are genetically distinct with different adaptations to their host plants (Jiang et al. 2003; De Barro et al. 2011). Characterization of the R1, R2 and R3 genotypes relative to S1 has led to five

significant discoveries about alfalfa's resistance and its impacts on members of the whitefly species complex.

First, the three highly resistant lines block 94-99% of the MEAM1 first-instar nymphs from progressing into their second instar. It is noteworthy the resistance phenotypes of our R1, R2 and R3 lines was similar to the most highly whitefly-resistant alfalfa plants (clones 3, 10, 27, and 37) characterized in Jiang et al. (2003). Surprisingly, our R1, R2 and R3 lines did not interfere with MED nymph development. These data suggest that the antibiotic traits that caused MEAM1 nymph mortality did not impact MED in a similar, despite the fact that these species both evolved from the Mediterranean (De Barro and Ahmed 2011; De Barro et al. 2011).

Second, the three R lines had different impacts on MEAM1 and MED adult longevity. Surprisingly, whitefly adult longevity was not strictly correlated with the antibiotic resistance trait(s) that impacted nymph development in R1, R2 and R3 with two exceptions. MEAM1 adults survived lived >2-fold longer on S1 than R3 plants and MED's lifespan was >2-fold longer on S1 than R2. In addition, differences whitefly longevity was discerned between the different resistant genotypes suggesting presence of different antibiotic traits in each of the R lines. For example, both MEAM1 and MED survived longer on R1 than R3 plants. In addition, MED lived longer on R1 relative to R2 plants.

Third, there were strong trends associated with MEAM1 resistance and adult choice. Choice decisions were evident at 24 hpi and beyond. Based on statistically significance and compelling trends, both MEAM1 and MED prefer S1 over the resistant genotypes. In addition, at MED prefers S1 over R2 at the 48 hpi interval and there is a non-choice phenomenon occurring with R3 from 8 – 48 hpi.

Fourth, the different *B. tabaci* species had different oviposition patterns on the S and R genotypes. Unlike Jiang et al (2003) who saw a weak correlation with MEAM1 nymph mortality and fecundity, there was no significant difference in oviposition between the resistant and susceptible genotypes for any of the whitefly species tested. However, there were differences in MED and NW1 oviposition when different resistant genotypes were compared. Significantly more eggs were deposited by NW1 females on R1 than on R2 ($p = 0.03$) plants. In addition, the number of MED eggs on R2 plants was 2-fold higher than the number on R1 or R3 plants. The decision of a female whitefly to deposit eggs on a host is partially a measure of host acceptance, as they feed and oviposit concomitantly (van Lenteren and Noldus 1990). However, oviposition rates were not well correlated with the host choice experiments, where antixenosis was clearly observed in resistant vs S1 plants for MEAM1 and MED. Therefore, we can conclude that alfalfa's whitefly resistance does not necessarily inhibit adult oviposition, but might greatly limit the number of emerged nymphs that become adults.

Finally, based on the nymph development, longevity and host choice assays, alfalfa is a suboptimal host for NW1. On S1 and the three resistant genotypes, NW1 nymphs did not develop, NW1 adults died within 3 d during the free-choice studies, and NW1 adults preferred to not to settle on any of the plants offered. Furthermore, while NW1 was capable of ovipositing, this is a suboptimal host choice and our host-choice experiments show that NW1 adults more than likely do not prefer alfalfa as a host. These data were surprising, as NW1 colonized alfalfa fields prior to its displacement by MEAM1 in the early 1990s (Toscano et al. 1994). There are several potential reasons for NW1's inability to thrive on alfalfa. First, as mentioned earlier, NW1 has a smaller host range than either MEAM1 or MED (Bellows et al. 1994). Second, the NW1 whiteflies that

were used to initiate our colonies were collected from a NW1 colony that has experienced several bottlenecks, potentially influencing its host range even further (J. Ng, personal communication).

Collectively, the data above indicate that R1, R2 and R3 impact the success of three *B. tabaci* species differently. Given the broad and continuous spectrum of resistance displayed in the populations from which R1, R2 and R3 were derived, whitefly resistance is likely to be multigenic. Therefore, the resistant individuals characterized here, while uniformly conferring an antibiosis that causes MEAM1 nymph mortality, must express different quantitative traits to explain differences in nymph mortality in MED and host-choice and fecundity in MEAM1, MED and NW1. To explore these differences, we have initiated a collaboration with Dr. Paul Fraser (Royal Holloway University London) to determine if the antibiosis and antixenosis to *B. tabaci* species that is displayed in R1, R2 and R3 are correlated with alfalfa specialized metabolites.

The discovery of differential resistance responses to the three *B. tabaci* species is distinct from the whitefly-resistance mechanisms in cassava and tomato. The adult and nymph mortality, lower fecundity, and repellence are all associated with the multigenic whitefly resistance in the cassava genotype ECU72 (Bellotti and Arias 2001; Perez-Fons et al. 2019). ECU72 confers a broad based resistance to seven whitefly species from four genera including: *Aleuotrachelus socialis*, *B. tabaci* SubSaharan African 1 (SSA1), *B. tabaci* SSA2, *B. tabaci* SSA3, *Bemisia tuberculata*, *Aleurothrixus aepim*, and *Trialeurodes variabilis (Lima et al. 2018; Bellotti and Arias 2001; Becerra Lopez-Lavalle ; Atim 2021; Barilli et al. 2019).* Furthermore, the apoplastic resistance to whiteflies expressed in *Mi1.2* tomatoes confers resistance to both MEAM1 and MED (Nombela et al 2003). *Mi1.2* is noteworthy as it confers resistance to nematodes (species), an aphid

(species), psyllids (species) and whiteflies (Kaloshian and Walling 2016). To date, the whitefly species specificity of *Brassica oleraceae*'s phloem-mediated antibiosis to the cabbage whitefly (*Aleyrodes protella*) has not yet been tested (Broekgaarden et al. 2012); in contrast, two specialists insects of *Brassica* spp.- the cabbage aphid (*Brevicoryne brassicae*) and caterpillars of the small cabbage white (*Pieris rapae*) – perform better on the whitefly-resistant *B.oleraceae* (Broekgaarden et al. 2009).

Given the precedent for superabundant whitefly populations on alfalfa in the past (Palumbo et al. 2000; Yee and Toscano 1996) and changing climate that may shift the geographic distribution of *B. tabaci* and its natural enemies (Ramos et al. 2018; Curnutte et al. 2014), deployment of host-plant resistance to whiteflies should be a high priority. While the whitefly resistance characterized in R1, R2, and R3 differentially impacts *B. tabaci* species, it has utility for protecting alfalfa from current and future damage from MEAM1, which is a current resident in fields in California, and from MED1, which is currently in greenhouses in California (McKenzie and Osborne 2017; McKenzie et al. 2012; Hodges and McKenzie 2008). As alfalfa lines are genetically diverse populations of plants, which are designed to be resilient with changes in biotic stresses, deploying multigenic resistance to *B. tabaci* is feasible and desirable. Considering the differences in resistance phenotypes of our R1, R2, and R3 lines, it might be beneficial to combine the traits of our resistant genotypes. It would be of particular advantage to combine R1 and R3. R1 has a potent antibiosis and antixenosis to MEAM1 that confers nymph mortality and repellence. While MEAM and MED adults have shorter lifespans on R3 than R1. The combination of the resistance traits from R1 and R3 could be used to develop alfalfa lines that are highly repellant to MEAM1 and MED adults and lethal to MEAM1 nymphs. Based on our results, R2 may not be a preferred parent for future

alfalfa breeding as MED adults are more fecund on R2 than R1 and R3. A breeding program to create to create a whitefly-resistant alfalfa cultivar (UC-Impalo-WF) was developed (https://fsp.ucdavis.edu/seed-catalog/alfalfa-varieties/uc-impalo-wf). UC-Impalo-WF is also resistant to multiple phyla of pathogens to varying degrees including Fusarium wilt (*Fusarium oxysporum*), Phytophthora root rot (*Phytophthora megasperma),* southern anthracnose (*Colletotrichum trifolii),* three species of aphids (*Threioaphis maculate, Acyrthosiphon kondoi, Acyrthosiphon pisum)*, and northern and southern root knot nematodes (*Meloidogyne incognita spp.*). In the future, it would be of interest to compare the relative resistance of UC-Impalo-WF to R1, R2 and R3 and determine its specificity to the three *B. tabaci* species studies here.

In the future, it may also be of interest to characterize R1 and R2 responses to other pathogens and pests of alfalfa. Like UC-Impalo-WF, the UC-2845 population, from which R1 and R2 are derived, incorporated many of the pathogen and pest resistance genes used in the development of UC-Impalo-WF. While resistance to aphids, nematodes, Phytophthora root rot, anthracnose, and fusarium wilt is genetically independent of whitefly resistance, understanding their relationships at the molecular level would provide novel insights into the molecular basis of resistance to multiple attackers. One outstanding question is if a single alfalfa genotype can express resistance to all pests/pathogens or if the UC-Impalo-WF individuals, R1 or R2 selective express one or more resistance mechanism. This addresses the compatibility/incompatibility of activating multiple resistances in the field.

Finally, understanding the basis of the antibiosis that delays MEAM1 development is of interest. R1, R2 and R3 plants, like the resistant clones studied by Jiang et al. (2003), are derived from individuals from the UC-2458 population. Therefore, the substantial

delay in nymph development observed in R1, R2 and R3 is likely to reflect the cessation of phloem feeding and subsequent nymph mortality as seen in clones 27 and 37 from Jiang et al. (2003) and Jiang and Walker (2007). It should also be noted that our criterion for identifying highly resistant plants was more rigorous than the criterion used in Jiang et al (2003). On highly resistant plants, less than 10% of MEAM1 nymphs progressed beyond the first instar; while the resistant clones 3, 10, 27 and 37 identified by Jiang et al (2003) progressed to the second instar and then ceased development. Currently, we interpret the delays in nymph development in R1, R2 and R3 as nymph mortality. In the future, this can be assessed by using vital dyes to assess nymph viability or electropenetration graphs to determine if the first instars that exist on R1, R2 and R3 plants at 21-28 d continued to feed to maintain their viability. In addition, it would be of interest to determine if nymphs on R1, R2, and R3 plants can progress beyond their first instar if given greater than 28 days to develop.

Some entomologists propose that 98-99% of nymphs must perish to effectively manage whitefly populations (Naranjo 2004). Whether the resistance in R1, R2 and R3 display is due to nymph mortality or a protracted first instar, this mechanism of resistance can have a profound impact on whitefly population expansion. First, the delays in nymph development may enhance natural biocontrol; longer windows of opportunity for predators and parasitoid wasps to identify whitefly nymphs are provided (Hagler et al. 2004; Gerling et al. 2001). Second, and perhaps more importantly, the reduced number of adults that emerge during a life cycle has a big impact on subsequent whitefly generations. Using our findings for MEAM1 on R1, we estimate that maximum lifespan of MEAM1 will be 22 d (Figure 10), females will deposit ~ 53 eggs (Figure 11), and no more than 1% of the instars will become adults R1 plants. For a

susceptible genotype, such as S1, a similar MEAM1 lifespan and fecundity is used, but we project that >70% of the eggs will survive to adulthood (Jiang et al 2003). This allows for a simplistic prediction of whitefly populations in one generation. If we assume 200 fertilized females infest an R1/S1 plant, they will lay 10600 eggs. For S1, 7420 adults will emerge. In contrast for R1, 106 adults will emerge; this translates to a >99% reduction in the whitefly population. For the second generation, we assume that ~50% of the adults that emerge will be female (Jiang et al. 2003) on both R1 and S1 plants. In the second generation, the S1 populations will increase to >137,600, while the R1 population would not exceed 28 insects; this translates to a 4900-fold difference in whitefly populations within two generations. This would have a massive impact on plant growth and honeydew deposition, with the subsequent growth of sooty mold, which impacts the value of alfalfa hay (Palumbo et al. 2000). However, these predictions need to be tempered with the facts that any whitefly-resistant commercial line would have 60% or less of its population expressing R1's potent resistance; however, whitefly survival might also be adversely effected by environmental conditions (Naranjo 2004). For this reason, we have established a collaboration to develop a comprehensive model that will more realistically predict the impact of R1's resistance on MEAM1 populations. To test this model, a large scale mutigenerational studies might be needed to assess if this theory is valid in practice. Finally, also have a considerable number of "moderately resistant" lines that might be useful for alfalfa breeders. We have not explored the nuanced difference between highly-resistant and moderately-resistant genotypes. For this reason, it is not clear if these genotypes express similar or different resistance traits that will impact the success of MEAM1, MED and NW1. Future studies would allow us to further distinguish these phenotypes. By understanding the molecular mechanisms and metabolites that

underly the resistance displayed in resistant and susceptible alfalfa, we hope to reveal the molecular and cellular events that control this novel nymph-based whitefly resistance mechanism.

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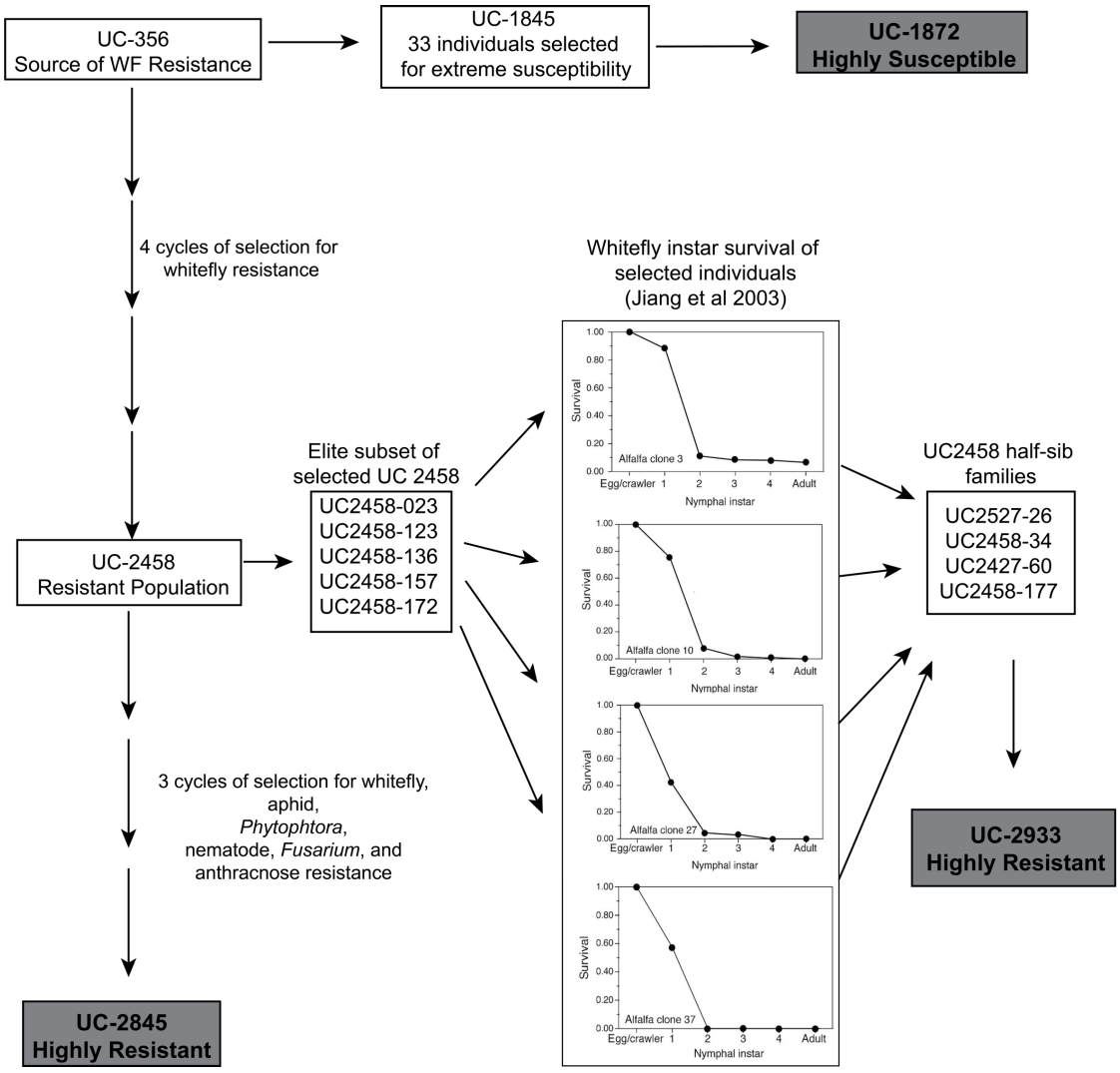


Figure 1.1 Breeding diagram for the UC WF^R program.

Alfalfa whitefly-resistant germplasm UC-356 was developed using resistant lines selected by Teuber et al (1997). This germplasm was used to create a resistant population (UC-2458) which was used to create two elite populations of WF^R alfalfa (UC-2933 and UC-2845). A highly susceptible population (UC-1872) was also made from the WF^R germplasm by selecting for 33 highly susceptible lines.

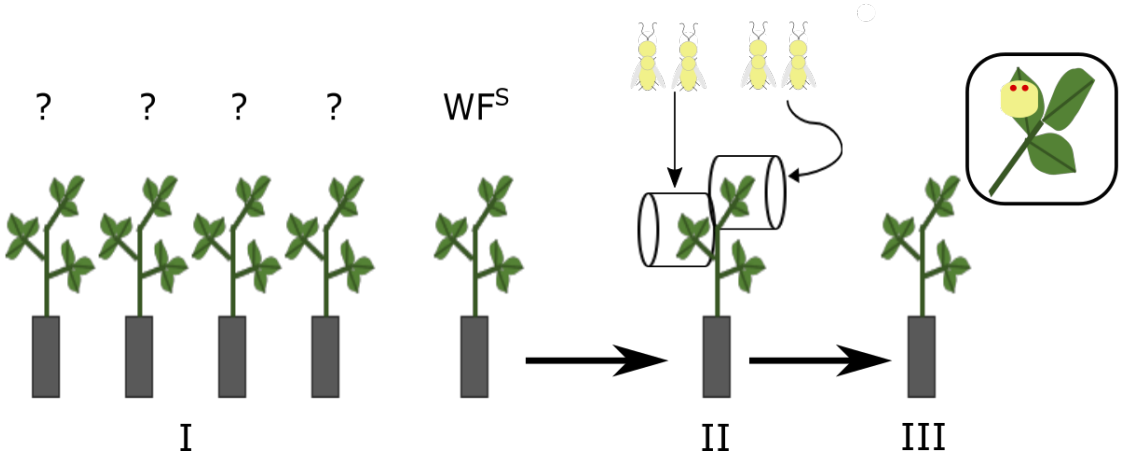


Figure 1.2 Schematic of the whitefly resistance screen used to phenotype alfalfa plants from the UC-1872, UC-2845 and UC2933 populations.

Individuals from each population were clonally propagated. (I) Clones from four or more alfalfa lines of an unknown phenotype (?) and a known susceptible alfalfa line (WF^S) were screened simultaneously. Five to ten plants per genotype were used in each bioassay (not shown). (II) Two trifoliate leaves were caged and infested with twelve *B. tabaci* MEAM1 adults (1:1 sex ratio) for 48 h (N =10 for five replicate plants). Whiteflies and cages were removed after 48 h. (III) Trifoliate leaves on the susceptible control line were checked daily until late-fourth instars were detected. Trifoliates were then excised from all plants and abaxial and adaxial surfaces of each leaf was photographed.

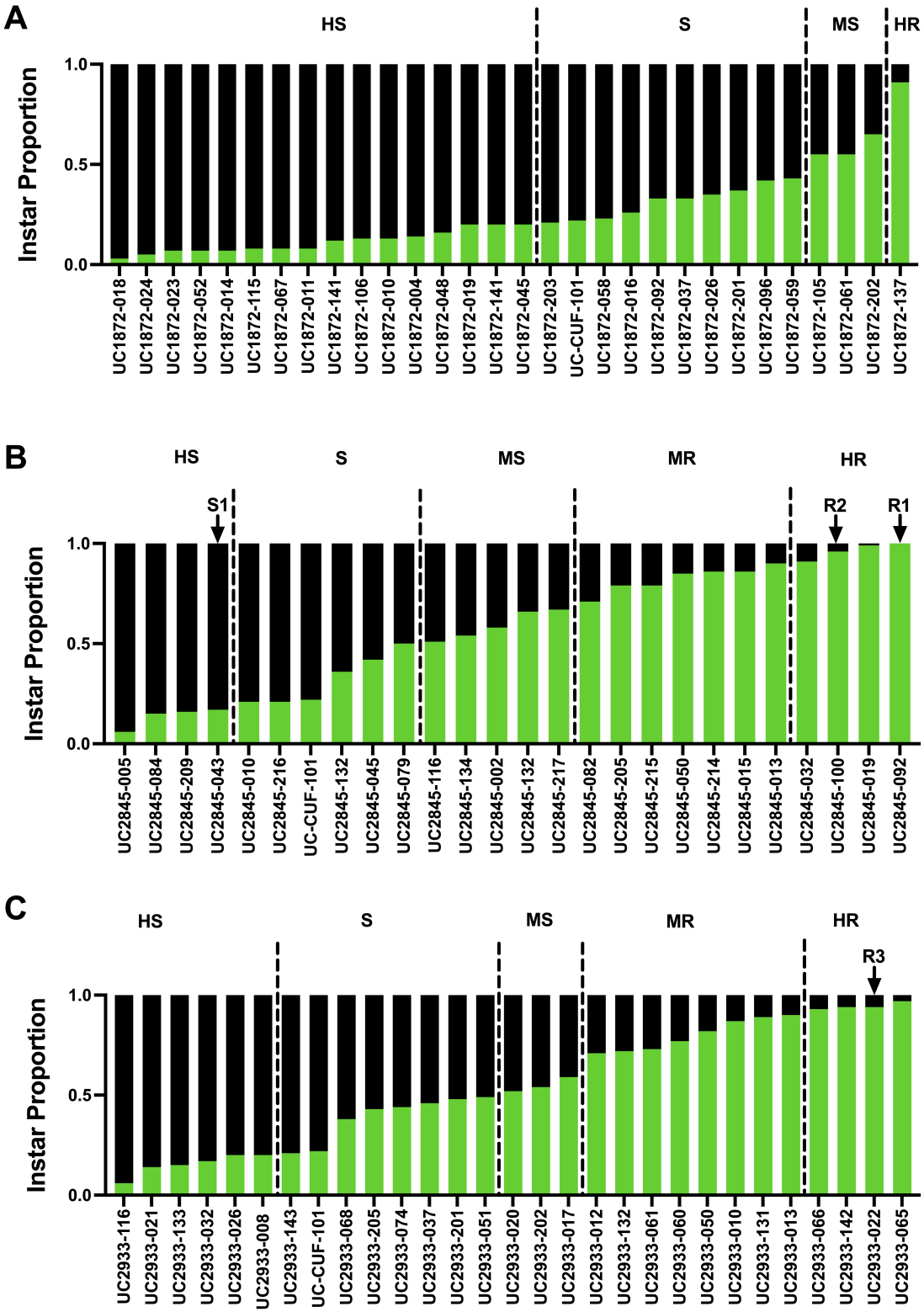

A
HS
S
MS
HR
Instar Proportion
1.0
0.5
0.0
UC1872-018
UC1872-024
UC1872-023
UC1872-052
UC1872-014
UC1872-115
UC1872-067
UC1872-011
UC1872-141
UC1872-106
UC1872-010
UC1872-004
UC1872-048
UC1872-019
UC1872-141
UC1872-045
UC1872-203
UC-CUF-101
UC1872-058
UC1872-016
UC1872-092
UC1872-037
UC1872-026
UC1872-201
UC1872-096
UC1872-059
UC1872-105
UC1872-061
UC1872-202
UC1872-137
B
HS
S
MS
MR
HR
S1
R2
R1
Instar Proportion
1.0
0.5
0.0
UC2845-005
UC2845-084
UC2845-209
UC2845-043
UC2845-010
UC2845-216
UC-CUF-101
UC2845-132
UC2845-045
UC2845-079
UC2845-116
UC2845-134
UC2845-002
UC2845-132
UC2845-217
UC2845-082
UC2845-205
UC2845-215
UC2845-050
UC2845-214
UC2845-015
UC2845-013
UC2845-032
UC2845-100
UC2845-019
UC2845-092
C
HS
S
MS
MR
HR
R3
Instar Proportion
1.0
0.5
0.0
UC2933-116
UC2933-021
UC2933-133
UC2933-032
UC2933-026
UC2933-008
UC2933-143
UC-CUF-101
UC2933-068
UC2933-205
UC2933-074
UC2933-037
UC2933-201
UC2933-051
UC2933-020
UC2933-202
UC2933-017
UC2933-012
UC2933-132
UC2933-061
UC2933-060
UC2933-050
UC2933-010
UC2933-131
UC2933-013
UC2933-066
UC2933-142
UC2933-022
UC2933-065

Figure 1.3 *B. tabaci* MEAM1 instar development on alfalfa genotypes used in the high-throughput screen.

MEAM1 nymph development in the UC-1872 (A), UC-2845 (B), and UC-2933 (C) populations based on the percentage of insects in their first instar vs later instars (second, third and fourth instar nymphs) are shown. Green represents the number of nymphs identified as first instars and black represents all others (2nds – exuvium). Resistance classes were defined based on the percentage of nymphs in their first instar at the end of the phenotypic screen. The classes included: highly resistant (HR, >90% first instars), moderately resistant (R, >70-90%), moderately susceptible (MS, >50-70%), susceptible (S, >20-50%), and highly susceptible (HS, 0 - 20%). The positions of CUF101, S1, R1, R2, and R3 genotypes along the susceptibility-resistance spectrum in these screens are indicated.

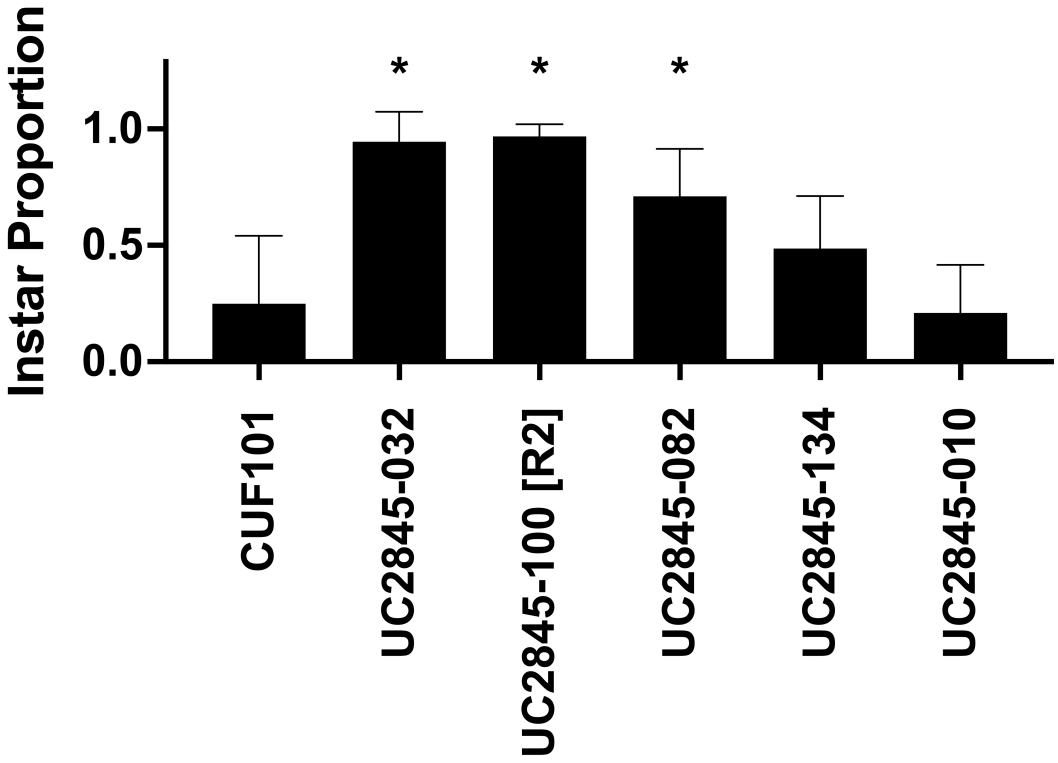


Figure 1.4 The first-instar proportion of six alfalfa lines in a representative whitefly resistance screen.

While lines for the phenotyping assays were chosen randomly, in this experiment all were of the UC2845 lineage. The mean proportion of insects in their first instar on each trifoliate leaf for a line was determined ($n \geq 12$). The proportions of first instar insects for each line were compared using a Kruskal-Wallis One-Way ANOVA after arcsin square root transformation of each mean. The experiment had significant differences in first-instar mortality ($p \leq .0001$). Resistant genotypes were confirmed by conducting a Dunn's multiple comparisons test against the known susceptible line CUF101. Resistant genotypes that passed the Dunn's multiple comparison threshold (p-value $\leq .05$) are indicated with an asterisk.

Figure 1.5 Images of alfalfa trifoliates from S1, R1, R2, and R3 plants.

Scale bar = 1 cm.

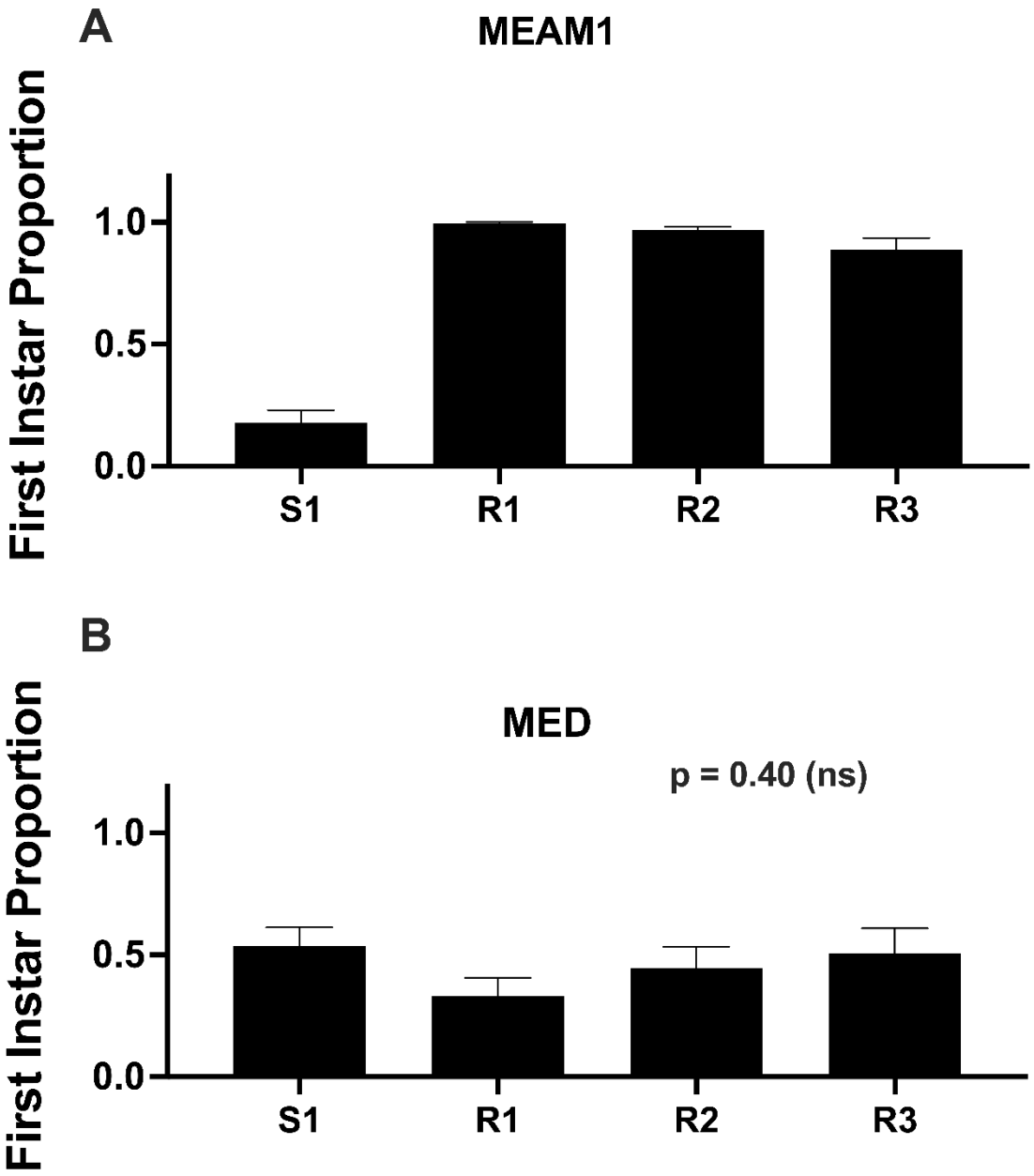

A
MEAM1
First Instar Proportion
1.0
0.5
0.0
S1
R1
R2
R3
B
MED
p = 0.40 (ns)
First Instar Proportion
1.0
0.5
0.0
S1
R1
R2
R3

Figure 1.6 The MEAM1 and MED nymph development of whitefly-susceptible and -resistant alfalfa.

The number of nymphs in their first, second, third and fourth instars, as well as exuvia, were determined on trifoliate leaves from a susceptible genotype (S1) and three resistant genotypes (R1, R2, R3) was determined. Insects and exuvia were counted on the day of the emergence of the first adult from the S1 line. (A) MEAM1 nymph development. (B) MED1 nymph development. The proportion of first-instar nymphs found on each genotype after screening. Lines were screened in separate experiments. Resistant genotypes were confirmed by conducting a Dunn's multiple comparison's test against a susceptible genotype. Five plants (ten trifoliates) were assayed in each experiment and the experiment was replicated twice ($N \geq 12$). Each experiment was compared using a Kruskal-Wallis one-way ANOVA on arcsin square root of the proportion of first-instars of each replicate.

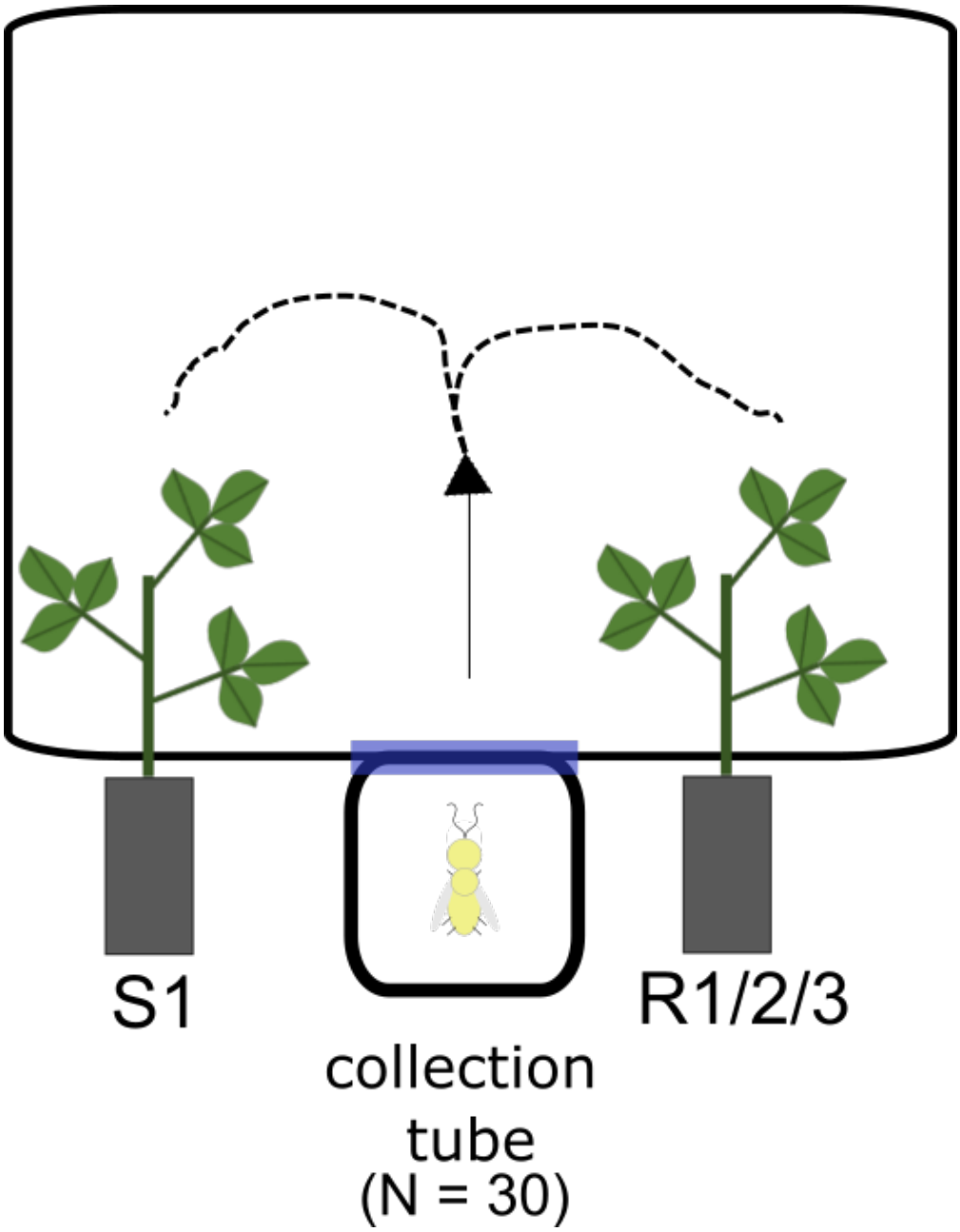


Figure 1.7 Free choice experiments.

Cages were designed to hold a susceptible and resistant shoot with trifoliate leaves that were secured to the cage with a foam plug. Whiteflies captured in collection tubes were released into the cage to initiate the choice experiment. Whiteflies residing on a susceptible (S1) plant or the resistant (R1/2/3) plant were designated as choice decisions. The remaining insects were considered as no-choice decisions. Insect locations were determined at 8-, 24-, 48-, and 72-hpi (h post-infestation) (N = 30).

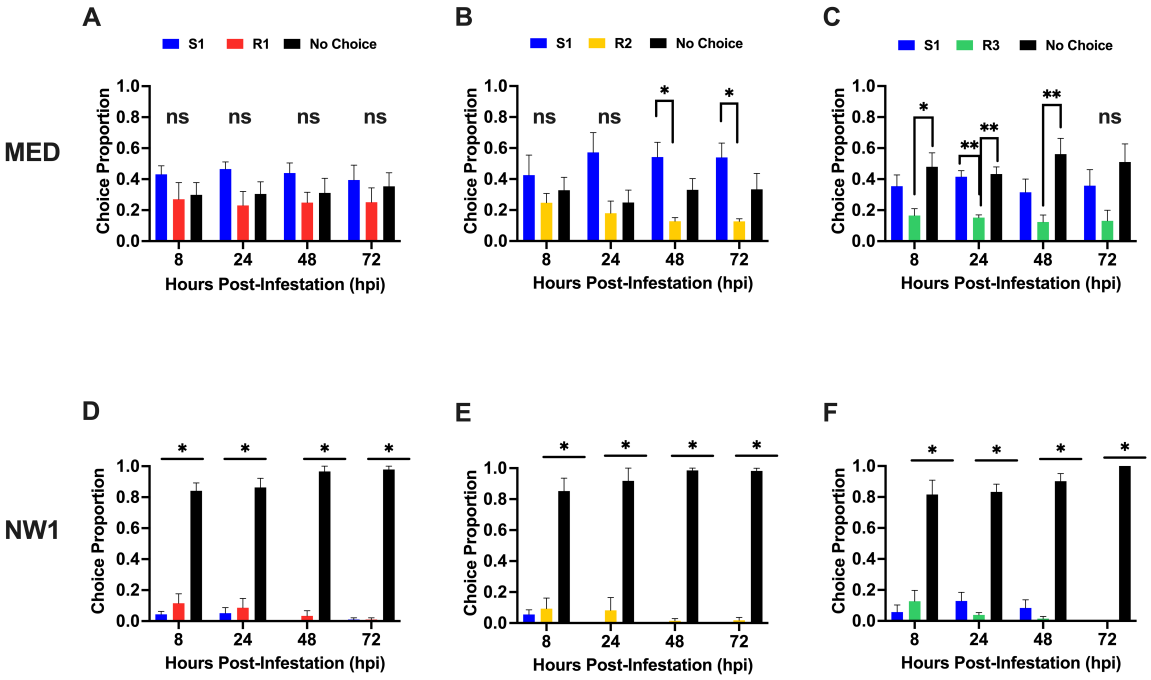


Figure 1.8 *B. tabaci* MEAM1 adult performance in pair-wise choice experiments between a whitefly-susceptible and three whitefly-resistant genotypes.

The proportion of adults that chose the susceptible genotype (S1) or a resistant genotype R1 (A), R2 (B) or R3 (C) or made no choice at 8, 24, 48 and 72 h post-infestation (hpi) is shown. The significance of choice or no choice decisions was determined using a two-way ANOVA analysis with Geisser-Greenhouse correction on arcsin transformed proportions for each time point. Each experiment was performed five times. Statistically significant comparisons between genotypes were confirmed with a Tukey's multiple correction test with individual variances calculated for each comparison. Differences in choice proportion that are statistically significant are marked with asterisks (* = .05, ** = .01, *** = .001, ns = not significant).

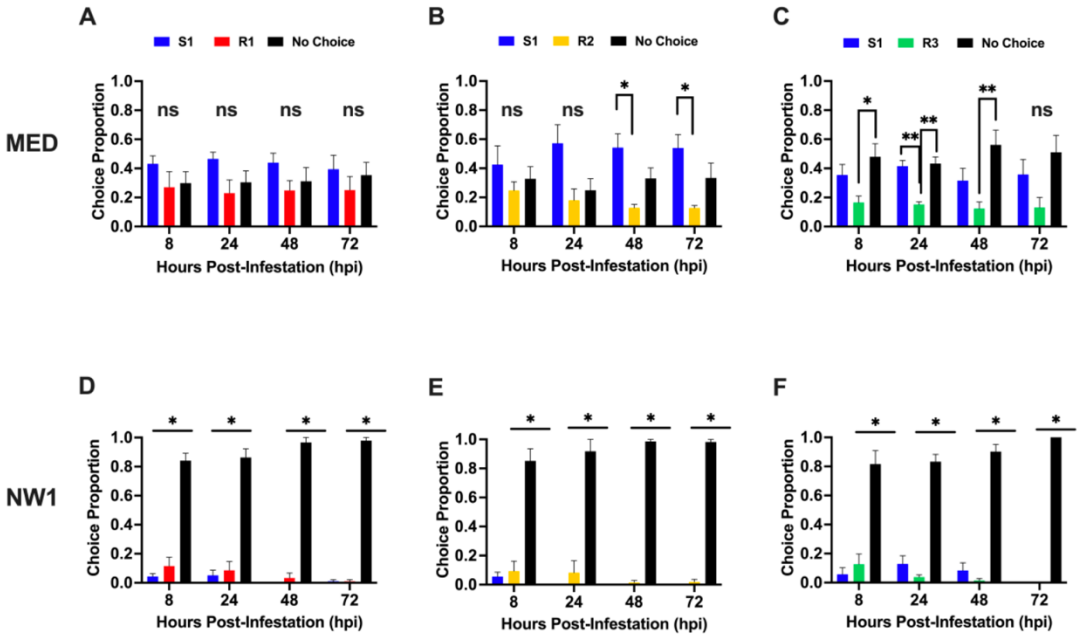
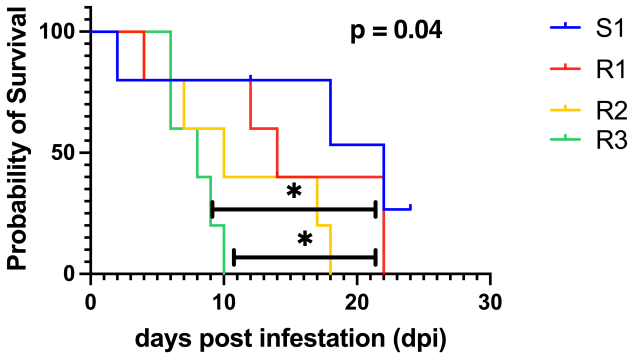


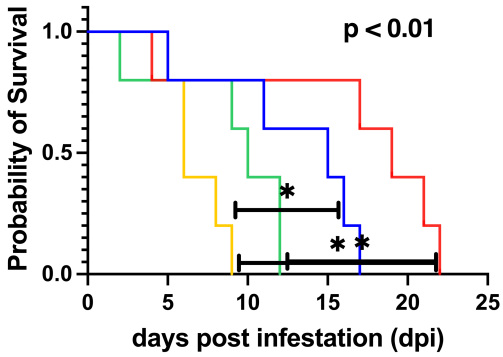
Figure 1.9 *B. tabaci* MED1 and NW1 adult performance in pair-wise free-choice experiments between a whitefly-susceptible and three whitefly-resistant genotypes.

The proportion of MED1 (A-C) or NW1 (D-F) adults that chose the susceptible genotype (S1) or a resistant genotype R1 (A, D), R2 (B, E) or R3 (C,F) or made no choice at 8, 24, 48 and 72 h post-infestation (hpi) is shown. The significance of choice or no choice decisions was determined using two-way ANOVA with Geisser-Greenhouse correction (N = 5) on the arcsin transformed proportions for each time point. Each experiment was performed five times. Statistically significant comparisons between genotypes were confirmed with a Tukey's multiple correction test with individual variances calculated for each comparison. Differences in choice proportion that are statistically significant are marked with asterisks (* = 0.05, ** = 0.01, ns = not significant).

A



B



C

MEAM1 Longevity on Resistant and Susceptible Alfalfa

Genotype	Median Survival (dpi)	Pairwise Comparison	P-value
		S1 - R1	0.39
		S1 - R2	0.08
S1	22	**S1 - R3**	0.03*
R1	14	**R1 - R2**	0.39
R2	10	**R1 - R3**	0.03*
R3	8	**R2 - R3**	0.23

MED Longevity on Resistant and Susceptible Alfalfa

Genotype	Median Survival (dpi)	Pairwise Comparison	P-value
		S1 - R1	0.06
		S1 - R2	0.03*
S1	15	**S1 - R3**	0.11
R1	19	**R1 - R2**	0.04*
R2	6	**R1 - R3**	0.03*
R3	10	**R2 - R3**	0.06

Figure 1.10 MEAM1 and MED adult longevity on S1, R1, R2, and R3 plants.

Ten newly emerged whiteflies (1:1 sex ratio) were introduced to caged alfalfa trifoliate leaves. The viability of MEAM1 (A) and MED (B) adults on susceptible (S1) and resistant (R1, R2 and R3) alfalfa was assessed daily for 24 d or until no viable whiteflies remained in a cage (n = 5). Survival curves were compared with a Mantel-Cox test. (C) Tables showing the median survival of MEAM1 or MED on each genotype and the pairwise comparison of survival. Pairwise comparisons were completed with a Mantel-Cox test of each survival curve at the 0.05 confidence interval.

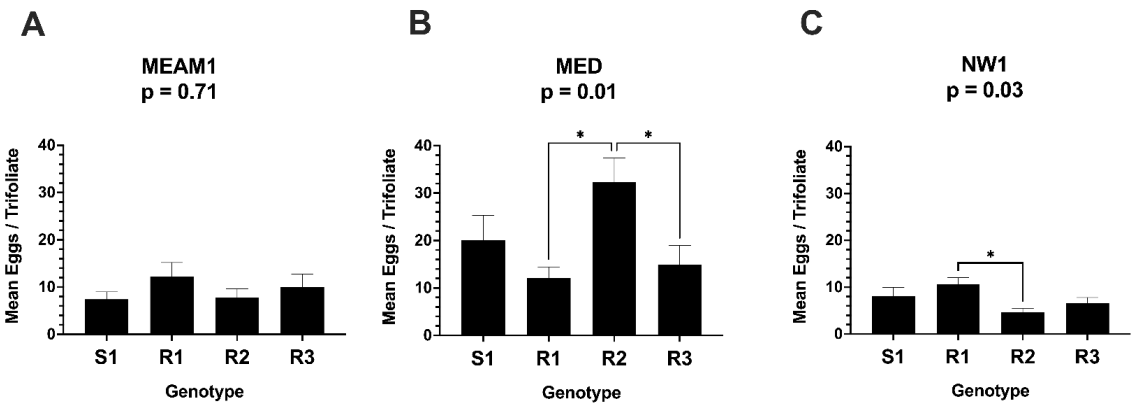


Figure 1.11 *B. tabaci* MEAM1, MED, and NW1 oviposition on susceptible and resistant alfalfa.

S1, R1, R2, and R3 trifoliate leaves were infested with five pairs of MEAM1 (A), MED (B) or NW1 (C) whiteflies (1:1 sex ratio) for 48 h (N ≧ 19) and the number of eggs oviposited on each trifoliate leaf was counted. The mean number of eggs/trifoliate leaf for each genotype was determined. Statistical significance of the means was assessed using a Kruskal-Wallis H test; p values appear in each panel. Statistically significant comparisons within the MED and NW1 data sets were determined with a Dunn's multiple comparison test are marked with an asterisk for significance at the 0.05 confidence interval.

Table 1.1 Development of NW1 whiteflies on susceptible and resistant alfalfa.

Alfalfa genotype	Whitefly-resistance class[A]	Proportion of whiteflies in each developmental stage					
		First Instar	Second Instar	Third Instar	Fourth Instar	Exuvium	Total Nymphs
CUF-101	S	0.49	0.25	0.22	0.04	0.00	51
2845-050	MR	1.00	0.00	0.00	0.00	0.00	21
2845-100	HR	0.85	0.15	0.00	0.00	0.00	39
2933-010	MR	0.57	0.26	0.17	0.00	0.00	82
2933-022	HR	0.94	0.06	0.00	0.00	0.00	17

[A] Primary data for whitefly-resistance classes is displayed in Figure 1.3.

Table 1.2 MEAM1 Longevity on Resistant and Susceptible Alfalfa

Genotype	Median Survival (dpi)	Pairwise Comparison	P-value[A]
		S1 - R1	0.39
		S1 - R2	0.08
S1	22	**S1 - R3**	0.03*
R1	14	**R1 - R2**	0.39
R2	10	**R1 - R3**	0.03*
R3	8	**R2 - R3**	0.23

[A] Comparisons significant at the 0.05 interval are marked with an asterisk

Table 1.3 MED1 Longevity on Resistant and Susceptible Alfalfa

Genotype	Median Survival (dpi)	Pairwise Comparison	P-value[A]
		S1 - R1	0.06
		S1 - R2	0.03*
S1	15	**S1 - R3**	0.11
R1	19	**R1 - R2**	0.04*
R2	6	**R1 - R3**	0.03*
R3	10	**R2 - R3**	0.06

[A] Comparisons significant at the 0.05 interval are marked with an asterisk

Chapter 2 Identification of candidate whitefly resistance loci in alfalfa using comparative *de novo* transcriptomics.

Abstract

Among Hemipteran insects, whiteflies are among the most devastating to agricultural crops. Their wide host range and myriad methods of damaging plants makes identify host plant resistance (HPR) mechanisms effective against whiteflies important. Alfalfa (*Medicago sativa*) has a nymph-based resistance mechanism. A whitefly-resistance mechanism was identified in alfalfa (*Medicago sativa*) that results in severely delayed nymph development. Here, we describe a comparative transcriptome analysis of a highly susceptible line (UC2845-043) and a highly resistant line (UC2845-092). Both lines were infested with MEAM1 whiteflies and samples were collected over the 22 day infestation at times correlated with MEAM1 stages in whitefly nymph development. *De novo* assembled transcriptomes were created and differentially expressed genes (DEGs) were identified based on differences between genotypes (gDEGs) and time (tDEGs), and using models that compensated for potential confounding effects of plant development (interaction DEGs, iDEGs). Principle component analysis of DEGs indicated that genotype was a stronger determinant of resistance than time. The rigorous iDEGs identified key processes associated with resistance that were further supported by the gDEG and tDEG analyses. Here, we describe a novel whitefly resistance mechanism in *M. sativa* that is correlated with induction of ethylene-signaling, suppression of JA, SA, and ABA signaling, changes in very long chain fatty acid (VLCFA) metabolism, suberin biosynthesis, and *ERECTA* induction.

Introduction

Whiteflies (*Bemisia tabaci*) are among the most devastating Hemipteran pests in agriculture worldwide. Whiteflies cause damage through phloem-feeding, virus vectoring, and honeydew secretion, which subsequently supports sooty mold growth making crops less valuable. While there are whiteflies (*B. tabaci* New World 1, NW1) native to North America known to cause moderate levels of damage, an invasive species (*B. tabaci* Middle Eastern Asia Minor 1, MEAM1) has become more prevalent in North America (Perring et al. 1993; Barinaga 1993; Perring et al. 1991). MEAM1 causes significant economic and agricultural losses in Southern California and at myriad agricultural hubs worldwide (De Barro and Ahmed 2011; Walling 2008). MEAM1 is a global pest that can be found on every continent except Antarctica (Perring 2001, De Barro, Liu et al. 2011). The cosmopolitan nature of this pest coupled with its propensity to develop insecticide resistance and limited success of biological control in most crop settings makes control of this pest through host-plant resistance (HPR), which is foundational for all integrated-pest management strategies, paramount (Naranjo and Ellsworth 2009).

While phloem-based resistance mechanisms to Hemipteran pests are known in many plant species, there are relatively few resistance (*R*) genes that have been cloned and characterized mechanistically (Walling and Thompson 2012). *R* genes identified and cloned to date include nine brown planthopper (*Nilaparvata lugens Stål*) resistance genes (*Bph2/26, 3, 6, 9, 14, 17, 18, 29, 32*) of rice (*Oryza sativa*), cotton-melon aphid (*Aphis gossypii*) resistance gene (*Vat*) of melon (*Cucumis melo*), and the multi-phyla resistance gene (*Mi-1.2*) of tomato (*Solanum lycopersicum*)(Rossi et al. 1998; Vos et al. 1998; Nombela et al. 2003; Casteel et al. 2006; Sani Haliru et al. 2020; Martin et al.

2003). The *Vat, Mi1-2,* and four *Bph* genes (*Bph2/26, 9, 14,* and *18)* encode for classical R proteins with coiled-coil (CC) nucleotide-binding site (NBS) leucine-rich repeat (LRR) domains (CC-NLR) (Dogimont et al. 2014; Milligan et al. 1998). BPH *R* genes have more functional and structural diversity. BPH3 and *BPH17* are membrane-localized lectin-domain receptor kinases (RK), BPH6 is an exocyst-localized, Bph29 is a B3 DNA-binding domain and BPH32 is a membrane-bound a small-copy repeat protein (Guo et al. 2018; Ren et al. 2016; Wang et al. 2015; Jairin et al. 2007; Liu et al. 2015).

The tomato *Mi-1.2* locus is distinct as it confers resistance to nematodes (*Melodogyne* spp.), potato aphid (*Macrosiphum euphorbiae*), tomato psyllid (*Bactericerca cockerelli*) and two species of whitefly (*Bemisia tabaci* MEAM1, *B. tabaci* Mediterranean (MED)) (Rossi et al. 1998; Vos et al. 1998; Nombela et al. 2003; Casteel et al. 2006). While resistance to aphids is antibiotic and phloem-localized and psyllids in antixenotic, resistance to whiteflies is apoplastic (Jiang and Walker 2007; Casteel et al. 2006; Kaloshian et al. 1997; Jiang et al. 2001). The effectiveness of these resistance mechanisms are also influenced by temperature, plant age, and plant species (Nombela et al. 2003; Goggin et al. 2006). *Mi-1.2* confers resistance to both aphids and whiteflies, aphid resistance is plant age-dependent, whereas whitefly resistance is both age-dependent and temperature-dependent. While these mechanisms are effective against whitefly, transgenic deployment of these hemipteran resistance mechanisms has not been successful. *Mi-1.2* was transformed into eggplant (*Solanum melongena*) and conferred resistance against root-knot nematode. In contrast, transgenic *Mi-1.2* eggplant were susceptible to aphid feeding (Goggin et al. 2006) and whitefly resistance in transgenic plants expressing *Mi-1.2* has yet to be determined.

In addition to *Mi-1.2*, sources of resistance to whiteflies have been identified in wild tomato, cassava, cotton, *Brassica*, melon, cowpea, soybean, and common bean; however, with a few exceptions these resistance genes and mechanisms are not characterized (Nombela et al. 2003; Firdaus et al. 2012; Rodriguez-Lopez et al. 2011; Bellotti and Arias 2001; Butter and Vir 1989; Farnham and Elsey 1995; Simmons and Levi 2002; Cruz et al. 2014; Da Silva et al. 2014; dos Santos et al. 2021). Several wild relatives of tomato have trichome-mediated antixenotic defenses against whiteflies and other insects (Liedl et al. 1995; Rodriguez-Lopez et al. 2011; Firdaus et al. 2012; McDaniel et al. 2016). In some cases, these multigenic resistance mechanisms have been successfully moved into cultivated tomato (Rodriguez-Lopez et al. 2011; McDaniel et al. 2016).

Brassica oleraceae possesses an antibiotic resistance mechanism to the whitefly (*Aleroydes proletella*) that is developmentally-regulated and is correlated with a rise in ABA and ABA-dependent gene expression (Broekgaarden et al. 2018). While the loci for cotton's whitefly resistance has not been mapped (Jin et al. 2018), resistance appears to be antixenotic and linked to upregulation of *WRKY40* and *MPK3* (Li et al. 2016). Finally, whitefly resistance based on nymph mortality and adult repellence in cassava (*Manihot esculenta*) and have also been identified (Perez-Fons et al. 2019). *Cassava's resistance mechanism is multigenic and appears to be linked to ABA and SA (Garceau 2021).*

Whitefly-resistance in alfalfa (*Medicago sativa*) appears to be multigenic as a spectrum of resistance is observed in whitefly-resistant alfalfa populations (Jiang et al. 2003). Furthermore, this resistance is phloem-based, blocks nymph development and influences fecundity (Teuber et al. 1997; Jiang and Walker 2007). Unfortunately, the initial lines characterized in Jiang et al (2003) and (Jiang and Walker 2007) were lost.

However, three alfalfa populations that were derived from the germplasm that were studied by Jiang et al and Jiang and Walling were used to create three new alfalfa populations that were segregating for whitefly-resistance (Chapter 1). These lines were screened for whitefly resistance and three highly-resistant (R1, R2 and R3) and one highly-susceptible (S1) lines were used to study the behaviors of three *B. tabaci* species: MEAM1, New World 1 (NW1), and MED). Each resistant line displayed antixenosis and antibiosis but the responses of the three *B. tabaci* species were distinct (Chapter 1).

With the foundational knowledge from Chapter 1, it is timely to pursue the molecular mechanisms that regulate whitefly resistance in alfalfa. Comparative transcriptomics experiments have been effective in identifying host plant resitance responses in the highly-resistant (R1) and highly-susceptible (S1) alfalfa lines upon infestation with MEAM1 whiteflies. In doing so, we accomplished the following objectives: (1) successfully assembled an alfalfa-whitefly response *de novo* transcriptome, (2) differentially expressed genes (DEGs) between genotypes and timepoints (e.g, genotype, temporal and interaction DEGs), and (3) demonstrated whitefly-resistance is associated with a significant reprogramming of ET, SA, JA and ABA phytohormone signaling, cell wall-mediated defenses, and suppression of PAMP/MAMP-triggered immunity and effector-triggered immunity.

Materials and Methods

Maintenance of B. tabaci MEAM1 colony

The *Bemisia tabaci* MEAM1 colony was maintained on 4-week old *Brassica napus* var 'Florida Broad Leaf' (W. Atlee Burpee & Co.) at 27°C, 55% relative humidity under long-day (16-h light:8-h dark) conditions in UC Soil Mix 3 in growth rooms within the Insectary and Quarantine Facility (IQF) at the University of California, Riverside.

Plant Growth

While, the genotypes studied by Jiang et al. (2003) and Jiang and Walker (2007) were lost, several alfalfa populations selected for WF^R and WF^S were available to pursue the mechanisms of alfalfa's potent nymph-mortality resistance. The whitefly-resistant R1 (UC-2845-092) and whitefly-susceptible S1 (UC-2845-043) alfalfa genotypes were identified in Chapter 1. Several R1 and S1 parent plants were maintained in 1-gallon pots in UC Soil Mix 3 at 26°C, 55% relative humidity under long-day (12-h light:12-h dark) conditions in a plant growth room or in a greenhouse with lighting as described in Chapter 1.

Stem cuttings (6-cm in length) from R1 and S1 parent plants were used to clonally propagate these genotypes. Stem cuttings were dipped in Clonex (Hydronamics International; Lansing, MI) gel-rooting media and dipped in Bonide (Tractor Supply) to minimize transfer of any insect pests that the parent plant acquired in the greenhouse environment. Three cuttings were placed in a UC soil mix 3 in a 2 x 2- inch well of a 72-well insert within a 1020 greenhouse tray (without holes) and covered with a humidity dome (Growers Solution; Cookeville, TN). Cuttings were misted daily to promote the high- humidity environment required for rooting. Dome vents were opened after cuttings had established roots (ca.10 – 14 d) and domes were removed after 21 d. To assure stem cuttings were well watered during the root establishment period, wells were watered from the top. Stem cuttings with established root systems were transferred to 5-inch pots with UC soil mix 3 and were grown in a growth room at 27°C, 35-50% relative humidity with a 12-h day:12-h night light cycle (300 µM light) inside thrip-proof bug dorms (MegaView Science Company).

***Bemisia tabaci* MEAM1 Infestations**

Young MEAM1 adults (2-3 days old) were collected individually into 49 mm x 6 mm glass test tubes sealed with corks and were sexed under a dissecting microscope. Males and females were pooled to establish short-term, sex-specific colonies with 1600 males and 1600 females in each colony. The sex-specific colonies were maintained on a *Brassica* plant in bug dorms in the greenhouse used for infestation experiments. On the day of infestation, trifoliate leaves were enclosed in cages as described by Jiang et al. (2003). Two to four insect cages were placed on each R1 and S1 plant. Each leaf with a cage was tagged with a jewelry tag. Infestations were initiated by releasing 20 whiteflies (1:1 sex ratio) using a customized aspirator and cages were sealed with a cork. Whiteflies were kept on plants for 24 h and were removed by aspiration. The number of viable and dead whiteflies were documented for each plant and cage. After WF removal, alfalfa plants were placed in clean thrips-proof bug dorms (300 µM light, 12-hr day, 25°C). R1 and S1 plants for each replicate were organized in a randomized block design.

Samples were collected at time points that correlated with *Bemisia tabaci* MEAM1 feeding/nymph development: 0 h post-infestation (hpi) (control), 1 day post-infestation (dpi) (adult feeding and egg deposition), 7 dpi (eggs and 1st-instar feeding), 14 dpi (2nd- and 3rd-instar feeding), and 22 dpi (4th-instar feeding and adult emergence). At each time point, alfalfa trifoliate leaves with their petiole were excised using a clean razor blade for each sample and were flash-frozen in liquid nitrogen and stored at -80°C until use. For each time point, the leaves from three alfalfa plants were pooled and five biological replicates of this experiment were performed, with three replicates used for RNA-seq library construction and all five replicates will be used in future metabolomics studies.

RNA Extraction

Alfalfa leaves were ground in liquid N_2 using a mortar and pestle . After N_2 evaporation, 300 µL extraction buffer (100 mM LiCl, 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, 1% β-mercaptoethanol) at 80°C and 300 µL of water-saturated phenol (80°C) were added. After vortexing for 30 sec, 300 µL chloroform:isoamyl alcohol (24:1) were added. The sample was vortexed for 30 sec and centrifuged for 5 min. The aqueous layer was removed and mixed with one volume of 4 M LiCl. After overnight precipitation at −80°C, total RNA was recovered by centrifugation in a microfuge (Eppendorf) at 12,000 × g at 4°C for 20 min. RNA pellets were dissolved in 250 µL diethyl pyrocarbonate (DEPC)-treated water for 30 min and washed with 25 µL 5 M NaCl and 500 µL 100% ethanol and centrifuged for 20 minutes. The pellet was then washed with 1 mL 70% EtOH and centrifuged for 20 minutes, resuspended in water, and stored at −80°C. RNAs were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA quality was assessed using 1% denaturing agarose gels and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) at the UCR Institute for Integrative Genome Biology (IIGB) Genomics Core.

RNA-seq library preparation, sequencing, and bioinformatics analyses

Three biological replicates from each time point were used to construct libraries. cDNA libraries were prepared at the IIGB Genomics Core. Strand-specific cDNA libraries were prepared using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (New England BioLabs; Ipswich, MA) using an input of 1 µg of RNA in 50 µL DEPC-treated water. Samples were multiplexed using NEBNext® Multiplex Oligos. RNA-seq libraries were constructed and sequenced using the Illumina NextSeq 500 platform (single-end 75-bp reads) at the Institute of Integrative Genome Biology Genomics Core

(UC Riverside). Libraries were multiplexed (12 libraries/lane) and sequenced resulting in 6.7 – 38 million reads per library. After trimming and fastq filtering, reads were used to construct three de-novo transcriptome assemblies with Trinity using default parameters: (1) a R1 transcriptome, (2) a S1 transcriptome, (3) and a transcriptome combining both genotypes (Grabherr et al. 2011). Reads were mapped to the *de novo* transcriptome using Bowtie2/2.2.5 and RSEM/1.3.1 (Li and Dewey 2011; Langmead and Salzberg 2012). Transcripts with mean of less than 10 reads across the time course for both genotypes were not included in the DEG analysis. DESeq2 was used to identify differentially-expressed gene (DEG) analysis (Love et al. 2014). DEGs were defined at the $|\log_2 FC| > 1$ and FDR < 0.05 thresholds using the Benjamini Hochberg method.

Temporal DEGs (tDEGs) were identified within a genotype by comparisons of 0 dpi vs infestation timepoints. Genotype DEGs (gDEGs) were classified as DEGs differentially expressed between genotypes at the same time point (for example, R0 vs -S0). Interaction DEGs (iDEGs) identified genotype-specific changes in gene expression using a series of models that were designed to account for any effect(s) of development over time might have on gene expression. Genotype and temporal heatmaps were organized using hierarchical k-means clustering and assembled using the R program *ComplexHeatmap* (Gu et al. 2016). Venn diagrams used to visualize DEGs were assembled using the R program *VennDiagram* (Chen and Boutros 2011). PCA was performed using default parameters in DESeq2 (Love et al. 2014).

Gene Annotation, Functional Analysis, and Ortholog Identification

DEGs were annotated using the Trinotate package and the following databases: Swissprot, Pfam, Mercator4 v2.0, Eggnog, HMMER, signalp, and tmHMM (Duvaud et al. 2021; Mistry et al. 2020; Schwacke et al. 2019; Almagro Armenteros et al. 2019; Huerta-Cepas et al. 2018; Eddy 2011, 2009, 2008; Krogh et al. 2001; Bryant et al. 2017) .

Homologs of alfalfa DEGs were identified in *Medicago truncatula* Mt4.0v1, and the *Arabidopsis thaliana* Araport11 genomes using BlastX. All NCBI-BLAST searches used an E-value cutoff at 10^{-5} for homolog identification. GO Term analysis was conducted using the Bioconductor package *goseq* (Young et al. 2010; Altschul et al. 1990) and assembled in heatmaps using *ComplexHeatmap* (Gu et al. 2016).

Results

Transcriptome analysis, defining DEG classes and DEG identification

Chapter 1 described a large-scale phenotypic screening for whitefly resistance/susceptibility using 84 alfalfa individuals (genotypes) from a whitefly-susceptible population (UC1872) and two whitefly-resistant populations (UC2933 and UC2845). Resistance was identified as the failure of nymphs to develop beyond the first instar. The most highly resistant genotype UC2845-095 (R1) and a highly susceptible genotype UC2845-043 (S1) from the UC2845 population were selected for study (Figure 1A). On R1 plants, 99% of the nymphs remained in their first instar at 21- 28 dpi, while the S1 genotype promoted nymph development. S1 had only 17% of nymphs remaining in the first instar and most insects were in their 2nd to 4th instars at the end of the assay. The disparate phenotypes between two half-sib individuals made them viable candidates for comparative transcriptomics.

RNA-seq libraries from three replicate time-course infestations (0, 1, 7, 14 and 22 dpi) of S1 and R1 plants were constructed and sequenced to identify the transcriptome profiles of the whitefly-resistant R1 and -susceptible S1 genotypes. Five time points were selected to correlate with significant whitefly behaviors: 0 dpi (uninfested control), 1 dpi (adult feeding and eggs), 7 dpi (eggs, instar emergence from eggs, and first instar translocation/probing/feeding), 14 dpi (second instar feeding), and 22 dpi (third- and

fourth instar feeding, adult emergence) (Zarate et al. 2007; Kempema et al. 2007) (Figure 1B).

Collectively the 30 RNA-seq libraries generated 485,865,149 reads, averaging ~16 M reads per library. Initially, reads from each library were mapped to the *Medicago truncatula* Mt4.0v1 and the diploid *Medicago sativa* CADL 1.0 reference genomes using the *systemPipeR* pipeline (Backman and Girke 2016). Due to poor alignment to each genome (~ 50% and 60%, respectively) (Supplemental Table 2.1.B and 2.1.C), the *de novo* assembler Trinity under default parameters was used to assemble three transcriptomes: R1, S1 and a combined (R1 + S1) assembly (Grabherr et al. 2011). Approximately 90 – 95% of the reads from the combined assembly mapped to the *de novo* transcriptome. The *de novo assembly* produced 190,627 transcripts and 124,435 genes with a mean contig size of ≈ 760 bp and a contig N50 of 1275 (Supplemental Table 2.1).

Bowtie2 and RSEM were used to align and quantify reads, respectively (Langmead and Salzberg 2012; Li and Dewey 2011). Transcripts with low total read counts ($\leq$10) were filtered out resulting in 45718 transcripts for these analyses. RSEM was used to identify differentially expressed genes (DEGs) between genotypes (genotype DEGs, gDEGs) or temporally within a genotype (temporal DEGs, tDEGs) with the criteria of $p < 0.05$ and 1.0-fold change (LFC) (Figure 2; Table 2.1 and 2.2). The PCA analysis (Figure 2.3) of the infestation time-course samples shows samples are clustered by genotype than by time (PC1 = 54%; PC2 = 9%).

The expression profiles of the gDEGs (Figure 2.4) supports the PCA analyses, as most gDEGs do not have profound temporal variation. There was a total of 8242 unique genotype DEGs (Supplemental Table 2.2). There were generally more down-

regulated gDEGs than up-regulated gDEGs at all time points, with the exception of the 22 dpi time point (Figure 2.5A). In the susceptible genotype (S1), there were 663 up-regulated and 1,236 down-regulated temporal DEGs, while in the resistant genotype (R1) there were 1,046 up-regulated and 692 down-regulated temporal DEGs across all time points (Figure 2.5B).

To better understand the magnitude of transcript changes over time after whitefly infestation or between the genotypes, the distribution of DEG log_2FC values was examined (Table 2.3; Figures 2.5C-D). A vast majority of genotype DEGs, both upregulated and downregulated, were within a log_2FC range of 1 – 2 or 2 – 3. However, the number of upregulated DEGs that had a higher log_2FC range (4 – 5 or > 5) increased as the whitefly infestation progressed. The number of downregulated DEGs in the higher ranges log2FC did not change much throughout the infestation. In S1 and R1, most temporal DEGs were at a FC value greater than five at all times except 22-dpi. At 22-dpi in S1, there were more upregulated DEGs between 1 – 2 FC (245 genes) than were > 5 FC (119 genes) and an equal number of between 1 – 2 FC (220 genes) and > 5 (220 genes) that were classified as downregulated. At 22-dpi in R1, there were more upregulated and downregulated genes within the 1 – 2 FC range (145 and 121, respectively) than in the > 5 FC range (123 and 104, respectively).

The 8242 gDEGs were organized into heatmaps with 10 expression clusters and two major expression trends were seen. Expression profiles were largely dependent on the alfalfa genotype gene expression trends established prior to infestation (0 h). Overall, gDEGs were either up-regulated (clusters 5-10) or down-regulated (clusters 1-4) throughout the infestation in R1 (Figure 2.4). Within these larger groups, there were temporal fluctuations, but gDEGs largely changed their magnitude of expression

opposed to their expression profile (up vs. down regulated). The 2404 tDEG heatmaps were grouped into nine different clusters that followed six patterns of regulation: (1) DEGs strongly downregulated during at least one time point in R1 (Clusters 1 – 4), (2) tDEGs downregulated from 7 – 22 dpi in R1 (Cluster 5), (3) tDEGs upregulated throughout infestation (Cluster 6), (4) tDEGs strongly induced at 7 dpi and beyond (Cluster 7), (5) tDEGs strongly upregulated in R1 at all times with most also being upregulated in S1 (Cluster 9), and (6) tDEGs strongly upregulated in R1 at all times with no particular expression pattern in S1 (Cluster 10) (Figure 2.6). The data from gDEGs and tDEG analyses, as well as the nymph developmental block at the first instar (Chapter 1), indicate that the genes contributing to whitefly resistance are likely be found at early timepoints in our datasets.

Definition and Identification of Interaction DEGs

While our data trends were compelling, it is possible that gDEGs and tDEGs might not identify all DEGs that were specifically responsive to different phases of whitefly infestation. As plant development was not accommodated in these analyses (Qiu et al. 2020). Therefore, we developed a series of more rigorous comparisons classified “interaction DEGs” (iDEGs) (Table 2.2) (Law et al. 2020). iDEGs were transcripts differentially expressed between genotypes and/or timepoints and their identification accounted for any differences in basal patterns of gene expression in R1 and S1 and/or differences in plant development at each timepoint. To this end, we designed 11 analysis models to identify iDEGs responsive to whitefly infestation (Table 2.2). These more stringent criteria significantly reduced the numbers of DEGs for most comparisons, which helped to highlight pathways and processes that distinguished R1

and S1 during whitefly infestation. For the 11 interaction models tested, a total of 12,949 cDNAs were classified as iDEGs in one or more models.

Eleven interaction models allowed the temporal differences between R1 and S1 responses to be resolved (Table 2.2; Figure 2.7). Genes that were differentially expressed in R1 vs S1 plants during with early phases of whitefly infestation were highlighted in Interaction models 1-4. Interaction 1 included 1-dpi activities spanning adult feeding and oviposition and Interaction 2 included responses at both 1 dpi and 7 dpi (the time of 1st instar feeding). Small numbers of iDEGs were identified in these comparisons. Interaction 3 identified DEGs associated with adults, eggs and 1st instar feeding (1 dpi-7 dpi, but basal expression in R1 and S1 was not accounted for); the largest number (8573) of iDEGs was revealed in this interaction. Finally, like Interaction 3, Interaction 4 identified DEGs associated with the transition to 1st instar feeding (1 dpi-7 dpi); however, in Interaction 4, the differences in basal expression in R1 and S1 were accounted for and this reduced the number of iDEGs to 1014. Interaction 5 revealed iDEGs that were expressed from 1 to 14 dpi (responses to adults, eggs, and 1st to 3rd instar feeding). Interaction 6 identified iDEGs associated with all of the periods of time when 1st to 4th instars were feeding (7 to 22 dpi).

We also identified iDEGs expressed only in later stages of whitefly feeding in three additional interaction models (Table 2.2; Figure 2.7). Interaction 7 and 8 identified iDEGs only associated with feeding by 2nd and 3rd instar nymphs (14 dpi) (4710 iDEGs) or 4th instar nymphs (22 dpi) (4556 iDEGs), respectively; while interaction 9 identified iDEGs expressed only at both 14 and 22 dpi. Finally, Interactions 10 and 11 were distinct. They identified temporal DEGs expressed only at 14 and 21 dpi in either R1 and S1 plants, respectively.

Because alfalfa's resistance to whiteflies blocks nymph development beyond the first instar, we emphasized on iDEGs identified early during the infestation (Interactions 1-4). iDEGs were examined for putative functions consistent with a role in resistance. In addition, as signaling pathways are modulated by the activity of both activators and repressors, iDEGs with either coordinate or reciprocal expression profiles during these early times were initially examined. These rigorous iDEG analyses enabled identified genes associated with phytohormone-regulated defense responses and the cuticle (see sections below). Collectively, they pointed to plausible mechanisms of alfalfa's whitefly resistance.

The challenges associated with analysis of a *de novo* transcriptome assembly of tetraploid alfalfa

The recent chromosome-assembled tetraploid alfalfa genome has 49,165 annotated genes (Shen et al. 2020). This paper analyzed 163 alfalfa populations from China and noted high genetic diversity within and between lines. As the heritage of the reference genome is distinct from the progenitors of the alfalfa lines R1 and S1 used in our study, we expect significant polymorphisms including gene family expansions and contractions. Currently our *de novo* transcriptome is likely over-estimating the number of DEGs, as it identified a total of 190,627 transcripts representing 124,435 gene sequences. This is in three-fold excess of the number of genes estimated from the reference genome (Shen et al. 2020). It should be noted that when genes that were expressed at very low levels were removed prior our DEG analyses, the number of genes was reduced to 45,718 genes, which is in better alignment with the estimated number of genes in the alfalfa genome. The larger number of transcripts that were detected is likely due to the mean contig size of ~ 760 bp and a contig N50 of 1275 (Supplemental Table 2.1), as well as the fact that many transcripts mapped to multiple

locations in the *de novo* transcriptome. Prior to publication, the quality of the *de novo* transcriptome should be improved. This means in the near future sequence redundancy must be minimized in the *de novo* transcriptome using a sequence clustering method, such as CD-HIT (Fu et al. 2012; Li and Godzik 2006).

The unanticipatedly large number of transcripts identified in my studies has impact on the interpretation of DEG roles in resistance or susceptibility. For example, for some DEG single gene transcripts were identified. For other genes, two or more transcripts were identified either as DEGs or not differentially regulated. Finally, as alfalfa is tetraploid and highly polymorphic, it would not be surprising to identify four different transcripts for a gene and these transcripts could be expressed in different manners as tetraploid genomes allow for genetic drift and neofunctionalization of genes (Cheng et al. 2018; Conant et al. 2014; Comai 2005; Flagel and Wendel 2009). We do not think that the future reanalysis of these data sets will change the discoveries about phytohormone pathways regulation in R1 versus S1 lines in any substantial manner; but we do think it will reduce the total number of DEGs. This might also allow us to make more accurate assessments of variation between gene paralogs that are DEGs.

For the DEGs we identified in the R1 and S1 lines with possible roles in resistance, we report all transcripts detected for transparency. Whenever possible, alfalfa genes were named based on their orthologs in *Arabidopsis thaliana*. As it is common practice to translate basic findings in the model plant Arabidopsis to crops (Studham and MacIntosh 2013; Ferrier et al. 2011; Chew and Halliday 2011; Zhang et al. 2004), in this Chapter, gene function is inferred based on the Arabidopsis ortholog. If multiple transcripts with identity to a single Arabidopsis locus were identified, each has

been given a letter designation. For example, two alfalfa transcripts to the *AtCOI1* genes were identified and designated as *MsCOI1-A* and *MsCOI1-B*.

Enriched gene ontology terms at 0 dpi point to cuticular-and cell wall-mediated defenses in whitefly response

gDEGs that are upregulated or down-regulated in R1 relative to S1 prior to infestation generally maintained that expression modality throughout the entire infestation time course. These data suggest that the defenses that are constitutively up-regulated in R1 may be associated with resistance and those that are constitutively down-regulated in R1 may be susceptibility factors. For this reason and the fact that first-instar nymph development was negatively impacted by alfalfa's whitefly resistance mechanism, we focused on early timepoints during whitefly infestation.

To this end, we determined if there were enriched gene ontology (GO) terms in R1 plants prior to infestation (0 dpi). We used the R package *goseq* to determine if up- and down-regulated genes were enriched for specific gene ontology (GO) terms (Young et al. 2010). Among the 1582 upregulated gDEGs in R1 at 0 dpi, we found sixteen enriched GO terms (0.05 FDR threshold) over-represented. In the "biological process" ontology, these terms included: anther wall tapetum development, long-chain fatty-acyl-CoA metabolic process, suberin biosynthetic process, and fatty-acyl-CoA metabolic process (Table 2.4; Figure 2.8; Supplemental Table 2.3). In addition, overrepresented GOs in the "molecular function" ontology included octadecanal decarbonylase activity, aldehyde decarboxylase activity, aldehyde oxygenase (deformylating) activity. In addition, three other molecular functions terms associated fatty metabolism (fatty acid-acyl-CoA reductase (alcohol-forming) activity, alcohol-forming fatty acid-acyl-CoA reductase, and long-chain fatty-acyl-CoA reductase activity) were identified. Among the

2028 downregulated gDEGs, we identified eight enriched GO terms. The six terms in biological process included GO terms associated with defense: defense response, defense response to fungus, protein autophosphorylation, and regulation of hydrogen peroxide metabolic process (Table 2.5; Figure 2.9; Supplemental Table 2.3).

There were 3610 gDEGs identified at 0 dpi. To determine if any 0-dpi upregulated gDEGs continued to be DEGs at other time points of the infestation, we compared the 0-dpi gDEGs, the 553 early-infestation response iDEGs (Interaction 3) and 511 late-infestation response iDEGs (Interaction 9). Among these gDEGs and iDEGs, we identified 174 DEGs upregulated in all three conditions (Figure 2.10; Supplemental Table 2.5). These upregulated DEGs could potentially mediate R1 alfalfa's resistance to whiteflies. Three GO terms were enriched: long-chain fatty-acyl-CoA metabolic process (FDR = 9.44E-06), suberin biosynthesis (FDR = 3.58E-05), and fatty-acyl-CoA metabolic process (FDR = 2.66E-04). All three GO term categories point to the cuticle and the cell wall's suberin having an important role in alfalfa's resistance to whiteflies.

Role of the cell wall and cuticle in whitefly resistance.

Enriched upregulated GO terms among the shared genes in Figure 2.8 and 2.10 suggested that changes in the cuticle and suberin were associated with alfalfa's whitefly resistance (Tables 2.4 and Supplemental Tables 2.3 and 2.4). The specific transcripts in the enriched GO classes included transcripts for *FATTY ACYL-COA REDUCTASE 1* (*FAR1*) and *FAR4* that are involved in the synthesis of C18-C22 and C18-C20 fatty acid alcohols used for cuticular wax esters and suberin biosynthesis (Vishwanath et al. 2013). In addition, *3-KETOACYL-COA SYNTHASE 2* (*KCS2*), which is part of the fatty acid elongation complex that synthesizes of very long-chain fatty acids (VLCFAs) ranging from C22 to C26 was identified (Trenkamp et al. 2004).

The cuticle forms the hydrophobic barrier that is closely associated with the epidermal cell wall. The cuticle has two layers: the cuticle proper that is primarily waxes and the cuticular layer that is sandwiched between the cuticle proper and the cell wall. The cuticular layer contains cutin, waxes and associated polysaccharides. Cutin and wax biosynthesis occurs in the endoplasmic reticulum (ER) and initiates with C16/C18 fatty acids derived from the chloroplasts. Within the ER, these fatty acids are converted into CoA esters that are used for cutin and very long-chain fatty acid biosynthesis (VLCFA) to produce the cuticular waxes and suberin. Along with cuticular waxes, cutin is a major component of the plant cuticle (Joubès and Domergue 2018). Cutin is a biopolyester interestified with hydroxy and epoxy-hydroxy fatty acids (C_{16} and C_{18}): these C_{16} and C_{18} fatty acids have a terminal hydroxyl and at least one mid chain oxygenation (Nawrath 2002; Fich et al. 2016b; Joubès and Domergue 2018). Cutin resides on top of the cell wall and is a thin, translucent, waterproof barrier that barricades water, solutes and gases (Nawrath 2002).

Suberin is a hydrophilic macromolecule in specialized cell walls that is synthesized in response to wounding, to protect against drought, and sealing for abscission (Graça 2015; Nawrath 2002). Suberin is a polymer formed from phenolic molecules, fatty acids or VLCFAs, and glycerol and are located at the interface of the cell wall and plasma membrane. Suberin levels can vary among different plant tissues and can also accumulate in apoplastic regions in non-cutinized boundary cell layers (Nawrath 2002; Fich et al. 2016b). *FAR1, FAR4 and KCS2* also have roles in suberin biosynthesis in leaves in response to wounding and *KCS2* is also involved in cuticular wax production, which improves responses to abiotic stresses such as drought (Lee et al. 2009; Domergue et al. 2010; Franke et al. 2009).The overrepresentation of DEGs

associated with VLCFAs and suberin among our GO terms pointed to cuticle fortification or suberin biosynthesis might be responsible for whitefly resistance in alfalfa.

Considering the importance of the cuticle and suberin for protection against biotic and abiotic stressors, we identified gDEGs and tDEGs associated with cutin or VLCFA biosynthesis. There are approximately 50 genes involved in cutin and suberin biosynthesis in *Arabidopsis* (Yeats and Rose 2013). Seventy transcripts corresponding to 27 different loci associated with cutin and suberin biosynthesis were identified. Cutin and wax biosynthesis is initiated when LONG CHAIN ACYL-COA SYNTHETASE 1 and 2 (*AtLACS1/2*) activate C_{16} and C_{18} fatty acids by conjugating CoA to these molecules. We identified a *MsLACS2* transcript, which was downregulated in the 22-dpi interaction; however was more upregulated in R1 than S1 at that time (Supplemental Table 2.5; Figure 2.11). We also identified a *MsLACS9* transcript, which was slightly upregulated throughout the infestation. In Arabidopsis, LACS9, along with the functionally redundant LACS4, is involved with channeling fatty acids and lipids from the ER to the plastid and is not directly linked to cutin or wax production in the ER (Jessen et al. 2015).
We identified transcripts for genes involved in the fatty acid elongase (FAE) complex, which is responsible for producing VLCFAs, as well as genes encoding proteins that are associated with the FAE core complex.

In Arabidopsis, four proteins comprise the core FAE complex: ECIFERUM 6 (CER6/CUT1), CER10/ECR**,** VERY-LONG-CHAIN (3R)-3-HYDROXYACYL-COA DEHYDRATASE PASTICCINO 2 (PAS2/HCD), and VERY-LONG-CHAIN 3-OXOACYL-COA REDUCTASE 1 (KCR1/GL8). Four *MsCUT1* and two *MsPAS2* transcripts were identified downregulated iDEGs and or gDEGs. In addition, a *MsKCR2* transcript was an upregulated gDEG; however, in Arabidopsis *AtKCR2* does not contribute to the

functional FAE core complex, while its homolog *AtKCR1* is active in the FAE complex (Nagano et al. 2019).

Furthermore, transcripts for several alfalfa genes that encode proteins that are associated with the FAE core complex were identified including *KSC2, KCS5, KCS11, and CER2* (Kim et al. 2021). In Arabidopsis, KCS2, KCS5, and KCS11 are associated with synthesis of C20, C26-C30, C16-C20 elongation, respectively (Franke et al. 2009; Trenkamp et al. 2004; Blacklock and Jaworski 2006). *MsKCS11, KCS5* and five of the six *KCS2* transcripts are gDEGs at 0 dpi. We found two *MsKCS2* (*MsKCS2-A and -B*) transcripts were upregulated as gDEGs and as iDEGs in several interactions (Supplemental Table 2.5.A; Figure 2.11). However, we also identified three MsKCS2 transcripts (*MsKCS2-C, -D,* and *-F*) as downregulated iDEGs and *MsKCS2-E* was identified as an upregulated and downregulated gDEG and iDEG. *MsKCS5* was identified as a downregulated iDEG at 22 di along with *MsKCS11-A* and *-B*. These data might indicate a trend for the elongation of these smaller LFCAs.

In the ER, FAR proteins utilize the long-chain fatty acids of VLCFAs to form the fatty acid alcohols needed for the synthesis of suberin or waxes; FARs have different substrate specificities based on the fatty acid chain length and saturation. FAR8, FAR4, and FAR1 using C16:0, C20:0, and fatty acids as substrates, respectively (Vishwanath et al. 2013; Domergue et al. 2010). The *MsFAR8*, *MsFAR4*, and ***s***ix *MsFAR1* and were upregulated gDEGs and/or iDEGs. The C18:0, C20:0, and C22:0 primary alcohols are used for suberin biosynthesis. In addition, two *MsFAR3/CER4* transcripts were upregulated gDEGs and/or iDEGs. FAR3/CER4 uses VLC acyl-CoA (C22:0 and C24:0/C26:0) to make VLC alcohols for wax biosynthesis. These data indicate a significant upregulation of genes associated with wax ester formation (Yeats and Rose 2013).

VLC-CoAs can also be converted to VLC aldehydes or VLC alkanes (Yeats and Rose 2013); *CER3* and CER1 control these consecutive biosynthetic steps, respectively.CER1 is a very long chain fatty acid decarbonylase essential for epicuticular wax biosynthesis (Mark et al. 1995; Bernard et al. 2012). Five *MsCER3* transcripts were downregulated throughout the time course. CER3 preferentially uses C30-CoAs as a substrate, but can also use C28, C32 and C34 coAs for aldehyde formation (Jenks et al. 1995; Chen et al. 2003). CER1 uses the CER3 generated aldehydes to form alkanes. Eleven *MsCER1* transcripts were upregulated gDEGs and iDEGs throughout the time course. In addition, two transcripts of for *MsCER1-like1* and one transcript of *MsCER1-like2* displayed similar trends (Supplemental Table 2.5.A; Figure 2.11). The differential regulation of CER3 and CER1 suggests that fewer aldehydes may be available for CER1, which could lead to a deficit of VLC alkanes, secondary alcohols, and ketones, which are important in plant waxes (Yeats and Rose 2013).

Transcripts for genes associated with cutin biosynthesis were also identified as DEGs (Fich et al. 2016a). A *MsHTH* transcript, six transcripts of *MsGPAT6*, and a transcript of *MsGPAT8* were upregulated gDEGs. *AtHTH* is involved in cutin monomer biosynthesis (Xu et al. 2017). AtGPAT6 and AtGPAT8, on the other hand, are acyltransferases which is as essential function of cutin biosynthesis (Yang et al. 2010). These data suggest that cutin biosynthesis maybe upregulated in R1 plants relative to S1.

We also identified transcripts of *MsABCG11* and *MsABCG32,* which encode transporters. In Arabidopsis *ABCG11* and *ABCG32* function as cutin transporters. In addition, *MsABCG11* and its paralog *MsABCG12,* and potentially apoplastic lipid transfer proteins transport waxes to the cuticle proper. While *MsABCG12* transcripts were detected in the transcriptome, none were DEGs. The *MsABCG11s* had three different

expression profiles. *MsABCG11-A* and *-C* were downregulated at early times, while *MsABCG11-B* and -D were upregulated gDEGs. In addition, *MsABCG32* was a downregulated iDEG, however was up at 0 dpi and fluctuates in expression. We also identified a transcript of the lipid transfer protein *MsLTPG1,* which was downregulated as an iDEG. In Arabidopsis, *LTPG1* gene controls cuticular lipid composition but mutants do not alter total wax and cutin monomers in the cuticle (DeBono et al. 2009; Kim et al. 2012). Collectively these data suggest that the wax and cutin composition of the cuticle maybe different in R1 plants than S1 plants.

Three transcription factors that influence cutin and wax biosynthesis genes were identified as DEGs: *ERF106, RAP2.4*, and *SNH3* (Kim et al. 2018a; Yang et al. 2020; Aharoni et al. 2004). In Arabidopsis, *ERF106* is a key negative transcriptional regulator of wax biosynthesis*. A*lso known as DEWAX2, AtERF106 downregulates several genes involved in cuticular wax biosynthesis (*CER1, ACLA2, LACS1, LACS2,* and *KCS12*) (Kim et al. 2018b). Two alfalfa *ERF106* transcripts (*MsERF106-A* and *-B*) were upregulated early and late iDEGs in **(**Supplemental Table 2.5.A; Figure 2.11). If similar in function to the *AtERF106*, the upregulation of *MsERF106* would point to downstream targets being downregulated. However, in our dataset *MsCER1* and *MsLACS2* transcripts were upregulated in our dataset suggesting that the role of MsERF106 in wax biosynthesis may be different in alfalfa. AtRAP2*.4* is a transcription factor that activates *KCS2* and *CER1* under drought conditions (Yang et al. 2020). *MsRAP2.4* was an early, upregulated iDEG; this is well correlated with the upregulation of several *MsKCS2 and MsCER1* transcripts in R1 plants. Finally, in Arabidopsis three SHINY genes (*SNH1-3)* are functionally redundant and induce of cuticular wax and cutin biosynthesis genes (Aharoni et al. 2004). One *MsSNH3* transcript was a DEG. While the *MsSNH3* transcript

was identified as a downregulated iDEG at 22 dpi, its transcript levels were elevated in R1 at 0 and 1 dpi; however, this difference did not meet the criteria as a gDEG. SNHs regulate genes involved in the early stages of VLCFA biosynthesis. The elevated (but not statistically significant) levels of *MsSNH3* transcripts early might point to increased production of waxes and cutins early with a repressed phase at later times after infestation.

Finally, while we have not fully explored genes associated with cell wall biosynthesis and modification; this will occur prior to publication. Two genes suggest that cell wall modification may be substantially different in R1 vs S1 plants. *MsRWA3-A* and *MsPMR5* transcripts are gDEGs that are 26 and 22 -fold higher in R1 than S1 plants. In Arabidopsis RWAs and PMR5 are involved in acetylation of xylan during secondary cell wall biosynthesis and pectin, respectively (Manabe et al. 2013). The elevated levels of these transcripts point to the fortification of the cell wall. We also identified several *POLYGALATURONAE INHIBITOR 1* transcripts (*MsPGIP1-A-G*) that were all upregulated as gDEGs and iDEGs throughout the infestation; PGIP1 have a positive role in cell wall integrity by inhibiting polygalacturonase activity in microbes and insects whi (Ferrari et al. 2006; De Lorenzo et al. 2001). The upregulation of PGIP1 points to another means of potentially inhibiting whitefly infestation.

These data point to increased VLCFA biosynthesis as well as cuticle and cell wall fortification in the R1 line. Collectively, these data strongly suggest that the physical barriers of the cuticle and cell wall may be modified in R1 plant prior to infestation and modulated after infestation. In addition, as elucidated in the next several section of the Dissertation, R1 plant have a profound reprogramming of JA-, SA-, ABA- and ET-

regulated defense signaling, as well as differences in transcripts associated with the PAMP/MAMP triggered immunity.

Jasmonic acid biosynthesis and signaling genes are downregulated in whitefly-resistant alfalfa.

Jasmonic acid (JA)-regulated responses are associated with defense against necrotrophic pathogens, as well as tissue-damaging and phloem-feeding herbivores, in numerous plant species (Yates-Stewart et al. 2020; Pré et al. 2008; Schuman et al. 2018). Precedent for the importance of JA-regulated defenses in antagonizing whitefly nymph development was provided by Zarate et al. (2007). Using JA- and SA-defense signaling mutants, Zarate et al. showed that *B. tabaci* MEAM1 induces SA-regulated and suppresses JA-regulated defense genes. They also showed that JA regulates the defenses that are critical for basal resistance to whiteflies and actively deter whitefly nymph development. For this reason, we looked for differences JA-biosynthesis and -signaling genes in R1 and S1 plants in our iDEG, gDEG and tDEG data sets. Surprisingly, several DEGs in our dataset point to repression of the JA-signaling pathway upon whitefly infestation of the R1 relative to S1 alfalfa plants.

One-hundred thirty DEGs with a role in JA biosynthesis, signaling, transcriptional regulation, or JA-mediated defenses were identified as gDEGs, tDEGs or iDEGs (Supplemental Table 2.5.B; Figure 2.12). Several genes with antagonistic or synergistic roles in JA signaling were upregulated or downregulated, respectively. While many of the genes involved with JA biosynthesis or JA modification were not identified as DEGs, a few were identified as DEGs. For example, R1 plants had lower levels of *MsLOX2-B, MsLOX6* and *MsACX1-A*.

Three genes involved in JA modifications were DEGs. *AtJAR1* conjugates Ile to JA to product the bioactive JA-Ile. Three of the four *MsJAR1* transcripts were downregulated suggesting lower levels of bioactive JA in R1 plants. In addition, all four *MsCYP94B1* transcripts, which encode the enzyme that converts JA-Ile into its inactive 12-hydroxy-JA-Ile form, were also downregulated at all times during whitefly infestation. In contrast, *MsJMT* that is critical for production of the volatile MeJA was upregulated.

In the resting state when JA levels are at low levels, MYC2-dependent JA-response genes are silent (Yang et al. 2019). These genes are suppressed by JAM2 proteins that competitively bind MYC2 binding sites. MYC2 is in a repressive complex with JAZ, NINJA, and TPL Activation of MYC2-dependent JA expression is dependent on recruitment of HDA6, a histone acetylase needed to open chromatin regions and for the tethering of the JA receptor COI via MED25 to the JAZ-NINJA-TPL complex. Upon binding its ligand JA-Ile, COI1 binds JAZ proteins and delivers them to SCF complex for ubiquitylation and JAZ protein turnover, thereby activating MYC2-dependent gene expression. Several of the regulatory components important for activation of JA-response genes (*MsMYC2A-C*, *MsMED25A-B, MsCOIA-B*, and *MsHDA*6) were DEGs (Supplemental Table 2.5.B; Figure 2.12). However, the reciprocal regulation of *MsMED25-A* and *-B* and *MsCOI1-A* and *-B* made their potential roles in resistance difficult to predict (Supplemental Table 2.5.B; Figure 2.12). For MYC2, *MsMYC2A* was identified as an iDEG, gDEG and tDEG and was down regulated > 22 fold in R1 at multiple times after whitefly infestation; while *MsMYC2B-C* were both up-regulated in R1.

In contrast, a more compelling picture was seen when the genes that negatively regulate JA-response genes were examined (Supplemental Table 2.5.B; Figure 2.11). All four *JAM* transcripts were up-regulated in R1. Eleven JAZ genes were identified as

DEGs including: *MsJAZ1-A-C, MsJAZ2, MsJAZ3, MsJAZ4, MsJAZ6,* and *MsJAZ12-A-D*. Seven of these transcripts were up-regulated, while *MsJAZ4, MsJAZ6, and MsJAZ12-C,and MsJAZ12-D* were down-regulated in R1. Of the three *MsNINJA* transcripts, two were up-regulated and one of the two *TPL* transcripts were upregulated. Collectively, these data suggest that R1 alfalfa has a repressed JA-signaling response.

This conclusion is further strengthened by the regulation of additional regulators of JA signaling. MPK4 is a negative regulator SA signaling and promotes JA/ET-dependent responses in a PAD4-dependent manner (Brodersen et al. 2006). Two *MsMPK4* transcripts are down-regulated in R1and two *MsPAD4* transcripts are upregulated, consistent with a lower level of JA signaling. More recently the LRR-RLK receptor *AtLIK1* was identified as a positive regulator of JA/ET-signaling and a negative regulator of chitin- and flg22-mediated PAMP-triggered immunity (Le et al. 2014). Twenty-one *MsLIK1* transcripts were identified and 18 are down-regulated in R1 relative to S1 plants in response to whitefly infestation. Collectively, these data also support the hypothesis that several components of the JA-signaling is down-regulated in the whitefly-resistant R1 plants

SA signaling is repressed in R1 alfalfa in response to whitefly feeding.

Given the reciprocity of JA and SA signaling in whitefly infestation in Arabidopsis (Zarate et al. 2007; Kempema et al. 2007; Zhang et al. 2013) and the apparent down-regulation of JA signaling in R1 plants, we examined the impact of whitefly feeding on SA biosynthesis and modification, signaling and response genes in R1 and S1 plants. We identified 149 SA-responsive transcripts in our dataset as either gDEGs, tDEGs or iDEGs (Supplemental Table 2.5.C; Figure 2.12).

AtCBP60g and AtSARD1 are central defense regulators serving as transcription factors that promote SA biosynthesis and also serve as major regulators of other SA-

responses (Sun et al. 2015; Tongjun et al. 2018; Wang et al. 2011). In addition, to their role in activating SA biosynthesis, AtCBP60g and AtSARD1 activate the genes essential for the synthesis of pipecolic acid (Pip) and the mobile SAR signal N-hydroxypipecolic acid (NHP) (Huang et al. 2020b). One *MsCBP60g* and three *MsSARD1-A-C* transcripts were identified. All but one (*MsSARD1-B*) of these transcripts were downregulated gDEGs and iDEGs in R1 versus S1 suggesting that SA, Pip and NHP synthesis and other SA-regulated defense responses may be impaired.

In plants, the ICS (isochorismate), PAL (phenylalanine ammonium lyase) and a minor, recently discovered PBS3/EPS1 pathway for SA biosynthesis are active (Peng et al. 2021b). In addition, eight genes are control in chemical modifications of SA (Peng et al. 2021b). To date, the pathway(s) used by alfalfa is unknown. We identified seven *PAL1* transcripts downregulated at 0 dpi, however they did not meet the statistical criteria as gDEGs. All nine *PAL1* transcripts were identified as downregulated iDEGs at 22 dpi in R1. The SA transporter (*EDS5*) was not identified as iDEGs or gDEGs. However, an ICS1-regulator gene *PHB3* was an upregulated gDEG and iDEG. PHB3 is one of several prohibitins that forms a complex with ISC1 to promote ISC1 accumulation (Seguel et al. 2018). In contrast, *MsICS2* and four *MsPAL1* genes were identified as tDEGs that were down regulated at all time points or at 7 dpi, respectively, in R1 relative to S1 plants (Supplemental Table 2.5.C; Figure 2.12). These data suggest that SA biosynthesis genes are not regulated differentially in R1 plants during whitefly infestation; this is distinct from the responses of Arabidopsis to whitefly feeding (Kempema et al. 2007).

SARD4, ALD1, and *FMO1* are responsible for three sequential steps in NHP biosynthesis (Huang et al. 2020a). Ms*SARD4* and Ms*ALD1*, which synthesize Pip, were

downregulated gDEGs and *MsFMO1*, which converts Pip to NHP, was an upregulated gDEG in the R1 genotype at 7 dpi. In addition, a Pip oxidase is a gDEG in R1, with lower levels in R1 initially and then increasing after whitefly infestation. Collectively, these data suggest that if protein levels reflect RNA levels, R1 plants may be deficient in the two local (SA and Pip) and the mobile SAR signal NHP, which are essential for activation of SA-responsive genes and induction of SAR. We are collaborating with Dr. Paul Fraser (Royal Holloway University London) to assess if there are changes in the levels of SA, Pip and NHP in R1 and S1 plants after *B. tabaci* infestation.

SA is perceived by NPR1, NPR3 and NPR4; none are DEGs in alfalfa. However, some of the TGA transcription factors that interact with NPR1 that are critically important in deploying SA-dependent defenses were gDEGs in alfalfa R1 (Gatz 2013; Peng et al. 2021b) (Supplemental Table 2.5.C; Figure 2.12). TGA1/TGA4 positively regulate SARD1 and CBP60g to activate SA-dependent defenses indirectly (Sun et al. 2015; Sun et al. 2018), TGA1 and TGA4 are downregulated iDEGs and gDEGs and consistent with their downregulated was the fact the *MsDLO1*, which is the major target of AtTGA1/4, was a downregulated early iDEG and gDEG. In addition, MsTGA6 was a down-regulated iDEG and gDEG. In Arabidopsis TGA2/5/6 are redundant and critical for SA-induced defenses and SAR (Zhang et al. 2003). Collectively, these data indicate that key regulators of SA-dependent defenses are down-regulated in alfalfa R1.

In Arabidopsis, indolic glucosinolates, camalexin and transport are critical components of defense (Lemarié et al. 2015; Stotz et al. 2011). While alfalfa does not produce glucosinolates nor camalexin, a number of genes suggest alfalfa may produce indolic compounds associated with defense and these compounds are down-regulated in R1 plants. For example, in Arabidopsis, AtPEN2 (a myrosinase), AtPEN3 (an SA-

induced transporter) and camalexin biosynthetic enzymes (CYP79B2, CYP79B3, PAD3) are SA-response genes. In alfalfa, *MsPEN2* and three of the four *MsPEN3* transcripts (*MSPEN3-A,-C, and -D*) were downregulated gDEGs; it is noteworthy that *MsPEN3-A* is downregulated over 25 fold (Supplemental Table 2.5.C; Figure 2.12). While alfalfa is not reported to produce glucosinolates, these data suggest that alfalfa may produce a glucose-conjugated indolic compound that is transported as cargo to the apoplast during alfalfa's SA-mediated defenses and these defenses are blocked in R1 plants.

Pattern-triggered immunity and Effector-triggered immunity are impaired in R1 plants

PAMP/MAMP-triggered immunity (PTI) is controlled by plasma membrane pattern-recognition receptors (PRRs), which recognize pest/pathogen-derived and modified plant host-molecules to activate defense (Zipfel 2009, 2008). PTI controls non-host responses and responses to non-adapted pathogens and pests. Effector-triggered immunity (ETI) is mediated by cytoplasmic nucleotide-binding leucine rich repeat (NLR) receptors, which recognize pathogen/pest effectors or changes in host-plant proteins that report the action of an effector. ETI controls host-plant resistance, which is often associated with localized cell death. The signaling components of ETI and PTI immune pathways are known and they activate a set of transcriptional and cellular defense responses (Chang et al. 2022; Martel et al. 2021; Bigeard et al. 2015; Zipfel 2009). Basal immunity describes the defense responses triggered by pathogens/pests that deploy effectors to impair PTI; this occurs in a majority of pest/pathogen interactions and is thought to reflect a diminished PTI response and weak ETI response (Dongus and Parker 2021a). As we outline below, our transcriptome evidence indicates that both PTI and ETI is impaired in R1 alfalfa plants.

We identified 61 alfalfa DEGs that were orthologs of Arabidopsis genes linked to PTI including four PRRs *(FLS2, EFR, LYK5, LYM1)* and co-regulators (*BAK1, BIR1, SOBIR1, IOS1, LYK4, CHIB1*) (Supplemental Table 2.5.D; Figure 2.13). In Arabidopsis, EFR and FLS2 perceive bacterial peptide motifs derived from elongation factor-Tu (elf18) and flagellin (flg22) (Zipfel et al. 2006; Chinchilla et al. 2006). The *MsEFR* and three *MsFLS2* transcripts were downregulated iDEGs and gDEGs in R1 relative to S1 at all timepoints prior to and after whitefly infestation. AtFLS2 and AtEFR use three co-receptors (BAK1, BKK1 and BIK1) (Li et al. 2019; Roux et al. 2011; Yuan et al. 2021; Wang et al. 2014; Liu et al. 2013; Lu et al. 2010). Three *MsBAK1* transcripts were detected but only *MsBAK-A* was downregulated at all times in R1. In contrast, transcripts encoding the LysM-domaining containing PRR receptors *MsLYM1* and four of five *MsLYS5* were up-regulated gDEGs. AtLYM1 and AtLYK5 are PRRs and are high-affinity receptors that bind peptidoglycans and chitin, respectively (Willmann et al. 2011; Cao et al. 2014). AtLYK5 uses the co-receptors CERK1, IOS1 and FERONIA (FER) for chitin perception (Cao et al. 2014). *MsCERK1, MsIOS1* and 16 of the 25 *MsFER* transcripts were down regulated iDEGs and gDEGs. Finally, AtLYK4 is a low affinity chitin-binding protein that forms complexes with LYK5 (Cao et al. 2014) (Supplemental Table 2.5.D; Figure 2.13). Two *MsLYK4* transcripts were downregulated DEGs in R1 plants. Collectively these data paint a complex portrait of PTI signaling. In R1 alfalfa, signaling by FLS2, ERF1 and the low-affinity chitin receptor LYK4 is likely to be impaired. The ability of the LYK5 PRR to perceive chitin and transduce signaling is harder to discern. as *MsLYK5* is upregulated but many of its co-receptors are down-regulated. As insect cuticles contain chitin and chitin polymers are shed during molts and line the canals of the stylets (Walker and Perring 1994; Rosell et al. 1995; Pollard 1955; Jiang and Walker

2003), the reciprocal regulation of *MsLYK5* and its co-receptor in R1 plants may reflect an autoregulatory loop to prevent hyperactivation of chitin-triggered PTI. The upregulation of *MsLYS3*, the peptidoglycan receptor, was well correlated with the several of the transcripts encoding the acidic endochitinase (*MsCHIA*). This endochitinase releases small peptidoglycans from longer polymers; the smaller peptidoglycans are the ligands for LYS3 (Liu et al. 2014). These data suggest that R1 plants maybe be primed for perception of pathogen and pest cell wall/cuticle components.

The impairment of PTI signaling is also reflected at the level of the downstream MAP kinase signaling cascades that are triggered after PRRs perceive their ligands. MEKK1 is phosphorylated by BIK1 to activate two down-stream signaling cascades. In Arabidopsis, the MEKK1-MKK4/5-MPK3/6 cascade is essential for inducing immune response genes, glucosinolate and camalexin biosynthesis and regulating ethylene biosynthesis (Wang et al. 2018; Han et al. 2010). *MsMEKK1A-B* and *MsMPK3* are upregulated DEGs. The MEKK1-MKK1/2-MPK4 negatively regulates SA biosynthesis and *PR* gene expression and enhances the expression of the ET/JA-defense response pathway in Arabidopsis (Gao et al. 2008). *MsMKK2-B* and *-C* are upregulated gDEGs across the whitefly infestation period and *MsMKK2-A* transcript levels are lower in R1 than S1 at 0 dpi but its RNA levels increase throughout the infestation, respectively (Supplemental Table 2.5.D; Figure 2.13). In addition, two *MsMPK4* transcripts are downregulated at all timepoints in R1 after whitefly infestation. As MAP kinase cascades are primarily controlled at the posttranscriptional level, the significance of the changes *MEKK1, MKK2, MPK3,* and *MPK4* gene expression in alfalfa is hard to predict. Downstream of MPK4 is MKS1, which regulates WRKY33 activity and the induction of

the indolic camalexin in Arabidopsis. While *MsMKS1* was not a DEG, *MsWRKY33-A-C* were down and upregulated DEGs in R1. Again, painting a complex picture of the impact of the MPK4-signaling pathway.

Associated with both innate immunity and ETI in Arabidopsis are the three related nucleocytoplasmic lipase-like proteins EDS1, SAG101 and PAD4 (Dongus and Parker 2021a). EDS1 and SAG101 interact to heterodimer and EDS1- PAD4 heterodimer to transduce SA-mediated defense responses. All five *MsEDS1* transcripts were down-regulated iDEGs and gDEGs (all timepoints). Nine of the 16 *MsSAG101* transcripts were also downregulated, while *MsPAD4* transcripts were upregulated. Since EDS1 and SAG101 are critical for ETI's transcriptional reprogramming and cell death with TIR NLRs (Lapin et al. 2019b), the *MsEDS1* and *MsSAG101* data suggest that ETI may be significantly impaired in R1 alfalfa.

ACTIVATED DISEASE RESISTANCE 1 (ARD1)-type and N REQUIREMENT GENE 1 (NRG1)-type are NLR helper proteins, which activate EDS1-mediated ETI triggered by TIR-domain NLR receptors (Lapin et al. 2019a). The ADR1 works with the EDS1-PAD4 complex and has a smaller role in ETI-induced cell death (Pruitt et al. 2021; Chini et al. 2004). *MsADR1* transcripts were not DEGs. EDS1-SAG101-NRG1 activate ETI and provokes host cell death. Two classes of NRG1 transcripts were detected in alfalfa *MsNRG1.1A-K* and *MsNRG1.2.* The majority of these transcripts were upregulated in R1 plants (either early or late) after infestation (Supplemental Table 2.5.D; Figure 2.13).

EDS-SAG101-mediated cell death is finely controlled to prevent serendipitous activation of this cell death pathway by SNC1, SRFR1, TCP8/14/15 and MOS1 (Dongus and Parker 2021b; Lapin et al. 2019b). Even small increases in the SNC1 causes

autoactivation of immunity and cell death (Gou and Hua 2012); reciprocally, loss-of-function *snc1* mutants prevents EDS1-dependent cell death. SNC1 is a TIR-NLR that activates the cell death pathway when over-expressed. SNC1 transcription is positively regulated by MOS1and TCP8/14/15; although none of these transcription factors were identified as DEGs after whitefly infestation. SNC1 is also negatively regulated by SRFR1 and TPR1/2/3. After whitefly infestation, two *MsSNC1* transcripts were downregulated gDEGs and iDEGs. The *MsTPR3* transcript was a downregulated gDEG in R1 at all times after infestation and one *MsTPR3* transcript was an upregulated gDEG. The downregulation of *MsSNC1* is unambiguous; therefore, it is not clear if the changes in the three *MsTPR3* transcripts are important in SNC1 regulation given their disparate regulation in R1 plants.

Finally, SNC1 triggers cell death by activating the DEFENSE NO DEATH1 (CNGC2, cyclic nucleotide-gated ion channel 2). *MsCNGC21* is a down-regulated gDEG; this is well correlated with the down-regulation of SNC1 (Supplemental Table 2.5.D; Figure 2.13) . As PTI and ETI pathways converge (Chang et al. 2022) and is also noteworthy that AtCNGC20 activity is carefully modulated by AtBAK1 as high levels of CNGC20 induce cell death (Yu et al. 2019a). Three *MsCNCG20* transcripts were all down-regulated gDEGs (Supplemental Table 2.5.D; Figure 2.13).

CC-NLRs provide host plant resistance to many pathogens and most hemipteran pests R genes are CC-NLRs (Kapos et al. 2019; Borrelli et al. 2018). Insights into the regulation of CC-NLRs in R1 and S1 were limited. Recently, the structure of the first CC-NLR resistosome was elucidated using the NLR ZAR1, which recognizes many pathogen effectors, its associated ZAR-associated pseudokinases (ZRKs), the pathogen effector, and PBL proteins that form a pore that promotes calcium influx and ROS

production (Ullrich 2021; Burdett et al. 2019; Wang et al. 2019). MsZAR1 was identified as a tDEG and the ZRK known as MsRKS1 was identified as a down-regulated gDEG at all times after whitefly infestation of R1 plants.

ABA Biosynthesis is repressed in resistant alfalfa in response to whitefly.

The trends for downregulation of genes associated with SA- and JA- signaling, as well as PTI and ETI components, in the whitefly resistant R1 line were unanticipated, as previous studies in the *B. tabaci*-Arabidopsis interactions demonstrated whiteflies induced SA-regulated defenses and suppressed the JA-regulated defenses that actively antagonized whitefly nymph development (Kempema et al. 2007; Zarate et al. 2007). As abscisic acid (ABA) regulates defense signaling, often in an antagonistic manner to JA- and SA-regulated responses (Checker et al. 2018; Yasuda et al. 2008), and has a critical role is adaptation to abiotic stresses such as drought, cold and salinity (Bharath et al. 2021; Munemasa et al. 2015), we interrogated the regulation of the ABA pathway. It is also noteworthy that ABA plays a significant role in *Brassica oleraceae's* host-plant resistance to the whitefly *Aleyrodes proletella* (the cabbage whitefly) (Broekgaarden et al. 2018; Cao et al. 2011). ABA has also been implicated to have a role in cassava's resistance to whiteflies (Garceau 2021).

To investigate if ABA-regulated processes were associated with alfalfa's resistance mechanism, 123 DEGs with a role in ABA biosynthesis, signaling or responses were identified from the literature. ABA is a 15-C compound that is derived from the chloroplast-synthesized isoprenoids, therefore the genes associated with the chloroplast MEP (2-C-methyl-D-erythritol 4-phosphate) pathway, carotenoid pathway and enzymes committed to ABA biosynthesis were examined and iDEGs, gDEGs and tDEGs identified (Supplemental Table 2.5.E; Figure 2.14). The methylerythritol 4-

phosphate (*MEP*) *pathway* (MEP) pathway is highly regulated at several levels (Banerjee and Sharkey 2014; Rodríguez-Concepción and Boronat 2015). In addition to providing the isoprenoid precursors for many downstream pathways, its intermediate MEcPP (methyl erythritol cyclopyrophosphate) is a key regulator of SA and JA responses (Lemos et al. 2016; Xiao et al. 2012). Genes encoding enzymes for six steps in the MEP pathway were DEGs (Supplemental Table 2.5.E; Figure 2.14). DXS controls flux into the MEP pathway, as upregulation of *DXS* increases ABA levels, as well as gibberellins, carotenoids, or tocopherols (Estévez et al. 2001). Two *DXS* orthologs (*MsDXS-A* and *-B*) were upregulated gDEGs. Increases in DXR can also increase isoprenoid production (Carretero-Paulet et al. 2002). In contrast to DXS, the two *MsDXR* transcripts were down regulated DEGs at all timepoints after whitefly infestation of R1. Finally, HDS, which is the penultimate step in the MEP pathway and catabolizes the defense signal MEcPP, was strongly up-regulated in R1 plants when whitefly adults are feeding and egg deposited (1 dpi). These data point to possible increased production of isoprenoids in R1; however not all transcripts are consistent with this pattern and there are other levels of control that are active in modulating this pathway.

Based on changes in alfalfa transcripts associated with carotenoid and ABA biosynthesis, these pathways were suppressed in whitefly-infested R1 plants (Supplemental Table 2.5.E; Figure 2.14). Transcripts for the alfalfa *PDS, ZDS1-A-B, CRTISO, LUT1,* CYP97B3, *ABA1-A-E, AAO1, AAO3A-B, ABA2, and ABA3-A-B* were all downregulated iDEGs and/or gDEGs at all times after whitefly infestation (Supplemental Table 2.5.E; Figure 2.14). In contrast, the *MsAAO2* and *MsAAO3-C* transcripts were only down-regulated at 0 h. Finally, the only gene up-regulated transcript was *MsCYP707A3*, which encodes for the major ABA catabolic enzyme - ABA 8'-hydroxylase (Okamoto et

al. 2011; Nambara and Marion-Poll 2005). Hydroxylated ABAs are further processed or are directly imported into the vacuole for storage and ready deployment under stress (Bharath et al. 2021; Kuromori et al. 2018). Collectively, these data indicate that ABA biosynthesis is likely impaired in the whitefly-resistant R1 plants prior to and after whitefly infestation (Supplemental Table 2.5.E; Figure 2.14).

The core module used for ABA perception and initiation of ABA signaling is composed of ABA receptors (PYR and PYLs), protein phosphatases (PP2C, *ABI1*), and a set of protein kinases (SnRK2/3/6/7/8 and CDPK) (Chen et al. 2020). DEGs associated with these functions were examined, no compelling conclusion about the modulation of ABA perception in R1 plants could be made based on transcript changes. This is because only a small number of ABA receptors were identified as DEGs and the variable regulation of the receptor DEGs (Supplemental Table 2.5.E; Figure 2.14). For example, while *MsPYL4-A/B* transcripts increased over time, *MsPYL8* transcripts declined across the whitefly infestation time-course. Similarly, *MsABI1, MsPP2CA, MsSnRK2E,* and *MsSnRK2C* were DEGs. Multiple transcripts for these genes were identified and were often reciprocally regulated. For example, *MsPP2C-A* was upregulated and *MsPP2C-B* was downregulated at all timepoints after infestation.

Subsequent steps in ABA signaling are regulated at the transcriptional level and by a complex series of post-translational events including phosphorylation/ dephosphorylation and ubiquitylation for degradation by the proteasome; this regulation includes activators and suppressors of signaling (Chen et al. 2020; Yu et al. 2019b). Of the activators, eight transcription factors were identified as DEGs and had variable expression patterns. We detected seven *OSMOTIN34* transcripts. *MsOSM34-A, -B* and *-C* were highly upregulated at 0 h and all other times after infestation; while other

MsOSM34 transcripts had less pronounced regulatory patterns. Of the genes associated with negative regulation of ABA that were detected as DEGs, two transcription factors (*MsATAF1, MsICE1*), two *MsCPL-A,B* (a kinase) and half of the *MsERD15* transcripts (a negative regulator with unknown mechanism) were upregulated; consistent with the hypothesis that ABA biosynthesis is suppressed in R1 plants. In contrast, *FERONIA(FER)* is a negative regulator of ABA signaling in Arabidopsis (Yu et al. 2012). *MsFER* transcripts were predominantly downregulated DEGs. It is noteworthy that *FERONIA* is also important in PTI and its downregulation appears to be linked to a defective SA and PTI response (see sections above).

From these data, we can conclude ABA biosynthesis and signaling are downregulated in R1 with several signaling and responsive genes showing confounding results.

Ethylene signaling is induced during alfalfa's resistance response to whiteflies.

As SA, JA, and ABA biosynthesis and/or signaling did not correlate with the whitefly resistance in R1 alfalfa, we investigated that last of the four major defense phytohormones - ethylene (ET). ET has a known role in basal immunity to herbivores and it was possible that ET biosynthesis, signaling or responses would have a role in whitefly resistance in alfalfa (Anstead et al. 2010; Broekgaarden et al. 2015; Louis et al. 2015a; Lu et al. 2014; Qi et al. 2020). Five genes involved in ET biosynthesis were identified including SAM synthase (*MsSAM1*-A,B), Ms*ACS1/6/8* and *MsACO3*; their transcript levels are not well correlated with whitefly resistance (Supplemental Table 2.5.F; Figure 2.15). However, the major ethylene biosynthetic gene ACS5 was not detected as a DEG. Two negative regulators of ET biosynthesis were detected as DEGs: *TARGET OF RAPAMYCIN* (*MsTORA-C*) and *MsETO1*. *MsETO1* was a downregulated

iDEG and gDEG. In Arabidopsis ETO1 binds ACS5 to inhibit its activity and target it for turnover by the 26S proteasome (Yoshida et al. 2005). As ACS5 is regulated post-translationally by ETO1, this suggests that this major rate limiting enzyme may increase the synthesis of ACC, the immediate precursor of ET. Supporting this premise was the fact that two of three *MsTOR* transcripts (MsTOR-*A and -C)* were strongly downregulated at all times pre and post whitefly infestation. TOR is a protein kinase with roles in growth and development. TOR suppression in *Arabidopsis* is also linked to induced phytohormone signaling (Fingar and Blenis 2004; Dong et al. 2015).

ET signaling is complex with many levels of transcriptional, posttranscriptional and posttranslational regulation (Broekgaarden et al. 2015). ET is perceived by five ET receptors (ETR1, ERS1, EIN4, ETR2, and ERS2). We detected three *MsETR1*, two *MsERS1* and one *MsEIN4* transcripts as gDEGs or iDEGs (Supplemental Table 2.5.F; Figure 2.15). There was no consistent trend in their regulation and a correlation with whitefly resistance was not possible. In the resting state, the kinase CTR1 phosphorylates EIN2 and EIN2 activity is suppressed and ETR1/2 promote EIN2 turnover to keep ET signaling at low levels (Sakai et al. 1998; Bisson and Groth 2010). *MsETR1/2* were not DEGs, but two *MsCTR1* transcripts were detected; one was upregulated and one downregulated. However, the chromatin associated protein EEN promotes EIN2 transcription (Zander et al. 2019) and *MsEEN* transcripts were upregulated gDEGs; however, *MsEIN2* was not detected as a DEG. With ET binding to its receptors, the plasma membrane bound EIN2 is cleaved and EIN2's C-terminal end (EIN2 C-end) is translocated to the nucleus to activate the transcription factors EIN3 and EIN3-like to inducde ET-regulated defenses. Two *MsEIN3-B* transcripts were detected; *MsEIN3B* is an upregulated gDEG and iDEG and *MsEIN3-A* transcript levels were also

elevated in R1 vs S1 plants and its levels increased over the whitefly infestation timecourse, although it was not designated as an upregulated DEG. This suggests that ET signaling may be enhanced in R1 plants.

In Arabidopsis, EIN3 is negatively regulated by EBF1 and EBF2, which are F box proteins that stimulate EIN3 turnover (Binder et al. 2007). Four *MsEBF1* transcripts were detected and three were downregulated across the entire whitefly infestation timecourse. These data suggest a rise in EIN3 proteins and therefore ET signaling would be expected in R1 plants. Furthermore, *AtEBF1* transcripts are negatively regulated by EIN2 C-end. EIN2 C-end binds *EBF1* mRNAs and delivers it to processing (P) bodies for turnover by the 5'-exoribonuclease EIN5/XRN4 and, by inference LARP1, which delivers EIN5 to its target transcripts (Olmedo et al. 2006; Merret et al. 2013). The levels of EBF1 are consistent with the dissipation of CTR1's negative role in modulated "free" EIN2 C-end. In contrast, the transcript levels of the four MsEIN5 transcripts, which were downregulated DEGs, and the LARP1 transcript, which was an upregulated DEG, were not consistent with the observed regulation of EBF1 transcripts in R1 alfalfa after whitefly infestation (Supplemental Table 2.5.F; Figure 2.15).

In addition, EER4, also known as TAF9, is an upregulated DEG. EER4 binds to EIN3 and positively regulates the activities of the EIN3 and EIN3-like transcription factors to activate ET signaling (Robles et al. 2007) (Supplemental Table 2.5.F; Figure 2.15). Consistent with the activation of ET signaling by EIN3 is the increased transcript levels for many ET-dependent genes. The most striking examples are the ET-dependent ERF transcription factors that were up-regulated DEGs. ERFs have been linked to transcription activation and repression of ET signaling (Binder 2020; Thirugnanasambantham et al. 2015).

We also identified large number proteins in the AP2/ERF (Ethylene responsive factor) transcription factor family as DEGs; however, only a subset of these genes were likely to be ET-responsive based on their Arabidopsis orthologs (Nakano et al. 2006; Raghavan et al. 2006; Huang et al. 2015). Several were upregulated DEGs including *ERF4/5/105/106* and *RAP2.3/2.4/2.6* (Supplemental Table 2.5.F; Figure 2.15). *ERF4* is a negative regulator of chitin signaling, while *ERF5* is known as a negative regulator of ET signaling (Yang et al. 2005; Babula et al. 2006). We identified an upregulated ortholog of *ERF4* (*MsERF4*) and two orthologs of *ERF5*, one upregulated as a gDEG and one downregulated as a gDEG and iDEG. *ERF105/106* are also positive regulators of ET signaling and were both identified as upregulated iDEGs early in infestation. We also identified several upregulated orthologs of *PR-3* (AT3G12500), a known basic endochitinase B responsive to JA and ET; these *MsPR-3* transcripts are upregulated gDEGs at all times after infestation or induced early upon whitefly infestation. Collectively, the repression of negative ET regulators and the induction of *EIN2*, *EIN3, CHI-B, PR-3s* and several ERFs suggest that ET-signals and downstream responses are induced and are well correlated with R1's whitefly resistance.

Discussion

The use of genomic and transcriptomic tools to identify the roles of specific plant defense pathways against pests and pathogens has been helpful across the plant kingdom. The advent of de-novo assemblers has enabled the analysis of non-model plant species, many of which hold significant agricultural value (Robertson et al. 2010; Ward et al. 2012). The use of transcriptomics to compare time courses of resistant and susceptible plants infested with Hemipteran insects, in particular, has been used recently

to better comprehend resistance mechanisms against these elusive plant pests. The number of transcriptomic analyses of resistant/susceptible plants in response to hemipteran pests has increased recently including numerous responses to aphids ((Studham and MacIntosh 2013; Chapman et al. 2018; Louis et al. 2015a; Tu et al. 2018a; An et al. 2019; Pingault et al. 2021) and planthoppers (Zhang et al. 2019a; Tan et al. 2020; Satturu et al. 2021) . The elucidation of whitefly resistance in *Brassica*, cotton, and cassava through transcriptomic and metabolomic analyses has provided some basis of understanding of whitefly-resistance mechanisms in model and non-model plant species (Broekgaarden et al. 2018; Li et al. 2016; Garceau 2021; Irigoyen et al. 2020; Perez-Fons et al. 2019).

Our goal was to investigate the potent whitefly resistance mechanism in alfalfa. To achieve this goal, we used *de-novo* transcriptome assembly to analyze differential gene expression during whitefly infestation of whitefly-resistant and -susceptible alfalfa. In Chapter 1, we identified a highly resistant (R1) and a highly susceptible (S1) alfalfa lines from the whitefly-resistant population (UC2845) (Teuber et al. 1997). We showed that R1 is an undesirable host for MEAM1 adults and their nymphs are severely delayed in development. Such a distinct phenotype between two closely related lines made them prime candidates for comparative transcriptomic analyses. Using gDEGs and iDEGs, the rigorous comparisons that compensated for developmental time, identified defense pathways and physical barrier modifications that were likely significant contributors to alfalfa's whitefly resistance mechanism.

Principal component analysis (PCA) and heatmaps that displayed transcript profiles across the 21-d infestation time course showed that resistance was driven primarily driven by genotype, not by temporal differences in gene expression. There is

precedence for a constitutively active resistance to phloem-feeding Hemipteran pests in the literature (Chiozza et al. 2010; Studham and MacIntosh 2013). For example, Chiozza et al. (2010) showed that amino acids levels were higher in soybeans resistant to the soybean aphid than their susceptible counterparts prior to infestation. Studham and MacIntosh (2013) extended these studies at the transcriptome levels and showed that many defense-related genes were expressed at higher levels in the resistant line prior to infestation. Other aphid-elicited defense responses in plants have been investigated including an antibiotic broad-based resistance against three species of aphids linked to an induction of JA, ABA, and ET-responsive (Leybourne et al. 2019; Chapman et al. 2018), a response dependent on the C-terminus of PAD4 (Dongus et al. 2020), a response induced by aphid saliva that deploys both SA and JA to confer antixenosis and antibiosis (reduced feeding) against future aphid infestations (Zhang et al. 2017). Constitutive resistance has been seen in aphid-monocot interactions in wheat, and barley (Delp et al. 2009; Han et al. 2009; Chiozza et al. 2010). While the constitutive resistance to MEAM1 whiteflies isn't unique amongst resistance mechanisms deployed against Hemipterans, other aspects distinguish alfalfa's whitefly resistance as an unorthodox approach to hemipteran control.

It is important to place alfalfa's resistance relative to what is known about whitefly basal immunity and Brassica's whitefly resistance. Zarate et al. (2007) discovered JA-mediated responses were essential for *Arabidopsis* to inhibit *Bemisia tabaci* MEAM1 nymph development and Broekgaarden et al. (2018) showed that host-plant resistance to the whitefly *A. proletella* was correlated with ABA levels and gene expression. In addition, both elevated JA and ABA levels were linked to soybean aphid tolerance in soybean and aphid resistance in *Medicago truncatula* (Kamphuis et al. 2016; Tu et al.

2018b). In contrast, SA has been associated with the resistance to aphids in tomato and planthoppers in rice (Coppola et al. 2013; Guo et al. 2018; Du et al. 2009). With these precedents, the SA, JA and/or ABA appeared to be prime candidates for controlling alfalfa's whitefly resistance. However, our transcriptome of R1 and S1 plants refutes these ideas – neither SA, JA or ABA appear to be key regulators of R1's MEAM1 resistance.

Examination of the expression trends JA biosynthesis and signaling genes, showed a compelling trend of JA-response down regulation. The biosynthesis genes included *ACX1* and *LOX6,* which is involved in the long-distance accumulation of JA. In addition, upregulation of repressors of JA-responsive gene expression (*JAZs, NINJA, JAM1/2*) also pointed to a suppression of JA responses in the whitefly-resistant R1 plants (Figure 9). Similar to JA, suppression of SA-signaling was suggested by our transcriptome analyses. Central regulators of SA biosynthesis, SAR and SA signaling (ie., *EDS1, SAG101, CBP60g*) as well as downstream transcription factor genes such as *TGA*s were down-regulated gDEGs suggesting the SA-modulated defenses were also downregulated in R1 plants (Figure 10). Finally, while ABA has a positive role in whitefly resistance in *Brassica* (Broekgaarden et al. 2018), R1 plants display a marked down-regulation of ABA biosynthesis gene transcripts and upregulation of central negative regulators of ABA signaling (*PP2CA* and *ABI1*) (Figure 11).

The downregulation of three defense-signaling pathways in pathogen/pest resistance is unprecedented. Analysis of the ET signaling pathway provided the first evidence of a defense pathway positively correlated with whitefly resistance. While the transcriptome did not implicate changes in ET biosynthesis in whitefly resistance, multiple ET signaling components and ET-responsive transcription factors were

upregulated (EIN3, EEN, RAP2.3, and ERF5) and multiple negative regulators of the ET-signaling pathway (ie, CTR1, EBF1 and TOR) (Figure 12).

Ethylene as a resistance mechanism against hemipteran pests is not as common as SA-, JA- or ABA-mediated responses. However, there are some instances of this phytohormone being associated with mechanisms that deter herbivory. For example, the maize *Mir1* gene encodes an endoprotease that confers resistance to the lepidopteran pest corn leaf aphid (*Rhopalosiphum maidis*) , as well as antibiosis and antixenosis towards corn leaf aphid (Pingault et al. 2021; Louis et al. 2015b). Mir1 acts via an ethylene-dependent and JA-independent mechanism (Pingault et al. 2021; Louis et al. 2015a). In addition, ET biosynthesis genes were preferentially induced in two aphid-host plant resistance responses. *Macrosiphum euphorbiae* and *Aphis gossypii* infestation of aphid resistant Mi-1.2 tomatoes and Vat melons, respectively, induced ET biosynthesis genes (Anstead et al. 2010). In the A. gossypii-*Vat* melon interaction increases in the ET receptor (*ETR2*), *EIN3* and *ETR1* transcripts were higher than in the interactions with susceptible plants. Another ET-mediated resistance to a hemipteran pest can be found in cucumber's basal response to aphid feeding which is induced by ET and ROS responses (Qi et al. 2020).

Several basal responses to chewing insects have been linked to positive regulation of ET in rice (Lu et al. 2014), chickpea (Pandey et al. 2017), and *Medicago truncatula* (Paudel and Bede 2015). A response conferred against aphids in wheat also utilized ET, among other phytohormones (Zhang et al. 2019b). Resistance genes with roles in ET-mediated responses have also been identified in rice in response to BPH (Ye et al. 2020) and in *Arabidopsis* in response to green peach aphid (Lü et al. 2013). *Spodoptera* resistance in *M. truncatula* was also linked to ET signaling which is also

involved in SA-JA crosstalk (Paudel and Bede 2015). That being said, we also identified some instances where ET is a negative regulator of herbivore resistance. Tian et al. (2014) identified JA is a positive regulator of tomato's resistance to *H. zea* while ET was identified as a negative regulator.

Analysis of phytohormone-associated DEGs provided several lines of data that implicate ET is a major player in whitefly resistance. One additional finding supports the premise of phytohormone reprogramming. While not discussed in Chapter 2, the master growth regulator *TOR* (*TARGET OF RAPAMYCIN*) is also modulated in R1 plants; one *TOR* transcript is strongly downregulated (20 to 50-fold) in R1 plants relative to S1 plants. TOR is a kinase that balances growth/development with stress signaling (McCready et al. 2020; Dong et al. 2015; Xiong and Sheen 2014). TOR is a known negative regulator of the ET-signaling pathway. EIN2 is a direct substrate of TOR and TOR phosphorylates EIN2, which renders EIN2 unable to stabilize EIN3 and EIL1, thereby inhibit ET responses (Fu et al. 2021; Zhuo et al. 2020). In addition, depleted levels of glucose or suppression of *TOR* releases EIN2 from TOR regulation and EIN2-C end can move to the nucleus where it stabilizes EIN3 and EIL1. This stabilization allows a host plant's ET-mediated responses to be deployed (Fu et al. 2021). Additionally, inhibition of *TOR* in *Arabidopsis* has been found to induce senescence- and ethylene-related DEGs (Fu et al. 2021; Zhuo et al. 2020; Fu et al. 2020). TOR also inhibits ET biosynthetic enzymes ACS2/6, and suppression of *TOR* induces *ACS2/6* accumulation (Zhuo et al. 2020). The patterns of induction and repression of ET pathway DEGs coupled with evidence in *Arabidopsis* made us conclude that ET is likely the phytohormone responsible for whitefly resistance in alfalfa. It should also be noted that TOR also impacts ABA, JA and SA signaling in its efforts to coordinate growth (ie.,

photosynthesis, carbon fixation, and chlorophyll fixation) with responses to abiotic/biotic stress responses (Dong et al. 2015). As ABA, JA and SA biosynthesis/signaling, as well as PTI, appear to be down-regulated in the whitefly-resistant R1, TOR's role in mediating ET signaling vs the other phytohormones remains speculative at this time.

Consistent with the impairment of SA and JA signaling, PTI may also be impaired in R1 plants. The transcripts for many PRR receptors/co-receptors (ie., FLS2, EFR, BIR1, CERK1, LYK4, IOS1, and FER) that perceive elicitors to trigger immune responses were gDEGs or iDEGs and were strongly downregulated in R1 plants. This suggests that recognition of phytopathogen elicitors may be impaired in downstream events such as SA and JA signaling may be dampened. This is consistent with the trends in our transcriptomes.

It is noteworthy, that two PRRs, which detect chitin (LYS5) and peptidoglycan (LYM1) were upregulated DEGs. Chitin is a component of insect exoskeletons, and chitin polymers are shed during insect development (Merzendorfer and Zimoch 2003). Whitefly stylets are chitinous and whitefly stylets are known to leave trace amounts of chitin in their host. Chitin is a MAMP detected by LYSM receptors (Cao et al. 2014; Wan et al. 2012; Petutschnig et al. 2010; Miya et al. 2007); LYK5 and LYK4 are high and low-affinity chitin receptors, respectively (Cao et al. 2014; Wan et al. 2012). In addition, IOS1 and FER are LYK5/LYK4 co-receptors and their transcripts are down-regulated in R1. It is hard to interpret at the RNA level the outcome of on chitin signaling. However, chitin-responsive *ChiB* transcripts were detected as upregulated DEGs, suggesting the chitin signaling can be activated during whitefly infestation. We do not know how to interpret the upregulation of the peptidoglycan PRR LYM1. Peptidoglycan are derived from microbial cell walls. There for is it possible cell wall fragments from B. tabaci resident

endosymbionts may generate these elicitors (Andreason et al. 2020); recognition of endosymbiont-derived effectors is well established in aphid-triggered defenses in host plants (Elzinga et al. 2014; Atamian et al. 2013; Kettles and Kaloshian 2016; Chaudhary et al. 2019).

The potential, even transient, suppression of PTI in R1 plants is intriguing. Pathogen effectors are known to target host proteins to impair deployment of PTI-triggered defenses (Martel et al. 2021; Kaloshian and Walling 2016; Kazan and Lyons 2014; Naalden et al. 2021). However, the global down regulation PTI, SA, JA, and ABA signaling is unusual and suggests more complex regulatory network that helps to prioritize the defenses essential for deterring *B. tabaci* MEAM1 development and settling is active in R1 plants. This rather surprising impairment of so many branches of host defense suggests that R1 plants could be more susceptible to other pathogens, which would not be a sustainable strategy for host plant survival. However, when grown in greenhouses, R1 plants are not more susceptible to greenhouse-associated pathogens/pests than other alfalfa resistant and susceptible lines that are grown beside the R1 plants. It is possible that R1 are not hypersusceptible to other phytopathogens, because there is a compensatory ETI response called ETI-Mediating and PTI-Inhibited Sector (EMPIS) (Hatsugai et al. 2017). We have not yet rigorously tested this hypothesis but it will be a future endeavor.

Finally, in addition to an altered defense signaling response, R1 plants may have substantial differences in its protective physical barriers to pathogen and pest attack - the cuticle and cell wall. As the cuticle stores phytochemicals and is the first surface contacted by whiteflies, alterations to the cuticle and underlying cell wall could influence both short-term and long-term whitefly interactions with its host. Cuticle composition

changes with plant development and the bayberry whitefly (*Parabemisia myricae*) can distinguish differences in the cuticles of young versus older citrus leaves (Walker 1988); cuticles from older citrus leaves deter whitefly feeding. While the differences in citrus cuticles was not explored, these data suggest that the chemistry of this protective layer may be a significant deterrent to whiteflies.

Therefore, the discovery that multiple genes (*KCS2, CER1, FAR1, FAR4, FAR6*, and *FAR3/CER4*) that influence the synthesis of long-chain (LC) or very long-chain (VLC) fatty acids and their derivatives were upregulated in R1 plants maybe significant in terms of whitefly resistance. The LC and VLC fatty acids are used for the synthesis of suberin for fortifying the cell wall (Vishwanath et al. 2013; Domergue et al. 2010) and cutin and waxes for the cuticle (Joubès and Domergue 2018; Domínguez et al. 2017). The upregulation of *FAR1*, *FAR4*, and *KCS2* indicate an increase in suberin biosynthesis suberin and *CER1, HTH,* and *GPAT6* indicate that there is an increase in cutin or wax biosynthesis. However, predicting changes in waxes and cutin may not be straight forward as *KCS2* and *CER3* transcripts are strongly downregulated DEGs. KSC2 is part of the fatty acid elongation complex essential for synthesis of VLCFAs. CER3 is critical for the production of VLC aldehydes that are using by CER1 to produce the alkane waxes of the cuticle. We hypothesize that the upregulation of *CER1* is due to the lower levels of CER3 transcripts, protein and therefore activity. It is possible CER1 and CER3 are part of a feedback loop to assure adequate wax production is maintained even when CER3 is limiting. These hypotheses can be tested. We will be providing cuticular extracts to our collaborators Drs. Paul Fraser and Laura Perez-Fons (Royal Holloway University London) to assess if there are differences in the levels of cutin, suberin, and waxes in R1 vs S1 plants.

The changes in enzymes important for cutin, suberin and wax biosynthesis is particularly interesting. In Chapter 1, we showed that R1 plants were a less desirable host compared to S1 in our adult choice studies. It is possible that biochemical changes in the cuticular proper, underlying cuticular layer and the cell wall mediate these choice studies. In addition, whitefly eggs are attached to the plant cell surface by a pedicel that penetrates the cuticle, cell wall and is imbedded in epidermal cells (Buckner et al. 2002); the pedicel mediates water and small molecule uptake from the plant (Byrne et al. 1990), it is possible the pedicel transfer phytochemicals to the egg to inhibit its development and to impair the development of the emerging first instar nymph. This is plausible as the cuticle and cell wall have numerous phytochemicals imbedded in these barriers and the pedicel is a permeable conduit. We have not yet explored this biochemistry. In the future, we will focus on secondary metabolites, phytochemistry of the cuticle and cell wall, and modifications of the cell wall; this will be enabled by further interrogation of our RNA-seq datasets in this Chapter and in Chapter 2, as well as our collaboration with the Fraser lab in the near future.

The whitefly timecourse transcriptomes from R1 and S1 plants have given us significant insights into the probably mechanisms deployed in R1's potent resistance against whiteflies. These studies have suggested steps for the future. First, we were not able to align these reads to the newly released alfalfa genomes (Li et al. 2020; Shen et al. 2020). Our first attempt to align a de novo transcriptome to these was not successful due to the differences between our alfalfa's population ancestry and that of the transcriptome. To enable this in the near future, we will compress the de novo transcriptome by removing overlapping and redundant sequences . This should allow for a more accurate evaluation of our transcriptome and therefore annotations of candidate

DEGs. It is clear that the current alfalfa genomes are in flux as new annotated versions are being posted at https://medicagohapmap2.org/.

Second, in Chapter 2, we focused on early gDEGs and iDEGs due to the fact that alfalfa's resistance impacts first instar nymphs and adult choice responses. It is also possible, that the gDEGs/iDEGs identified at later times of infestation, or even tDEGs, might provide the longer lasting component to alfalfa's resistance *B. tabaci* MEAM1. In addition, as mentioned above, we have not focuses on the genes associated with secondary metabolism, which are expressed across the infestation timecourse, and these genes might provide significant insights into the chemistries of R1 plants that cause the delays or mortality in whitefly nymphs and make R1 plants a less desired host for settling.

Third, we would like to determine if PTI is impaired in R1 plants and if this has ramifications on susceptibility to alfalfa pathogens. In the future, we will assess if R1 plants have altered ROS bursts, callose deposition, ion fluxes, and altered MAP kinase cascades in response to elicitors such as flg22, elf18 and chitin. We would therefore expect our R1 plants to be susceptible to bacterial pathogens and others that elicit PTI.

Fourth, in Chapter 1, we identified a large number of alfalfa lines that were highly resistant and susceptible to whiteflies. As R1's resistance appears to be expressed prior to and during whitefly infestation, this gives us an excellent opportunity to assess if the R1's mechanism of resistance is also used in other resistant alfalfa. To do this, we are currently constructing RNA-seq libraries from four resistant and four lines. These transcriptomes will let us assess if the 0 dpi status of other whitefly resistant and susceptible plants are aligned with R1 and S1 discoveries. In addition, metabolites from the R1 and S1 time courses and in 0 dpi leaves from the additional resistant and

susceptible lines will be examined in collaboration with Dr. Paul Fraser's lab. Using UCR's Multi-Omics Correlation Analysis tool developed by Manhoi Hur (IIGB, UCR), we will be able to correlate transcript and metabolite profiles. We expect that there may be differences. We know the block to MEAM1's nymph development is present in all resistant lines based on the screen that was used to identify them and R1, R2 and R3 alfalfa also have similar impacts on adult host plant choice (Chapter 1). However, we also know there are differences in *B. tabaci* MED and NW1 interactions on R1, R2 and R3 suggesting differences in the metabolites, physical barriers, or defense signaling in these lines.

Finally, we would like to test our defense hypothesis *in planta*. Unfortunately, alfalfa does not have the advantage of having an array of hormone biosynthesis or signaling mutants, as is found in Arabidopsis (Kumar 2014; Tuteja 2007; Vishwakarma et al. 2017; Ng et al. 2014; Checker et al. 2018; Ruan et al. 2019; Chen et al. 2020; Binder 2020; Peng et al. 2021a; Bisson and Groth 2010). In addition, while making transgenic alfalfa is feasible (Hawkins and Yu 2018; Shi et al. 2017; Prosperi et al. 2014; Li and Brummer 2012), not all alfalfa genotypes are capable of regenerating in culture, providing a bottle neck for future studies. If S1 plants were capable of regeneration in culture, we might be able to impair defense signaling using dsRNA constructs to recapitulate the down-regulation the SA, JA and ABA pathways and upregulation of the ET pathway in isolation to assess their specific roles in defense. Alternatively, we can use Arabidopsis mutants to test candidate genes. This is particularly attractive for testing the role suberin, cutin and waxes in whitefly interactions. Given the profound differences in SA and JA signaling in alfalfa and Arabidopsis as will be revealed in the next chapter

and the different roles of JA and SA in basal immunity to whiteflies in these plants, testing the impacts of defense hormone signaling in Arabidopsis may not be fruitful.

The data compiled and analyzed in this Chapter have provided us a more comprehensive understanding of alfalfa's response to whiteflies and how it varies between lines. This information will enable us to make more strategic decisions as to how we further unravel this response and how it varies within the alfalfa population and across other plant species. The comprehension of the cuticle's role in whitefly response in alfalfa also provides the foundation to launch studies to investigate how cuticle composition might impact whitefly feeding and other behaviors.

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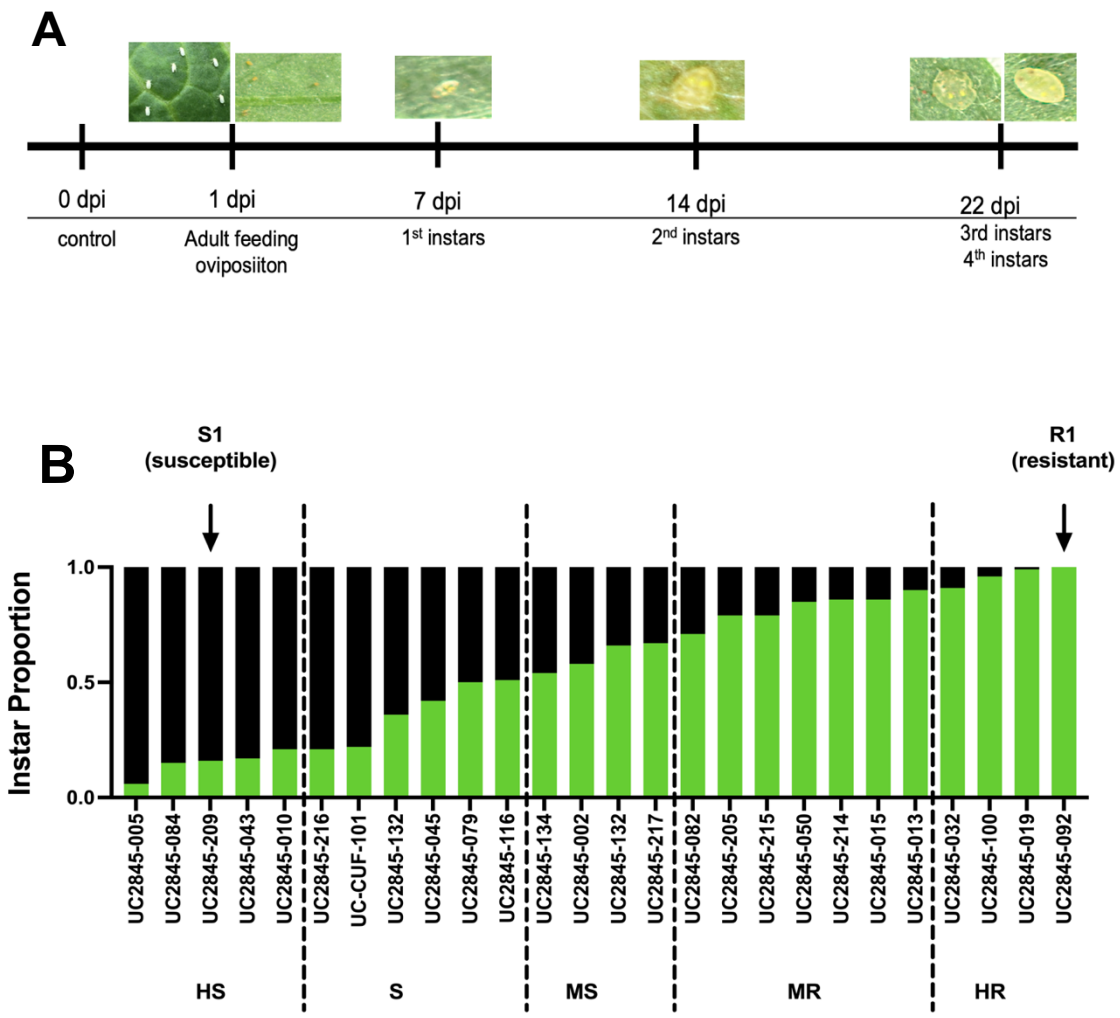


Figure 2.1 Identification of resistant genotypes and experimental design.

(A) Bar graph showing the first instar mortality of alfalfa genotypes in a resistant population (UC2845). Genotypes were grouped into one of five phenotypic classes: highly susceptible (HS), susceptible (S), moderately susceptible (MS), moderately resistant (MR) and resistant (R). Genotypes used in transcriptomics studies are highlighted above. (B) A timeline showing all of the times when alfalfa trifoliate samples were collected. Each timepoint correlated with a behavior in whitefly development essential to feeding and development.

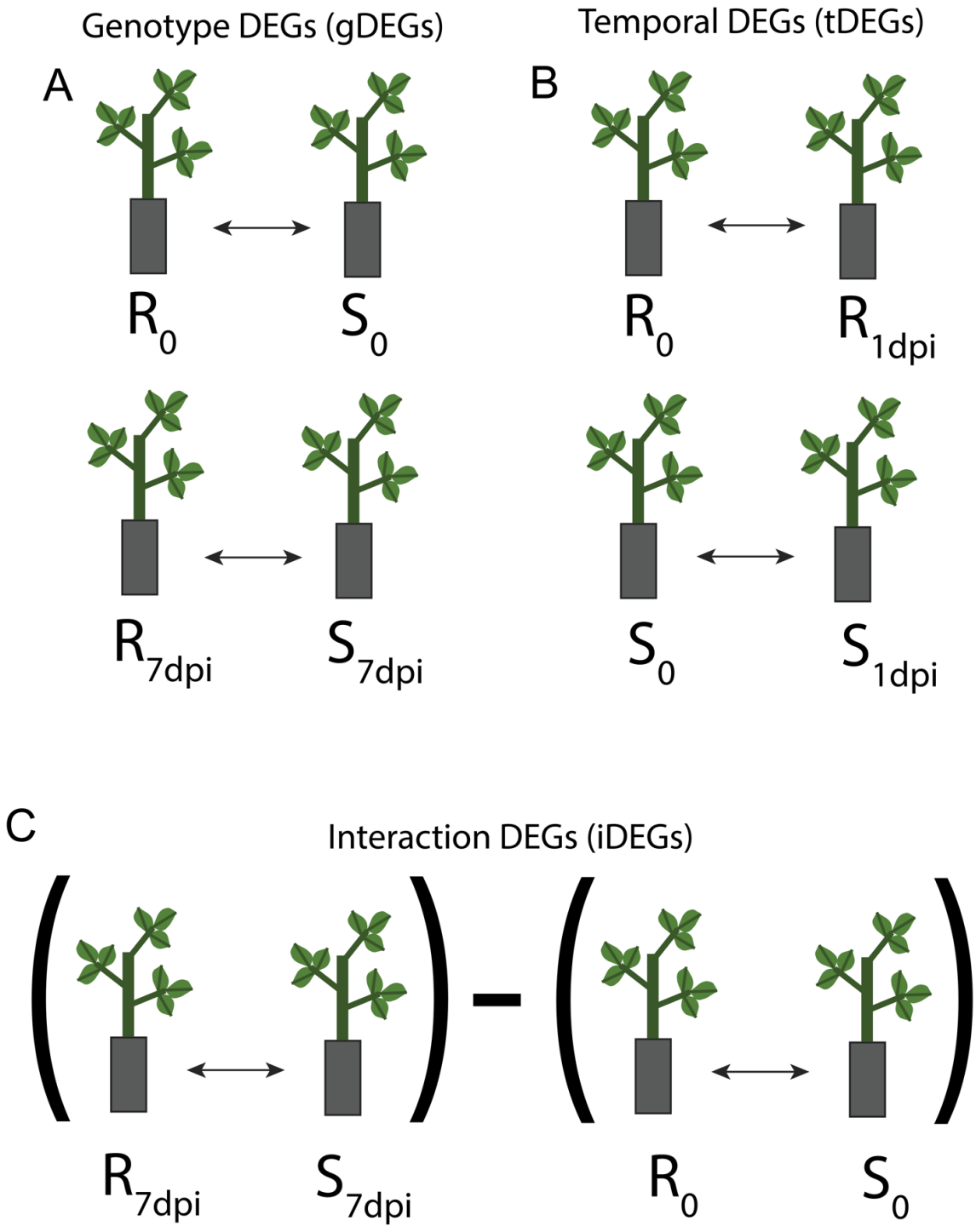

Genotype DEGs (gDEGs)
A
R0
S0
R7dpi
S7dpi
Temporal DEGs (tDEGs)
B
R0
R1dpi
S0
S1dpi
C
Interaction DEGs (iDEGs)
R7dpi
S7dpi
R0
S0

Figure 2.2 Schematic representation of differentially expressed gene (DEG) classification.

DEGs were identified as either (A) genotypic (gDEGs) based on changes in expression profile at the same time in different genotypes, (B) temporal (tDEGs) based on changes in expression profile in samples of the same genotype at different time points, or (C) differentially expressed upon fulfilling specific genotypic, temporal, or developmental conditions (interaction DEGs, iDEGs). One example of an iDEG is shown. Models for iDEGs are found in Table 2.2.

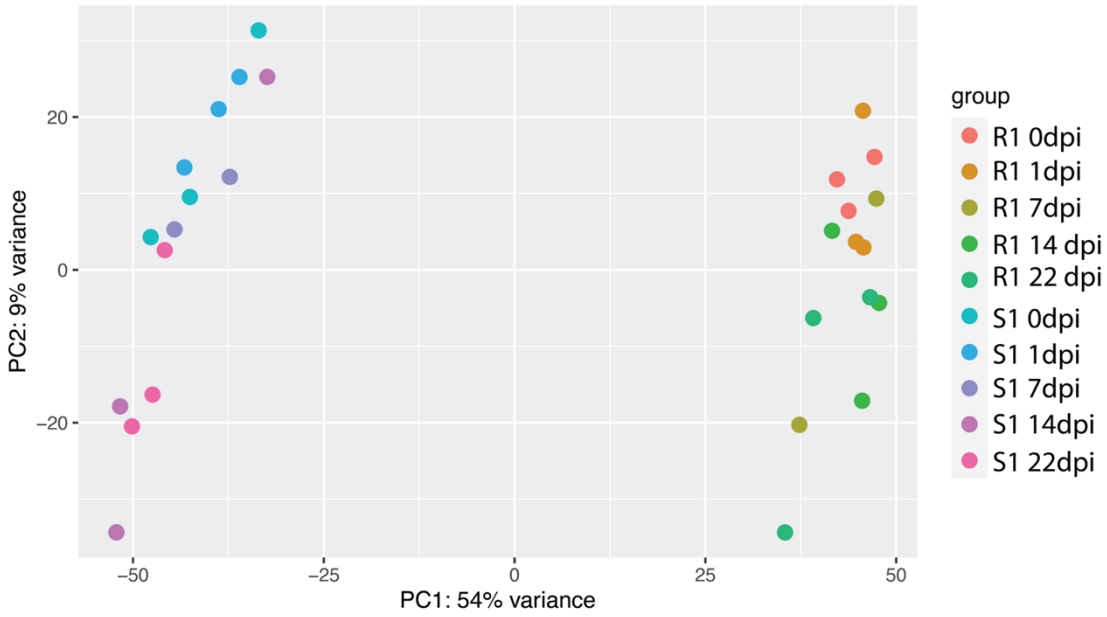


Figure 2.3 Alfalfa – WF Transcriptome PCA Analysis.

PCA analysis was conducted using default parameters in DESeq2.

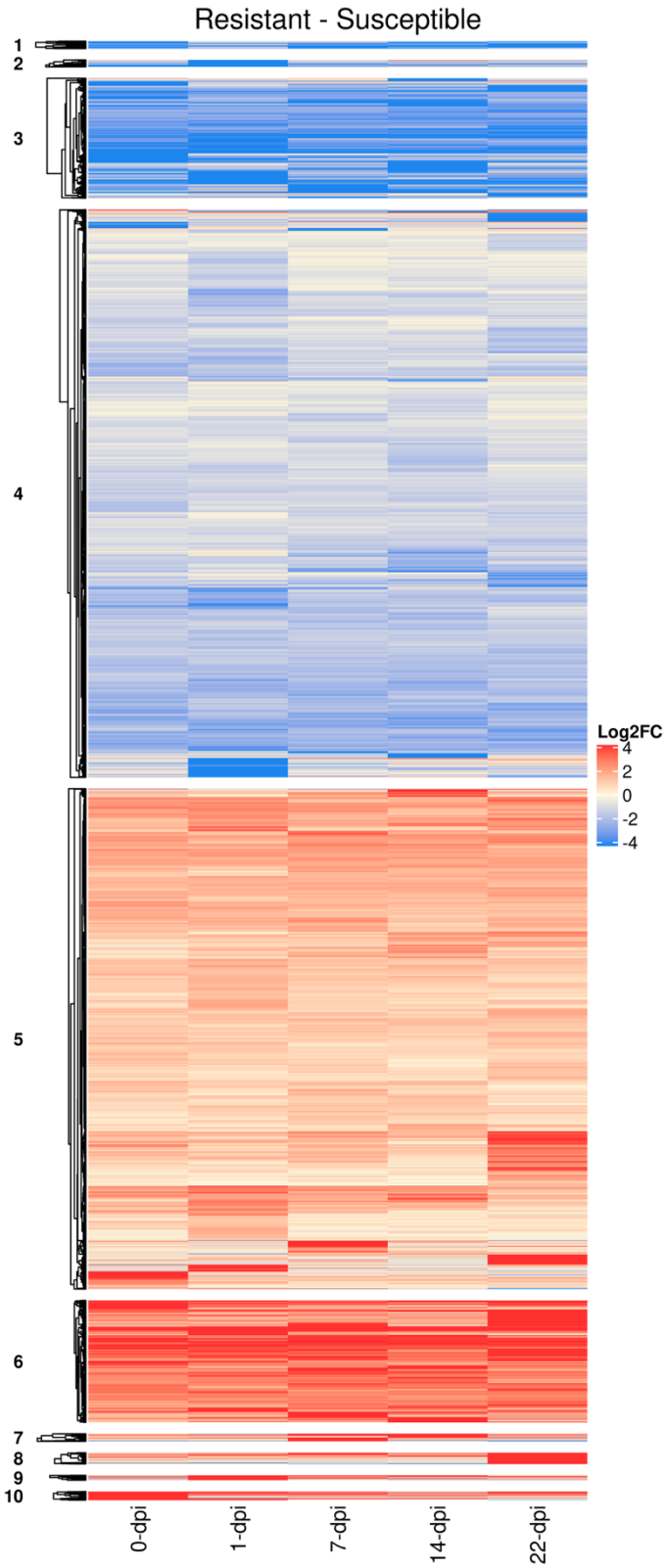

Resistant - Susceptible
1
2
3
4
5
6
7
8
9
10
Log2FC
4
2
0
-2
-4
0-dpi
1-dpi
7-dpi
14-dpi
22-dpi

Figure 2.4 Heatmap of Genotype DEGs.

Genotype DEG expression during the whitefly-alfalfa infestation. Genotype DEGs were identified with a $|\log_2 \text{foldchange}| > 1$ and a FDR ≤ 0.05. Heatmap displays resistant (R1) log2expression in comparison to the susceptible (S1) $\log_2$FC. DEGs were clustered along the y-axis based on their expression profile.

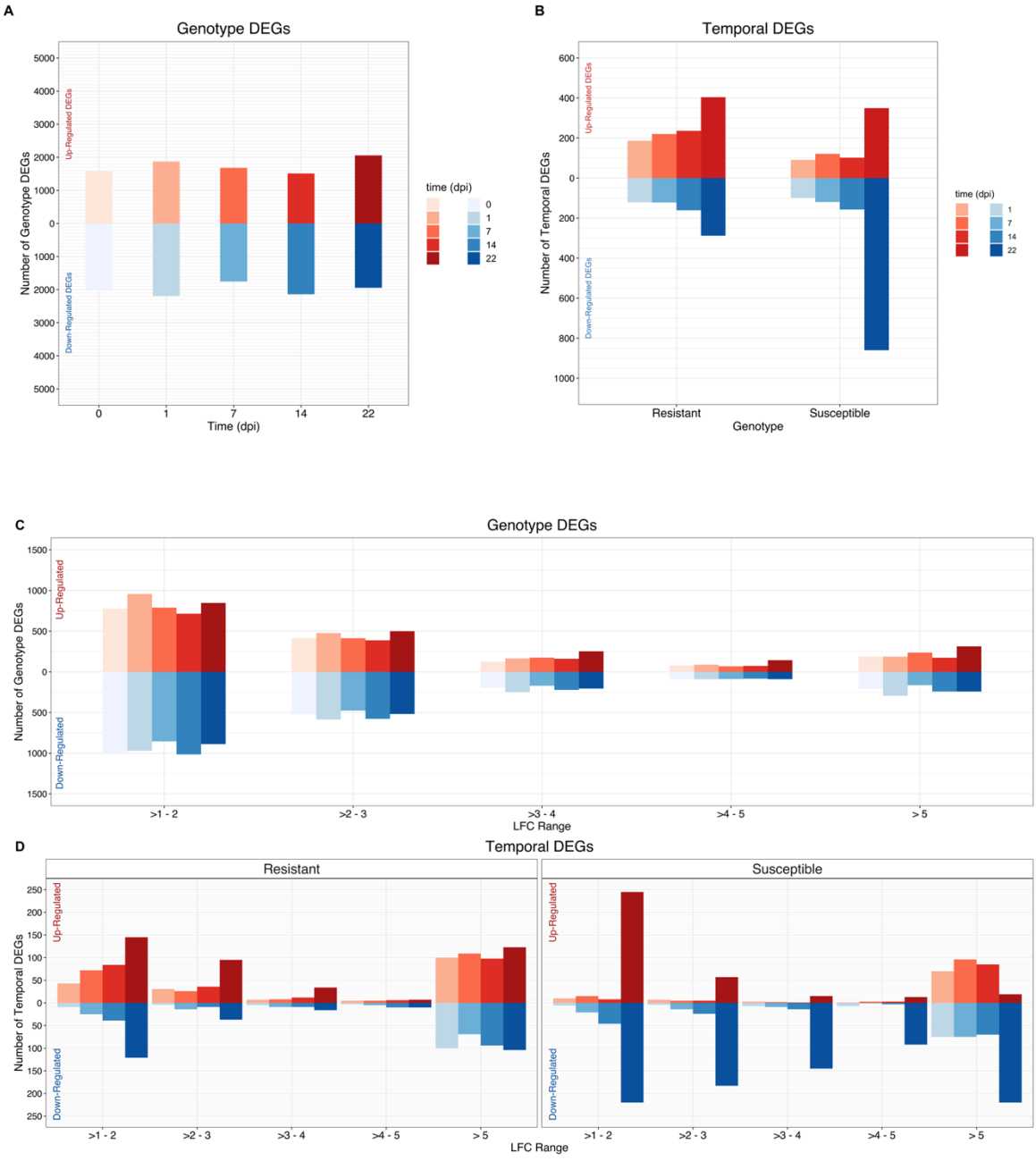

A
Genotype DEGs
Number of Genotype DEGs
Up-Regulated DEGs
Down-Regulated DEGs
Time (dpi)
time (dpi)
B
Temporal DEGs
Number of Temporal DEGs
Genotype
Resistant
Susceptible
C
Genotype DEGs
Up-Regulated
Down-Regulated
LFC Range
D
Temporal DEGs
Resistant
Susceptible

Figure 2.5 Bar plot of Genotype and Temporal DEG Counts for Alfalfa –WF Transcriptome Analysis.

Bar plots showing the number of upregulated or downregulated genotype and temporal DEGs. Bar plots show number of (A) Genotype DEGs at each time point, (B) Temporal DEGs for each genotype at each time point, and the distribution of DEGs based on their log2 fold change (LFC) for either genotype (C) or temporal (D) comparisons.

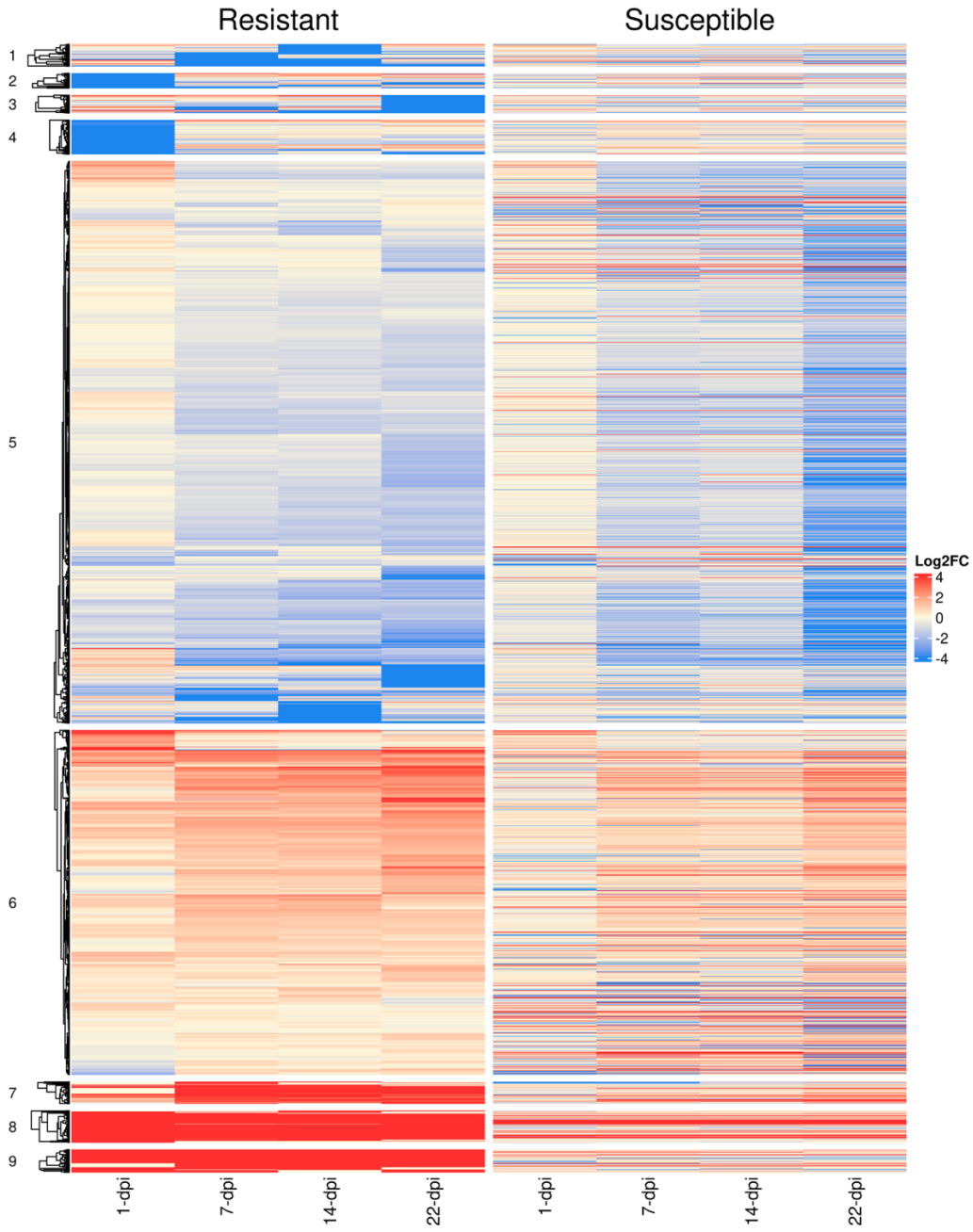


Figure 2.6 Heatmap of Temporal DEGs.

Temporal DEG expression during the whitefly-alfalfa infestation. Heatmap displays DEGs in the susceptible (S1) and the resistant (R1) genotype. DEGs were grouped along the y-axis by expression pattern during the time course in R1. Expression values are shown as relative expression compared to the 0-dpi time point for each genotype.

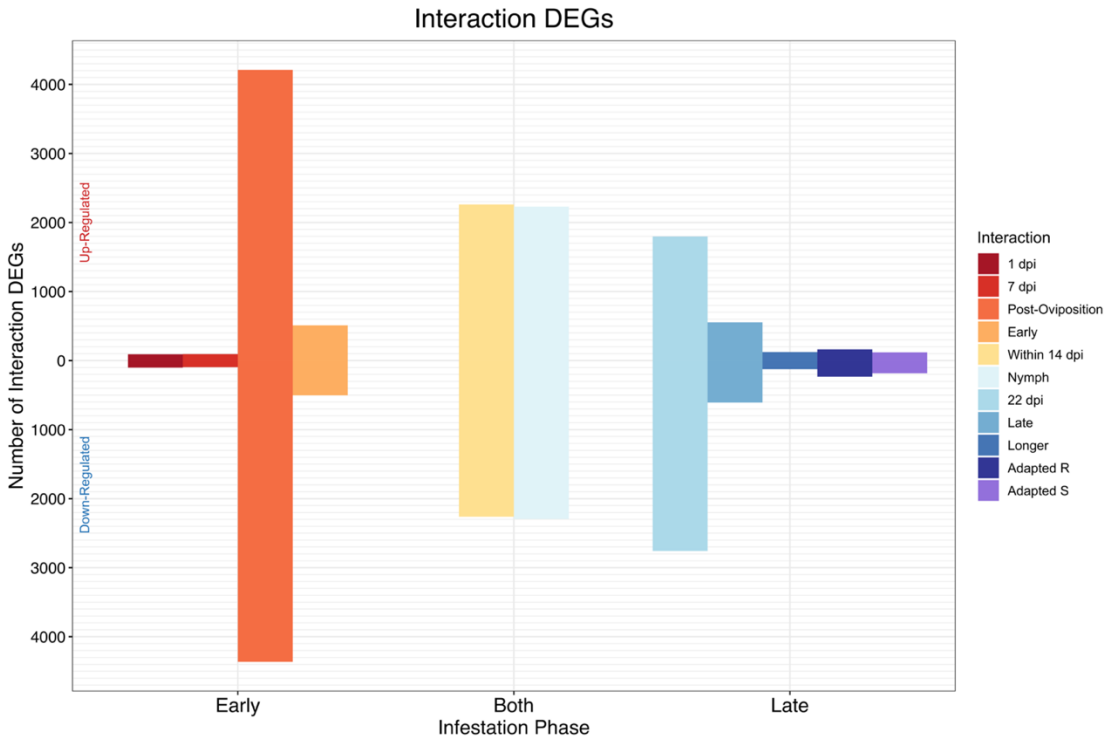


Figure 2.7 Bar plot of DEG Counts for Alfalfa – WF Transcriptome Analysis.

Bar plots showing the number of upregulated or downregulated DEGs for each interaction. Interactions encapsulating early time points (Interactions 1 – 4), interactions encapsulating both infestation phases (Interactions 5 & 6), and interactions encapsulating both later time points (Interactions 7 - 11).

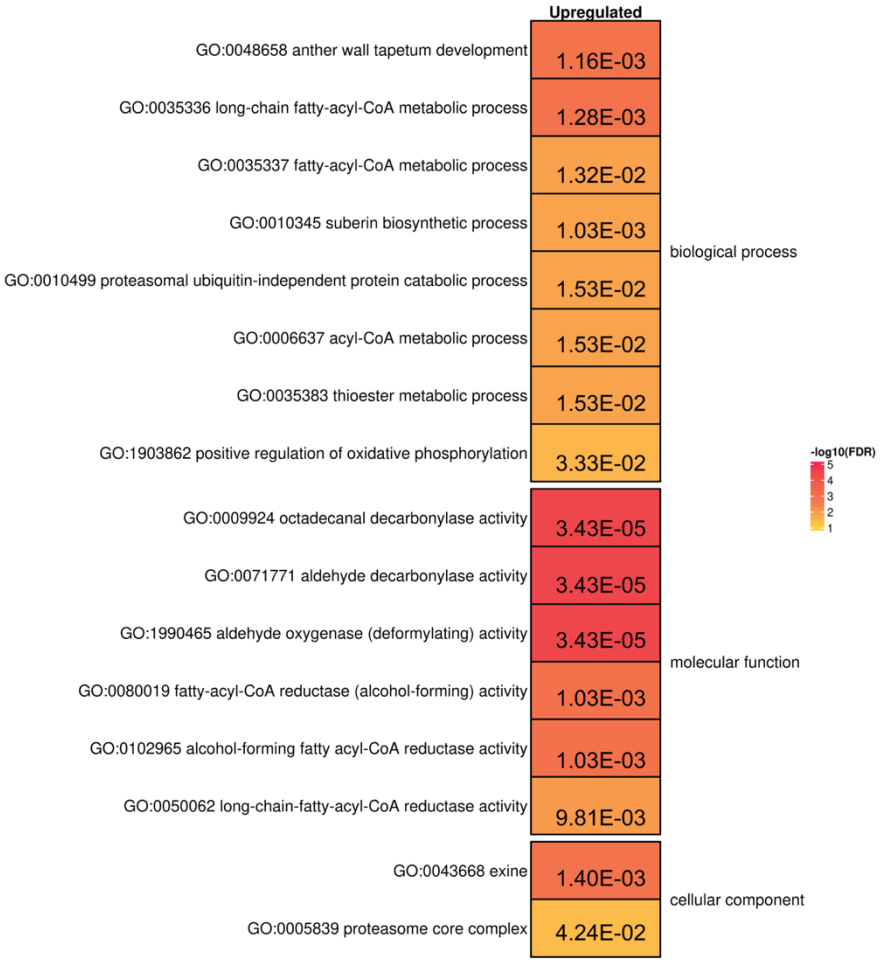


Figure 2.8 GO Terms associated with 0-dpi gDEGs.
Heatmap of “biological process” GO terms for upregulated gDEGs in uninfested R1 alfalfa (0-dpi gDEGs). GO terms were identified using *goseq* and passed the 0.05 FRD threshold. FDRs are plotted as -log10(FDR).

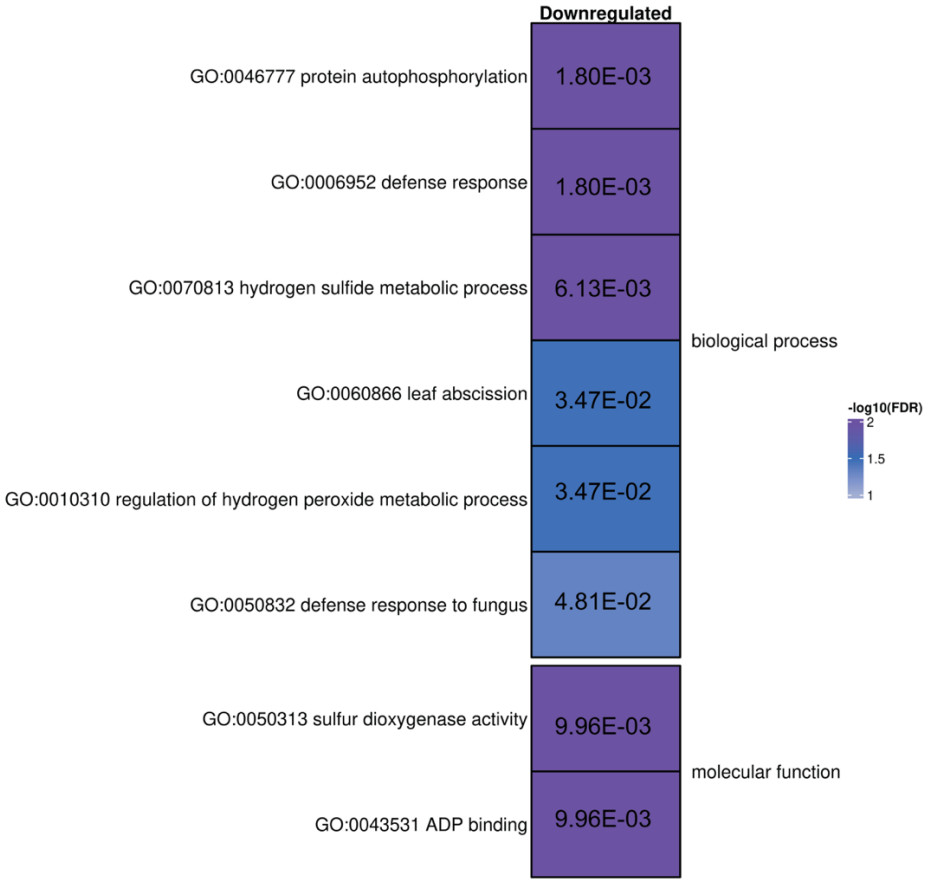

Downregulated
GO:0046777 protein autophosphorylation
1.80E-03
GO:0006952 defense response
1.80E-03
GO:0070813 hydrogen sulfide metabolic process
6.13E-03
GO:0060866 leaf abscission
3.47E-02
GO:0010310 regulation of hydrogen peroxide metabolic process
3.47E-02
GO:0050832 defense response to fungus
4.81E-02
GO:0050313 sulfur dioxygenase activity
9.96E-03
GO:0043531 ADP binding
9.96E-03
biological process
molecular function
-log10(FDR)
2
1.5
1

Figure 2.9 GO Terms associated with 0-dpi gDEGs.

Heatmap of “biological process” GO terms for downregulated gDEGs in uninfested R1 alfalfa (0-dpi gDEGs). GO terms were identified using *goseq* and passed the 0.05 FRD threshold. FDRs are plotted as -log10(FDR).

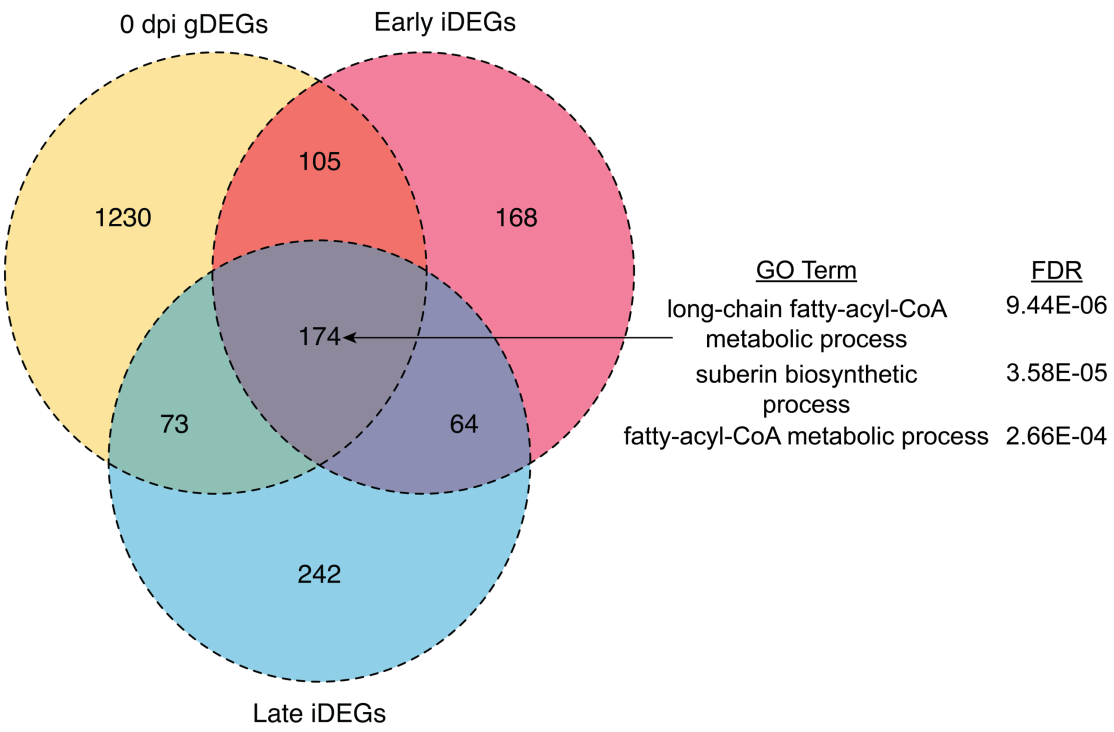
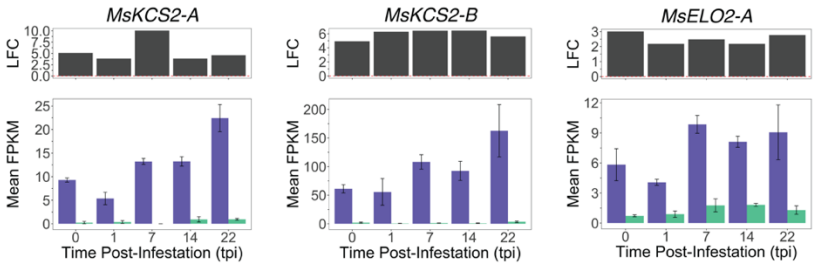


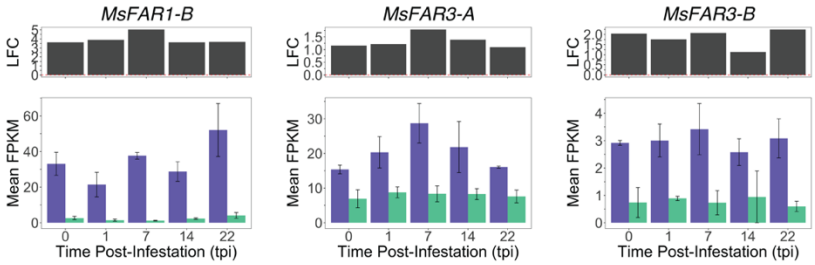
Figure 2.10 DEGs upregulated throughout infestation are involved in very long-chain fatty acid (VLCFA) and suberin synthesis.

The overlap of transcripts identified as gDEGs at 0 dpi, iDEGs associated with adults, eggs and 1st instar feeding (Interaction 3, early iDEGs), and iDEGs associated with nymph feeding at 14 and 22 dpi (Interaction 9, late iDEGs) are displayed. The enriched biological process GO terms associated with DEGs identified with all three phases of infestation are shown to the right. GO terms were identified using the goseq package at an FDR of 0.05 using the Benjamini Hochberg method. The identity of the overlapping genes are found in Supplemental Table 2.4.

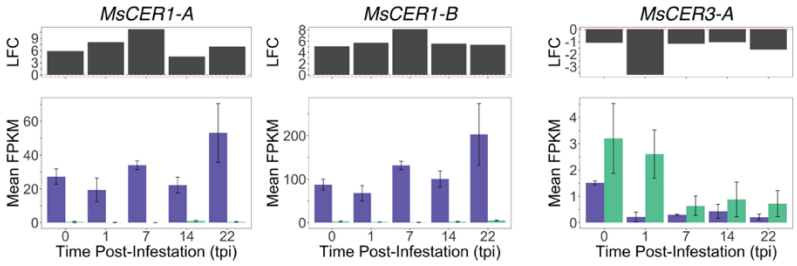
FAE Complex



Suberin Biosynthesis



Wax Biosynthesis



Cutin Transporters

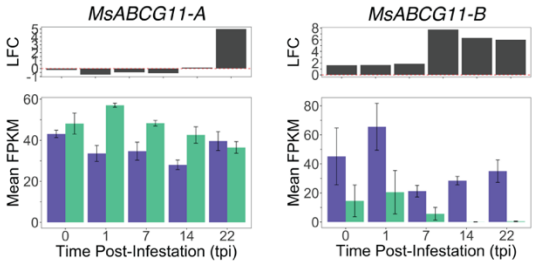


Figure 2.11 Expression of Cuticle and Suberin Biosynthesis DEGs.

Expression of DEGs associated with very-long chain fatty acid, wax, or suberin biosynthesis and cutin/wax transport. Log2-fold change (LFC) barplot shows the LFC difference between R1 and S1 at a given time point. The mean FPKM of transcripts during the 22 d whitefly infestation period in R1 (purple bars) and S1 (green) are displayed.

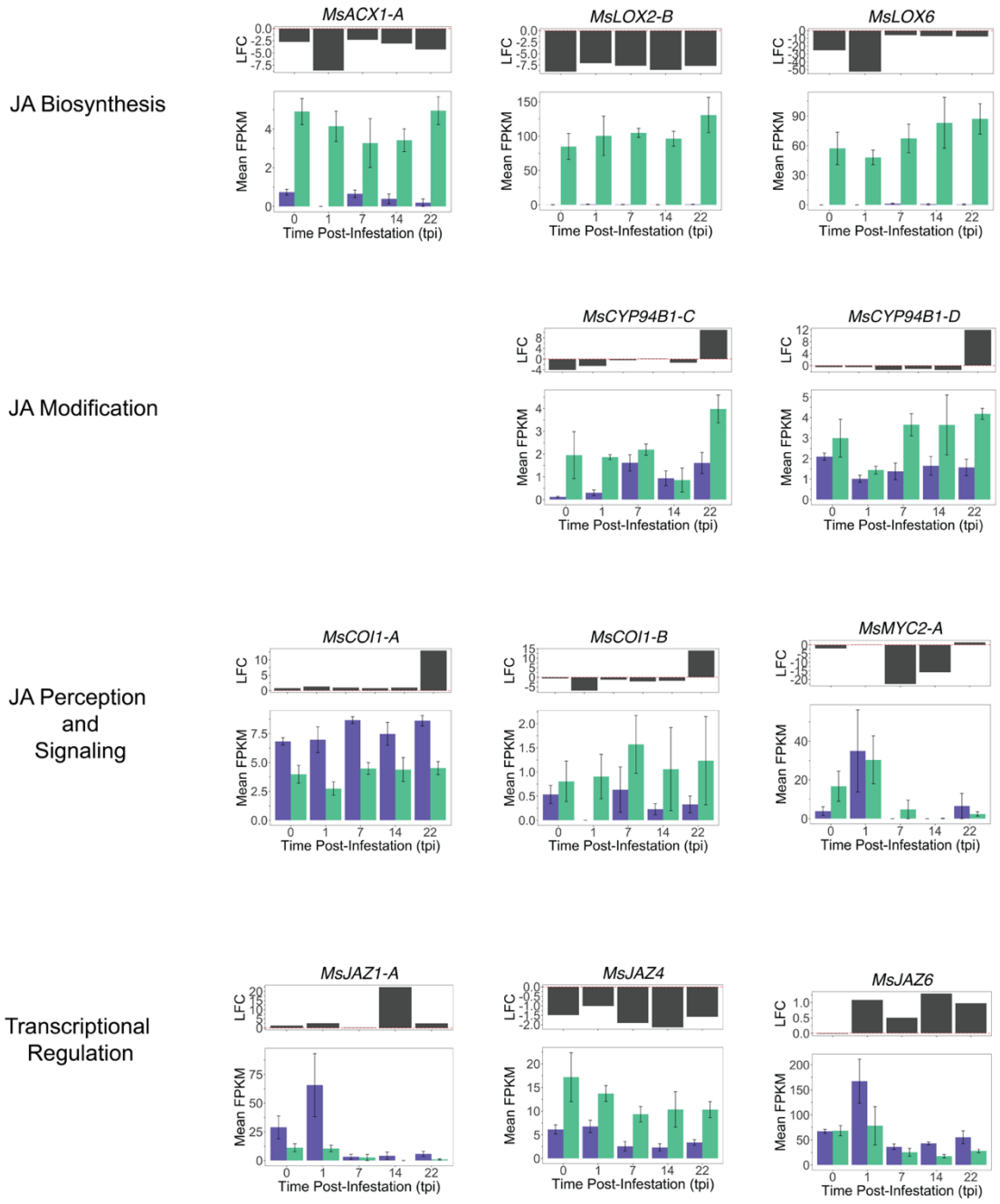
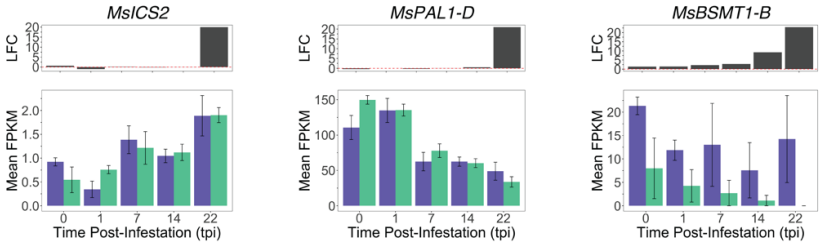


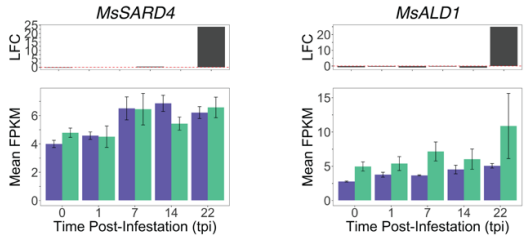
Figure 2.12 Expression of JA Pathway DEGs.

Expression of DEGs associated with JA biosynthesis, modification, perception and signaling or transcriptional control are shown. DEGs are either gDEGs, tDEGs or iDEGs. Log2-fold change (LFC) barplot shows the LFC difference between R1 and S1 at a given time point. The mean FPKM of transcripts during the 22 d whitefly infestation period in R1 (purple bars) and S1 (green) are displayed.

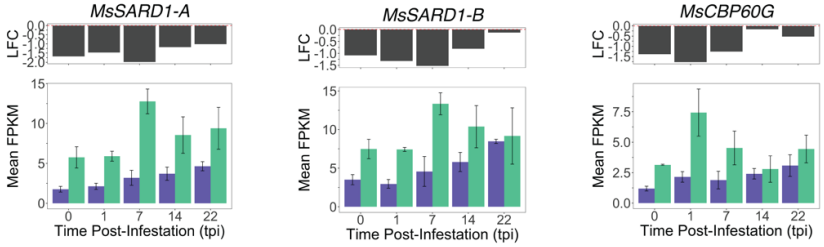
SA Biosynthesis and Modification



NHP Biosynthesis



SARD1 and CBP60G



Transcriptional Regulators

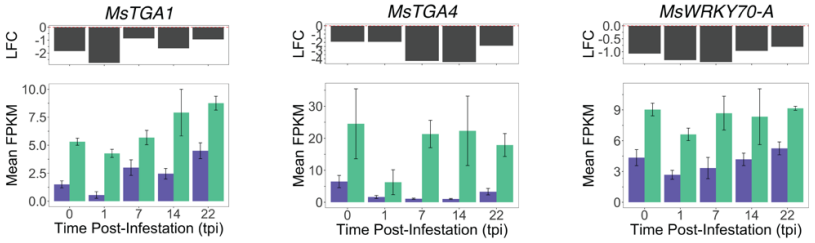
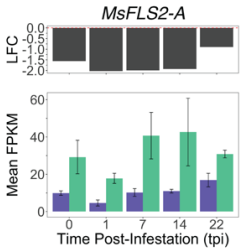
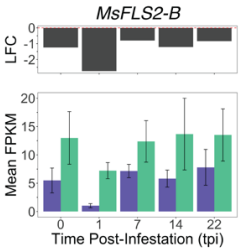


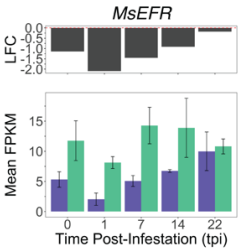
Figure 2.13 Expression of SA and SAR Pathway DEGs.

Expression of DEGs associated with SA biosynthesis, modification, pipecolic acid synthesis, and transcriptional control of SA-regulated defenses are shown. DEGs are either gDEGs, tDEGs or iDEGs. Log2-fold change (LFC) barplot shows the LFC difference between R1 and S1 at a given time point. The mean FPKM of transcripts during the 22 d whitefly infestation period in R1 (purple bars) and S1 (green) are displayed.

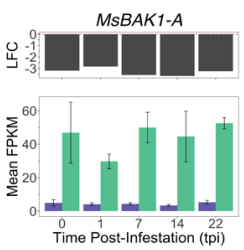
PRR Receptors

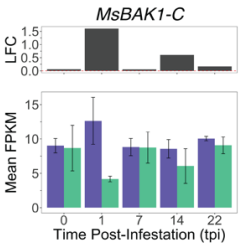


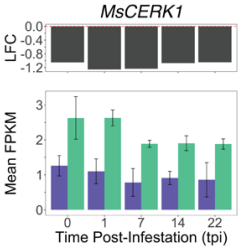




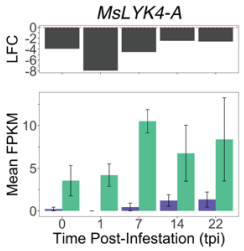
PRR Co-receptors

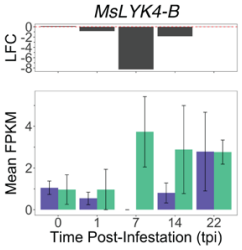


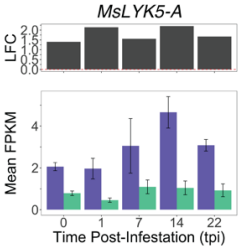




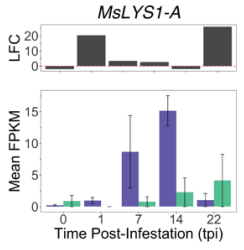
Chitin Perception

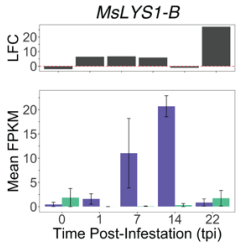






Other PTI Interactors





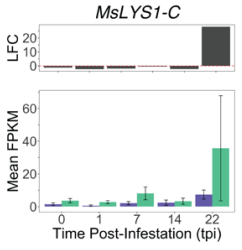
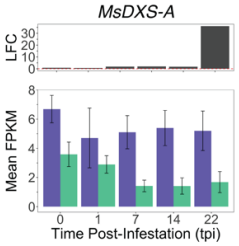
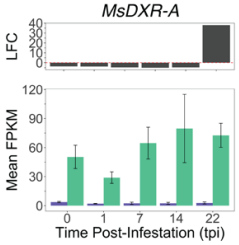


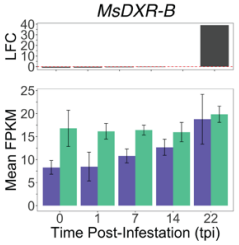
Figure 2.14 Expression of PTI-associated DEGs.

Expression of DEGs for PRR receptors for flg22 and elf18 perception, PRR co-receptors, chitin perception, and other PTI interactors are shown. DEGs are either gDEGs, tDEGs or iDEGs. Log2-fold change (LFC) barplot shows the LFC difference between R1 and S1 at a given time point. The mean FPKM of transcripts during the 22 d whitefly infestation period in R1 (purple bars) and S1 (green) are displayed.

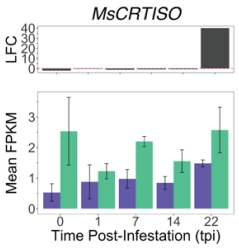
MEP Pathway

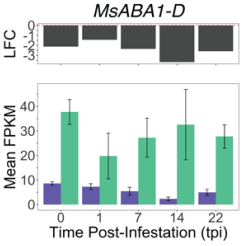


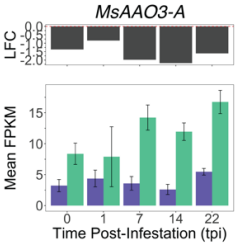




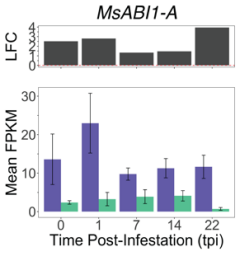
ABA Biosynthesis

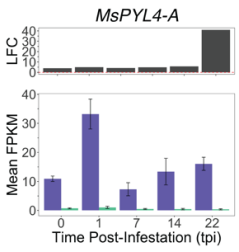


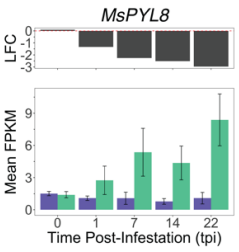




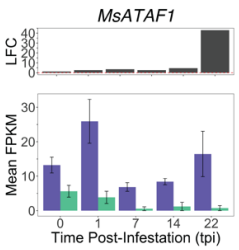
ABA Receptors

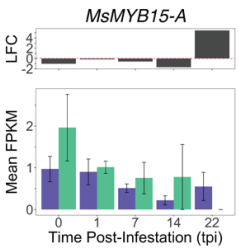






ABA Suppresors





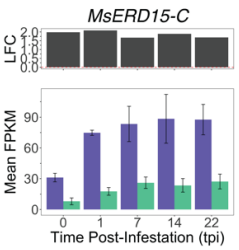


Figure 2.15 Expression of ABA Pathway DEGs.

Expression of DEGs associated isoprenoid biosynthesis (MEP pathway), ABA biosynthesis, ABA receptors, and ABA-pathway repressors are shown. DEGs are either gDEGs, tDEGs or iDEGs. Log2-fold change (LFC) barplot shows the LFC difference between R1 and S1 at a given time point. The mean FPKM of transcripts during the 22 d whitefly infestation period in R1 (purple bars) and S1 (green) are displayed.

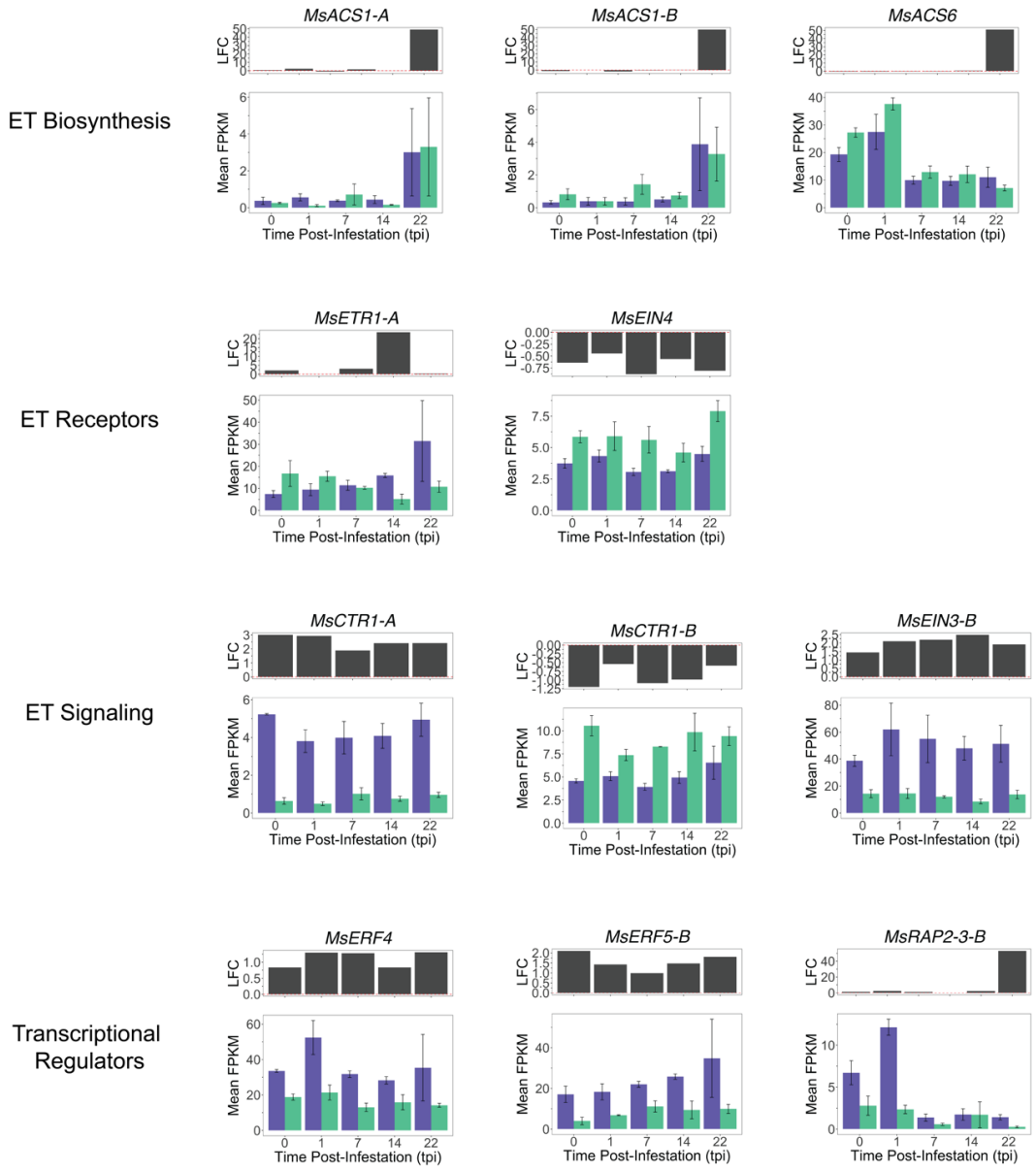

ET Biosynthesis
MsACS1-A
MsACS1-B
MsACS6
ET Receptors
MsETR1-A
MsEIN4
ET Signaling
MsCTR1-A
MsCTR1-B
MsEIN3-B
Transcriptional Regulators
MsERF4
MsERF5-B
MsRAP2-3-B
LFC
Mean FPKM
Time Post-Infestation (tpi)

Figure 2.16 Expression of ET-Pathway DEGs.

Expression of DEGs associated with ET biosynthesis, ET perception, negative regulation of ET signaling (CTR1), and ET-responsive transcription factors are shown. DEGs are either gDEGs, tDEGs or iDEGs. Log2-fold change (LFC) barplot shows the LFC difference between R1 and S1 at a given time point. The mean FPKM of transcripts during the 22 d whitefly infestation period in R1 (purple bars) and S1 (green) are displayed.

Table 2.1 Alfalfa gDEG and tDEG Model Design

RNA-seq Model Design					
	Model	**Meaning**	**Comparison Name**	**Notes**	**DEGs**
	R0 - S0	baseline/constitutive resistance gene expression	Genotype DEGs (0 dpi)	Snapshot of how resistance plants perform without stress.	3610
	R1 - S1	effects of feeding for 1d in resistant vs susceptible	Genotype DEGs (1 dpi)	Similar feeding responses between R and S might not be detected.	4061
	R7 - S7	effects of feeding for 7d in resistant vs susceptible	Genotype DEGs (7 dpi)		3435
	R14 - S14	effects of feeding for 14d in resistant vs susceptible	Genotype DEGs (14 dpi)		3650
	R22 - S22	effects of feeding for 22d in resistant vs susceptible	Genotype DEGs (22 dpi)		4000
	S0 - S1	effects of feeding for 1d (Susceptible)	S1 Temporal DEGs	Confounds any defense interactions with development (At 7, 14, or 22d are these plants developing the same? If feeding induces a similar response in R and S plants, this will not be detected.).	190
	S0 - S7	effects of feeding and development for 7d (Susceptible)			241
	S0 - S14	effects of feeding and development for 14d (Susceptible)			259
	S0 - S22	effects of feeding and development for 22d (Susceptible)			1209
	R0 - R1	effects of feeding for 1d (Resistant)	R1 Temporal DEGs		307
	R0 - R7	effects of feeding and development for 7d (Resistant)			342
	R0 - R14	effects of feeding and development for 14d (Resistant)			397
	R0 - R22	effects of feeding and development for 22d (Resistant)			692

Table 2.2 iDEG Model Design

RNA-seq Model Design					
Interaction#	**Model**	**Meaning**	**Comparison Name**	**Notes**	**DEGs**
1	(R1 - S1) - (R0 - S0)	effects of feeding without constitutive expression	Induced 1-dpi	Should remove constitutive expression and show only induced expression	193
2	(R7 - S7) - (R0 - S0)	effects of time on 7 dpi sample	Induced 7-dpi	Should show genes induced by WF nymphs 7 dpi, also accounting for development.	190
3	(R7 - S7) - (R1 - S1)	effects of first instar feeding vs adult feeding/oviposition	Post-Oviposition	Might account for the morphological differences between adults, eggs, and nymphs and the potential triggers they may deploy for defense responses.	8573
4	[(R7 + R1) - (S7 + S1)] - (R0 - S0)	effects of adult feeding/oviposition and early nymph establishment	Regulated Early	Combines both the response to adults/oviposition and 1st instar establishment compared to uninfested alfalfa	1014
5	[(R1 + R7 + R14) - (S1 + S7 + S14)] - (R0 - S0)	effects of adult infestation and nymph feeding through 14 days of infestation	Regulated within 14-dpi	Should capture the genes that are primarily responsible for conferring R to nymphs within the first 14 days of infestation	4710
6	[(R7 + R14 + R22) - (S7 + S14 + S22)] - (R0 - S0)	effects of all nymph feeding throughout infestation	Nymph Induced	Identifies only those genes that are induced by WF nymphs, independent of time, but might miss genes that are induced by WF adults that also explain nymph R.	4524
7	(R22 + S22) - (R0 + S0)	effects of time on all samples	Regulated 22-dpi	Should show how plant development changed over time and the conserved response to feeding	4556
8	[(R14 + R22) - (S14 + S22)] - (R0 - S0)	effects of later-staged nymph feeding	Induced Late	Similar effect as above, but focuses on later-staged nymphs.	1161
9	[(R22 + R14) - (R7 + R1)] - [(S22 + S14) - (S7 + S1)]	effects of longer duration of feeding in resistant plants relative to susceptible	Longer Feeding	Accounts for development of both genotypes and should only show those genes responsive to later stage WF feeding.	249
10	(R22 + R14) - (R7 + R1)	effects of longer duration of feeding	Adapted DEGs (R)	Should show more systemic and acclimation response of the plant to feeding relative to initial recognition and signaling	396
11	(S22 + S14) - (S7 + S1)	effects of longer duration of feeding	Adapted DEGs (S)	Should show more systemic and acclimation response of the plant to feeding relative to initial recognition and signaling	303

Table 2.3 gDEG and tDEG LFC Ranges

	Up-regulated Genotype DEGs (Resistant - Susceptible)					
	> 1 - 2	**>2 - 3**	**>3 - 4**	**>4 - 5**	**> 5**	**Total**
0-dpi	776	417	125	78	186	1582
1-dpi	957	476	165	87	186	1871
7-dpi	789	413	175	68	236	1681
14-dpi	715	388	161	73	173	1510
22-dpi	847	500	253	143	313	2056
	Down-regulated Genotype DEGs (Resistant - Susceptible)					
0-dpi	1012	523	196	91	206	2028
1-dpi	971	585	249	91	294	2190
7-dpi	855	476	171	88	164	1754
14-dpi	1015	577	222	84	242	2140
22-dpi	888	518	206	90	242	1944

Table 2.3 Continued

Up-regulated Temporal DEGs (Susceptible)						
	>1 - 2	**>2 - 3**	**>3 - 4**	**>4 - 5**	**> 5**	**Total**
1-dpi	10	7	3	1	70	91
7-dpi	15	5	2	3	96	121
14-dpi	8	5	1	3	85	102
22-dpi	245	57	15	13	19	349
Down-regulated Temporal DEGs (Susceptible)						
	>1 - 2	**>2 - 3**	**>3 - 4**	**>4 - 5**	**> 5**	**Total**
1-dpi	10	7	3	1	70	91
7-dpi	15	5	2	3	96	121
14-dpi	8	5	1	3	85	102
22-dpi	245	57	15	13	19	349
Up-regulated Temporal DEGs (Resistant)						
	>1 - 2	**>2 - 3**	**>3 - 4**	**>4 - 5**	**> 5**	**Total**
1-dpi	43	31	7	5	100	186
7-dpi	72	26	8	5	109	220
14-dpi	84	36	12	6	98	236
22-dpi	145	95	34	7	123	404
Down-regulated Temporal DEGs (Resistant)						
	>1 - 2	**>2 - 3**	**>3 - 4**	**>4 - 5**	**> 5**	**Total**
1-dpi	9	4	5	3	100	121
7-dpi	25	14	9	5	69	122
14-dpi	39	9	9	10	94	161
22-dpi	121	37	16	10	104	288

Table 2.4 GO Terms among gDEGs upregulated at 0 dpi

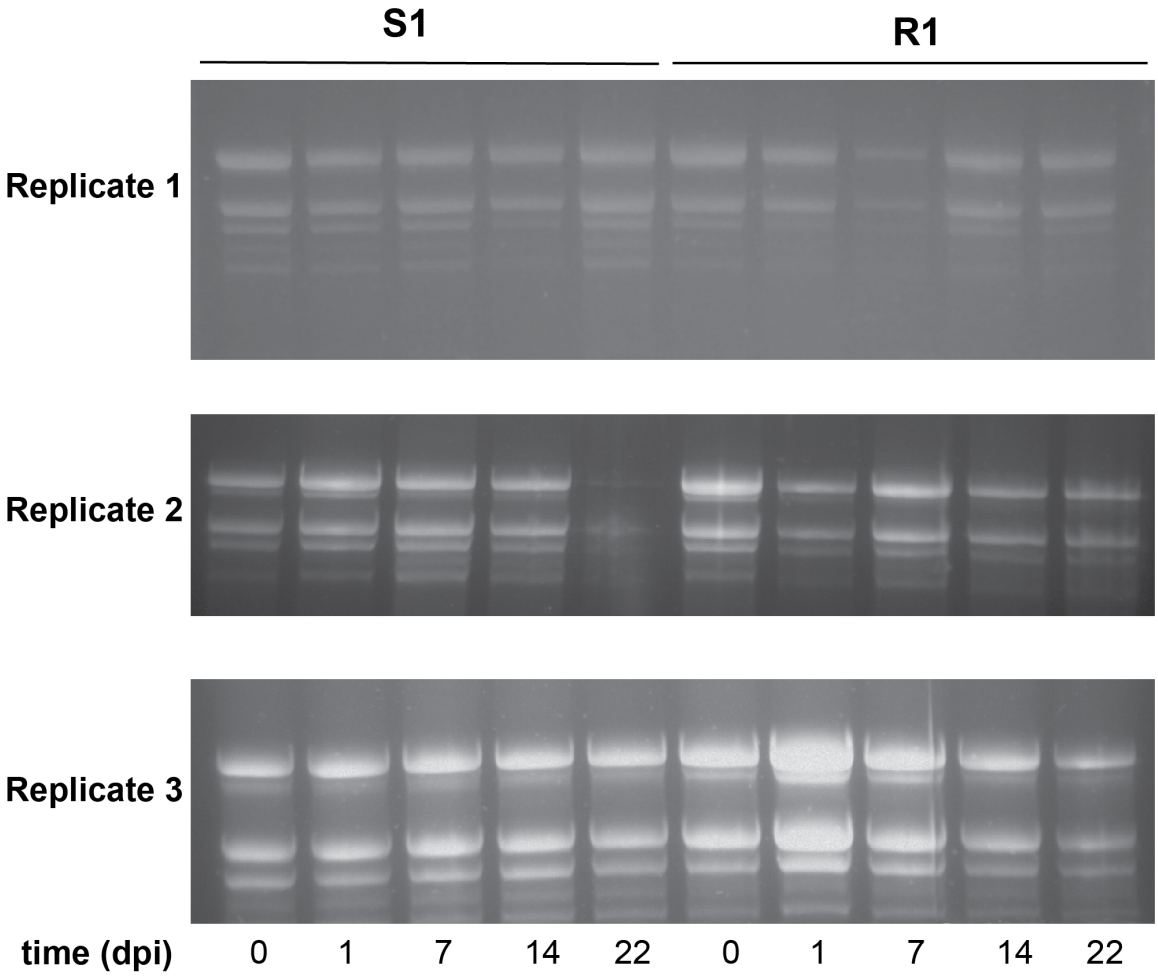
GO Term	Ontology	False Discovery Rate (FDR)
octadecanal decarbonylase activity	MF	3.43E-05
aldehyde decarbonylase activity	MF	3.43E-05
aldehyde oxygenase (deformylating) activity	MF	3.43E-05
fatty-acyl-CoA reductase (alcohol-forming) activity	MF	1.03E-03
alcohol-forming fatty acyl-CoA reductase activity	MF	1.03E-03
long-chain-fatty-acyl-CoA reductase activity	MF	9.81E-03
exine	CC	1.40E-03
proteasome core complex	CC	4.24E-02
anther wall tapetum development	BP	1.16E-03
long-chain fatty-acyl-CoA metabolic process	BP	1.28E-03
fatty-acyl-CoA metabolic process	BP	1.32E-02
suberin biosynthetic process	BP	1.53E-02
proteasomal ubiquitin-independent protein catabolic process	BP	1.53E-02
acyl-CoA metabolic process	BP	1.53E-02
thioester metabolic process	BP	1.53E-02
positive regulation of oxidative phosphorylation	BP	3.33E-02

A. MF = molecular function; BP = biological process; CC = cellular component

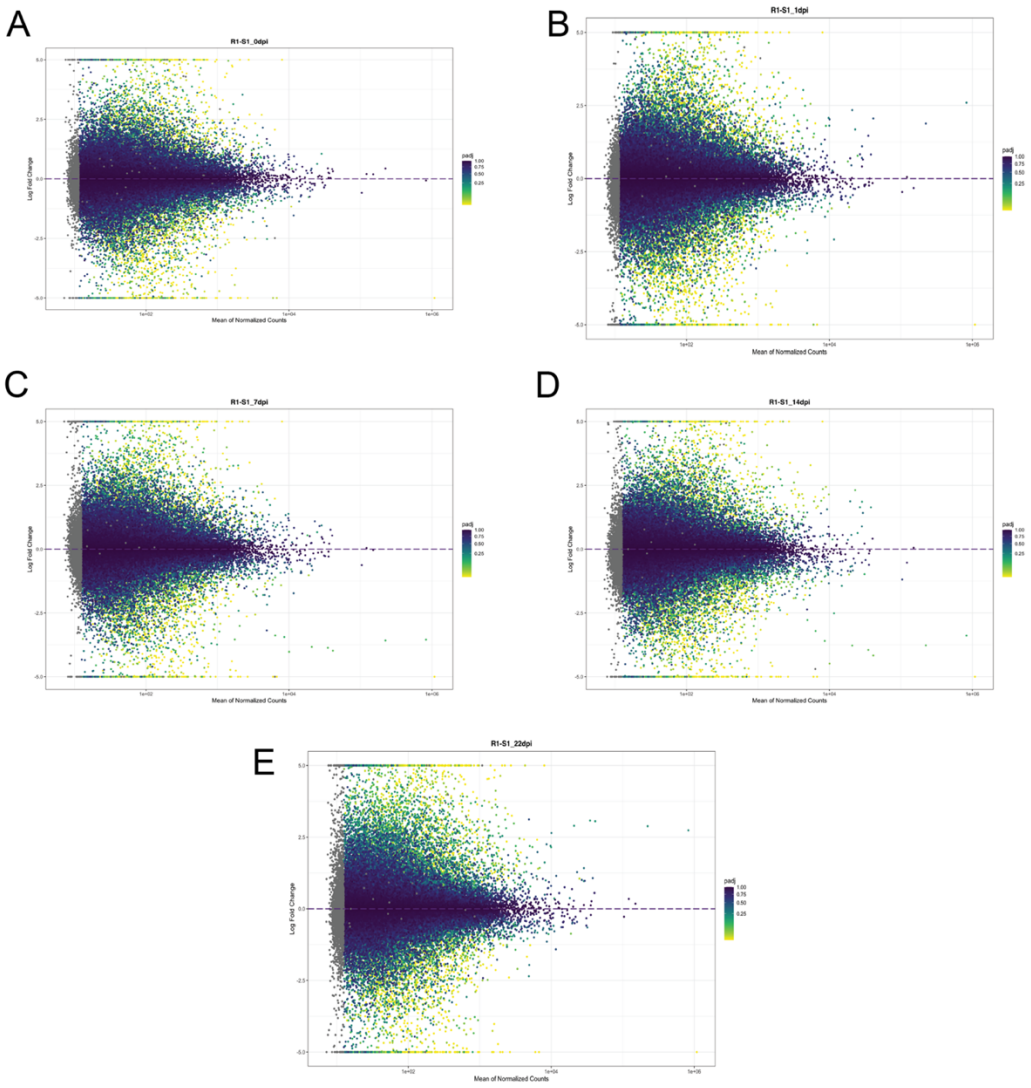
Table 2.5 GO Terms among gDEGs downregulated at 0 dpi

GO Term	Ontology	False Discovery Rate (FDR)
sulfur dioxygenase activity	MF	9.96E-03
ADP binding	MF	9.96E-03
protein autophosphorylation	BP	1.80E-03
defense response	BP	1.80E-03
hydrogen sulfide metabolic process	BP	6.13E-03
leaf abscission	BP	3.47E-02
regulation of hydrogen peroxide metabolic process	BP	3.47E-02
defense response to fungus	BP	4.81E-02

A. MF = molecular function; BP = biological process; CC = cellular component

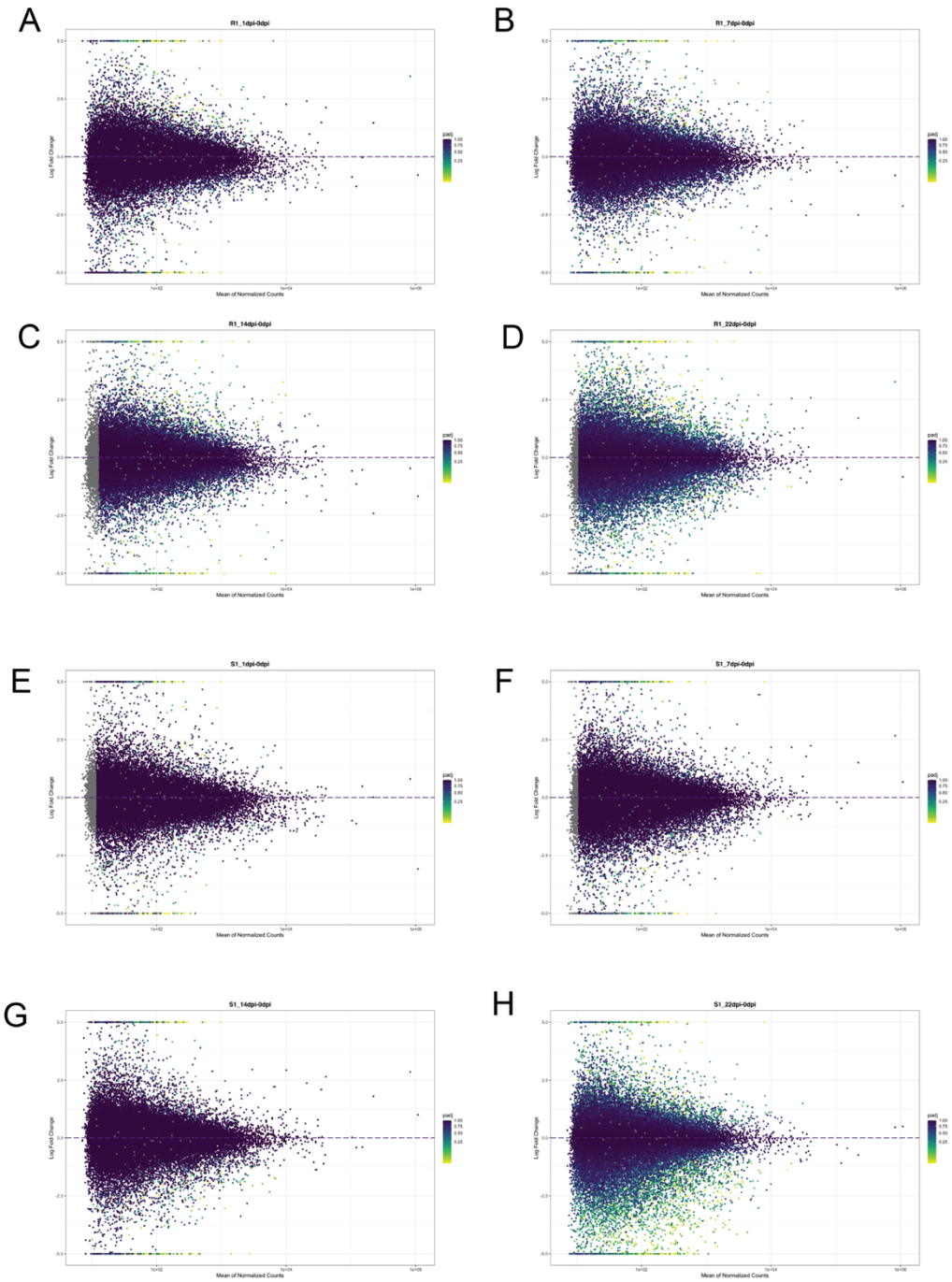


Supplemental Figure 2.1 RNA denaturing gel of transcriptome samples



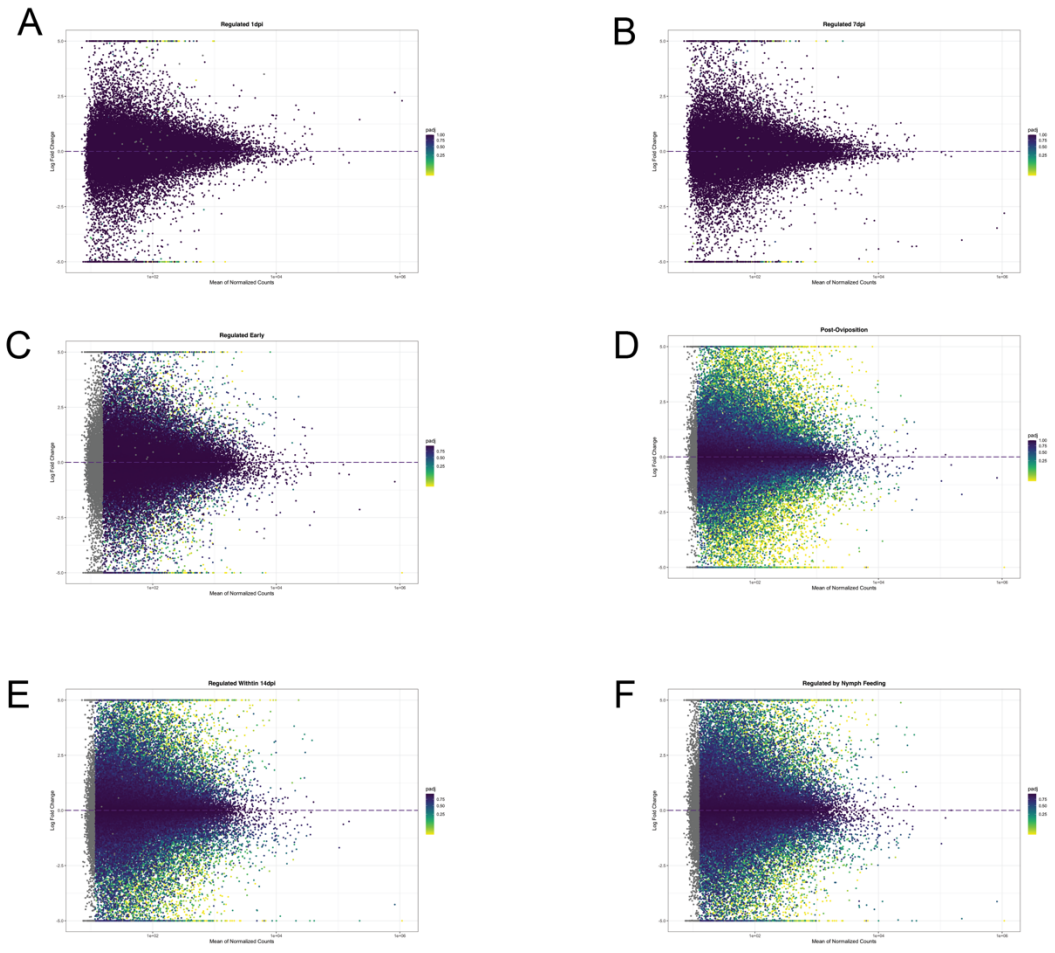
Supplemental Figure 2.2 MA Plots of gDEG analyses.

MA Plots are ordered by time points (0 – 22 dpi) in the time course (Supplemental 2.2.A – 2.2.E).


A
R1_1dpi-0dpi
B
R1_7dpi-0dpi
C
R1_14dpi-0dpi
D
R1_22dpi-0dpi
E
S1_1dpi-0dpi
F
S1_7dpi-0dpi
G
S1_14dpi-0dpi
H
S1_22dpi-0dpi
Log Fold Change
Mean of Normalized Counts
padj

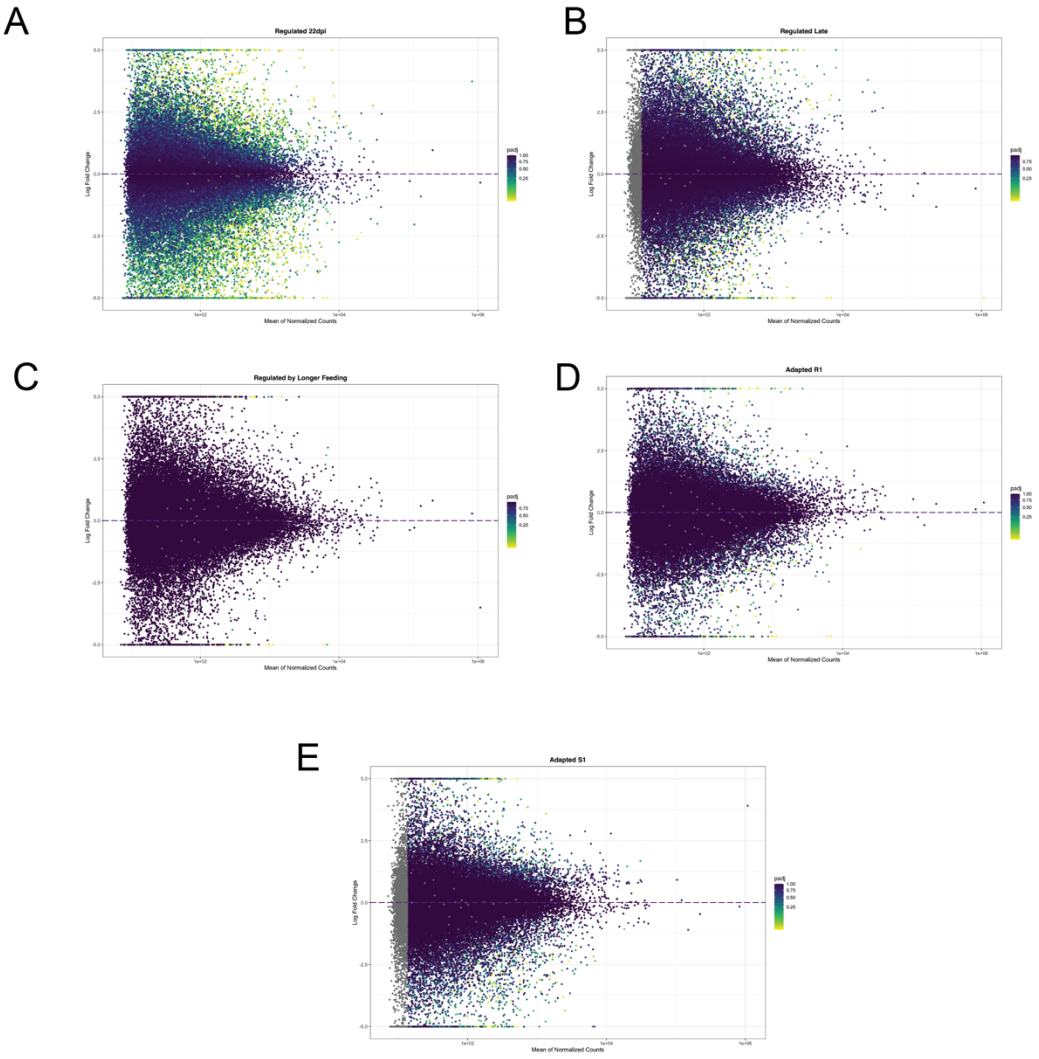
Supplemental Figure 2.3 MA Plots of tDEG analyses.

MA Plots are ordered by time points (1 – 22 dpi) in the time course for the resistant (Supplemental 2.3.A – 2.3.D and susceptible (Supplemental 2.3.E – 2.2.3H) lines.



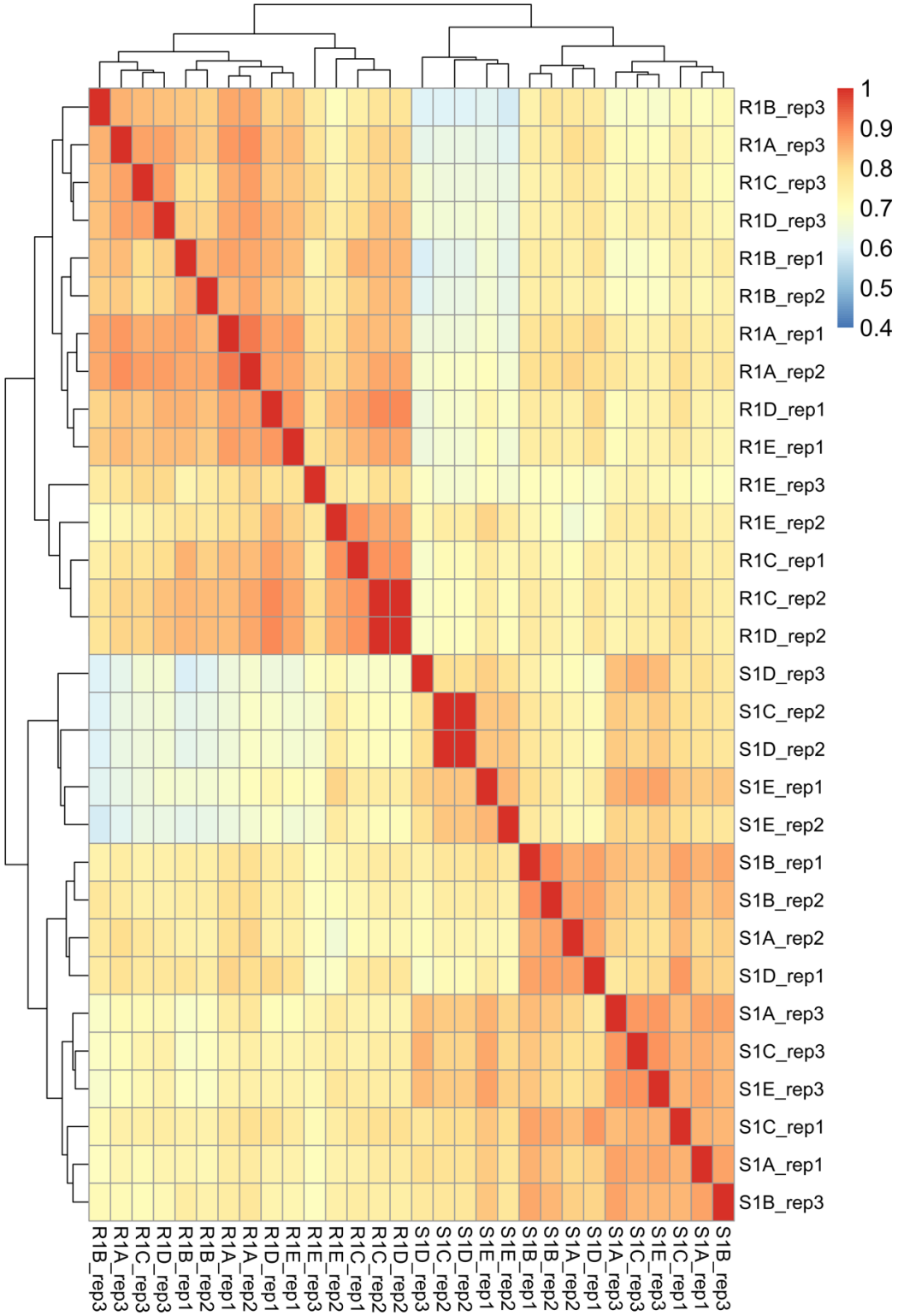
Supplemental Figure 2.4 MA Plots of iDEG analyses 1 – 6

MA Plots for Interactions 1 – 6 (Supplemental 2.4.A – 2.4.F) as described in Table 2.2


A
Regulated 22dpi
B
Regulated Late
C
Regulated by Longer Feeding
D
Adapted R1
E
Adapted S1
Log Fold Change
Mean of Normalized Counts
padj

Supplemental Figure 2.5 MA Plots of iDEG analyses 7 – 11

MA Plots for Interactions 7 – 11 (Supplemental 2.5.A – 2.5.E) as described in Table 2.2



Supplemental Figure 2.6 Pearson correlation analysis of alfalfa-whitefly libraries

Chapter 3 Alfalfa's Phytohormone Response and Its Correlation to Whitefly Infestation

Abstract

Phytohormone signaling is a critical component of plant immunity as different pathogens can elicit different phytohormone signaling pathways. Two of the most prominent phytohormones are salicylic acid and jasmonic acid. SA-mediated defenses are usually associated with biotrophic pathogens and JA-mediated defenses are associated with necrotrophs. While there is some synergy between these pathways, they are generally antagonistic and some pathogens possess the ability to manipulate signaling of either SA or JA to promote their growth on a plant host. Understanding crosstalk between SA and JA is important to comprehending the fundamental of plant defense. While elucidating the phytohormone signaling pathways of *Arabidopsis* has been a focus of the plant community, there is a relative dearth of knowledge about alfalfa's phytohormone signaling pathways. Here, we unraveled the complexity behind alfalfa's phytohormone signaling responses by performing a 24-h SA and JA treatment for a comparative transcriptomic analysis. Utilizing sentinel genes canonical to SA and JA signaling in *Arabidopsis*, we identified 1 and 8 h as viable time points representing early and later responses for transcriptome sequencing. In our transcriptomic analyses, we observed a larger number of SA- and JA-responsive genes at 8 h compared to 1 h. Unlike Arabidopsis, there was evidence of reciprocity between phytohormone responses in alfalfa. Upon gene ontology (GO) term-enrichment analyses of SA and JA responses, defense-related terms were associated with upregulated SA- and JA-DEGs at 1 h, while terms associated with metabolism were enriched in the 8 h DEGs with both hormone treatments. SA and JA's role in defense was further supported by the overrepresentation

of growth and photosynthesis genes among DEGs downregulated at 8 h. Finally, we anchored an alfalfa-whitefly transcriptome described in Chapter 2 to these phytohormone libraries to reveal the identity of SA/JA-responsive genes during alfalfa's response to whitefly infestation. There was a weak correlation between whitefly and JA responses among genotype DEGs (gDEGs) and a weak correlation between whitefly and SA response among temporal DEGs (tDEGs) in whitefly-susceptible alfalfa. From these data, we can conclude alfalfa's SA and JA responses are similar but distinct and alfalfa's response to whitefly is largely independent of both phytohormones.

Introduction

Phytohormone-signaling pathways play essential roles in plant life and functions. Plant defense is one function where phytohormone signaling and crosstalk are essential for optimal operation. Plant defense is antagonistic to growth and development, as a plant must shift C and N resources from growth, development and reproduction to defense upon attack by a pest or pathogen (Huot et al. 2014). Each defense phytohormone regulates a cascade of regulatory events resulting in transcriptional reprogramming, production of secondary metabolites, and, in some cases, systemic acquired resistance (SAR) and programmed cell death (PCD) (Grant and Jones 2009b; Fu and Dong 2013; Checker et al. 2018). Virtually every phytohormone interacts with one or more defense-signaling pathways as a major or minor player. The major players of defense signaling include: salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), and ethylene (ET). The minor players include auxin, gibberellic acid (GA) and cytokinins, among others.

Two of the best-studied defense hormones are SA and JA (Zhang et al. 2020; Erb et al. 2012; Peng et al. 2021a). Each hormone regulates different networks of genes that that have different defense outputs. For example, SA regulates defenses against biotrophic pathogens, JA regulates defense to herbivorous insects and JA and ET collaborate to mediate resistance to necrotrophic pathogens (Yang et al. 2019b; Klessig et al. 2018; Yang et al. 2015). As plants often encounter a suite of herbivores/pathogens (phytopathogens) simultaneously in the field, plant have evolved complex mechanisms to prioritize defense-signaling pathways to deploy the defenses targeted to their attackers. Depending on the timing and magnitude of increases in SA and JA after pathogen attack, JA- and SA-signaling pathways might act antagonistically, additively or synergistically (Checker et al. 2018; Yang et al. 2015; Mur et al. 2006). For this reason, mechanisms for the SA and JA pathways to communicate (ie., crosstalk) is essential for deployment of defense to single or multiple pathogens (Vos et al. 2015; Erb et al. 2012).

JA and SA crosstalk is controlled by several genes including: *NON EXPRESSOR OF PATHOGENESIS RESISTANCE 1* (*NPR1*), *WRKY70*, *MITOGEN-ACTIVATED PROTEIN KINASE 4, MYC2,* and *NAC domain-containing proteins 19, 55, and 72 (ANAC19/55/72)* (Rayapuram and Baldwin 2007; Ren et al. 2008; Li et al. 2004; Brodersen et al. 2006; Kazan and Manners 2013; Yang et al. 2019b). *NPR1* and transcription factor *WRKY70* work to stimulate SA signaling and attenuate JA signaling (Ren et al. 2008; Li et al. 2006; Li et al. 2004; Spoel et al. 2003). *MYC2, MPK4*, and *ANAC19/55/72* dampen SA signaling, while inducing JA signaling (Zheng et al. 2012; Petersen et al. 2000; Brodersen et al. 2006).

To promote their success, phytopathogens introduce proteinaceous or chemical effectors to influence defense-signaling pathway activation (Huang et al. 2021;

Kaloshian and Walling 2016). Many phytopathogens introduce effectors that are virulence factors. These effectors interfere with the ability of a host plant to perceive an attacker or to induce the molecular signaling events critical for defense trait expression (Naalden et al. 2021; Stahl et al. 2018; Kaloshian and Walling 2016). Other phytopathogens introduce effectors to manipulate defense-signaling pathways that are activated upon attack. By activating a “mismatched” set of defenses, the phytopathogen enhances its own performance on a host plant (Martel et al. 2021; Grant and Jones 2009a). One example of this is the cosmopolitan hemipteran whitefly *Bemisia tabaci*. During *B. tabaci* infestation of *Arabidopsis thaliana*, genes associated with the SA-signaling pathway are induced, while genes associated with JA-signaling are suppressed (Kempema et al. 2007; Zarate et al. 2007). SA-pathway activation causes Arabidopsis to be a better host for *B. tabaci,* as mutants that disrupt phytohormone perception and synthesis show that the JA-mediated responses, which are repressed, antagonize whitefly nymph development in *Arabidopsis* (Zarate et al. 2007; Kempema et al. 2007). This cross-talk between SA and JA in whitefly-Arabidopsis interactions has been verified by Zhang et al. (2013).

Our understanding of the genes regulated by different defense-signaling pathways, defense hormone cross-talk, and the differential activation/suppression of phytohormone pathways by phytopathogens with different infection and infestation strategies have largely been gleaned from model diploid systems such as Arabidopsis, tomato and rice (Nishimura and Dangl 2010; De Vleesschauwer et al. 2013; Berens et al. 2017; Yang et al. 2013). Far less is known about phytohormone-defense signaling non-model crops. To date, comprehensive transcriptome studies after exogenous SA treatments have been performed on *Psammosilene tunicoides* (a medicinal plant) and *Salvia miltiorrhiza*

*(*Chinese sage) (Zhang et al. 2016; Su et al. 2021a). In addition, exogenous MeJA treatments have also been performed on sugarbeet, ryegrass, and pigeon pea (Su et al. 2021b; Fugate et al. 2017; Du et al. 2021). To date, one comprehensive study of SA, JA, ET and ABA responses in a non-model crop (*Capsicum annum*) has been reported (Lee et al. 2020). Experiments done in non-model plant species would provide significant insights to the conversation and diversification of phytohormone responses in land plants. Polyploids and non-model plants might have evolved different signaling responses based on paralog duplication events or deletions, gene dosage, and neofunctionalization of signaling genes(Cheng et al. 2018; Conant et al. 2014; Birchler and Veitia 2014). In addition, it is not clear if the principles of phytohormone signaling established in model plants will be extended to non-model systems and if the cohort of genes that are SA, JA and dually or reciprocally regulated by SA and JA will be similar or distinct (Comai 2005; Flagel and Wendel 2009).

Among non-model plants, alfalfa (*Medicago sativa*) is a perennial tetraploid legume, which is a high-acreage and high-value seed crop that is a host for numerous pathogens and pests (Teuber et al. 1997). Prior to the development of alfalfa genomics resources (ie., transcriptomes and genomes) (Hawkins and Yu 2018; Li and Brummer 2012), all genomic and transcriptomic analyses were performed using the model legume *Medicago truncatula's* genome or its transcriptomes as references. This is due to high levels of synteny of *M. truncatula* with alfalfa (Li et al. 2014). While diploid and tetraploid alfalfa genomes were recently released (Li et al. 2020; Shen et al. 2020), the genomics resources available to understand this crop is, at best, underwhelming (Brummer 2004; Kumar 2011; Li and Brummer 2012; Hawkins and Yu 2018).

Regardless, there have still been transcriptomic studies performed on *Medicago truncatula* and alfalfa to understand their responses to (a)biotic stresses. Several *M. truncatula* transcriptomes have been analyzed to understand responses to abiotic stressors including salt stress, heat stress, drought, and aluminum toxicity (Chandran et al. 2008; Sańko-Sawczenko et al. 2019; Chen et al. 2021; Iyer et al. 2013; Vu et al. 2015; Gruber et al. 2009).

A limited number of transcriptomic studies have been performed to explore biotic stress response in *M. truncatula.* These studies explored the response of *M. truncatula* resistant to *Rhizoctonia* and *Fusarium oxysporum* and identified an upregulation of ERF transcription factors in resistant lines and an induction of cell wall metabolism genes, respectively (Thatcher et al. 2016; Anderson et al. 2018).

There have also been some transcriptomic studies performed on alfalfa to comprehend (a)biotic stress. Transcriptomes have been analyzed for salt, cold and aluminum stress along with several biotic stressors (aphids, thrips and bacterial stem blight) (Lei et al. 2018; Tu et al. 2018b; Tu et al. 2018a; Shu et al. 2017; Wang et al. 2016). Transcriptomes of aphid-resistant and -susceptible alfalfa plants at 72 h after spotted alfalfa aphid (*Therioaphis trifolii*) feeding were determined (Tu et al. 2018b); β-alanine, fatty acid degradation, flavonoid biosynthesis, and phenylalanine metabolism were correlated with aphid resistance in alfalfa. RNA-seq analyses of thrips (*Odontothrips loti*)-resistant and -susceptible alfalfa were also performed (Tu et al. 2018a). These studies focused on biochemical pathways that were deployed by both resistant and susceptible plants to thrips. The shared genes were enriched for KEGG terms associated with phenylpropanoid biosynthesis, linolenic acid metabolism and flavonoid biosynthesis. The authors presumed the changes in phenylpropanoid

biosynthesis and linolenic acid metabolism were correlated with the phytohormones SA and JA, respectively. In addition, transcriptomes of alfalfa that are resistant and susceptible after infection with the causal agent of bacterial stem blight *Pseudomonas syringae pv. syringae* were determined (Nemchinov et al. 2017). The 24- and 72-h transcriptomes showed an accelerated change in transcripts in resistant vs susceptible plants and showed that WRKYs were early responses to *Pss* in both resistant and susceptible plants (Nemchinov et al. 2017). There have also been some analyses of alfalfa after phytohormone treatments. SA treatments were found to relieve heat stress symptoms and exogenous ABA treatments were found to repress SA, JA, and ET signaling (Luo et al. 2019; Wassie et al. 2020). It is important to note that the identity of SA-, JA- and ET- biosynthesis and -response genes were inferred from the model plant Arabidopsis.

In Chapter 2, we described the first whitefly-responsive transcriptomes of whitefly-resistant and -susceptible alfalfa. We conducted a 22-d infestation time course with *Bemisia tabaci* MEAM1 on a resistant (UC2845-092 or “R1”) and a susceptible (UC2845-043 or “S1”) alfalfa lines. We collected time points associated with whitefly nymph development (0, 1, 7, 14, and 22 d post-infestation (dpi)) and assembled a *de novo* transcriptome. In our analysis, we identified the induction of ET signaling and a repression of SA, JA and ABA signaling, as well as repression of key signaling components associated with pattern-triggered immunity. We also showed that changes in genes associated with long-chain and very long-chain fatty acids were associated with R1 plants. These changes would impact cutin, and waxes of the cuticle and suberin within the cell wall. While we identified several DEGs orthologous to phytohormone-responsive *Arabidopsis* loci, we currently lack empirical evidence demonstrating that

these DEGs are phytohormone regulated in alfalfa. Considering the potential for alfalfa to face superabundant *B. tabaci* infestations and other pathogenic stressors in the face of climate change, more knowledge of alfalfa's hormone-signaling mechanisms would improve host-plant resistance in alfalfa (Curnutte et al. 2014).

In this study, we provide the first insights into alfalfa's phytohormone-signaling programs. Based the temporal expression of sentinel genes that are known to be SA or JA regulated in Arabidopsis and tomato, we identified two time points (1 and 8 h) to assess the number of differentially regulated genes (DEGs) after SA and JA treatment. To determine whether or not SA or JA is a major regulator of whitefly-responsive genes alfalfa, we compared the whitefly-responsive transcriptomes to the MeJA- and SA-responsive transcriptomes. We were able to determine the alfalfa transcriptome is more responsive to JA than SA and both hormones elicit defense responses rapidly. We also identify considerable overlap between hormones at both time points, particularly later. We did not find evidence of SA and JA cross talk at the timepoints chosen for study. Finally, we also show that relatively few alfalfa DEGs are responsive to both whitefly and SA/JA.

Methods

Plant Growth

The alfalfa genotype UC-2845-043 (S1) parent plants were maintained at 26°C, 55% relative humidity under long-day (12-h light:12-h dark) conditions. S1 was clonally propagated by stem cuttings (6-cm in length), which were dipped in Clonex (Hydronamics International; Lansing, MI) gel rooting media to promote rooting and dipped in Pyrentic insecticide to minimize transfer of any insect pests that the parent

plant acquired in the greenhouse environment. The stem cuttings were placed in a UC soil mix in a 2 x 2-inch well of a 72-well insert within a 1020 greenhouse tray (without holes) covered with a humidity dome (Growers Solution; Cookeville, TN). Cuttings were misted daily to promote the high humidity environment required for rooting. Domed vents were opened after cuttings had established roots (ca. 10 – 14 d) and domes were removed after 21 d. To assure stem cuttings were well watered during the root establishment period, wells were watered from the top. Stem cuttings with established root systems were grown in a growth room at 27°C, 35-50% relative humidity with a 12-h day:12-h night light cycle (300uM light) inside thrip-proof bug dorms (MegaView Science Company). Plants were fertilized with MiracleGro every two weeks. Plants were four- to six-weeks old from day of cloning at the time of treatment and had approximately three to five pairs of trifoliates.

Phytohormone Treatments

Four- to six-weeks old alfalfa plants were transported to a treatment room 24 – 48 h before treatments for acclimation to the new environment (26°C; 16-h light/8-h dark cycles; 200 – 300 μE). On the day of the treatments, 0-h samples were collected immediately. Plants to be treated with JA and SA were move to different locations to no cross-contamination of hormone treatments. Alfalfa plants were sprayed with either 100 μM SA (FisherSci, Waltham, MA) or 100 μM MeJA (Fisher Scientific, Waltham, MA) in a solution of 0.1% EtOH and 0.01% Tween 20 using a 100-ml spray bottle. Sprays were performed until droplets saturated the leaf surface (de Wit et al. 2013). Treated alfalfa leaves were then collected at 0.5, 1, 2, 4, 8, 12, and 24 h post-treatment. Four to six leaves from three plants were pooled at each time point (one biological replicate). Each treatment was repeated for a total of three biological replicates per time point. Treated

leaves were excised and placed in 50-mL Falcon tubes and immediately immersed in liquid nitrogen; leaf samples were stored at -80°C until use.

RNA Extraction

Leaves were ground to a fine powder in liquid N_2 using a mortar and pestle. Total RNAs were extracted from 50 mg leaves. After N_2 evaporation, 300 μL extraction buffer (100 mM LiCl, 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, 1% β-mercaptoethanol) at 80°C and 300 μL of water-saturated phenol (80°C) were added. After vortexing for 30 sec, 300 μL chloroform: isoamyl alcohol (24:1) were added. The sample was vortexed for 30 sec and centrifuged for 5 min. The aqueous layer was pulled and mixed with one volume of 4 M LiCl. After overnight precipitation at 4°C, total RNA was recovered by centrifugation for 20 min. The pellet was diluted in 250 μL DEPC water for 30 min. Diluted RNA was then mixed with 25 μL 5 M NaCl and 500 μL 100% ethanol and centrifuged for 20 min. Once the supernatant was removed, the pellet was mixed with 1 mL 70% EtOH and centrifuged for 20 min, resuspended in 40 μL water and incubated for 30 minutes at 4°C. All centrifugation was completed at 12,000 × *g* at 4°C. RNAs were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA quality was assessed using 1% denaturing agarose gels and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All RNAs were stored at −80°C.

Marker Gene RT-PCR

One μg of RNA in 2 μl DEPC-treated water was DNAse treated using the RQ1 RNAse-Free DNAse (Promega, Madison, WI) at 37°C for 30 min. The DNAse reaction was stopped using RQ1 RNAse-Free DNAse Stop Solution (Promega, Madison, WI) at

65°C for 10 min followed by 2 min at 4°C. 2 µM oligo dT per RNA were added to the DNAse reaction for a total volume of 25 µL and was incubated at 70°C for 5 min followed by 4°C for 5 min. The RNAs were reverse transcribed with Promega ImProm-II Reverse Transcriptase (Promega, Madison, WI) using 28µL ImPromII 5X, 4 mM $MgCl_2$, 10µM dNTPs, 5 µL reverse transcriptase, and DEPC-treated water to a volume of 100 µL. The reaction was incubated for 10 min at 25°C, 60 min at 42°C, 15 min at 70°C, followed by a 4°C chill. cDNAs were cleaned with phenol-chloroform DNA precipitation with TE-buffered phenol and chloroform. cDNAs were mixed with phenol and chloroform a ratio of 2:1:1. cDNAs were vortexed for 30 seconds and spun in a minicentrifuge at 14000 rpm for 20 min at 4°C. The supernatant was recovered and was mixed with 1 volume chloroform and vortexed for 30 seconds. The cDNA-chloroform mixture was spun in a minicentrifuge at 14000 rpm for 20 min at 4°C. The supernatant was recovered and 1/10 volume 3 M sodium acetate and 3 volumes of cold ethanol were added and the cDNAs incubated at -20°C for one hour. cDNAs were spun in a minicentrifuge at 14000 rpm for 15 min at 4°C and the pellet was recovered. The pellet was washed with 70% ethanol at 4°C at 14000 rpm for 15 min. The pellet was dried with a vacuum aspirator and bench dried for 10 min. The pellet was diluted in 100 µL TE buffer and incubated at 4°C overnight. cDNA concentrations were calculated using a Nanodrop. cDNAs were diluted to a working concentration of 300 ng/µl for RT-PCRs. Each cDNA for each sample was verified using a PCR of UBQ5 primers (Supplemental Table 3.1). RT-PCRs for each sample were performed twice. RT-PCRs were conducted using 5X GoTaq Reaction Buffer (Promega, Madison, WI) following manufacturer instructions and 0.5 µM of each

primer at a final reaction volume of 30 µL. PCRs were conducted using an initial denaturing at 95°C for 2 min, a denaturing at 95°C for 30 seconds, and a final extension at 72°C for 5 min followed by a hold at 12°C. Primer-specific annealing temperatures/times and extension times were customized for each sentinel gene (Supplemental Table 3.1). PCR products were run on a 2% TBE agarose gel at 150 V for 20 min.

RNA-seq library preparation, sequencing, and bioinformatics analyses

RNA-seq libraries were made from 0 h (untreated control plants), 1-h and 8-h SA treated, and 1-h and 8-h JA treated samples. Three biological replicates for each time point were used to construct libraries. cDNA libraries were prepared at the IIGB Genomics Core. Strand-specific cDNA libraries were prepared using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (New England BioLabs; Ipswich, MA) using an input of 1 µg of RNA in 50 µL DEPC-treated water. Samples were multiplexed using NEBNext® Multiplex Oligos for Illumina. Libraries were sequenced using the Illumina NextSeq 500 platform (single-end 75-bp reads) at the IIGB Genomics Core. Libraries were multiplexed (12 libraries/lane) and sequenced resulting in 19956728 reads per library (Supplemental Table 3.2) .

After trimming and fastq filtering, reads were used to construct a *de novo* transcriptome assembly. To enable comparisons between the phytohormone libraries (a total of 15 libraries) with the whitefly-infested samples from Chapter 2 (a total of 30 libraries), the 45 libraries were combined for *de novo* transcriptome assembly using Trinity under default parameters, except for k=2 (Grabherr et al. 2011b). Reads from each library were mapped to the *de novo* transcriptome using Bowtie2/2.2.5 and RSEM/1.3.1 (Li and Dewey 2011; Langmead and Salzberg 2012). Transcripts with an

average of less than 5 reads across the time course for a phytohormone treatment (SA or MeJA) or less than 10 reads across the whitefly treatment (S1-WF and R1-WF) were removed from their analyses. Separate analyses were performed for the phytohormone-dependent and whitefly-dependent genes identified in the transcriptome. DESeq2 was used to identify differentially-expressed gene (DEG) (Love, Huber et al. 2014). DEGs were defined at the $|\log_2 FC| > 1$ and FDR < 0.05 thresholds using the Benjamini Hochberg method.

Gene Annotation, Functional Analysis, and Ortholog Identification

DEGs were annotated using the Trinotate package and the following databases: Swissprot, Pfam, Mercator4 v2.0, Eggnog, HMMER, signalp, and tmHMM (Duvaud et al. 2021; Mistry et al. 2020; Schwacke et al. 2019; Almagro Armenteros et al. 2019; Huerta-Cepas et al. 2018; Eddy 2011, 2009, 2008; Krogh et al. 2001; Bryant et al. 2017) . Homologs of alfalfa DEGs were identified in *Medicago truncatμa* Mt4.0v1, and the *Arabidopsis thaliana* Araport11 genomes using BlastX. All NCBI-BLAST searches used an E-value cutoff at 10^{-5} for homolog identification. GO Term analysis was conducted using the Bioconductor package *goseq* (Young et al. 2010; Altschul et al. 1990).

Results

Sentinel genes identify early and late phases of phytohormone responses

Phytohormone treatment time-courses were performed to assess the temporal expression of two SA and two JA sentinel genes and established the early and late phases in phytohormone gene expression. Alfalfa (UC2845-043, S1) plants were treated with 100 μM SA or 100 μM JA and samples were collected at 0.5, 1, 2, 4, 8, 12, and 24

h. Based on expression profiles for *Arabidopsis* phytohormone-responsive genes in the Arabidopsis EFP Browser (Winter et al. 2007), two SA-responsive (*PHENYLALANINE AMMONIA LYASE 1* (*PAL1*) and *PATHOGENESIS RELATED 2* (*PR2*)) and two JA-responsive (*ARGINASE 2* (*ARAGH2*) and *LIPOXYGENASE 3* (*LOX3*)) genes were chosen for study. *PAL1* and *LOX3* were putative "early-response" and *PR2* and *ARAGH2* were putative "late-response" genes. The expression of these genes was examined in the SA- or JA-time course experiments by RT-PCRs using gene-specific primers (Supplemental Table 3.1). In the SA treatment, *PAL1* transcript levels peaked by at 2 - 4 h and was suppressed from 8 – 24 h. There was more variation in *PR2* RNA accumulation. In all three replicates, PR2 transcripts were was induced by 4 h and levels fluctuated throughout the remainder of the time course (Supplemental Figure 3.1). In the MeJA treatment, *LOX3* RNAs peaked at 2 h and was suppressed at later time points. In contrast, *ARAGH2* transcripts had a biphasic pattern of accumulation peaking within 1 -2 h and again later at 8 – 12 h. We determined the best time points for capturing early and late phytohormone responses were 1 and 8 h, respectively.

Transcriptome analyses

To understand alfalfa's SA- and JA- responsive transcriptomes and relate these responses to the whitefly-responsive transcriptomes described in Chapter 2, we assembled a *de novo* transcriptome using reads from the SA and JA treatment time courses, and the 30 whitefly-infested RNA-seq libraries from Chapter 2. Collectively, these 45 RNA-seq libraries represented 898,052,761 reads, averaging ~ 20 M reads per library (Supplemental Table 3.2). The *de novo* assembly produced 179,717 transcripts and 116,828 genes with a mean contig size of ~ 453 bp and a contig N_{50} of 1566. An average of 91.5% of reads from all 45 libraries mapped to the transcriptome (Table

Supplemental Table 3.2). After filtering for lowly expressed transcripts, 100,499 transcripts from phytohormone treatments and 49,331 transcripts in our whitefly-response analysis remained.

Alfalfa's SA and JA signaling transcriptome is distinct from *Arabidopsis*

We identified the number of phytohormone-responsive DEGs for each treatment and timepoint. DEGs were defined as transcripts with a |Log2-fold change (LFC)| > 1 and a FDR ≥ 0.05. After 1 h of phytohormone treatment, there were 432 and 891 upregulated DEGs responsive to SA or JA, respectively (Figure 3.1A; Supplemental Table 3.3). There were also 244 and 200 downregulated SA- or JA-responsive DEGs identified at the same time point. At 8 h, we identified 1543 upregulated SA-responsive DEGs and 1473 upregulated JA-responsive DEGs. At the same time point, we also identified 1890 and 2060 downregulated DEGs for the SA and JA treatments, respectively (Figure 3.1A; Supplemental Table 3.3). A total of 3730 and 4247 unique genes were identified as SA- or JA-responsive, respectively, representing a total 3.7% and 4.2% of the phytohormone-responsive transcripts analyzed (Figure 3.1B).

Many genes that are currently considered hallmarks of SA and JA signaling were present in out our phytohormone-responsive dataset (Peng et al. 2021a; Klessig et al. 2018; Wasternack and Song 2016). We identified upregulated transcripts of several SA sentinel genes (*CAMODULIN BINDING PROTEIN 60E/G* (*CBP60E/G*)*, PATHOGENESIS RELATED 2* (*PR2*)*, WRKY40/70, CONSTITUTIVE DISEASE RESISTANCE 1* (*CDR1*)*, GLUTAREDOXIN 9* (*GRXC9*)*,* and PHENYLALANINE AMMONIA-LYASE 1 (*PAL1*)) (Table 3.1) among our 1-h SA DEG; fewer of these SA sentinel genes were present in the 8-h SA dataset. For example, only identified *PAL1*, *CBP60G*, *CDR1*, and *PR2* transcripts were detected at both time point and *PAL1* as

downregulated at 8 h. There were no upregulated transcripts of the SA biosynthesis genes *ICS1/2* or the pipecolic acid biosynthesis genes *SARD4* or *ALD1.* However, there was downregulated *FMO1* transcript among the 8 h SA DEGs. *FMO1* is important for the synthesis of the mobile N-hydroxy-pipecolic acid that is the mobile SAR signal.

We observed a similar trend in the JA dataset. Hallmark JA-response genes were also identified in the 1-h JA dataset (*JASMONATE-ZIM DOMAIN 1 and 3* (*JAZ1/3*), ACYL-COA OXIDASE (*ACX1*), *LIPOXYGENASE 2 and 5 (LOX2/5)*) in our dataset, while we only identified *LOX1/2* as upregulated among the 8-h JA DEGs (Table 3.1).

We also looked for the SA and JA sentinel gene transcripts in the JA and SA treatment datasets, respectively, to understand if these sentinels were strictly regulated by SA or JA. Among our SA DEGs, we also identified an ortholog of the JA-responsive *LOX1* as an upregulated DEG at the 1 h time point. We also identified orthologs of SA-responsive TGA transcription factors (*TGA3/6*) as upregulated DEGs in the 8-h JA samples . While *TGA2/5* are linked to positive regulation of JA-mediated responses, the induction of *TGA3/6* was not anticipated based on the Arabidopsis (Guo et al. 2018).

This cursory analysis indicates that the SA and JA treatments were effective in inducing known hallmark genes and imply that the 1-h treatments were more likely to capture phytohormone defense responses. In addition, only a small proportion of the alfalfa transcriptome was SA or JA responsive.

To further understand the alfalfa's temporal responses to both SA and JA, we determine the number of DEGs regulated by SA, JA or co-regulated by both hormones at 1 and 8 h (Figure 3.2). After the 1-h treatment, a set of gene responsive to both SA and JA (coregulated genes) were identified with 262 upregulated DEGs and 22 downregulated DEGs (Figure 3.2A). These co-regulated DEGs constituted 24.7% and

5.2% of all up- or downregulated DEGs identified at 1 h. For the SA treatment, the SA/JA co-regulated genes represented a substantial part of the SA-induced reprogramming (60.6%), while these coregulated genes only represented 29.4% of the JA response. It is noteworthy that a majority of the downregulated 1-h DEGs were solely regulated by SA or JA.

At the 8-h time point, the magnitude of the overlap in the transcript profiles had changed substantially. There were 1125 up-regulated and 1093 downregulated DEGs (Figure 2B) regulated by both JA and SA. These DEGs constituted a greater proportion of upregulated (59.5%) and downregulated (37.3%) DEGs identified at 8-h. In fact, the number of SA/JA co-regulated genes exceeded the number of DEGs solely regulated by SA or JA alone.

In Arabidopsis, SA and JA often regulate defense genes in a reciprocal manner (Liu and Timko 2021; Yang et al. 2019a; Li et al. 2019). Although, SA and JA can also co-regulate genes in an additive or synergistic manner (Mur et al. 2006). To determine if alfalfa uses a phytohormone regulatory strategy similar to that deployed in Arabidopsis, we determined if there was reciprocal regulation of genes at 1 or 8 h post-phytohormone treatments. Surprisingly, there were no DEGs that were reciprocally regulated by SA and JA at either 1 h or 8 h (Figure 3.2). We can conclude that in alfalfa, SA and MeJA transcriptional reprogramming is different than the model plant Arabidopsis. Based on the 1-h and 8-h DEGs in alfalfa, DEGs are: (1) solely regulated by SA; (2) solely regulated by JA; (3) responsive to both phytohormones; and (4) not reciprocally regulated SA and JA.

GO Term Association of Phytohormone-Responsive DEGs

We utilized GO term analyses from *goseq* and REVIGO to garner an understanding of the biological processes associated with each treatment and time point. We identified 51 GO terms associated with SA-responsive upregulated DEGs at 1 h (Figure 3.3; Table 3.2; Supplemental Table 3.4). The GOs most-overrepresented encompass response to stress (FDR = 1.66E-07), response to fungus (FDR = 3.6E-05), and response to stimulus (FDR = 3.6E-05). We also identified GOs associated with phytohormones including regulation of SA metabolism (FDR = 0.01), response to SA (FDR = 0.04), and regulation of ABA-activated signaling pathway (FDR = 0.04).

A broader spectrum of responses were identified in upregulated 1-h JA-responsive DEGs with 161 enriched GO terms (Figure 3; Table 3.2; Supplemental Table 3.4). However, the top GO terms were similar to those associated with the 1-h upregulated SA DEGs. These included response to stress (FDR = 4.87E-24), oxazole or thiazole biosynthetic process (FDR = 4.87E-24), response to stimulus (FDR = 2.75E-18), and defense response (FDR = 4.44E-18). We also identified several GO terms associated with phytohormones among the 1-h upregulated JA DEGs including regulation of SA metabolic process (1.37E-06), ET-activated signaling pathway (3.9E-05), ABA-activated signaling pathway (FDR = 0.01), and regulation of JA-mediated signaling pathway (FDR = 0.01).

We found 12 enriched GO terms associated DEGs upregulated by both hormones at 1 h and ten of these GO terms were also over-represented among the DEGs that were responsive to SA or JA alone. These shared GO terms include response to stress (FDR = 4.72E-06), response to stimulus (FDR = 8.48E-04), response to abiotic stimulus (FDR = 9.07E-03), and response to other organism (FDR = 9.07E-03) (Figure 3.3; Table 3.3; Supplemental Table 3.3). Some of the 1-h DEGs that were

responsive to both SA and JA in the GO categories mentioned above include orthologs of *NDR1 HIN1 LIKE PROTEIN 13* (*NHL13*), *ARGONAUTE 4* (*AGO4*), *CAMODULIN BINDING PROTEIN 60E* (*CBP60E*), *PENETRATION 3 (PEN3), FERONIA* (*FER*), *CALMODULIN BINDING TRANSCRIPTION FACTOR 3* (*CAMTA3*) and *CONSTITUTIVE DISEASE RESISTANT 1* (*CDR1*) (Table 3.4).

At 8 h, 64 and 74 GO terms associated with upregulated SA- and JA-responsive DEGs, respectively, were enriched (Figure 3.4, Table 3.2; Supplemental Table 3.4). There were no GO terms associated with phytohormones among either SA or JA set of DEGs. Several remaining GO terms were associated with light, RNA biogenesis and synthesis/catabolism of metabolites. GO terms over-represented at 8 h among upregulated SA-responsive DEGs included: oxazole or thiazole biosynthetic process (FDR = 6.07E-48), thiamine metabolic process (FDR = 5.93E-31), pyrimidine-containing compound metabolic process (FDR = 1.92E-17), and vitamin biosynthetic process (FDR = 8.24E-13)(Figure 3.4; Table 3.2; Supplemental Table 3.4). We also identified similar GO terms among the upregulated JA-responsive DEGs at 8 h; the most over-represented terms included: oxazole or thiazole biosynthetic process (FDR = 1.67E-50), thiamine metabolic process (FDR = 1.22E-31), pyrimidine-containing compound metabolic process (FDR = 6.26E-20), and vitamin biosynthetic process (FDR = 3.20E-14). We found 76 GOs associated with DEGs co-regulated by both SA and MeJA and the most overrepresented GOs were similar to those found among SA-responsive and JA-responsive DEGs. Collectively, these data indicate that by 8 h after phytohormone treatments, alfalfa has reprogrammed its biosynthetic machinery to produce metabolites key to defense and recovery and SA, JA and SA/JA responsive transcripts are involved in this massive cellular reprogramming. Finally, there were three enriched GO terms with

links to defense: regulation of cellular defense (FDR = 3.73E-03), negative regulation of cellular defense response (FDR = 3.73E-03), and regulation of stomatal opening (FDR = 4.67E-04). Genes in these GO categories include *MPK3/6-TARGETED VQ-MOTIF-CONTAINING PROTEIN 1* and *3* (*MVQ1/3*), *PROTEIN KINASE 1B* (*PK1B*), *CHY ZINC-FINGER AND RING PROTEIN 1* (*CHYR1*), and *OPEN STOMATA 1* (*OST1*).

Surprisingly, we did not find any over-represented GO terms associated with the downregulated 797 SA- or 967 JA-responsive DEGs at 1 h. In contrast, 141 and 151 GO terms were over-represented among the 8-h SA- and JA-downregulated DEGs, respectively (Figure 3.5; Table 3.5; Supplemental Table 3.3). There was a major overlap in the GO term categories for SA-, JA- and SA/JA-responsive genes (Figure 5). Many terms were associated with lipids, light photosynthesis, and carbohydrate metabolism and we found several genes associated with growth and photosynthesis; several of these gene (*FBA1/2*, *RCA*, *LHCB3*, *SBPase*) are regulated by *TARGET OF RAPAMYCIN* (*TOR*), which is a master growth regulator antagonistic to phytohormone signaling (Dong et al. 2015).

The GO terms most-overrepresented among the downregulated SA-responsive DEGs at 8 h include pyruvate metabolic process (FDR = 2.75E-26), glycolytic process (FDR = 7.62E-19), circadian rhythm (FDR = 3.17E-15), and phospholipid biosynthetic process (FDR = 3.84E-14) (Figure 3.5; Table 3.2; Supplemental Table 3.4). The most over-represented GO terms among the downregulated JA-responsive DEGs at 8 h included photosynthesis, light harvesting (FDR = 4.03E-49), generation of precursor metabolites and energy (FDR = 3.71E-47), protein-chromophore linkage (FDR = 2.85E-42), and inositol biosynthetic process (FDR = 1.09E-34).

We identified 170 GO terms associated with DEGs downregulated by both SA and JA at 8 h. These DEGs include pyruvate metabolic process (FDR = 2.83E-33), glycolytic process (FDR = 2.12E-25), generation of precursor metabolites and energy (FDR = 4.88E-19), and phospholipid biosynthetic process (FDR = 7.84E-19). The overlap of the down-regulated molecular and cellular responses to SA, JA and both SA/JA was emphasized by the fact that 113 enriched GO terms were shared among these three groups of DEGs.

Given the large number of SA/JA-coregulated DEGs (2502), the overlap in GO terms in both down- and upregulated DEGs, it was important to determine if there were GO terms exclusively associated with DEGs solely responsive to SA or JA. There were no enriched GO terms associated exclusively among upregulated SA-responsive DEGs. However, we identified 127 enriched GO terms among DEGs that were only responsive to JA at 1 h including oxazole or thiazole metabolic process (FDR = 6.75 E-21), response to stress (FDR = 1.08E-16), and defense response (FDR = 1.88E-15) (Table 3.6; Supplemental Table 3.5).

We also found GO terms that were exclusively associated SA and JA for the 8-h downregulated DEGs. The only GO term found among the downregulated DEGs responsive to SA was protein tetramerization (FDR = 0.01). These genes include *PHOSPHOETHANOLAMINE/PHOSPHOCHOLINE PHOSPHATASE 1* (*PEPC1*) and *PHOSPHATE STARVATION-INDUCED GENE 2* (*PS2*). Whereas, there were 28 enriched GO terms found among the downregulated JA-responsive DEGs. The most over-represented included photosynthesis (FDR = 5.65E-56), light harvesting (FDR = 5.66E-56), protein-chromophore linkage (FDR = 7.39E-46), and generation of precursor metabolites and energy (FDR = 1.22E-27) (Table 3.6; Supplemental Table 3.3).

Collectively, the GO term enrichment analysis of phytohormone-regulated genes indicates that: (1) more GO terms are associated with JA treatment than SA; (2) defense-mediated responses are most extant at 1 h compared to 8 h and both phytohormones elicit defense responses; (3) GO terms at the 8-h time point are more closely associated with general metabolic processes, heterocycle metabolism, and RNA metabolic processes; and (4) DEGs downregulated at 8 h in both genotypes were associates with growth and carbohydrate metabolism. We can also conclude that based on enriched GO term categories, SA and JA-elicited defense responses in alfalfa have overlap.

There are similar expression profiles among co-regulated phytohormone-responsive DEGs

Given the fact that GO term enrichment analyses suggested there was some overlap in responses to the two phytohormones in alfalfa, we assessed the number of DEGs responsive to a single phytohormone or to both SA and JA. This analysis looked at up- and downregulated DEGs simultaneously. When 1-h and 8-h SA- and/or JA-responsive DEGs are pooled, 5506 DEGs were identified. Of these DEGs, 2471 DEGs were regulated by both hormones (Figure 6; Supplemental Table 3.4). The expression profile of these 2471 DEGs were organized by heatmaps into ten distinct k-means clusters (Figure 7; Supplemental Table 3.5). The clusters revealed that the SA/JA-responsive DEGs had six general patterns of gene expression. Four down-regulated clusters were identified (Clusters 1 to 4). Cluster 1 contains DEGs that were strongly downregulated by both SA and JA throughout the time course. Cluster 2 and 3 DEGs were strongly downregulated during at least one time point. Finally, Cluster 4 DEGs had modest upregulation at one time point.

The remaining six clusters had upregulated DEGs. While differing in the magnitude of upregulation at 1 and 8 h, Clusters 5, 6 and 7 had DEGs that were upregulated at 8 h. Cluster 9 DEGs were highly upregulated at one time point. Finally, Clusters 8 and 10 were highly upregulated by SA or JA throughout the time course.

For the 2471 DEGs regulated by both phytohormones, GO term enrichment was determined (Figure 7, Table Supplemental Table 3.5). There were no GO terms overrepresented among Clusters 1, 2 and 3, perhaps due to the smaller number of DEGs associated with this cluster, which totaled 76, 21 and 50, respectively. Cluster 4 with 1002 DEGs had 178 GO terms. These GO terms are associated with pyruvate metabolic process (FDR = 1.76E-35), inositol biosynthetic process FDR = (FDR = 2.40E-32), and glycolytic process (FDR = 3.85E-27) (Figure 7; Supplemental Table 3.6). Genes in these categories were associated with growth and photosynthesis and included *FBA1/2*, *PPDK* and *LCHB3/6* (Dong et al. 2015). We also identified the GOs callose deposition in cell wall (FDR = 4.31E-03) and several associated with reactive oxygen species (ROS) [(regulation of response to oxidative stress (FDR = 2.91E-04), positive regulation of response to oxidative stress (FDR = 1.48E-02), reactive oxygen species biosynthetic process (FDR = 4.93E-02)]. Genes in these clusters include *GLYCOLATE OXIDASE 2* (*GLO2*), *NITRATE REDUCTASE 1* and *2* (*NR1/2*), *TEMPERATURE-INDUCED LIPOCALIN-1* (*TIL*) and *ABC2 HOMOLOG PROTEIN 13* (*ATH13*) associated with ROS response.

The DEGs in clusters 5 and 6 differed primarily in the magnitude of their transcript changes and 71 and 26 GO terms were enriched in these clusters, respectively. The top four enriched GO terms in Clusters 5 and 6 were the same and included oxazole or thiazole metabolic process (FDR = 2.69E-25), thiazole biosynthetic

process, thiamine biosynthetic process, and pyrimidine-containing compound metabolic process (FDR = 1.84E-10) (Figure 7; Supplemental Table 3.6). Thiamine is a water-soluble vitamin found abundantly in green plants thiamine is essential to all kingdoms and has roles in enzymatic reactions and also functions as a cofactor for enzymes (Machado et al. 1996; Feng et al. 2019) Genes found in our GO category include *THIAMINE THIAZOLE SYNTHASE* (*THI1*) and ARF-GAP DOMAIN-CONTAINING PROTEIN 2 (*AGD2*). The remaining top enriched GO terms in Clusters 5 and 6 were distinct (Figure 9). Unique cluster 5 GOs include ncRNA processing (FDR = 9.24E-09), response to oxidative stress (FDR = 2.85E-04), regulation of cellular defense response (FDR = 3.93E-04) and negative regulation of cellular defense response (FDR = 3.93E-04). Unique cluster 6 GOs include regulation of stomatal opening (FDR = 4.91E-03), polysaccharide catabolic process (FDR = 2.07E-02), and cellular nitrogen compound biosynthetic process (FDR = 1.22E-02).

Finally, while Cluster 7 had an overall program of gene expression similar to Clusters 5 and 6, the DEGs associated with this cluster were distinct. There were 12 enriched GO terms identified. Top five GO terms were associated with the light and movement. These terms include rhythmic process (FDR = 7.72E-08), circadian rhythm (3.54E-04), circumnutation (FDR = 5.08E-04), multicellular organismal movement (FDR = 7.51E-04), and negative regulation of long-day photoperiodism, flowering (FDR = 1.18E-03). Several of the genes found in these GO terms are associated with the circadian clock and the temporal regulation of development including *COLD REGULATED PROTEIN 27* (*COR27*), *CONSTANS LIKE 9* (*COL9*), *TIMING OF CAB EXPRESSION 1* (*TOC1*), *JMJC DOMAIN CONTAINING PROTEIN 30* (*JMJ30*), and *EARLY FLOWERING 4* (*ELF4*) (Figure 7, Supplemental Table 3.6). From these data, we

can conclude DEGs upregulated by both phytohormones are associated with growth, metabolism, and defense while those downregulated by both phytohormones are associated with ROS response and metabolism.

SA-JA coregulated genes with sustained expression share a similar pattern of gene expression

There are 169 DEGs that respond to both SA and JA at both treatment timepoints (ie., 1 h and 8 h) and these DEGs could be either up or down regulated based on the analysis (Figure 8A; Supplemental Table 3.8). To assess if these co-regulated genes share the same or distinct temporal expression programs, the DEGs were organized by heatmaps and two k-means clusters were sufficient to explain the data. Surprisingly, none of these genes were down-regulated by SA or JA. All of the genes were up-regulated at both 1 and 8 h after SA or JA treatments (Figure 8B). The two clusters served to separate genes with different overall magnitudes of gene expression with one cluster ranging from 0 – 10 fold and the other ranging from 10 to 20-fold. Upon further exploration of these DEGs, we found no enriched GO terms associated with this core of JA and SA co-regulated DEGs.

The alfalfa-whitefly response is largely independent of JA and SA.

Chapter 2 described the transcriptome analyses of susceptible (S1) and resistant (R1) alfalfa after infestation by *Bemisia tabaci* MEAM1. Given the roles of SA and JA in modulating defenses during whitefly infestation of Arabidopsis (Kempema et al. 2007; Zarate et al. 2007), we wanted to investigate if alfalfa's whitefly response was correlated with alfalfa's SA- or JA-responsive genes. In Chapter 2, we inferred the functions and regulation of alfalfa's hormone-and defense-regulated DEGs based on orthologs in Arabidopsis. We concluded that whitefly resistance was largely driven by genotype, with many ABA-, SA- and JA-regulated defenses being downregulated in the resistant R1

alfalfa, while ethylene-associated responses were positively correlated with alfalfa's whitefly resistance.

With an understanding of alfalfa's response to SA and JA, we turned to elucidating the role of SA and JA in alfalfa's whitefly response. This analysis presumes that the SA- and JA-responsive genes identified in the whitefly-susceptible S1 described in this Chapter will be regulated in a similar manner in the whitefly-resistant R1. This is a reasonable assumption, since S1 and R1 have a similar parentage (Chapter 1). New de novo transcriptome assemblies were needed to allow the comparisons of the whitefly time-course with the SA and JA treatment time-courses. As anticipated, the number of DEGs induced and suppressed in R1 and S1 plants showed similar patterns to that seen in Chapter 2's Figure 5 and 6 (Figures 9 and 10). Overall, the expression profile of gDEGs remained unchanged throughout the infestation and the number of up- and downregulated DEGs was similar. Additionally, the tDEG expression profile was also similar, with the exception of more downregulated tDEGs in S1 at 22 dpi.

Of the whitefly-responsive gDEGs, small numbers of genes were SA and/or JA responsive. Overall, the numbers of upregulated and downregulated SA and JA regulated gDEGs did not change dramatically over the 22-d whitefly infestation time course (Figure 11A; Supplemental Table 3.9). For example, the number of upregulated SA-responsive gDEGs ranged 62 to 113 and the numbers of upregulated JA-responsive gDEGs were similar, ranging from 69 to 125 Phytohormone-responsive downregulated gDEGs followed a similar pattern of expression, although the numbers were larger than upregulated gDEGs (Figure 11A). The upregulated and down-regulated gDEGs responsive to SA or JA made up a small percentage of the total whitefly-responsive gDEGs in alfalfa. For example, largest number of phytohormone upregulated and

downregulated gDEGs were identified in the 22-dpi and 14-dpi samples, respectively. There were slightly more JA-responsive DEGs than SA-responsive DEGs at these timepoints. Therefore, the upregulated JA gDEGs at 22 dpi (5.3%) and downregulated JA gDEGs at 14 dpi (9.3%) provide maximal estimates of the whitefly-responsive DEGs that were phytohormone responsive at any timepoint in the infestation (Figure 11B). Collectively, these data suggest that a relatively small proportion of alfalfa's genome response to whitefly infestation is regulated by SA and/or JA.

Based on this data, we can make several general conclusions: (1) few whitefly-responsive gDEGs were SA-, JA- or SA/JA -responsive, (2) there is very little modulation in the number or percentage of whitefly gDEGs that are also phytohormone-responsive throughout the whitefly time course, and (3) a small number of gDEGs are induced by SA, JA, and whiteflies, with some of these genes having roles in defense.

In the analysis above, we identified phytohormone-responsive gDEGs using the entire set of SA- and JA-response DEGs (both 1-h and 8-h DEGs that were up- or downregulated). As the majority of hormone-responsive DEGs with GO terms associated with defense-related functions were identified in the 1-h SA and JA treatments, we used up- and down-regulated DEGs from the 1-h SA and 1-h JA timepoints to identify SA-responsive and JA-responsive gDEGs. These data are a subset of the data in Figure 11 (Supplemental Table 3.10). The temporal profiles of the SA-responsive and JA-responsive gDEGs were different. Overall, the number of up-regulated SA- and JA-responsive gDEGs increased over time (Figure 12). In contrast, the number of down-regulated SA- and JA-responsive gDEGs were highest in 0- and 1-h dpi. In addition, it is clear that JA has the potential to regulate a larger number of gDEGs than SA at each infestation timepoint.

With knowledge of the SA- and JA-responsive gDEGs in our transcriptome, we wanted to see if there was any correlation between expression in response to whitefly and our phytohormone treatments. Chapter 2 and Figure 3.9 established that the majority of gDEGs were expressed both pre-and post-infestation. Therefore, we focused on an early time after whitefly infestation (1 dpi). We correlated the magnitude and directionality of 1-h SA-responsive gDEGs, 8-h SA-responsive gDNAs and 1-dpi whitefly gDEGs (Figure 13); a similar analysis was performed for JA-responsive gDEGs. We found a moderate correlation between gene expression in response to either SA (R_2 = 0.52, $p < 0.05$) or JA (R_2 = 0.56, $p < 0.05$) throughout our phytohormone treatment (Figure 13). In contrast, the JA-whitefly analysis showed there was a weak, negative correlation in the JA-whitefly gDEG comparison (R_2 = -0.17, $p < 0.05$). However, for the SA-whitefly gDEG analysis, a weaker, negative and non-significant correlation was detected (R_2 = -0.06, $p = 0.17$). We can conclude that the genotype response to whitefly infestation in our transcriptome was negatively correlated to JA, which supports the hypothesis in Chapter 2 that ET is a major player in the response to whitefly.

tDEGs show a similar response to hormones

While time is not a strong determinant of resistance in alfalfa-whitefly interactions, there still might be different phytohormone-associated temporal responses to whiteflies in R1 and S1 alfalfa. With this in mind, we identified whitefly-responsive temporal DEGs (tDEGs) in the Chapter 3 *de novo* assembly (Figure 14A). The number of tDEGs at each timepoint in R1 and S1 plants was similar to the tDEG profile analyzed in Chapter 2 (Figure 3.10), with the exception of a larger number of downregulated tDEGs in S1 (Figure 3.14A). The whitefly-responsive tDEGs that were phytohormone-responsive were identified (Figure 13.4B; Supplemental Table 3.11). Across the 22-d

whitefly-infestation period, there were a small number of tDEGs that were up- or down-regulated by SA or JA. When both R1 and S1 tDEGs were examined, no more than 34 tDEGs were phytohormone upregulated at any timepoint. Similar trends were seen for down-regulated tDEGs. Even smaller numbers of phytohormone-responsive down-regulated tDEGs were identified at 1, 7 and 14 dpi and these numbers increased at 22 dpi, ranging from 17 to 30 in RI and 68 to 97 in S1. Overall, the phytohormone-responsive tDEGs are a small percentage of the whitefly tDEGs identified, with no more than 12.7% of the tDEGs (S1 7-dpi) being phytohormone responsive at any one infestation timepoint.

We also determined if any tDEGs in S1 or R1 were coregulated SA and JA. In the S1 line, we did not identify any upregulated tDEGs that co-regulated by SA and JA (Supplemental Table 3.11). However, in the R1 line, we identified three upregulated tDEGs responsive to both SA and JA: a protein kinase superfamily protein (*PBL37*), a CHY-type/CTCHY-type/RING-type Zinc finger protein (*AT5G25560*), and *SUGAR TRANSPORT PROTEIN 7* (*STP7*). From these data, we can conclude there are: (1) few phytohormone-responsive tDEGs in our dataset, (2) more phytohormone-responsive tDEGs in S1 than in R1, (3) more phytohormone-responsive tDEGs later in S1 alfalfa-whitefly treatment (22 dpi) than at the earlier time points, and (4) more downregulated than up-regulated phytohormone-responsive tDEGs in S1.

Similar to the whitefly gDEG analyses (Figure 3.12), we examined the number of tDEGs that were also DEGs after 1 h of SA or JA treatment (Figure 3.15). A very small number of upregulated and down-regulated tDEGs were SA or JA responsive, in the R1 line (Figure 3.15; Supplemental Table 3.12); the largest number of up- and downregulated tDEGs was identified at 1 dpi and 22 dpi, respectively, and were JA-

responsive. In S1, we saw a similar trend with very small numbers of up- and downregulated tDEGs at 1, 7 and 14 dpi that were SA- or JA-responsive. However, there were significantly higher numbers of downregulated tDEGs at 22 dpi (Figure 3.15).

As with our phytohormone responsive gDEGs, we wanted to determine if there was a correlation between the alfalfa response to phytohormones and whiteflies in the tDEG dataset. In R1, we found there was no significant correlation between whitefly response and response to SA (R_2 = 0.05, p = 0.80) or JA (R_2 = 0.03, p = 0.20) (Figure 3.16). In S1, we found a weak negative correlation between the SA response and whitefly response (R_2 = -0.26, p < 0.05), but did not find this correlation among tDEGs responsive to JA (R_2 = -0.02, p < 0.77) (Figure 3.16). We can conclude there is no correlation between phytohormone response and whitefly response in R1, but there is a weak, negative correlation between SA and whitefly response in S1. This likely points to S1 utilizing a defense response antagonistic to SA over time in response to whitefly.

Among all the upregulated SA-responsive and MeJA-responsive tDEGs (Figure 14), we identified only one DEG that was co-regulated by SA and JA in R1 (AT2G28940, PBL37) (Supplemental Table 3.12). PBL37 is member of a protein kinase superfamily protein and has a probably role in immune signaling (Rao et al. 2018). From these data, we can conclude (1) few gDEGS and tDEGs are responsive to SA or JA within 1 h, (2) a larger proportion of the tDEGs in S1 are also phytohormone-responsive DEGs at 1 h than those in R1, (3) a larger proportion of phytohormone-responsive tDEGs are JA responsive at 1 h in R1, (4) the S1 line had more upregulated tDEGs later (22 dpi) than at earlier time points, (5) R1 had an induction of phytohormone-responsive tDEGS after 1 d, particularly 1 h JA-responsive tDEGs, and (6) there were weak, negative

correlations between JA-whitefly response among gDEGs and SA-whitefly among tDEGs in S1.

Discussion

Phytohormone signaling is an essential component of plant defense (Huot et al. 2014; Erb et al. 2012; Pieterse et al. 2009; López et al. 2008). The two phytohormones, SA and JA, are well-known hallmarks of defense against phytopathogens and/or pests (Loake and Grant 2007; Yang et al. 2019a; Spoel and Dong 2008). SA is associated with defenses against biotrophic pathogens, while JA is associated with defenses against herbivorous insects and necrotrophic pathogens (Glazebrook 2005; Erb et al. 2012). SA and JA often have an antagonistic relationship with each other in defense, but there are instances when they function additively or synergistically to control gene expression (Liu and Timko 2021; Yang et al. 2019b; Li et al. 2019; Klessig et al. 2018). While there is a considerable amount of knowledge known about the SA- and JA-signaling pathways and associated genes in the model plants Arabidopsis, tomato and rice (Yang et al. 2013; Tamaoki et al. 2013; Peng et al. 2021b; Liu and Timko 2021; Zhang et al. 2020; Wasternack et al. 2006; Lefevere et al. 2020), there is a paucity of such data in non-model plants. In addition, there is virtually no knowledge about phytohormone-responsive genes in alfalfa and if these genes share analogous functions with Arabidopsis.

As a tetraploid, the increased number of loci and allelic variation provides a more complex genetic system for alfalfa breeders and molecular biologists than encountered in diploid systems (Hawkins and Yu 2018). Alfalfa is more likely to have loci that have neofunctionalized or become nonfunctional than a diploid capable of incrossing (Comai 2005). Therefore, it is possible that alfalfa's transcriptome response to SA and JA could

be profoundly different to model plants, where these responses have been studied to date. Due to the fact that few phytohormone time-courses have been conducted and analyzed from crops (Lee et al. 2020; Su et al. 2021b; Zhang et al. 2016) and the abundance of rigorous data in the model plant Arabidopsis (Peng et al. 2021a; Liu and Timko 2021; Peng et al. 2021b; Zhang et al. 2020; Zhang and Li 2019; Yang et al. 2019b; Ruan et al. 2019; Yang et al. 2019a; Volodarsky et al. 2009), the hormone responsiveness of genes in crops is often inferred from studies in Arabidopsis. This translational approach has been used extensively in the field of plant pathogen/pest interactions (Studham and Macintosh 2012; Tzin et al. 2017; Jacques et al. 2020; Li et al. 2016).

In Chapter 2, we used orthologs from Arabidopsis to identify putative phytohormone regulatory programs in whitefly-resistant R1 and -susceptible S1 plants. We concluded that induction of ET-regulated responses and suppression of SA, JA, and ABA, as well as PTI, appears to orchestrate alfalfa's resistance response to whitefly (Chapter 2). To provide empirical evidence for the response of alfalfa genes to SA and JA, we established transcriptome reprogramming at 0, 1 and 8 h after SA or JA treatments. These studies provided snapshots of putative early and later responses to these central defense hormones. We preformed these studies in the whitefly-susceptible line S1. We presume that the SA and JA-mediated responses in S1 and R1 plants will be similar since these lines are derived from the same population (UC-2845). We leveraged these data to assess the contributions of both SA and JA to whitefly-resistant and -susceptible plant responses to whitefly infestation.

The phytohormone treatments of S1 revealed several general principles about alfalfa's response to SA and JA. In response to SA and JA, 3.7% (3730 DEGs) and 4.2%

(4247 DEGs) of the alfalfa transcriptome was altered. This is within the lower range of transcriptome responses (4-20%) to JA or JA agonists observed in Arabidopsis (Yang et al. 2017; Hickman et al. 2017; Pauwels et al. 2008) and salicylic acid where 14% of the genome is responsive to SA early in a manner independent of NPR1 (Blanco et al. 2009). The alfalfa response was more robust relative to maize (Wang et al. 2017; Wu et al. 2013) and rice (Garg et al. 2012), where 1 – 2% of the transcriptome observed was SA- or JA-responsive. Finally, alfalfa's transcriptome remodeling response to SA and JA pales relative to the responses of pepper (*Capsicum annuum*). In pepper, 6% and 10% of the transcriptome was SA- or JA-responsive, respectively (Lee and Choi 2013).The difference in the number of DEGs responsive to phytohormones in alfalfa and other plants can be attributed to a number of factors including: treatment conditions (ie., hormone concentrations, treatment times, method of hormone application), plant age and growth conditions, inherent differences in the ability of each species to take up and perceive SA and JA, and parameters set for the transcriptome analyses.

The temporal responses to SA and JA in alfalfa were distinct as evidenced by the magnitude of the response (ie., number of DEGs), identity of DEGs and overlap of the transcriptome responses at 1 and 8 h. Furthermore, GO term enrichment analyses emphasized the differences in the 1-h and 8-h SA- and JA-responsive transcriptomes. At 1 h, enriched GO terms associated with SA- and JA-upregulated DEGs were distinct. While some GO terms were shared, others were hormone-specific or the number of DEGs associated with a GO term differed substantially (Figures 3.3 – 3.5). In addition, many GO terms enriched in the 1-h upregulated DEGs focused on defense-associated processes. However, these upregulated GO terms were deprioritized by 8 h and insights into the biochemical gene reprogramming that occurred in response to SA and JA were

gleaned. Both treatments upregulated heterocycle metabolism (nucleic acid metabolism process, thiazole biosynthetic process, thiamine biosynthesis process), pointing to essential metabolic processes (Feng et al. 2019). Whereas, GO terms associated with 8 -h downregulated DEGs from both phytohormone treatments were associated with photosynthesis and metabolism. It is clear that by 8 h after SA and JA treatments, alfalfa plants were attempting to return to the homeostasis of its resting state. Unfortunately, as there are few studies that have compared SA and JA transcriptomes using the same treatment conditions and plant age. For this reason, there are few current datasets that shed light onto whether the temporal responses we see in alfalfa are iterated in other plants. While these studies have been performed in *Capsicum*, only the raw data is available (Lee et al. 2020).

Two other important discoveries about alfalfa's SA and JA responses were revealed. First, numerous genes were responsive to both phytohormones (the SA/JA coregulated DEGs). Second, there was little evidence for a cohort of genes that were reciprocally regulated by SA and JA. At both 1 and 8 h after SA and JA treatments coregulated genes were identified and these DEGs constituted 25% (262 DEGs) to 60% (1125 DEGs) of the phytohormone-regulated DEGs at 1 and 8 h, respectively. Evidence for genes that are co-regulated by both SA and JA is not abundant in the literature but there are examples. A recent meta-analysis of Arabidopsis transcriptome data by Zhang et al. (2020) showed identified genes responsive to both SA and JA. They identified 363 and 2608 genes that were up- and down-regulated by both SA and JA Another example are the *PR* genes in cassava, many of which are both JA and SA responsive (Irigoyen et al. 2020), unlike their counterparts in Arabidopsis or tomato. Finally, *PR1* and *WRKY45* are positively regulated by both SA and JA in rice, and *WRKY89* in poplar is negatively

regulated by both phytohormones (Tamaoki et al. 2013; Jiang et al. 2016). However, there is evidence of SA-JA synergism in rice (Yang et al. 2013).

Second, we did not identify SA-JA crosstalk in alfalfa, as at both 1 and 8 h, we found no reciprocity between DEGs induced or repressed by either phytohormone. This is unique as other systems that explore plant responses to insect pathogens have pointed to phytohormone crosstalk in multiple plant species. For example, the meta-analysis of Zhang et al (2020) identified 1646 genes that were reciprocally regulated by SA and JA. Furthermore, Kempema et al. (2007) showed that whiteflies take advantage of SA-JA crosstalk in Arabidopsis to promote their success through the induction of SA-regulated defenses and repression of JA-regulated defenses. Similar antagonistic phenomena have been seen in tobacco between JA-ABA and JA-ET crosstalk in tobacco (Lackman et al. 2011; Onkokesung et al. 2010), and JA-ET crosstalk in rice (Ma et al. 2020). While there is reason to believe there is no crosstalk between SA and JA in alfalfa, it is possible that SA-JA crosstalk occurs in the earlier time points (0.5 to 2 h) after hormone treatments.

This variation in SA and JA responses may not be surprising as there is a precedent of defense gene orthologs having different expression programs in different Arabidopsis accessions. van Leeuwen et al. (2007b) and Proietti et al. (2018) identified SA-responsive genes that had different temporal, magnitude and directionality responses when seven Arabidopsis accessions were examined in 21 pairwise comparisons. Also, Proietti et al. (2018) compared SA-JA and ABA-JA crosstalk in 360 different accessions and loci associated with variations in crosstalk were identified (van Leeuwen et al. 2007a; Proietti et al. 2018).

Therefore, synergism between hormones in other species is plausible and has been hinted at in other studies. SA and JA induction have been linked to mitigating *H. virescens* damage in *Arabidopsis* and in enhanced ROS response (Mur et al. 2006; Schweiger et al. 2014). While there is some preliminary evidence of coregulation of genes by SA/JA in alfalfa, this needs to be tempered by the fact the concentrations of phytohormones used were adapted from *Arabidopsis* and tomato (de Wit et al. 2013; Garceau 2021). Additionally, some crops like rice produce higher levels of a phytohormone which might confound results depending on the crop (Kakei et al. 2015; Silverman et al. 1995). Modifying the concentrations of SA/JA used might elicit different responses and might yield different DEGs and temporal responses.

While the SA and JA-dependent transcriptomes indicate that there is little reciprocity in SA- and JA-regulated responses, it is possible that one or both signaling pathways control important defense traits associated with alfalfa's resistance mechanism in R1 plants or in the basal immunity displayed in S1 plants. Having identified a cohort of genes that respond to SA and JA treatments, we used these data to interrogate our alfalfa-whitefly transcriptomes and determine the proportion of the whitefly-regulated transcriptome that is SA and/or JA dependent. Over the whitefly-infestation time course of R1 and S1 plants, a small but consistent proportion of gDEGs (~4%) were SA- or JA-responsive and more downregulated gDEGs were responsive to SA (6.8%) or JA (8.3%). When we correlated the phytohormone response to the response to whiteflies with our gDEGs, we found a weak, negative correlation between JA and whitefly response. Considering in Chapter 2, we postulate ET is the phytohormone responsible for conferring whitefly resistance in alfalfa and there is evidence of crosstalk between JA and ET (Ma et al. 2020; Onkokesung et al. 2010), these data supports our hypothesis.

While alfalfa's whitefly resistance is primary associated with genotype and is not time dependent, it is possible that SA- and JA-regulated DEGs expressed at distinct times during whitefly infestation of R1 and S1 plants are important in HPR or basal immunity, respectively. Again, very small number of SA or JA responsive tDEGs were identified. In addition, while there were no correlations between phytohormones and whitefly-resistance response in R1, there was a weak, negative correlation between SA and whitefly tDEGs in S1 alfalfa. Collectively these data indicate that SA and JA have a limited role in alfalfa's response to *B. tabaci* in either whitefly-resistant or -susceptible plants.

In closing, the interrogation of alfalfa SA- and JA-responsive transcriptomes has provided one of the first insights into phytohormone signaling in non-model tetraploid plants. These are novel discoveries emphasized for additional studies. The experiments in Chapter 3 were fiscally constrained and limited us to the analysis of only two time points (0, 1 and 8 h) and a single SA and JA concentration. Such studies would benefit immensely from a high-resolution analysis of SA and JA responses in alfalfa or another plant. To date, only Pauwels et al. (2008) have performed such a study using MeJA in Arabidopsis collecting data at 15 timepoints over a 16-h period. This fine time resolution and their use of mutants in signaling pathways allowed for informative JA networks and subnetworks to be established. Our obvious second constraint is the total lack of defense mutants in alfalfa that are needed to verify our findings.

In the future, we would like to test our hypothesis that elevated ET defenses are associated with whitefly resistance. ET treatments of S1 plants may allow us to test this hypothesis. If mutants in JA, SA, ET, and ABA perception and signaling were available in alfalfa, they would be a fantastic resource to test our hypothesis. However, in the

absence of these genetic resources, other strategies will need to be pursued. By utilizing additional phytohormones (ie., ABA, ET) treatments, performing higher resolution time courses for SA, JA, ET and ABA, and possibly testing our hypothesis in a model species such as *Medicago truncatula*, we should be able to test our hypothesis that ET is associated with whitefly resistance in alfalfa.

In addition, we have made one inherent assumption in our studies. We have assumed that the cohort of SA- and JA-responsive genes in S1 and R1 alfalfa are the same. Given their similar heritage, this assumption is valid, but should be verified. Optimally, phytohormone treatments of R1 plants determine if the phytohormone response in both R1 and S1 is the same or if they also have distinct phytohormone responses. Together, the data presented provide a better understanding of phytohormone responses in alfalfa, how these responses compare to other plant species, and how they also relate to alfalfa's response to pests and pathogens.

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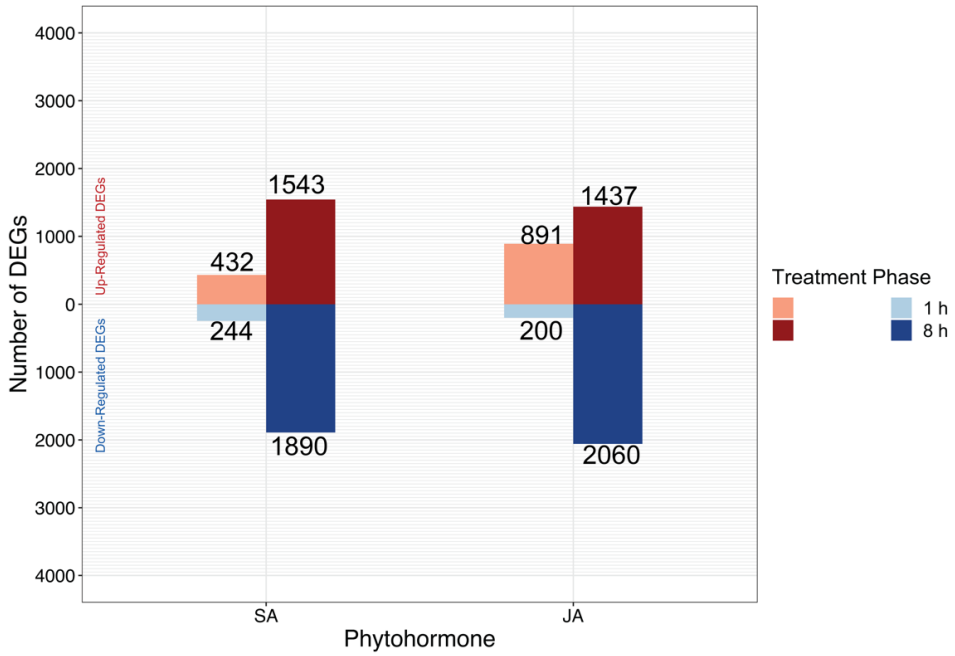
A


4000
3000
2000
1000
0
1000
2000
3000
4000
Number of DEGs
Up-Regulated DEGs
Down-Regulated DEGs
1543
432
244
1890
1437
891
200
2060
SA
JA
Phytohormone
Treatment Phase
1 h
8 h

B

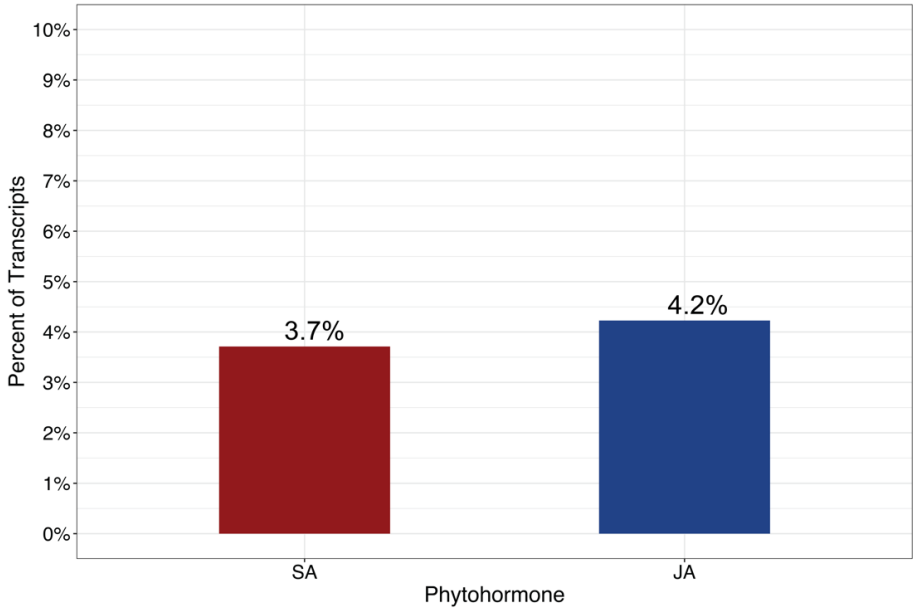
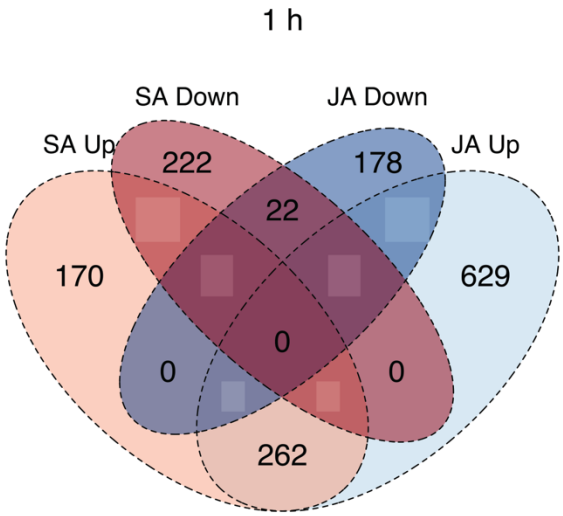

10%
9%
8%
7%
6%
5%
4%
3%
2%
1%
0%
Percent of Transcripts
3.7%
4.2%
SA
JA
Phytohormone

Figure 3.1 Bar plot of Up- and Downregulated DEG Counts and Percentages for Alfalfa – Phytohormone Transcriptome Analysis.

Bar plots show (A) number of SA and MeJA-responsive DEGs at each time point, (B) percentage of phytohormone DEGs in the phytohormone-dependent transcriptome for each phytohormone.

A


1 h
SA Down
JA Down
SA Up
JA Up
222
178
22
170
629
0
0
0
262

B

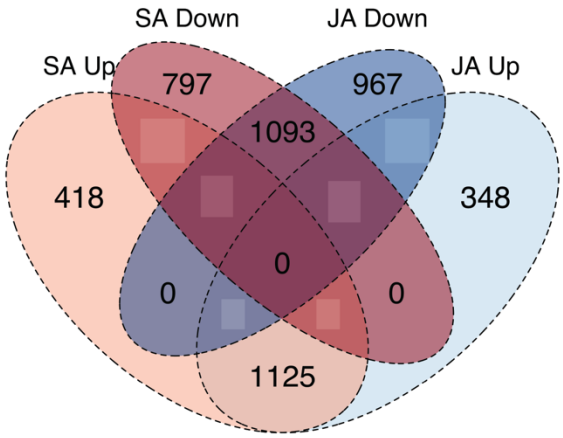

8 h
SA Down
JA Down
SA Up
JA Up
797
967
1093
418
348
0
0
0
1125

Figure 3.2 SA- and JA-responsive DEGs in whitefly-susceptible alfalfa.

The numbers of up- and downregulated SA- and JA-responsive DEGs at 1 h (A) and 8 h (B) after treatments.

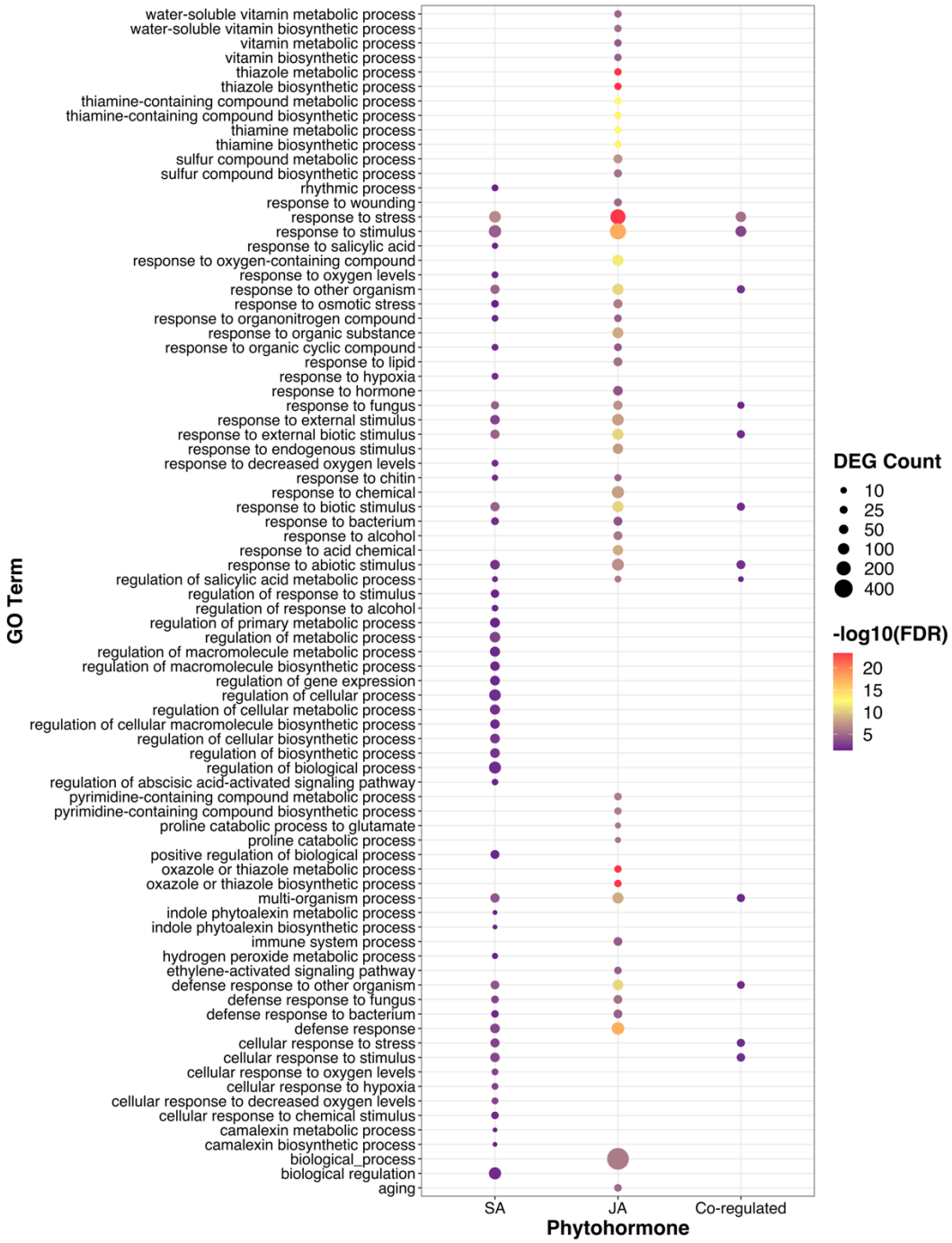

water-soluble vitamin metabolic process
water-soluble vitamin biosynthetic process
vitamin metabolic process
vitamin biosynthetic process
thiazole metabolic process
thiazole biosynthetic process
thiamine-containing compound metabolic process
thiamine-containing compound biosynthetic process
thiamine metabolic process
thiamine biosynthetic process
sulfur compound metabolic process
sulfur compound biosynthetic process
rhythmic process
response to wounding
response to stress
response to stimulus
response to salicylic acid
response to oxygen-containing compound
response to oxygen levels
response to other organism
response to osmotic stress
response to organonitrogen compound
response to organic substance
response to organic cyclic compound
response to lipid
response to hypoxia
response to hormone
response to fungus
response to external stimulus
response to external biotic stimulus
response to endogenous stimulus
response to decreased oxygen levels
response to chitin
response to chemical
response to biotic stimulus
response to bacterium
response to alcohol
response to acid chemical
response to abiotic stimulus
regulation of salicylic acid metabolic process
regulation of response to stimulus
regulation of response to alcohol
regulation of primary metabolic process
regulation of metabolic process
regulation of macromolecule metabolic process
regulation of macromolecule biosynthetic process
regulation of gene expression
regulation of cellular process
regulation of cellular metabolic process
regulation of cellular macromolecule biosynthetic process
regulation of cellular biosynthetic process
regulation of biosynthetic process
regulation of biological process
regulation of abscisic acid-activated signaling pathway
pyrimidine-containing compound metabolic process
pyrimidine-containing compound biosynthetic process
proline catabolic process to glutamate
proline catabolic process
positive regulation of biological process
oxazole or thiazole metabolic process
oxazole or thiazole biosynthetic process
multi-organism process
indole phytoalexin metabolic process
indole phytoalexin biosynthetic process
immune system process
hydrogen peroxide metabolic process
ethylene-activated signaling pathway
defense response to other organism
defense response to fungus
defense response to bacterium
defense response
cellular response to stress
cellular response to stimulus
cellular response to oxygen levels
cellular response to hypoxia
cellular response to decreased oxygen levels
cellular response to chemical stimulus
camalexin metabolic process
camalexin biosynthetic process
biological_process
biological regulation
aging
GO Term
SA
JA
Co-regulated
Phytohormone
DEG Count
10
25
50
100
200
400
-log10(FDR)
20
15
10
5

Figure 3.3 Enriched GO terms associated 1-h upregulated phytohormone-responsive DEGs.

Dot plots display the top 50 biological process GO terms for SA-responsive, JA-responsive, and SA/JA co-regulated DEGs that were upregulated after 1 h of phytohormone treatment. GO Terms were identified using the *goseq* package using a false discovery rate of 0.05 and the Benjamini Hochberg method. FDR values are displayed as a -log10(FDR). The number of DEGs associated with each GO term are indicated by shape size.

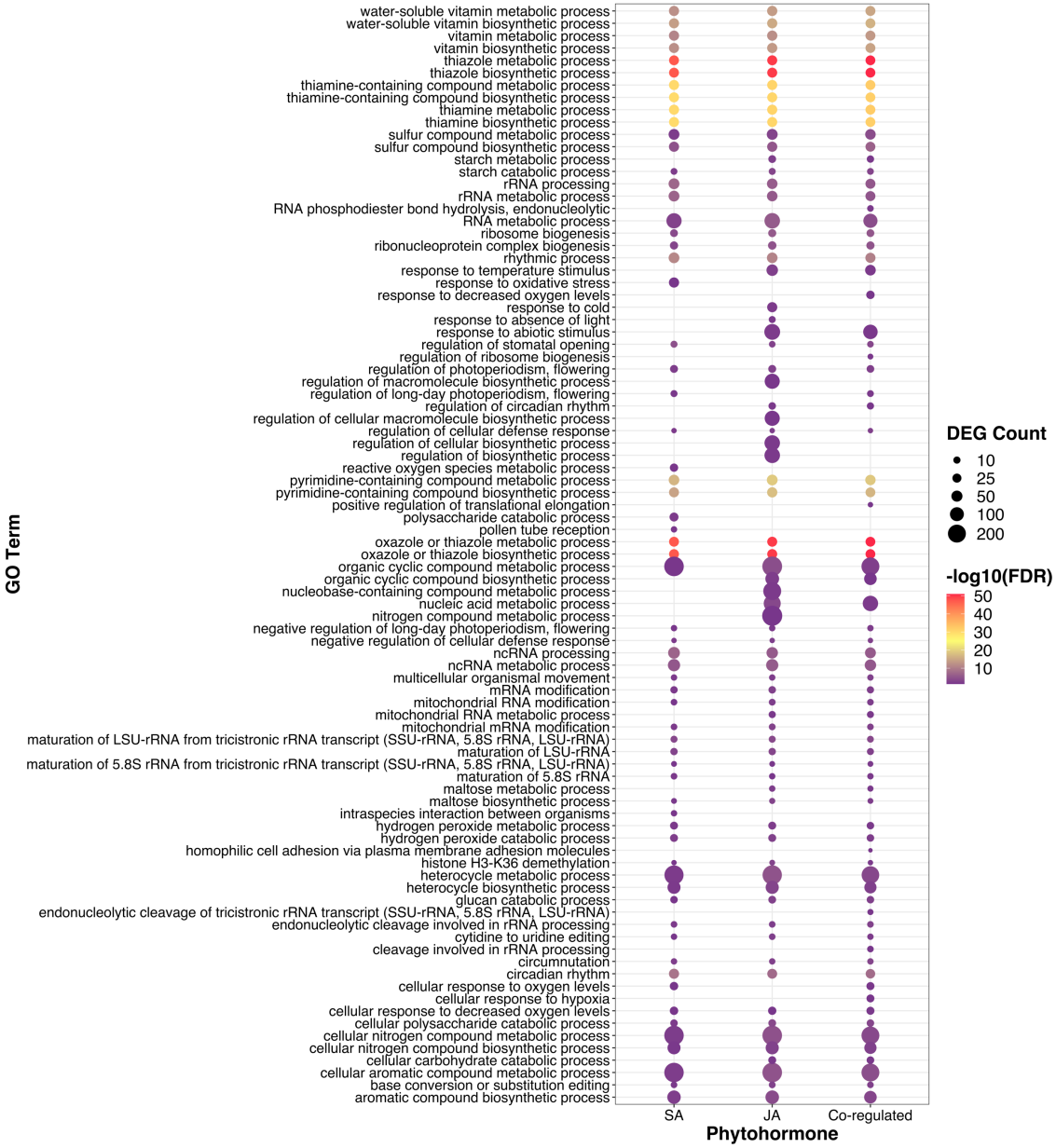

water-soluble vitamin metabolic process
water-soluble vitamin biosynthetic process
vitamin metabolic process
vitamin biosynthetic process
thiazole metabolic process
thiazole biosynthetic process
thiamine-containing compound metabolic process
thiamine-containing compound biosynthetic process
thiamine metabolic process
thiamine biosynthetic process
sulfur compound metabolic process
sulfur compound biosynthetic process
starch metabolic process
starch catabolic process
rRNA processing
rRNA metabolic process
RNA phosphodiester bond hydrolysis, endonucleolytic
RNA metabolic process
ribosome biogenesis
ribonucleoprotein complex biogenesis
rhythmic process
response to temperature stimulus
response to oxidative stress
response to decreased oxygen levels
response to cold
response to absence of light
response to abiotic stimulus
regulation of stomatal opening
regulation of ribosome biogenesis
regulation of photoperiodism, flowering
regulation of macromolecule biosynthetic process
regulation of long-day photoperiodism, flowering
regulation of circadian rhythm
regulation of cellular macromolecule biosynthetic process
regulation of cellular defense response
regulation of cellular biosynthetic process
regulation of biosynthetic process
reactive oxygen species metabolic process
pyrimidine-containing compound metabolic process
pyrimidine-containing compound biosynthetic process
positive regulation of translational elongation
polysaccharide catabolic process
pollen tube reception
oxazole or thiazole metabolic process
oxazole or thiazole biosynthetic process
organic cyclic compound metabolic process
organic cyclic compound biosynthetic process
nucleobase-containing compound metabolic process
nucleic acid metabolic process
nitrogen compound metabolic process
negative regulation of long-day photoperiodism, flowering
negative regulation of cellular defense response
ncRNA processing
ncRNA metabolic process
multicellular organismal movement
mRNA modification
mitochondrial RNA modification
mitochondrial RNA metabolic process
mitochondrial mRNA modification
maturation of LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)
maturation of LSU-rRNA
maturation of 5.8S rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)
maturation of 5.8S rRNA
maltose metabolic process
maltose biosynthetic process
intraspecies interaction between organisms
hydrogen peroxide metabolic process
hydrogen peroxide catabolic process
homophilic cell adhesion via plasma membrane adhesion molecules
histone H3-K36 demethylation
heterocycle metabolic process
heterocycle biosynthetic process
glucan catabolic process
endonucleolytic cleavage of tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)
endonucleolytic cleavage involved in rRNA processing
cytidine to uridine editing
cleavage involved in rRNA processing
circumnutation
circadian rhythm
cellular response to oxygen levels
cellular response to hypoxia
cellular response to decreased oxygen levels
cellular polysaccharide catabolic process
cellular nitrogen compound metabolic process
cellular nitrogen compound biosynthetic process
cellular carbohydrate catabolic process
cellular aromatic compound metabolic process
base conversion or substitution editing
aromatic compound biosynthetic process
GO Term
SA
JA
Co-regulated
Phytohormone
DEG Count
10
25
50
100
200
-log10(FDR)
50
40
30
20
10

Figure 3.4 Enriched GO terms associated 8-h upregulated phytohormone-responsive DEGs.

Dot plots display the top biological process GO terms for SA-responsive, JA-responsive, and SA/JA co-regulated DEGs that were upregulated after 8 h of phytohormone treatment. GO Terms were identified using the *goseq* package using a false discovery rate of 0.05 and the Benjamini Hochberg method. FDR values are displayed as a -log10(FDR). The number of DEGs associated with each GO term are indicated by shape size.

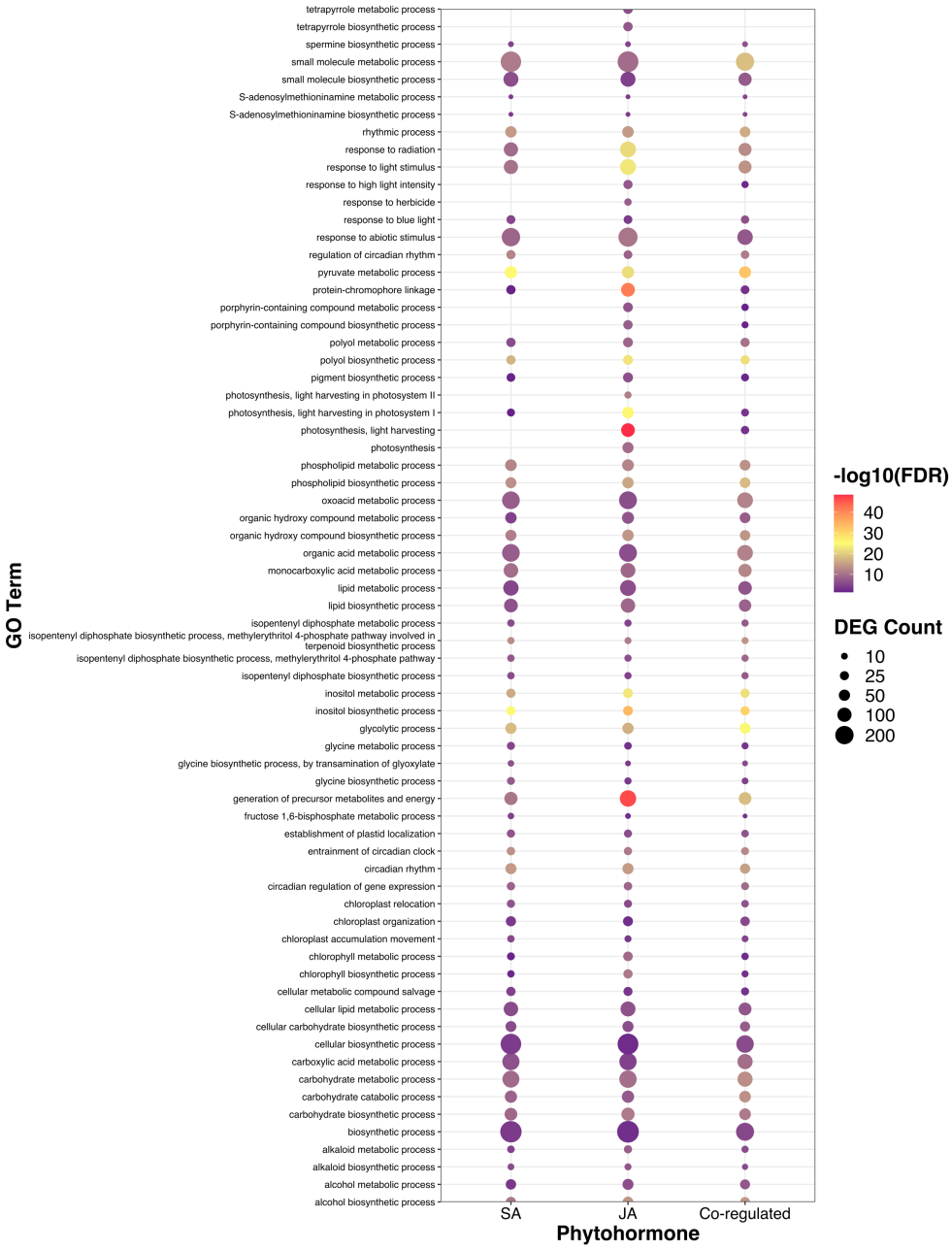

tetrapyrrole metabolic process
tetrapyrrole biosynthetic process
spermine biosynthetic process
small molecule metabolic process
small molecule biosynthetic process
S-adenosylmethioninamine metabolic process
S-adenosylmethioninamine biosynthetic process
rhythmic process
response to radiation
response to light stimulus
response to high light intensity
response to herbicide
response to blue light
response to abiotic stimulus
regulation of circadian rhythm
pyruvate metabolic process
protein-chromophore linkage
porphyrin-containing compound metabolic process
porphyrin-containing compound biosynthetic process
polyol metabolic process
polyol biosynthetic process
pigment biosynthetic process
photosynthesis, light harvesting in photosystem II
photosynthesis, light harvesting in photosystem I
photosynthesis, light harvesting
photosynthesis
phospholipid metabolic process
phospholipid biosynthetic process
oxoacid metabolic process
organic hydroxy compound metabolic process
organic hydroxy compound biosynthetic process
organic acid metabolic process
monocarboxylic acid metabolic process
lipid metabolic process
lipid biosynthetic process
isopentenyl diphosphate metabolic process
isopentenyl diphosphate biosynthetic process, methylerythritol 4-phosphate pathway involved in terpenoid biosynthetic process
isopentenyl diphosphate biosynthetic process, methylerythritol 4-phosphate pathway
isopentenyl diphosphate biosynthetic process
inositol metabolic process
inositol biosynthetic process
glycolytic process
glycine metabolic process
glycine biosynthetic process, by transamination of glyoxylate
glycine biosynthetic process
generation of precursor metabolites and energy
fructose 1,6-bisphosphate metabolic process
establishment of plastid localization
entrainment of circadian clock
circadian rhythm
circadian regulation of gene expression
chloroplast relocation
chloroplast organization
chloroplast accumulation movement
chlorophyll metabolic process
chlorophyll biosynthetic process
cellular metabolic compound salvage
cellular lipid metabolic process
cellular carbohydrate biosynthetic process
cellular biosynthetic process
carboxylic acid metabolic process
carbohydrate metabolic process
carbohydrate catabolic process
carbohydrate biosynthetic process
biosynthetic process
alkaloid metabolic process
alkaloid biosynthetic process
alcohol metabolic process
alcohol biosynthetic process
GO Term
SA
JA
Co-regulated
Phytohormone
-log10(FDR)
40
30
20
10
DEG Count
10
25
50
100
200

Figure 3.5 Enriched GO terms associated 8-h downregulated phytohormone-responsive DEGs.

Dot plots display the top 50 biological process GO terms for SA-responsive, JA-responsive, and SA/JA co-regulated DEGs that were downregulated after 8 h of phytohormone treatment. GO Terms were identified using the *goseq* package using a false discovery rate of 0.05 and the Benjamini Hochberg method. FDR values are displayed as a -log10(FDR). The number of DEGs associated with each GO term are indicated by shape size.

Figure 3.6 SA-, JA- and SA/JA-regulated DEGs.

All SA- and/or JA-responsive DEGs identified at 1 and/or 8 h after treatment are displayed. The intersection represents SA/JA co-regulated genes.

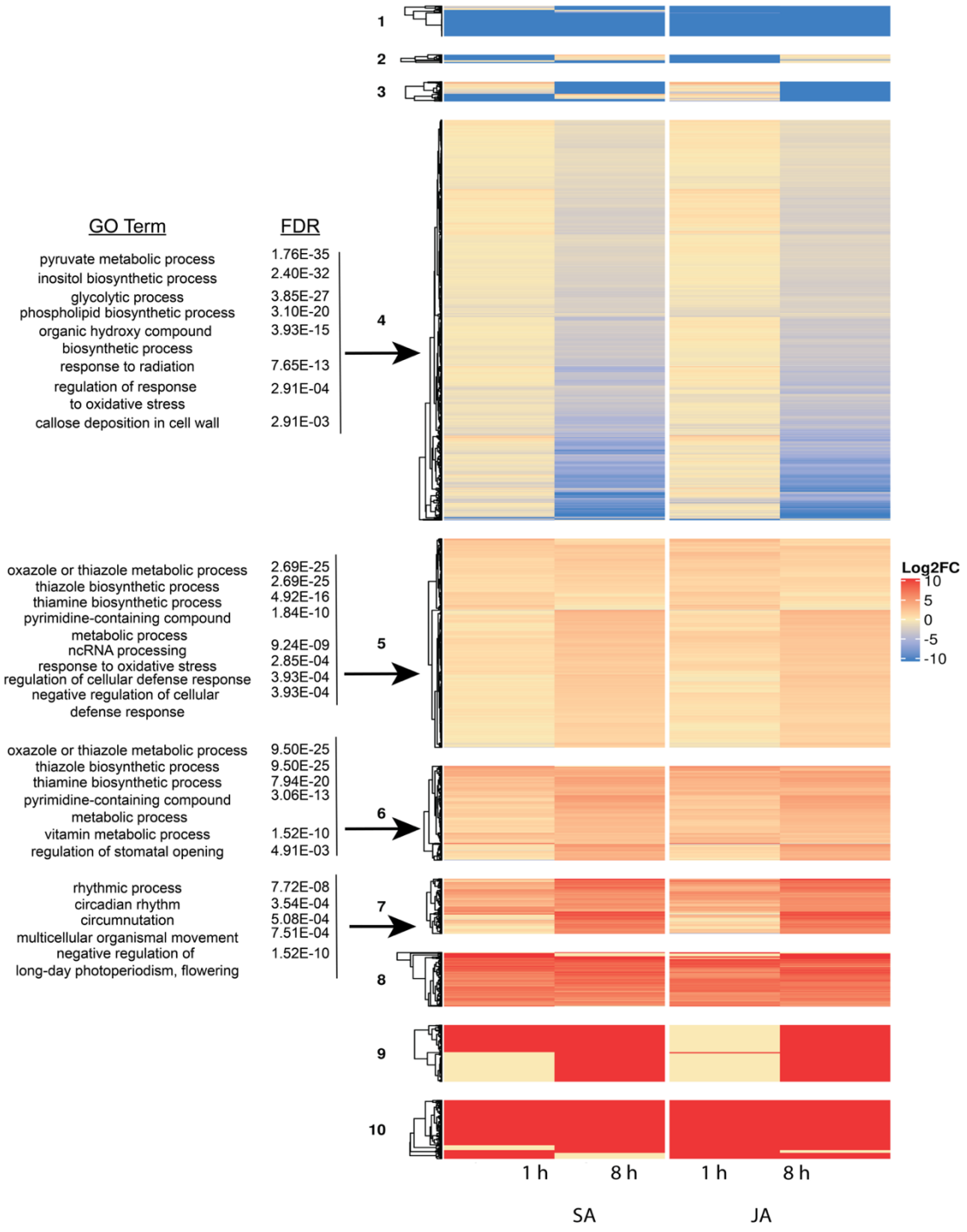
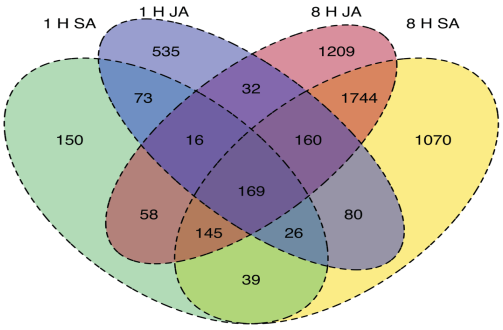

GO Term
FDR
pyruvate metabolic process
inositol biosynthetic process
glycolytic process
phospholipid biosynthetic process
organic hydroxy compound biosynthetic process
response to radiation
regulation of response to oxidative stress
callose deposition in cell wall
1.76E-35
2.40E-32
3.85E-27
3.10E-20
3.93E-15
7.65E-13
2.91E-04
2.91E-03
oxazole or thiazole metabolic process
thiazole biosynthetic process
thiamine biosynthetic process
pyrimidine-containing compound metabolic process
ncRNA processing
response to oxidative stress
regulation of cellular defense response
negative regulation of cellular defense response
2.69E-25
2.69E-25
4.92E-16
1.84E-10
9.24E-09
2.85E-04
3.93E-04
3.93E-04
oxazole or thiazole metabolic process
thiazole biosynthetic process
thiamine biosynthetic process
pyrimidine-containing compound metabolic process
vitamin metabolic process
regulation of stomatal opening
9.50E-25
9.50E-25
7.94E-20
3.06E-13
1.52E-10
4.91E-03
rhythmic process
circadian rhythm
circumnutation
multicellular organismal movement
negative regulation of long-day photoperiodism, flowering
7.72E-08
3.54E-04
5.08E-04
7.51E-04
1.52E-10
1
2
3
4
5
6
7
8
9
10
Log2FC
10
5
0
-5
-10
1 h
8 h
1 h
8 h
SA
JA

Figure 3.7 DEGs that respond to both SA and JA treatments.

Heatmap and k-means clustering were completed using *ComplexHeatmap*. Only Clusters 4, 5, 6, and 7 had GO terms that were identified by *goseq* and are shown adjacent to their respective cluster. GO terms were concatenated using REVIGO at 0.7 and GO terms with top FDRs are shown above. A complete list of GO terms and GO term concatenations can be found in Supplemental Table 3.5.

A


1 H SA
1 H JA
8 H JA
8 H SA
535
1209
32
73
1744
150
16
160
1070
169
58
80
145
26
39

B

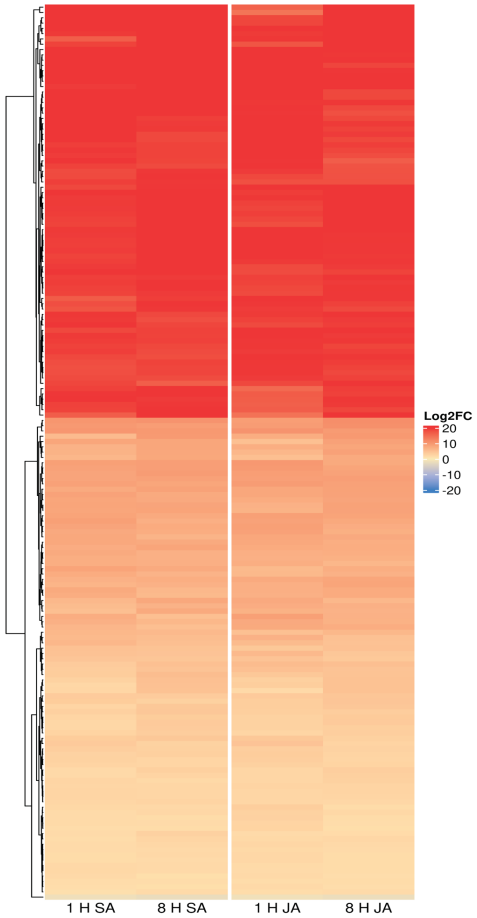

Log2FC
20
10
0
-10
-20
1 H SA
8 H SA
1 H JA
8 H JA

Figure 3.8 Heatmap of co-regulated DEGs.

(A) Venn Diagram of DEGs responsive to SA and MeJA at 1 and 8 H. (B) Heatmap of DEGs shared between both treatments at all times. Heatmap was constructed using *ComplexHeatmap*.

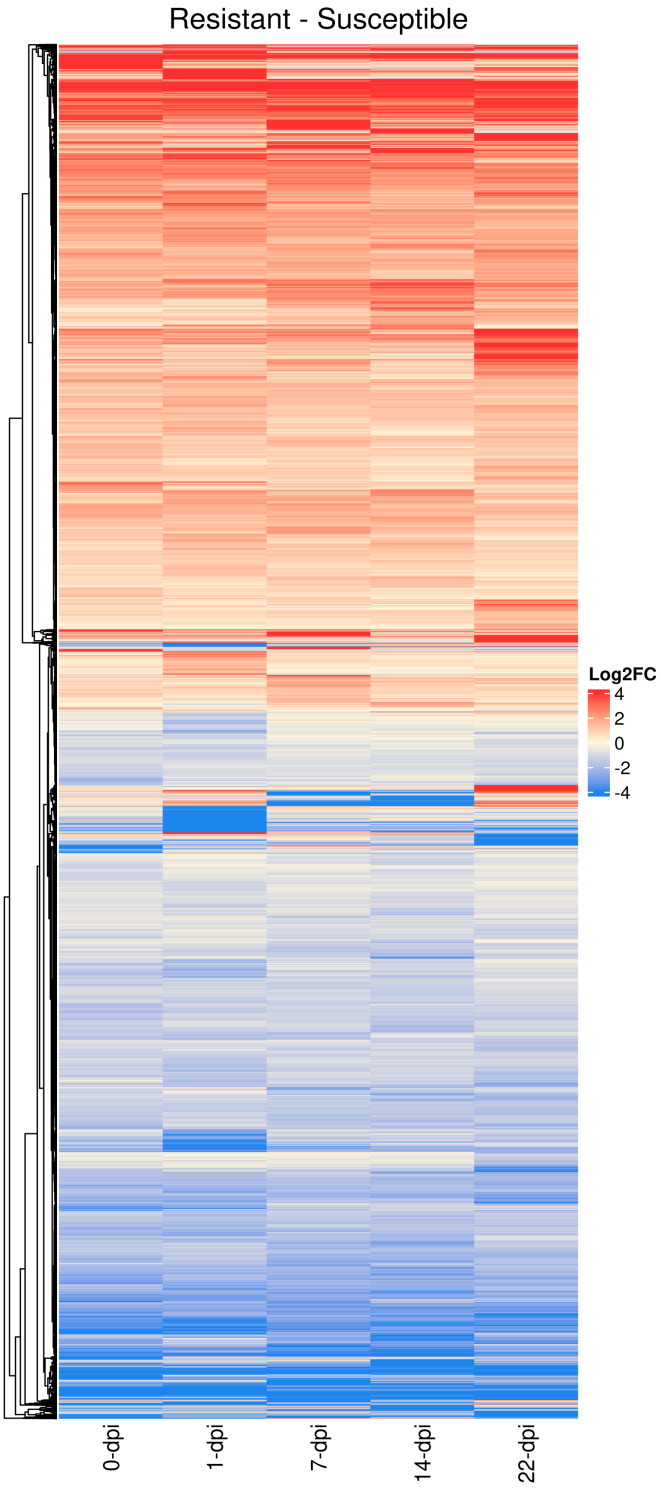

Resistant - Susceptible
Log2FC
4
2
0
-2
-4
0-dpi
1-dpi
7-dpi
14-dpi
22-dpi

Figure 3.9 Expression profile of Genotype DEG identified in analysis of Meta-Transcriptome resemble transcriptome profile of Chapter 2.

All gDEGs identified in analysis of meta-transcriptome assembly in a heatmap constructed with *ComplexHeatmap*.

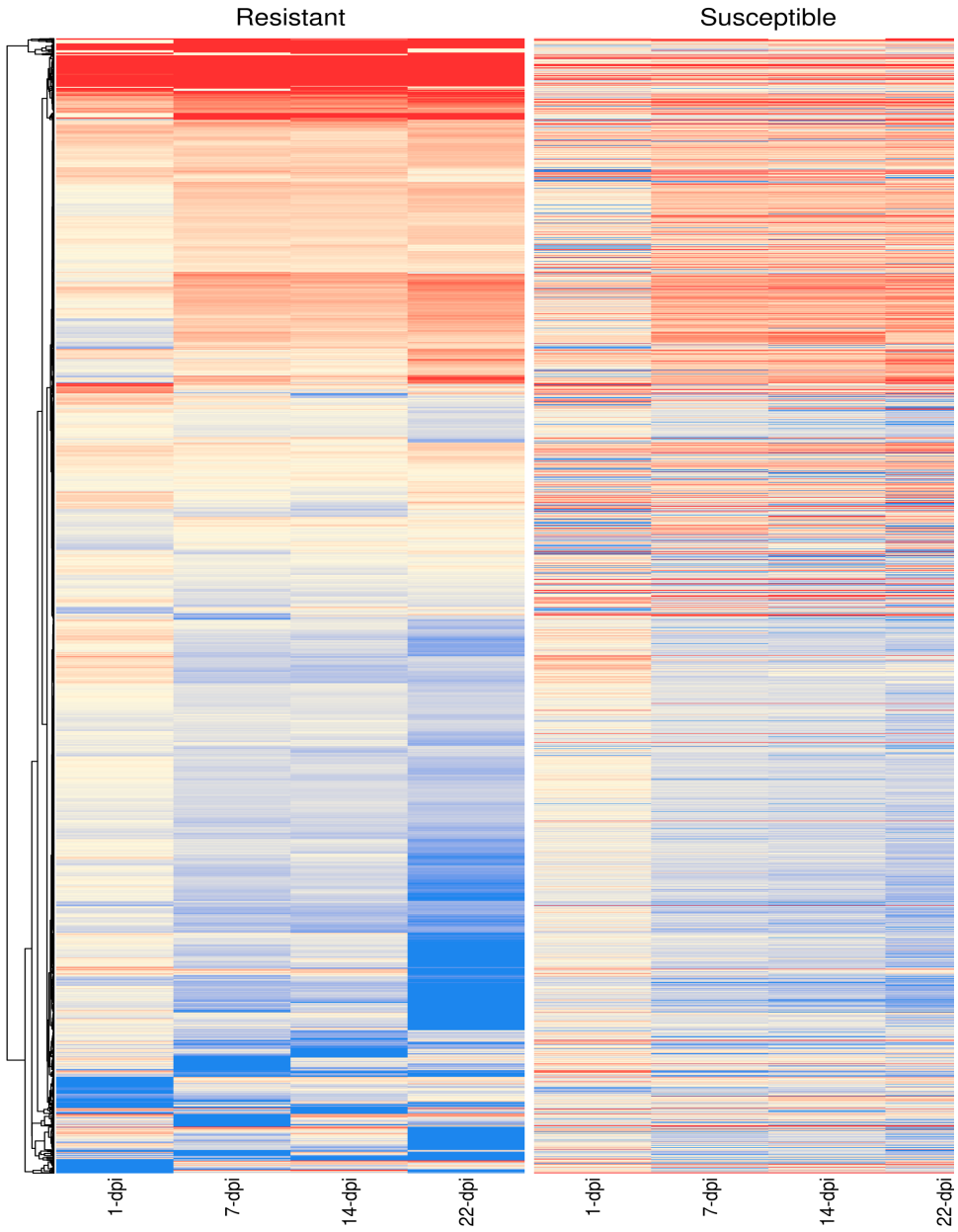
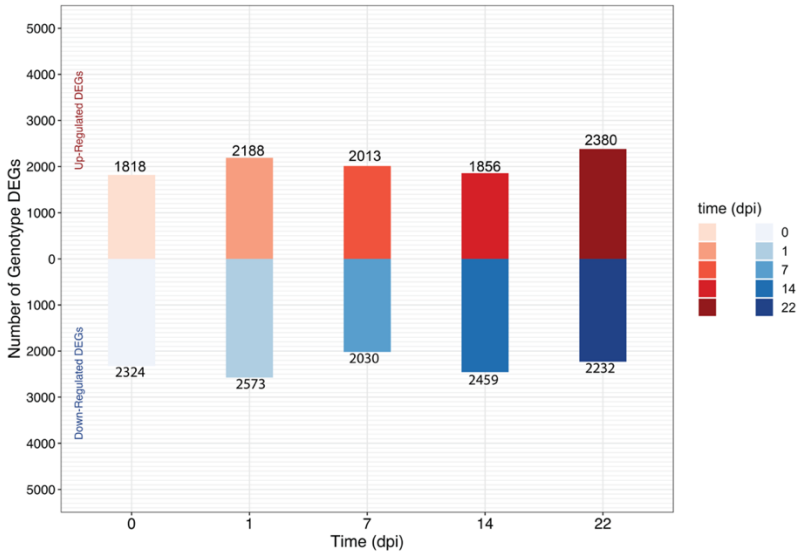

Resistant
Susceptible
1-dpi
7-dpi
14-dpi
22-dpi
1-dpi
7-dpi
14-dpi
22-dpi

Figure 3.10 Expression profile of Temporal DEG identified in analysis of Meta-Transcriptome resemble transcriptome profile of Chapter 2.

All tDEGs identified in analysis of meta-transcriptome assembly in a heatmap constructed with *ComplexHeatmap*.

A


Number of Genotype DEGs
Up-Regulated DEGs
Down-Regulated DEGs
5000
4000
3000
2000
1000
0
1000
2000
3000
4000
5000
1818
2188
2013
1856
2380
2324
2573
2030
2459
2232
0
1
7
14
22
Time (dpi)
time (dpi)
0
1
7
14
22

B

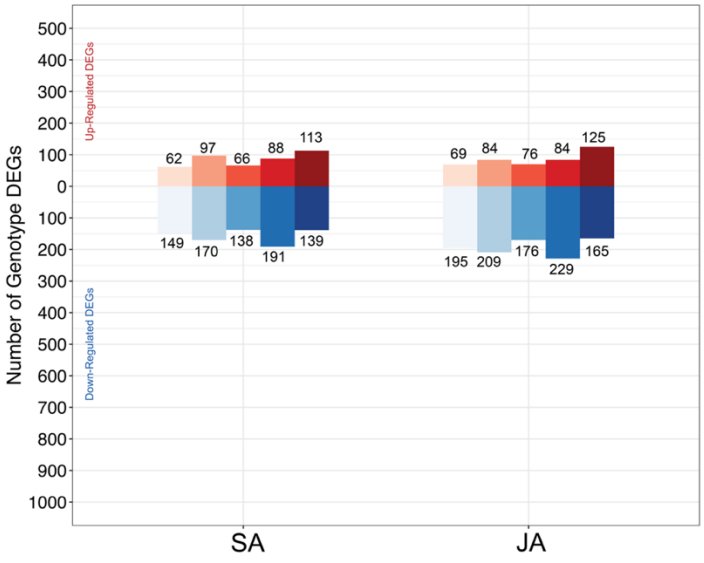

Number of Genotype DEGs
Up-Regulated DEGs
Down-Regulated DEGs
500
400
300
200
100
0
100
200
300
400
500
600
700
800
900
1000
62
97
66
88
113
149
170
138
191
139
69
84
76
84
125
195
209
176
229
165
SA
JA

Figure 3.11 The Genotype Response of Alfalfa to Whitefly is Largely Independent of SA and JA.

Barplots of the number of upregulated and downregulated genotype DEGs (gDEGs) (A) and the number of gDEGs responsive to SA and JA at each time point (B).

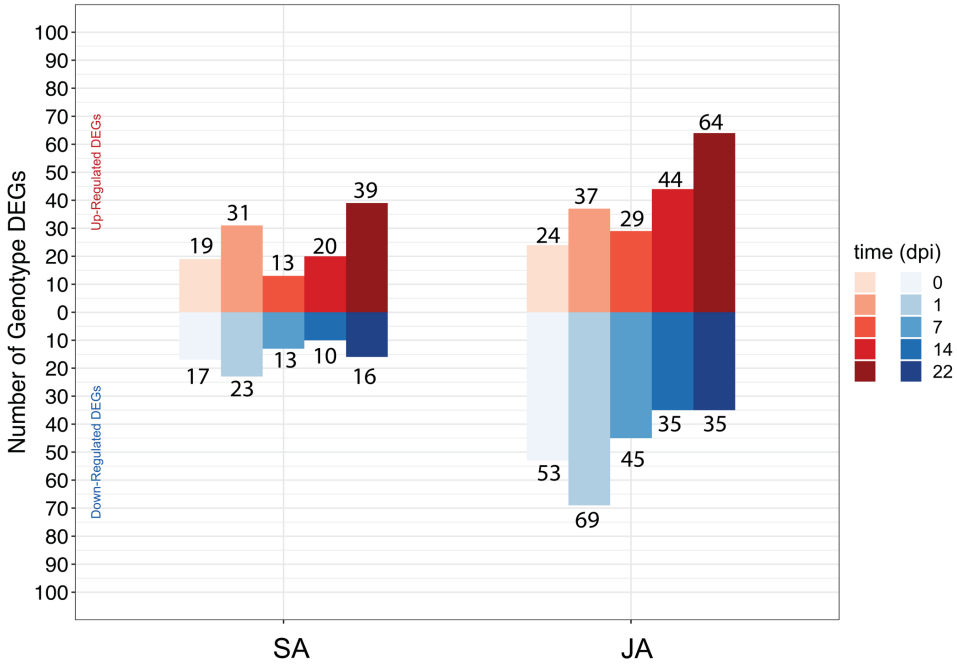
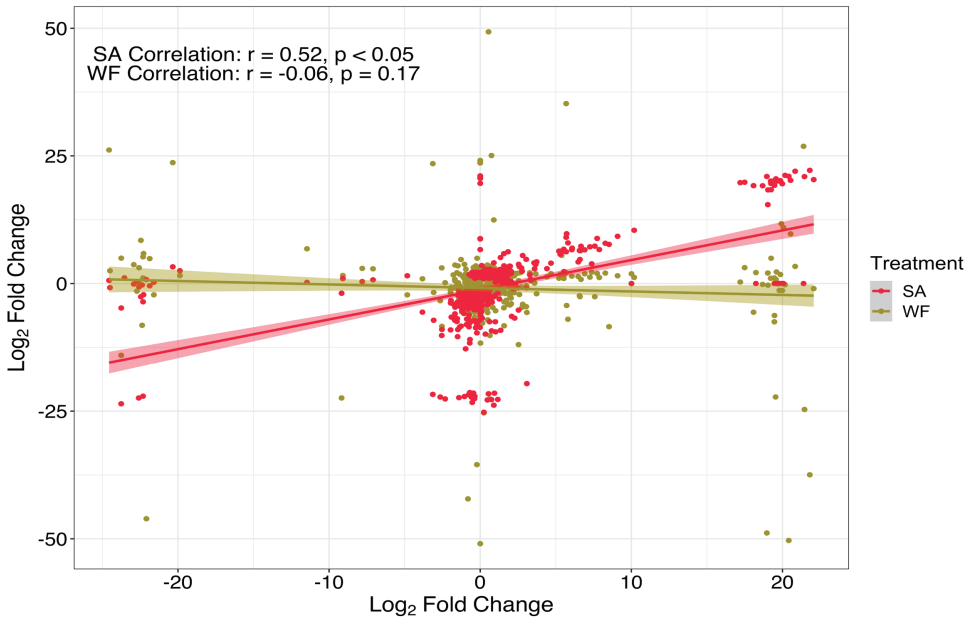

Number of Genotype DEGs
Up-Regulated DEGs
Down-Regulated DEGs
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
19
31
13
20
39
17
23
13
10
16
24
37
29
44
64
53
69
45
35
35
SA
JA
time (dpi)
0
1
7
14
22

Figure 3.12 There are more whitefly-responsive gDEGs responsive to JA than SA.

Barplot of the number of upregulated and downregulated gDEGs responsive to both SA and JA at 1 h.

A


SA Correlation: r = 0.52, p < 0.05
WF Correlation: r = -0.06, p = 0.17
Log2 Fold Change
Log2 Fold Change
50
25
0
-25
-50
-20
-10
0
10
20
Treatment
SA
WF

B

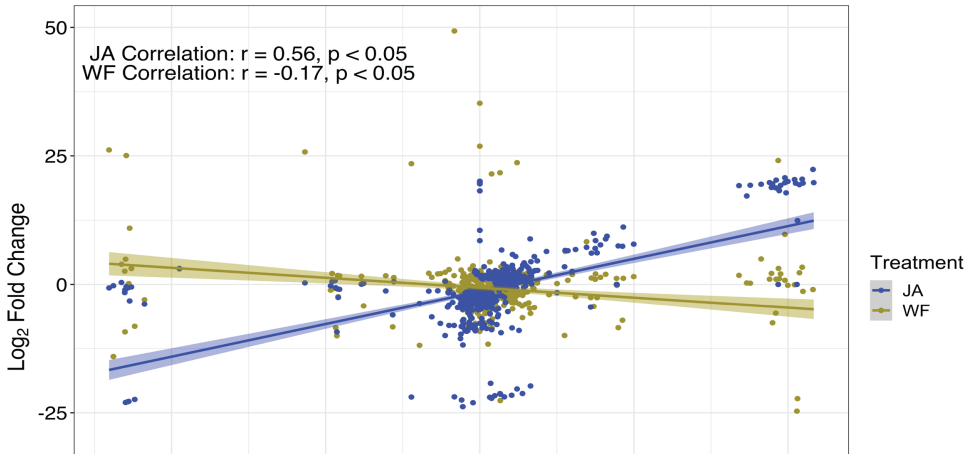
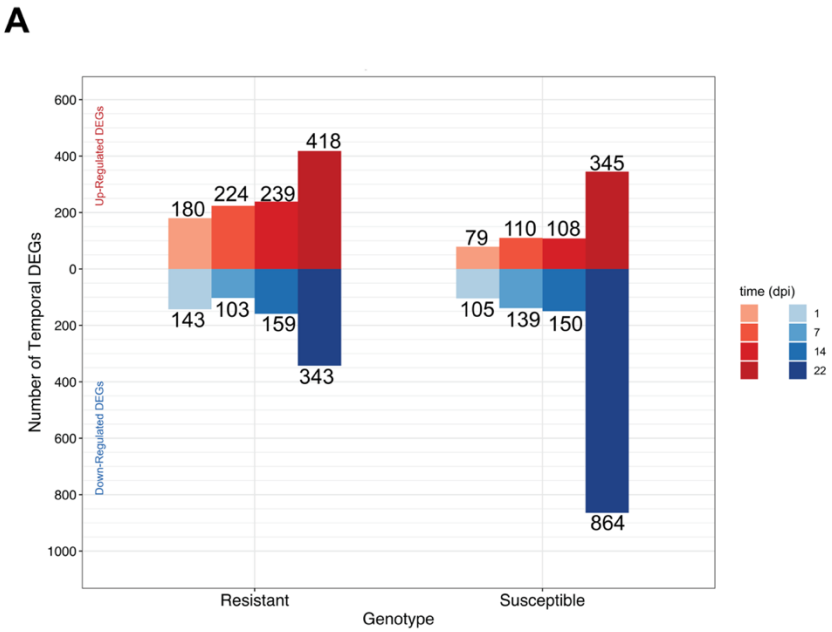

JA Correlation: r = 0.56, p < 0.05
WF Correlation: r = -0.17, p < 0.05
Log2 Fold Change
50
25
0
-25
Treatment
JA
WF

Figure 3.13 Whitefly-responsive Genotype DEGs (gDEGs) in alfalfa are more correlated to JA than SA.

Scatterplot of (A) SA- and (B) JA-responsive gDEGs. The x-axis represents the Log_2 Fold Change (LFC) in response to hormone treatment at 1 h. The y-axis represents the LFC at either 8 h (SA or JA treatments) or at 1 dpi in the whitefly treatment. The correlation co-efficient and p-value for each trend line was completed using a Pearson correlation.


A
Number of Temporal DEGs
Up-Regulated DEGs
Down-Regulated DEGs
600
400
200
0
200
400
600
800
1000
180
224
239
418
143
103
159
343
79
110
108
345
105
139
150
864
Resistant
Susceptible
Genotype
time (dpi)
1
7
14
22

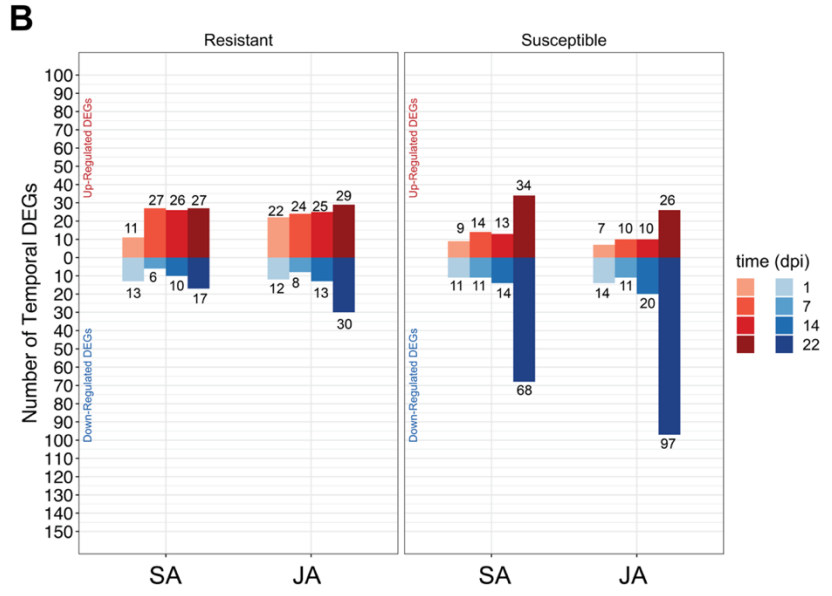

B
Resistant
Susceptible
Number of Temporal DEGs
Up-Regulated DEGs
Down-Regulated DEGs
11
27
26
27
13
6
10
17
22
24
25
29
12
8
13
30
9
14
13
34
11
11
14
68
7
10
10
26
14
11
20
97
SA
JA
SA
JA
time (dpi)
1
7
14
22

Figure 3.14 The Temporal Response of Alfalfa to Whitefly is Largely Independent of SA and JA.

(A) The number of upregulated and downregulated temporal DEGs (tDEGs) in R1 and S1 over the 22-d whitefly infestation. (B) The number of tDEGs responsive to SA orJA at each infestation time pointin R1 and S1 plants.

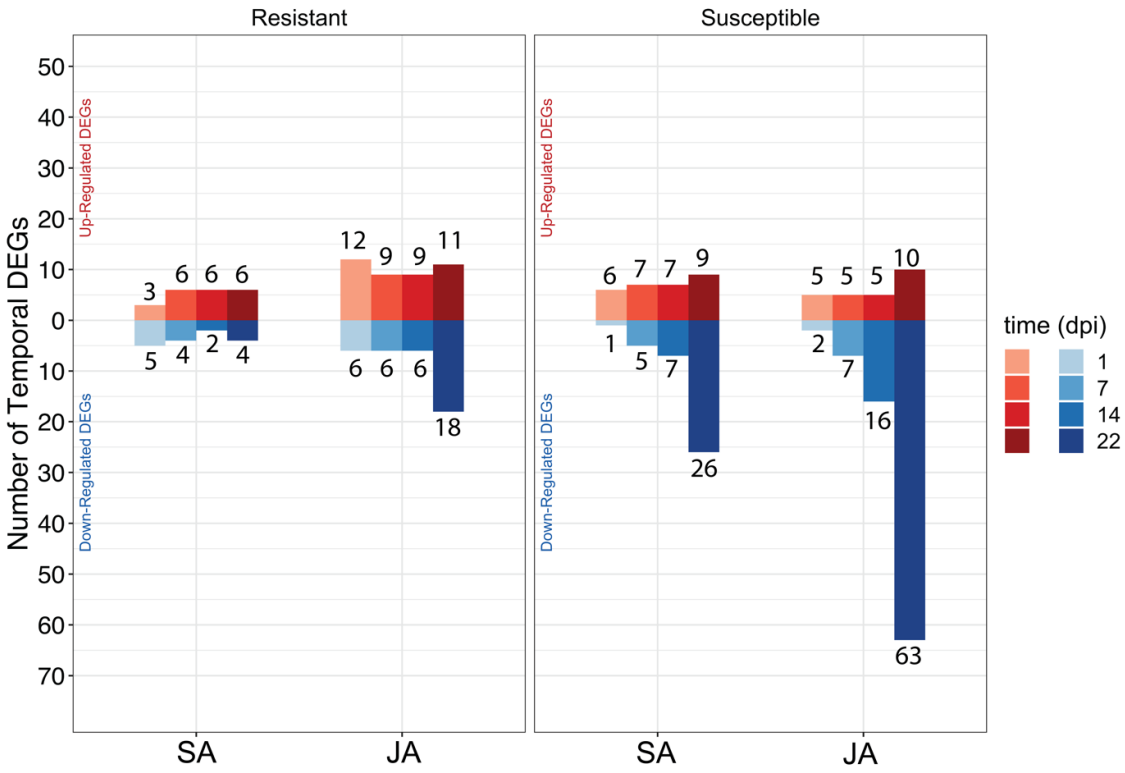

Resistant
Susceptible
Number of Temporal DEGs
Up-Regulated DEGs
Down-Regulated DEGs
50
40
30
20
10
0
10
20
30
40
50
60
70
3
6
6
6
5
4
2
4
12
9
9
11
6
6
6
18
6
7
7
9
1
5
7
26
5
5
5
10
2
7
16
63
SA
JA
SA
JA
time (dpi)
1
7
14
22

Figure 3.15 There are Few Temporal DEGs Responsive to SA or JA at 1 h.

The number of upregulated and downregulated temporal DEGs (tDEGs) that correspond to 1-h SA-and JA-DEGs are shown.tDEGs for the whitefly resistant (R1) and whitefly-susceptible (S1) alfalfa plants afterB. tabaciinfestation.

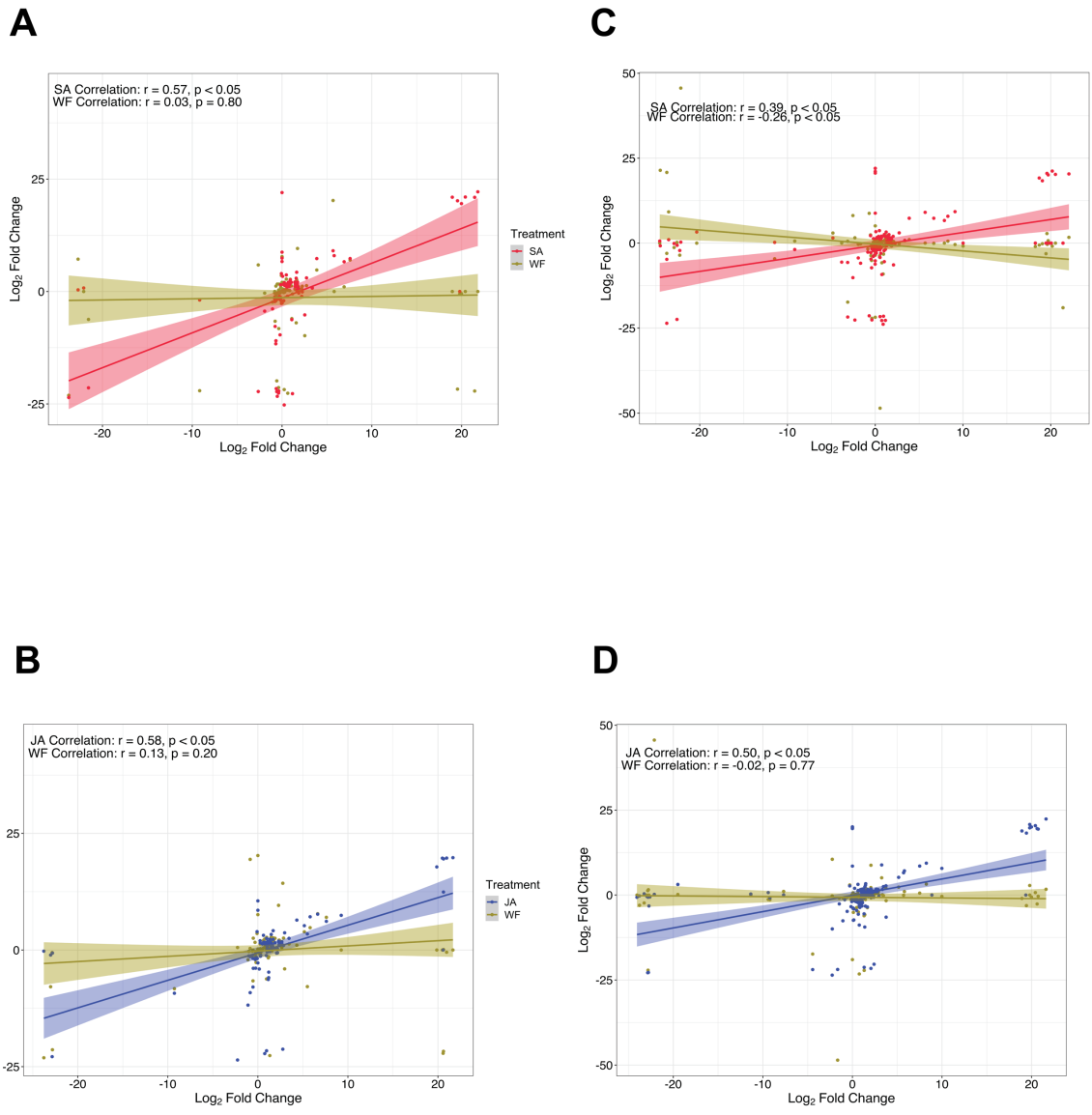

A
SA Correlation: r = 0.57, p < 0.05
WF Correlation: r = 0.03, p = 0.80
Log2 Fold Change
Log2 Fold Change
Treatment
SA
WF
C
SA Correlation: r = 0.39, p < 0.05
WF Correlation: r = -0.26, p < 0.05
Log2 Fold Change
Log2 Fold Change
B
JA Correlation: r = 0.58, p < 0.05
WF Correlation: r = 0.13, p = 0.20
Log2 Fold Change
Log2 Fold Change
Treatment
JA
WF
D
JA Correlation: r = 0.50, p < 0.05
WF Correlation: r = -0.02, p = 0.77
Log2 Fold Change
Log2 Fold Change

Figure 3.16 Whitefly-responsive Temporal DEGs (gDEGs) in alfalfa are more correlated to SA in S1.

Scatterplot of phytohormone- and whitefly-responsive tDEGs in R1 (A-B) and S1 (C-D) . The x-axis represents the Log_2 Fold Change (LFC) in response to SA (A and C) or MeJA (B and D) at 1 h in each genotype and the y-axis represents the LFC at either 8 h (SA or MeJA treatment) or at 1 dpi in the whitefly treatment. The correlation co-efficient and p-value for each trend line was completed using a Pearson correlation.

Table 3.1 Select Phytohormone-Responsive DEGs

SA-Responsive - 1 h

Transcript	LFC	FDR	TAIR ID	TAIR Gene Name
TRINITY_DN5499_c0_g1_i17	19.79	5.32E-03	AT3G57260	*BGL2*
TRINITY_DN5499_c0_g1_i28	18.09	8.00E-05	AT3G57260	*BGL2*
TRINITY_DN328_c0_g1_i1	7.79	4.35E-05	AT5G26920	*CBP60G*
TRINITY_DN2589_c0_g1_i1	3.41	8.20E-12	AT1G28480	*GRXC9*
TRINITY_DN8268_c0_g1_i1	2.50	4.85E-02	AT1G28480	*GRXC9*
TRINITY_DN8373_c0_g1_i3	3.34	6.44E-05	AT1G01720	*NAC002*
TRINITY_DN8329_c0_g1_i1	1.61	3.15E-03	AT1G01720	*NAC002*
TRINITY_DN181_c0_g1_i6	2.19	6.82E-03	AT3G56400	*WRKY70*
TRINITY_DN12466_c0_g1_i3	1.88	4.94E-02	AT3G56400	*WRKY70*
TRINITY_DN2579_c0_g2_i1	1.31	1.95E-04	AT2G37040	*PAL1*
TRINITY_DN12828_c0_g1_i2	20.71	2.53E-03	AT5G57580	*CBP60B*
TRINITY_DN6616_c0_g1_i1	18.04	3.13E-07	AT5G33340	*CDR1*
TRINITY_DN3178_c0_g2_i2	1.86	4.50E-02	AT2G24300	*CBP60E*

JA-Responsive - 1 h

Transcript	LFC	FDR	TAIR ID	TAIR Gene Name
TRINITY_DN4814_c1_g1_i2	1.07	3.25E-02	AT4G16760	*ACX1*
TRINITY_DN4814_c1_g1_i9	1.11	4.97E-03	AT4G16760	*ACX1*
TRINITY_DN228_c0_g1_i3	1.65	1.56E-02	AT2G27150	*AAO3*
TRINITY_DN103585_c1_g1_i1	-24.15	7.70E-10	AT1G55020	*LOX1*
TRINITY_DN38279_c0_g1_i1	7.69	3.09E-04	AT1G55020	*LOX1*
TRINITY_DN349_c0_g1_i1	2.88	2.71E-02	AT3G45140	*LOX2*
TRINITY_DN5550_c0_g1_i4	1.69	2.24E-02	AT1G17420	*LOX3*
TRINITY_DN33482_c0_g2_i3	2.63	3.85E-02	AT3G22400	*LOX5*
TRINITY_DN15521_c0_g1_i17	2.05	4.72E-02	AT1G19180	*TIFY10A*
TRINITY_DN15521_c0_g1_i2	2.00	1.63E-02	AT1G19180	*TIFY10A*
TRINITY_DN4577_c0_g1_i1	1.37	1.86E-02	AT3G17860	*TIFY6B*
TRINITY_DN12361_c0_g2_i2	1.24	3.08E-02	AT5G42650	*CYP74A*

SA-Responsive - 8 h

Transcript	LFC	FDR	TAIR ID	TAIR Gene Name
TRINITY_DN5499_c0_g1_i17	19.88	1.41E-03	AT3G57260	*BGL2*
TRINITY_DN5499_c0_g1_i28	19.17	3.69E-06	AT3G57260	*BGL2*
TRINITY_DN328_c0_g1_i1	7.01	1.28E-04	AT5G26920	*CBP60G*

Table 3.1 Continued

TRINITY_DN328_c0_g1_i3	6.33	1.03E-02	AT5G26920	*CBP60G*
TRINITY_DN6616_c0_g1_i1	18.90	1.00E-08	AT5G33340	*CDR1*
TRINITY_DN8482_c0_g1_i4	-6.97	2.80E-03	AT4G10500	*DLO1*
TRINITY_DN614_c3_g2_i2	-1.39	2.75E-02	AT4G05320	*UBQ10*
TRINITY_DN888_c1_g2_i1	-1.14	1.52E-03	AT4G05320	*UBQ10*
TRINITY_DN22069_c0_g1_i1	-1.18	8.08E-03	AT2G37040	*PAL1*
TRINITY_DN2579_c0_g1_i1	-1.30	1.76E-02	AT2G37040	*PAL1*
TRINITY_DN80982_c0_g1_i1	1.71	3.95E-03	AT2G37040	*PAL1*

JA-Responsive - 8 h

Transcript	LFC	FDR	TAIR ID	TAIR Gene Name
TRINITY_DN9608_c2_g1_i4	-20.76	7.58E-04	AT2G27150	*AAO3*
TRINITY_DN38279_c0_g1_i1	7.38	3.76E-04	AT1G55020	*LOX1*
TRINITY_DN25138_c1_g1_i7	2.49	3.60E-02	AT1G55020	*LOX1*
TRINITY_DN349_c0_g1_i2	-1.37	2.21E-02	AT3G45140	*LOX2*
TRINITY_DN4072_c0_g1_i3	-2.05	2.50E-03	AT3G45140	*LOX2*
TRINITY_DN102070_c0_g1_i5	1.75	9.07E-03	AT3G22400	*LOX5*
TRINITY_DN14304_c0_g1_i2	1.03	1.59E-02	AT4G01370	*MPK4*
TRINITY_DN1611_c0_g1_i6	8.58	4.38E-04	AT1G19640	*JMT*

Table 3.2 Top 20 GO Terms Among Upregulated SA/JA DEGs in Alfalfa

SA 1 h - Upregulated	
GO Term	**False Discovery Rate (FDR)**
response to stress	1.66E-07
response to other organism	1.89E-05
response to external biotic stimulus	2.47E-05
response to biotic stimulus	3.13E-05
response to stimulus	3.60E-05
response to fungus	3.60E-05
defense response to other organism	1.12E-04
multi-organism process	1.13E-04
cellular response to stimulus	6.80E-04
defense response	6.80E-04
defense response to fungus	8.82E-04
response to external stimulus	9.54E-04
cellular response to hypoxia	9.54E-04
cellular response to stress	9.54E-04
cellular response to decreased oxygen levels	9.54E-04
cellular response to oxygen levels	1.10E-03
regulation of metabolic process	1.16E-03
regulation of cellular biosynthetic process	5.29E-03
regulation of biosynthetic process	6.80E-03
response to abiotic stimulus	7.25E-03

JA 1 h - Upregulated	
GO Term	**False Discovery Rate (FDR)**
response to stress	4.87E-24
oxazole or thiazole biosynthetic process	4.87E-24
oxazole or thiazole metabolic process	4.87E-24
thiazole biosynthetic process	4.87E-24
thiazole metabolic process	4.87E-24
response to stimulus	2.75E-18
defense response	4.44E-18
thiamine biosynthetic process	9.81E-14
thiamine-containing compound biosynthetic process	9.81E-14

Table 3.2 Continued

thiamine metabolic process	2.47E-13
thiamine-containing compound metabolic process	2.47E-13
response to oxygen-containing compound	1.90E-12
defense response to other organism	2.04E-11
response to other organism	2.14E-11
response to external biotic stimulus	2.72E-11
response to biotic stimulus	2.75E-11
multi-organism process	2.87E-09
response to acid chemical	3.34E-09
response to organic substance	4.24E-09
response to external stimulus	1.50E-08

SA 8 h - Upregulated

GO Term	False Discovery Rate (FDR)
oxazole or thiazole biosynthetic process	6.07E-48
oxazole or thiazole metabolic process	6.07E-48
thiazole biosynthetic process	6.07E-48
thiazole metabolic process	6.07E-48
thiamine metabolic process	5.95E-31
thiamine-containing compound metabolic process	5.95E-31
thiamine biosynthetic process	1.11E-30
thiamine-containing compound biosynthetic process	1.11E-30
pyrimidine-containing compound metabolic process	1.92E-17
pyrimidine-containing compound biosynthetic process	2.78E-15
water-soluble vitamin biosynthetic process	1.17E-14
water-soluble vitamin metabolic process	5.14E-13
vitamin biosynthetic process	8.24E-13
rhythmic process	4.72E-12
vitamin metabolic process	1.66E-11
circadian rhythm	1.11E-09

Table 3.2 Continued

rRNA processing	8.27E-08
ncRNA processing	1.58E-07
rRNA metabolic process	1.71E-07
ncRNA metabolic process	5.31E-06
sulfur compound biosynthetic process	2.50E-05

JA 8 h - Upregulated

GO Term	False Discovery Rate (FDR)
oxazole or thiazole biosynthetic process	1.67E-50
oxazole or thiazole metabolic process	1.67E-50
thiazole biosynthetic process	1.67E-50
thiazole metabolic process	1.67E-50
thiamine metabolic process	1.22E-31
thiamine-containing compound metabolic process	1.22E-31
thiamine biosynthetic process	2.40E-31
thiamine-containing compound biosynthetic process	2.40E-31
pyrimidine-containing compound metabolic process	6.26E-20
pyrimidine-containing compound biosynthetic process	1.18E-18
water-soluble vitamin biosynthetic process	3.73E-16
water-soluble vitamin metabolic process	1.98E-14
vitamin biosynthetic process	3.20E-14
vitamin metabolic process	8.07E-13
rhythmic process	1.57E-11
circadian rhythm	2.24E-08
rRNA processing	1.47E-06
ncRNA metabolic process	1.95E-06
RNA metabolic process	2.07E-06
ncRNA processing	2.32E-06

Table 3.2 Continued

rRNA metabolic process	2.50E-06

Table 3.3 Top 20 GO Terms Among Coregulated SA/JA DEGs in Alfalfa

1 h Coregulated - Upregulated	
GO Term	**False Discovery Rate (FDR)**
response to stress	4.72E-06
response to stimulus	8.48E-04
response to abiotic stimulus	9.07E-03
response to other organism	9.07E-03
response to external biotic stimulus	1.09E-02
cellular response to stimulus	1.23E-02
response to biotic stimulus	1.29E-02
cellular response to stress	1.29E-02
defense response to other organism	1.71E-02
regulation of salicylic acid metabolic process	2.17E-02
multi-organism process	2.19E-02
response to fungus	2.37E-02

8 h Coreglated - Upregulated	
GO Term	**False Discovery Rate (FDR)**
oxazole or thiazole biosynthetic process	1.17E-51
oxazole or thiazole metabolic process	1.17E-51
thiazole biosynthetic process	1.17E-51
thiazole metabolic process	1.17E-51
thiamine metabolic process	7.76E-33
thiamine-containing compound metabolic process	7.76E-33
thiamine biosynthetic process	2.04E-32
thiamine-containing compound biosynthetic process	2.04E-32
pyrimidine-containing compound metabolic process	1.01E-19
pyrimidine-containing compound biosynthetic process	1.48E-17
water-soluble vitamin biosynthetic process	4.54E-17
water-soluble vitamin metabolic process	1.33E-15
vitamin biosynthetic process	3.01E-15
vitamin metabolic process	4.59E-14

Table 3.3 Continued

	5.30E-11
rhythmic process	
circadian rhythm	1.58E-08

Table 3.3 Continued

	3.16E-07
sulfur compound biosynthetic process	
ncRNA processing	2.13E-06
rRNA processing	6.52E-06
ncRNA metabolic process	6.52E-06
ribosome biogenesis	6.52E-06

8 h Coregulated - Downregulated

GO Term	False Discovery Rate (FDR)
pyruvate metabolic process	2.82E-33
inositol biosynthetic process	2.23E-31
glycolytic process	2.12E-25
inositol metabolic process	7.68E-23
polyol biosynthetic process	8.80E-23
small molecule metabolic process	2.82E-19
generation of precursor metabolites and energy	4.88E-19
phospholipid biosynthetic process	7.84E-19
rhythmic process	3.85E-17
circadian rhythm	5.85E-16
alcohol biosynthetic process	8.22E-15
organic hydroxy compound biosynthetic process	1.18E-14
isopentenyl diphosphate biosynthetic process, methylerythritol 4-phosphate pathway involved in terpenoid biosynthetic process	1.30E-14
response to light stimulus	2.15E-14
phospholipid metabolic process	2.70E-14
carbohydrate catabolic process	2.94E-14
carbohydrate metabolic process	4.45E-14
response to radiation	1.09E-13

Table 3.3 Continued

entrainment of circadian clock	2.29E-13
monocarboxylic acid metabolic process	4.32E-13
oxoacid metabolic process	1.07E-12

Table 3.4 DEGs shared among SA and JA at 1 h identified in GO term analysis

Transcript	TAIR ID	Gene Name	TAIR Description
TRINITY_DN14419_c0_g1_i2	AT3G20820	-	Leucine-rich repeat (LRR) family protein [Source:UniProtKB/TrEMBL;Acc:Q9LT39]
TRINITY_DN6589_c0_g1_i2	AT1G01300	APF2	Aspartyl protease family protein 2 [Source:UniProtKB/Swiss-Prot;Acc:Q9LNJ3]
TRINITY_DN1615_c0_g1_i1	AT2G27080	NHL13	NDR1/HIN1-like protein 13 [Source:UniProtKB/Swiss-Prot;Acc:Q9ZVD2]
TRINITY_DN1698_c0_g1_i2	AT5G14620	DRM2	DNA (cytosine-5)-methyltransferase DRM2 [Source:UniProtKB/Swiss-Prot;Acc:Q9M548]
TRINITY_DN1626_c0_g1_i5	AT5G18830	SPL7	Squamosa promoter binding protein-like 7 [Source:UniProtKB/TrEMBL;Acc:F4JZI4]
TRINITY_DN4731_c0_g1_i5	AT4G35740	RECQL3	ATP-dependent DNA helicase Q-like 3 [Source:UniProtKB/Swiss-Prot;Acc:Q9FT72]
TRINITY_DN937_c0_g1_i4	AT5G13680	ELP1	Elongator complex protein 1 [Source:UniProtKB/Swiss-Prot;Acc:Q9FNA4]
TRINITY_DN561_c0_g1_i30	AT2G34930	-	Disease resistance family protein / LRR family protein [Source:UniProtKB/TrEMBL;Acc:O64757]
TRINITY_DN2375_c1_g1_i11	AT3G05600	-	Alpha/beta-Hydrolases superfamily protein [Source:UniProtKB/TrEMBL;Acc:Q9M9W5]
TRINITY_DN13233_c0_g1_i1	AT5G44640	BGLU13	Beta-glucosidase 13 [Source:UniProtKB/Swiss-Prot;Acc:Q9LU02]
TRINITY_DN9001_c0_g1_i29	AT5G15080	PIX7	Probable serine/threonine-protein kinase PIX7 [Source:UniProtKB/Swiss-Prot;Acc:Q9LFP7]
TRINITY_DN8168_c0_g1_i1	AT4G10180	DET1	Light-mediated development protein DET1 [Source:UniProtKB/Swiss-Prot;Acc:P48732]
TRINITY_DN9462_c0_g1_i3	AT5G06370	-	AT5g06370/MHF15_11 [Source:UniProtKB/TrEMBL;Acc:Q93V51]
TRINITY_DN4554_c0_g1_i4	AT3G01610	CDC48C	Cell division cycle 48C [Source:UniProtKB/TrEMBL;Acc:A0A1I9LNC6]
TRINITY_DN2375_c1_g1_i6	AT3G05600	-	Alpha/beta-Hydrolases superfamily protein [Source:UniProtKB/TrEMBL;Acc:Q9M9W5]
TRINITY_DN3637_c0_g1_i1	AT4G04950	GRXS17	Monothiol glutaredoxin-S17 [Source:UniProtKB/Swiss-Prot;Acc:Q9ZPH2]
TRINITY_DN591_c0_g1_i22	AT2G27040	AGO4	Protein argonaute 4 [Source:UniProtKB/Swiss-Prot;Acc:Q9ZVD5]
TRINITY_DN5583_c0_g1_i10	AT2G26330	ERECTA	LRR receptor-like serine/threonine-protein kinase ERECTA [Source:UniProtKB/Swiss-Prot;Acc:Q42371]
TRINITY_DN707_c0_g1_i9	AT2G30110	UBA1	UBA1 [Source:UniProtKB/TrEMBL;Acc:A0A178VN59]
TRINITY_DN7806_c0_g1_i1	AT5G61910	-	DCD (Development and Cell Death) domain protein [Source:UniProtKB/TrEMBL;Acc:F4K518]
TRINITY_DN6616_c0_g1_i1	AT5G33340	CDR1	Aspartic proteinase CDR1 [Source:UniProtKB/Swiss-Prot;Acc:Q6XBF8]
TRINITY_DN34586_c0_g1_i9	AT1G15820	LHCB6	Chlorophyll a-b binding protein, chloroplastic [Source:UniProtKB/TrEMBL;Acc:Q9LMQ2]
TRINITY_DN20027_c0_g1_i4	AT5G26860	LON_ARA_AR A	lon protease 1 [Source:TAIR;Acc:AT5G26860]
TRINITY_DN3594_c0_g1_i10	AT1G72390	PHL	CONTAINS InterPro DOMAIN/s: Spt20 family (InterPro:IPR021950); Ha. [Source:TAIR;Acc:AT1G72390]
TRINITY_DN6126_c0_g1_i1	AT2G42690	-	Phospholipase A1-IIdelta [Source:UniProtKB/Swiss-Prot;Acc:Q9SJI7]
TRINITY_DN2060_c0_g1_i3	AT1G30755	-	Elongation factor G, putative (DUF668) [Source:UniProtKB/TrEMBL;Acc:Q8L5Y3]
TRINITY_DN839_c0_g1_i12	AT5G60800	-	Heavy metal transport/detoxification superfamily protein [Source:UniProtKB/TrEMBL;Acc:F4K0H3]

Table 3.4 Continued

TRINITY_DN8195_c0_g1_i5	AT5G60920	COB	Protein COBRA [Source:UniProtKB/Swiss-Prot;Acc:Q94KT8]
TRINITY_DN328_c0_g1_i1	AT5G26920	CBP60G	Calmodulin-binding protein 60 G [Source:UniProtKB/Swiss-Prot;Acc:F4K2R6]
TRINITY_DN38390_c0_g1_i9	AT4G21960	PER42	Peroxidase 42 [Source:UniProtKB/Swiss-Prot;Acc:Q9SB81]
TRINITY_DN595_c0_g1_i2	AT2G03150	emb1579	Protein SHORT ROOT IN SALT MEDIUM 1 [Source:UniProtKB/Swiss-Prot;Acc:F4IS91]
TRINITY_DN4879_c0_g1_i8	AT5G17680	-	Disease resistance protein (TIR-NBS-LRR class) [Source:UniProtKB/TrEMBL;Acc:Q9FN83]
TRINITY_DN15112_c0_g1_i3	AT2G18760	CHR8	chromatin remodeling 8 [Source:TAIR;Acc:AT2G18760]
TRINITY_DN11761_c0_g1_i8	AT1G14870	PCR2	PCR2 [Source:UniProtKB/TrEMBL;Acc:A0A178WDU8]
TRINITY_DN13802_c0_g1_i2	AT5G14040	MPT3	Mitochondrial phosphate carrier protein 3, mitochondrial [Source:UniProtKB/Swiss-Prot;Acc:Q9FMU6]
TRINITY_DN30073_c0_g1_i1	AT2G41480	PER25	Peroxidase 25 [Source:UniProtKB/Swiss-Prot;Acc:O80822]
TRINITY_DN20909_c0_g1_i4	AT4G20860	FAD-OXR	Berberine bridge enzyme-like 22 [Source:UniProtKB/Swiss-Prot;Acc:Q9SUC6]
TRINITY_DN25963_c0_g1_i6	AT3G54420	EP3	EP3 [Source:UniProtKB/TrEMBL;Acc:A0A178VE44]
TRINITY_DN413_c0_g1_i3	AT1G24100	UGT74B1	Glycosyltransferase [Source:UniProtKB/TrEMBL;Acc:A0A178WKT6]
TRINITY_DN10991_c0_g1_i20	AT3G09270	GSTU8	Glutathione S-transferase U8 [Source:UniProtKB/Swiss-Prot;Acc:Q9SR36]
TRINITY_DN3926_c0_g1_i2	AT5G56750	NDL1	Protein NDL1 [Source:UniProtKB/Swiss-Prot;Acc:Q9FJT7]
TRINITY_DN2632_c0_g1_i5	AT5G01090	-	Concanavalin A-like lectin family protein [Source:UniProtKB/TrEMBL;Acc:Q9LFC7]
TRINITY_DN16801_c0_g1_i1	AT2G39210	-	At2g39210/T16B24.15 [Source:UniProtKB/TrEMBL;Acc:O80960]
TRINITY_DN13474_c0_g1_i1	AT3G27890	NQR	NQR [Source:UniProtKB/TrEMBL;Acc:A0A384KSW6]
TRINITY_DN313_c0_g1_i1	AT4G34240	ALDH3I1	Aldehyde dehydrogenase [Source:UniProtKB/TrEMBL;Acc:A0A178UV01]
TRINITY_DN2589_c0_g1_i1	AT1G28480	GRXC9	Glutaredoxin-C9 [Source:UniProtKB/Swiss-Prot;Acc:Q9SGP6]
TRINITY_DN8373_c0_g1_i3	AT1G01720	NAC002	NAC domain-containing protein 2 [Source:UniProtKB/Swiss-Prot;Acc:Q39013]
TRINITY_DN426_c0_g1_i1	AT5G36930	-	Disease resistance protein (TIR-NBS-LRR class) family [Source:UniProtKB/TrEMBL;Acc:B3H776]
TRINITY_DN14050_c0_g1_i1	AT1G80840	WRKY40	Probable WRKY transcription factor 40 [Source:UniProtKB/Swiss-Prot;Acc:Q9SAH7]
TRINITY_DN0_c5_g1_i1	AT3G51550	FER	Receptor-like protein kinase FERONIA [Source:UniProtKB/Swiss-Prot;Acc:Q9SCZ4]
TRINITY_DN1979_c0_g1_i1	AT1G78600	LZF1	Light-regulated zinc finger protein 1 [Source:UniProtKB/TrEMBL;Acc:F4IBS4]
TRINITY_DN9761_c0_g1_i5	AT2G37130	PER21	Peroxidase 21 [Source:UniProtKB/Swiss-Prot;Acc:Q42580]

Table 3.4 Continued

TRINITY_DN4354_c0_g1_i1	AT5G42500	DIR2	Dirigent protein 2 [Source:UniProtKB/Swiss-Prot;Acc:Q9FIG7]
TRINITY_DN6145_c0_g1_i1	AT2G22500	PUMP5	Mitochondrial uncoupling protein 5 [Source:UniProtKB/Swiss-Prot;Acc:Q9SJY5]
TRINITY_DN16657_c0_g1_i7	AT5G36930	-	Disease resistance protein (TIR-NBS-LRR class) family [Source:UniProtKB/TrEMBL;Acc:B3H776]
TRINITY_DN5161_c0_g1_i1	AT3G56880	-	VQ motif-containing protein [Source:UniProtKB/TrEMBL;Acc:Q9LES0]
TRINITY_DN3914_c2_g1_i1	AT2G21660	RBG7	Glycine-rich RNA-binding protein 7 [Source:UniProtKB/Swiss-Prot;Acc:Q03250]
TRINITY_DN13133_c0_g1_i1	AT5G66880	SRK2I	Serine/threonine-protein kinase SRK2I [Source:UniProtKB/Swiss-Prot;Acc:Q39193]
TRINITY_DN11778_c0_g1_i1	AT1G21651	-	Putative SecA-type chloroplast protein transport factor [Source:UniProtKB/TrEMBL;Acc:Q8VZ06]
TRINITY_DN9065_c0_g1_i2	AT3G03300	DCL2	Endoribonuclease Dicer homolog 2 [Source:UniProtKB/Swiss-Prot;Acc:Q3EBC8]
TRINITY_DN3178_c0_g2_i2	AT2G24300	-	Calmodulin-binding protein [Source:TAIR;Acc:AT2G24300]
TRINITY_DN243_c0_g1_i2	AT3G08510	PLC2	Phosphoinositide phospholipase C [Source:UniProtKB/TrEMBL;Acc:C0Z2P3]
TRINITY_DN9669_c0_g1_i1	AT2G22300	CAMTA3	Calmodulin-binding transcription activator 3 [Source:UniProtKB/Swiss-Prot;Acc:Q8GSA7]
TRINITY_DN12447_c0_g1_i1	-	-	-
TRINITY_DN2308_c0_g1_i2	AT2G46210	SLD2	Delta(8)-fatty-acid desaturase 2 [Source:UniProtKB/Swiss-Prot;Acc:Q3EBF7]
TRINITY_DN689_c0_g1_i4	AT1G04220	KCS2	3-ketoacyl-CoA synthase 2 [Source:UniProtKB/Swiss-Prot;Acc:Q5XEP9]
TRINITY_DN4162_c3_g1_i1	AT3G10985	SAG20	Senescence associated gene 20 [Source:UniProtKB/Swiss-Prot;Acc:Q94AK6]
TRINITY_DN36397_c0_g1_i2	AT2G45180	-	At2g45180/T14P1.1 [Source:UniProtKB/TrEMBL;Acc:Q42044]
TRINITY_DN1155_c0_g1_i7	AT2G28940	-	At2g28940 [Source:UniProtKB/TrEMBL;Acc:O81064]
TRINITY_DN8682_c0_g1_i6	AT2G28930	APK1B	At2g28930 [Source:UniProtKB/TrEMBL;Acc:A1L4W8]
TRINITY_DN6059_c0_g1_i1	AT2G17840	ERD7	Protein EARLY-RESPONSIVE TO DEHYDRATION 7, chloroplastic [Source:UniProtKB/Swiss-Prot;Acc:O48832]
TRINITY_DN8329_c0_g1_i1	AT1G01720	NAC002	NAC domain-containing protein 2 [Source:UniProtKB/Swiss-Prot;Acc:Q39013]
TRINITY_DN8195_c0_g1_i4	AT5G60920	COB	Protein COBRA [Source:UniProtKB/Swiss-Prot;Acc:Q94KT8]
TRINITY_DN2422_c0_g1_i5	AT3G17980	CAR4	Protein C2-DOMAIN ABA-RELATED 4 [Source:UniProtKB/Swiss-Prot;Acc:Q9LVH4]
TRINITY_DN8767_c0_g1_i1	AT1G33590	-	Leucine-rich repeat (LRR) family protein [Source:TAIR;Acc:AT1G33590]
TRINITY_DN4997_c0_g1_i1	AT4G31550	WRKY11	Probable WRKY transcription factor 11 [Source:UniProtKB/Swiss-Prot;Acc:Q9SV15]
TRINITY_DN2154_c0_g1_i1	AT1G59870	ABCG36	ABC transporter G family member 36 [Source:UniProtKB/Swiss-Prot;Acc:Q9XIE2]
TRINITY_DN16237_c0_g1_i7	AT1G09070	SRC2	Protein SRC2 homolog [Source:UniProtKB/Swiss-Prot;Acc:O04023]
TRINITY_DN22164_c0_g1_i1	AT3G57520	RFS2	Probable galactinol--sucrose galactosyltransferase 2 [Source:UniProtKB/Swiss-Prot;Acc:Q94A08]
TRINITY_DN2017_c0_g1_i2	AT3G45640	MPK3	MPK3 [Source:UniProtKB/TrEMBL;Acc:A0A384L050]
TRINITY_DN593_c0_g1_i5	AT1G01140	CIPK9	CBL-interacting protein kinase 9 [Source:TAIR;Acc:AT1G01140]

Table 3.4 Continued

TRINITY_DN2877_c0_g1_i6	AT3G24550	PERK1	Proline-rich receptor-like protein kinase PERK1 [Source:UniProtKB/Swiss-Prot;Acc:Q9LV48]
TRINITY_DN1186_c1_g1_i1	AT5G56000	HSP90-4	Hsp81.4 [Source:UniProtKB/TrEMBL;Acc:A0A178UQ52]
TRINITY_DN15107_c0_g1_i2	AT1G64060	RBOHF	Respiratory burst oxidase homolog protein F [Source:UniProtKB/Swiss-Prot;Acc:O48538]
TRINITY_DN9422_c1_g1_i1	AT3G17410	-	Protein kinase superfamily protein [Source:UniProtKB/TrEMBL;Acc:Q9LUT0]
TRINITY_DN50087_c0_g1_i1	AT1G19570	DHAR1	Glutathione S-transferase DHAR1, mitochondrial [Source:UniProtKB/Swiss-Prot;Acc:Q9FWR4]

Table 3.5 Top 20 GO Terms Among Downregulated SA/JA DEGs in Alfalfa

SA 8 h - Downregulated	
GO Term	**False Discovery Rate (FDR)**
pyruvate metabolic process	2.75E-26
inositol biosynthetic process	2.08E-25
glycolytic process	7.62E-19
polyol biosynthetic process	5.34E-18
inositol metabolic process	4.89E-17
rhythmic process	3.17E-15
circadian rhythm	3.17E-15
entrainment of circadian clock	3.36E-14
phospholipid biosynthetic process	3.84E-14
isopentenyl diphosphate biosynthetic process, methylerythritol 4-phosphate pathway involved in terpenoid biosynthetic process	7.49E-14
phospholipid metabolic process	6.99E-13
regulation of circadian rhythm	6.99E-13
organic hydroxy compound biosynthetic process	4.21E-12
small molecule metabolic process	7.91E-12
alcohol biosynthetic process	2.63E-11
generation of precursor metabolites and energy	2.90E-11
response to light stimulus	9.66E-11
monocarboxylic acid metabolic process	2.50E-10
response to radiation	5.79E-10
carbohydrate metabolic process	6.64E-10
carbohydrate biosynthetic process	1.35E-09
JA 8 h - Downregulated	
GO Term	**False Discovery Rate (FDR)**
photosynthesis, light harvesting	4.03E-49
generation of precursor metabolites and energy	3.71E-47
protein-chromophore linkage	2.85E-42
inositol biosynthetic process	1.09E-34

Table 3.5 Continued

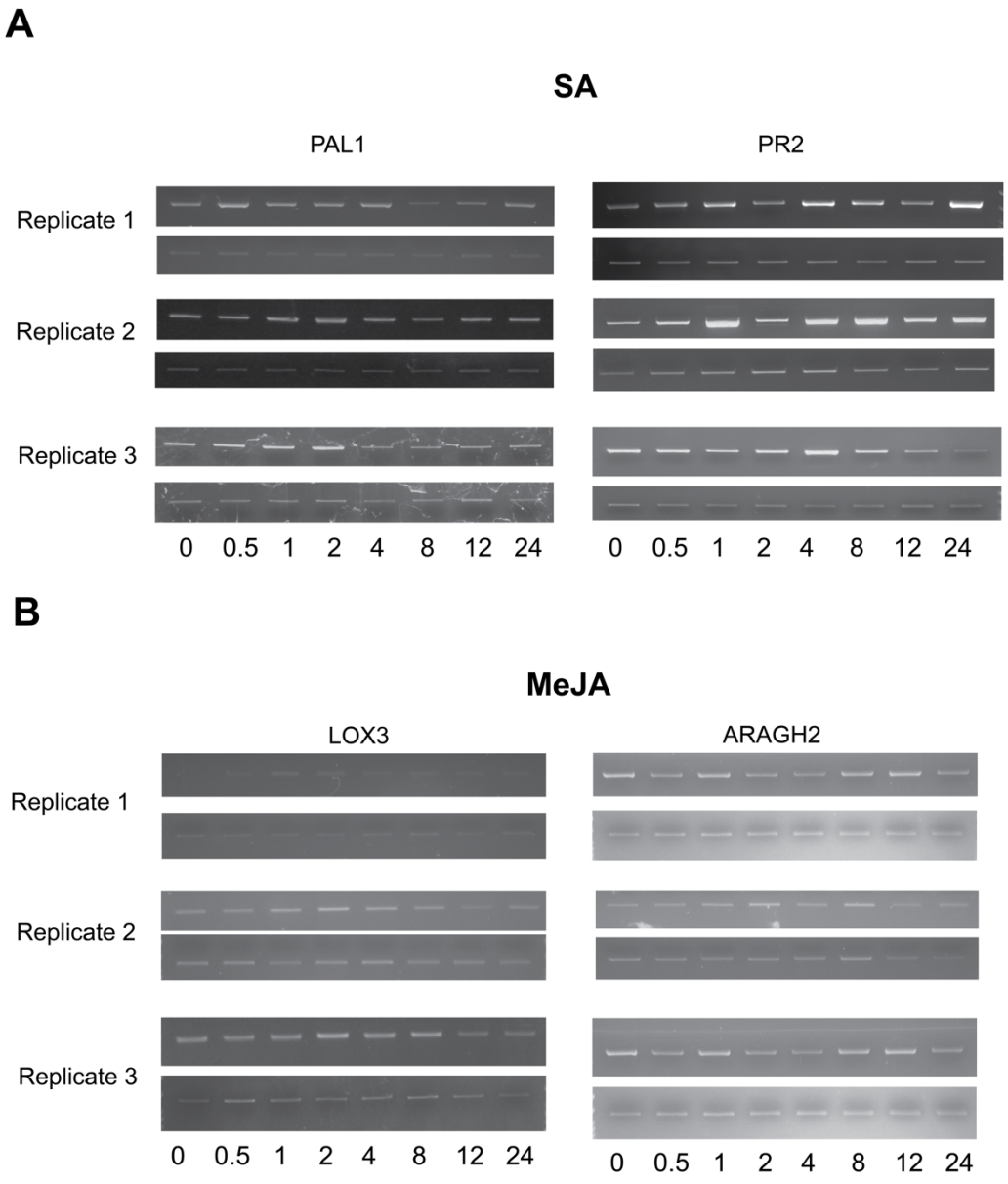
photosynthesis, light harvesting in photosystem I	1.86E-26
inositol metabolic process	1.51E-23
response to light stimulus	1.61E-23
polyol biosynthetic process	3.07E-23
pyruvate metabolic process	2.31E-22
response to radiation	3.37E-22
glycolytic process	1.30E-17
phospholipid biosynthetic process	1.56E-16
circadian rhythm	1.79E-15
rhythmic process	2.70E-15
organic hydroxy compound biosynthetic process	9.36E-15
alcohol biosynthetic process	1.04E-14
phospholipid metabolic process	9.01E-13
photosynthesis, light harvesting in photosystem II	3.73E-12
isopentenyl diphosphate biosynthetic process, methylerythritol 4-phosphate pathway involved in terpenoid biosynthetic process	5.41E-12
carbohydrate biosynthetic process	9.97E-12
chlorophyll biosynthetic process	1.21E-11

Table 3.6 Top 20 GO Terms Among Unique SA/JA DEGs in Alfalfa

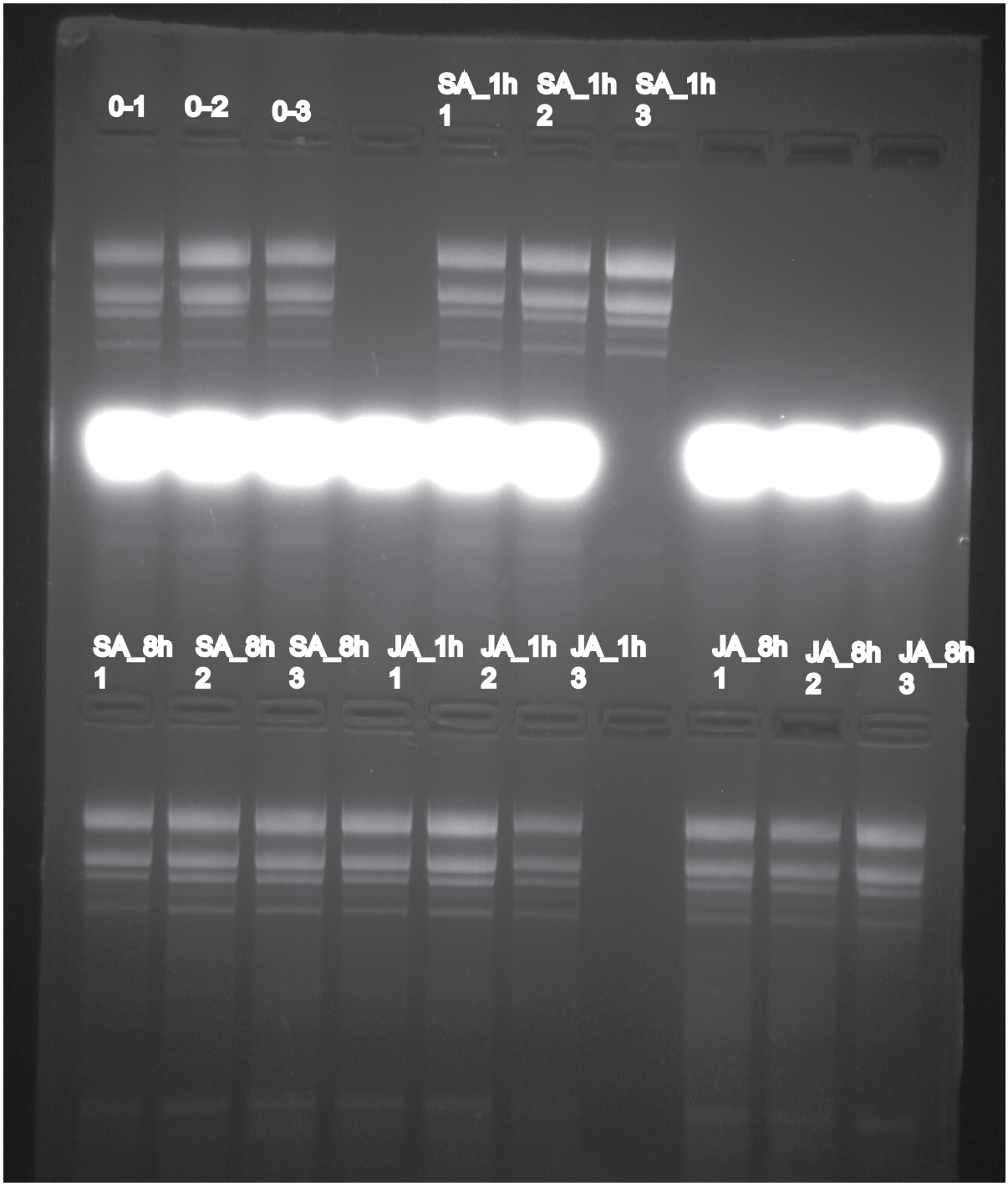
Unique JA 1 h - Upregulated	
GO Term	**False Discovery Rate (FDR)**
oxazole or thiazole biosynthetic process	6.75E-21
oxazole or thiazole metabolic process	6.75E-21
thiazole biosynthetic process	6.75E-21
thiazole metabolic process	6.75E-21
response to stress	1.08E-16
defense response	1.88E-15
response to stimulus	2.17E-13
thiamine biosynthetic process	2.20E-13
thiamine-containing compound biosynthetic process	2.20E-13
thiamine metabolic process	4.69E-13
thiamine-containing compound metabolic process	4.69E-13
response to oxygen-containing compound	1.03E-11
response to organic substance	6.06E-08
response to acid chemical	6.66E-08
defense response to other organism	9.80E-08
response to chemical	1.09E-07
response to biotic stimulus	1.85E-07
response to external biotic stimulus	2.19E-07
response to endogenous stimulus	2.19E-07
response to other organism	2.19E-07
proline catabolic process	2.19E-07
Unique SA 8 h - Downregulated	
GO Term	**False Discovery Rate (FDR)**
protein tetramerization	1.32E-02

Table 3.6 Continued

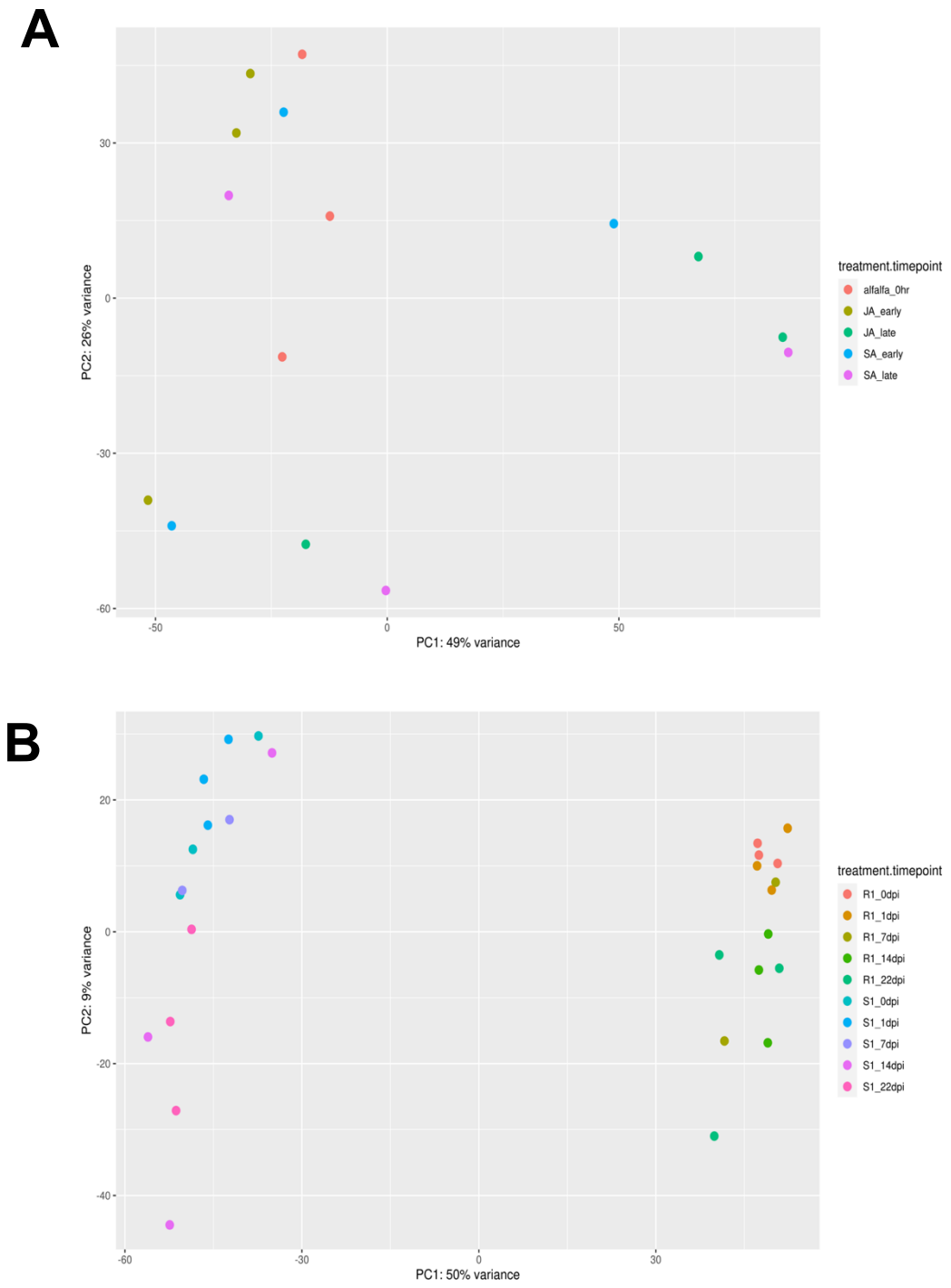
Unique JA 8 h - Downregulated	
GO Term	**False Discovery Rate (FDR)**
photosynthesis, light harvesting	5.66E-56
protein-chromophore linkage	7.39E-46
generation of precursor metabolites and energy	1.22E-27
photosynthesis, light harvesting in photosystem I	1.57E-25
photosynthesis, light harvesting in photosystem II	2.39E-12
photosynthesis	2.80E-11
response to herbicide	6.73E-08
response to light stimulus	7.84E-08
cellular protein modification process	9.90E-08
protein modification process	9.90E-08
chlorophyll biosynthetic process	1.55E-07
response to radiation	2.79E-07
chlorophyll metabolic process	5.62E-06
porphyrin-containing compound biosynthetic process	9.48E-06
macromolecule modification	1.21E-05
tetrapyrrole biosynthetic process	2.63E-05
cellular protein metabolic process	3.63E-05
protein metabolic process	7.69E-05
porphyrin-containing compound metabolic process	1.03E-04
tetrapyrrole metabolic process	2.05E-04
response to high light intensity	2.24E-04



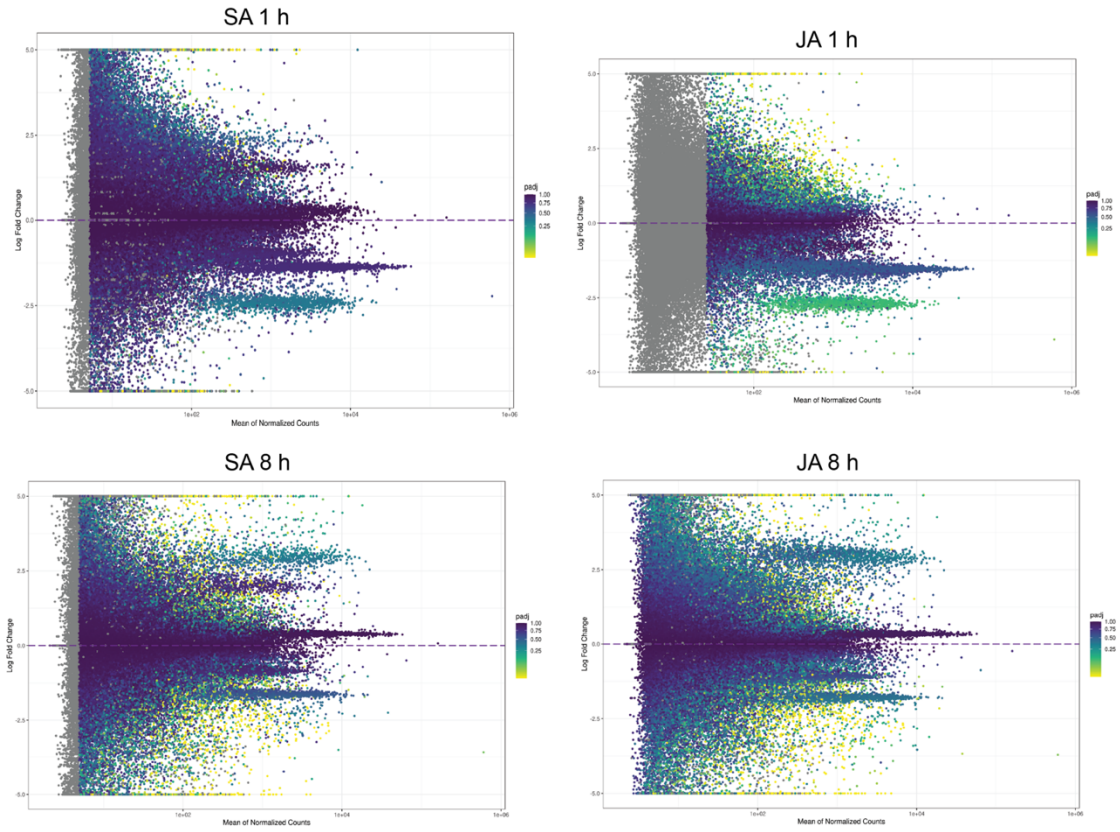
Supplemental Figure 3.1 RT PCR of SA and JA sentinel genes after phytohormone treatment in alfalfa.



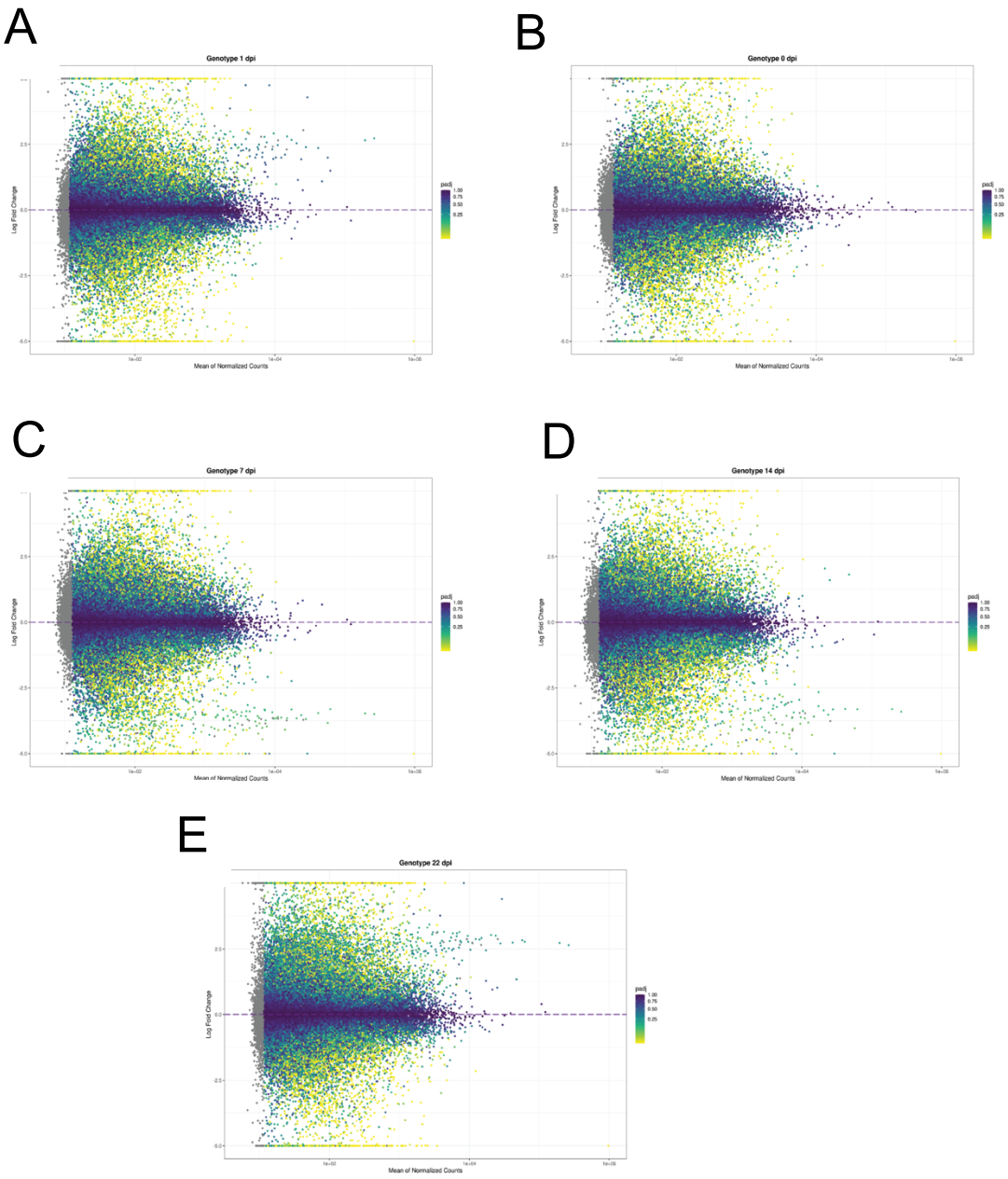
Supplemental Figure 3.2 RNA Gel of Phytohormone treated alfalfa samples for transcriptome libraries


A
30
0
-30
-60
PC2: 26% variance
-50
0
50
PC1: 49% variance
treatment.timepoint
alfalfa_0hr
JA_early
JA_late
SA_early
SA_late
B
20
0
-20
-40
PC2: 9% variance
-60
-30
0
30
PC1: 50% variance
treatment.timepoint
R1_0dpi
R1_1dpi
R1_7dpi
R1_14dpi
R1_22dpi
S1_0dpi
S1_1dpi
S1_7dpi
S1_14dpi
S1_22dpi

Supplemental Figure 3.3 PCA Plots of (A) Phytohormone RNAseq analysis and (B) alfalfa-whitefly RNAseq re-analysis.

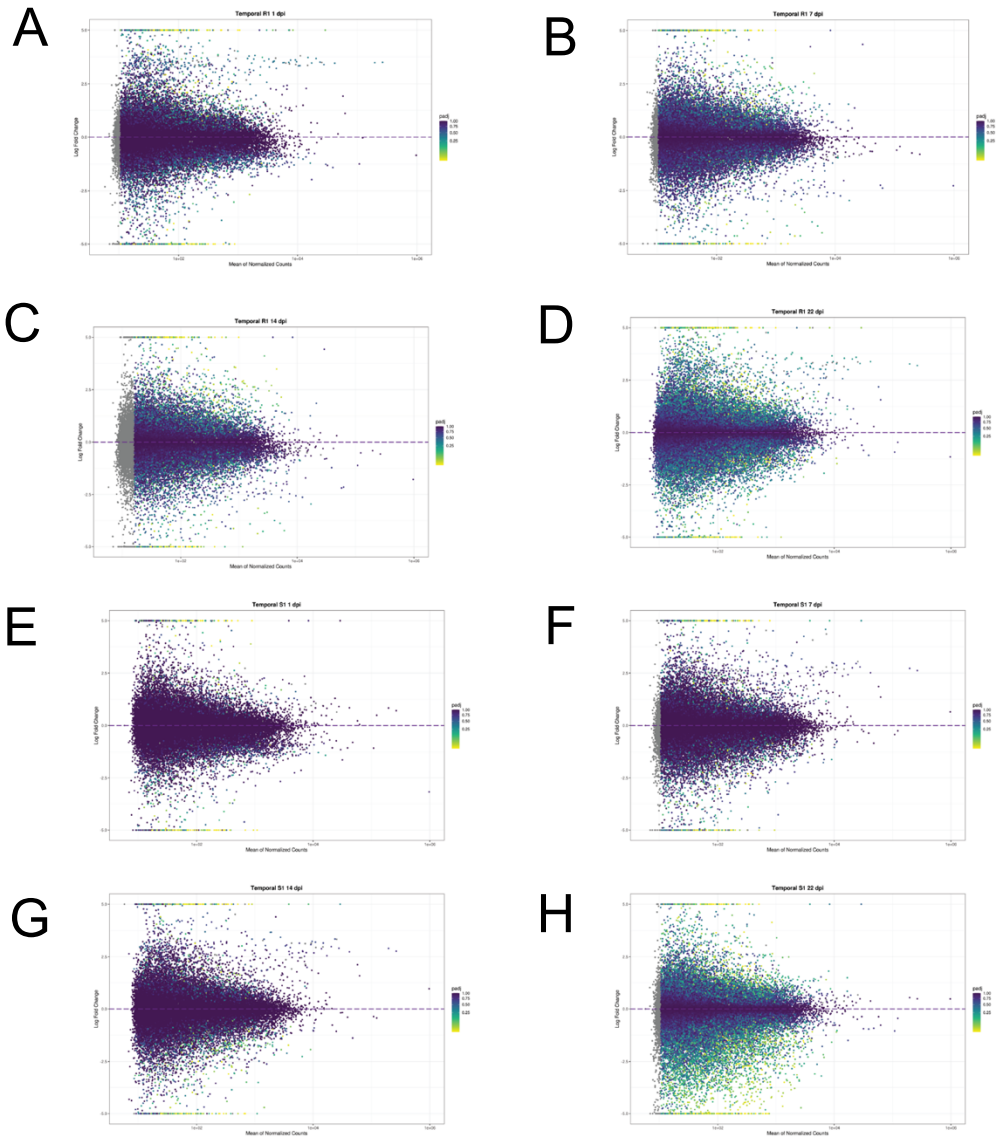


Supplemental Figure 3.4 MA Plots of Phytohormone Analyses



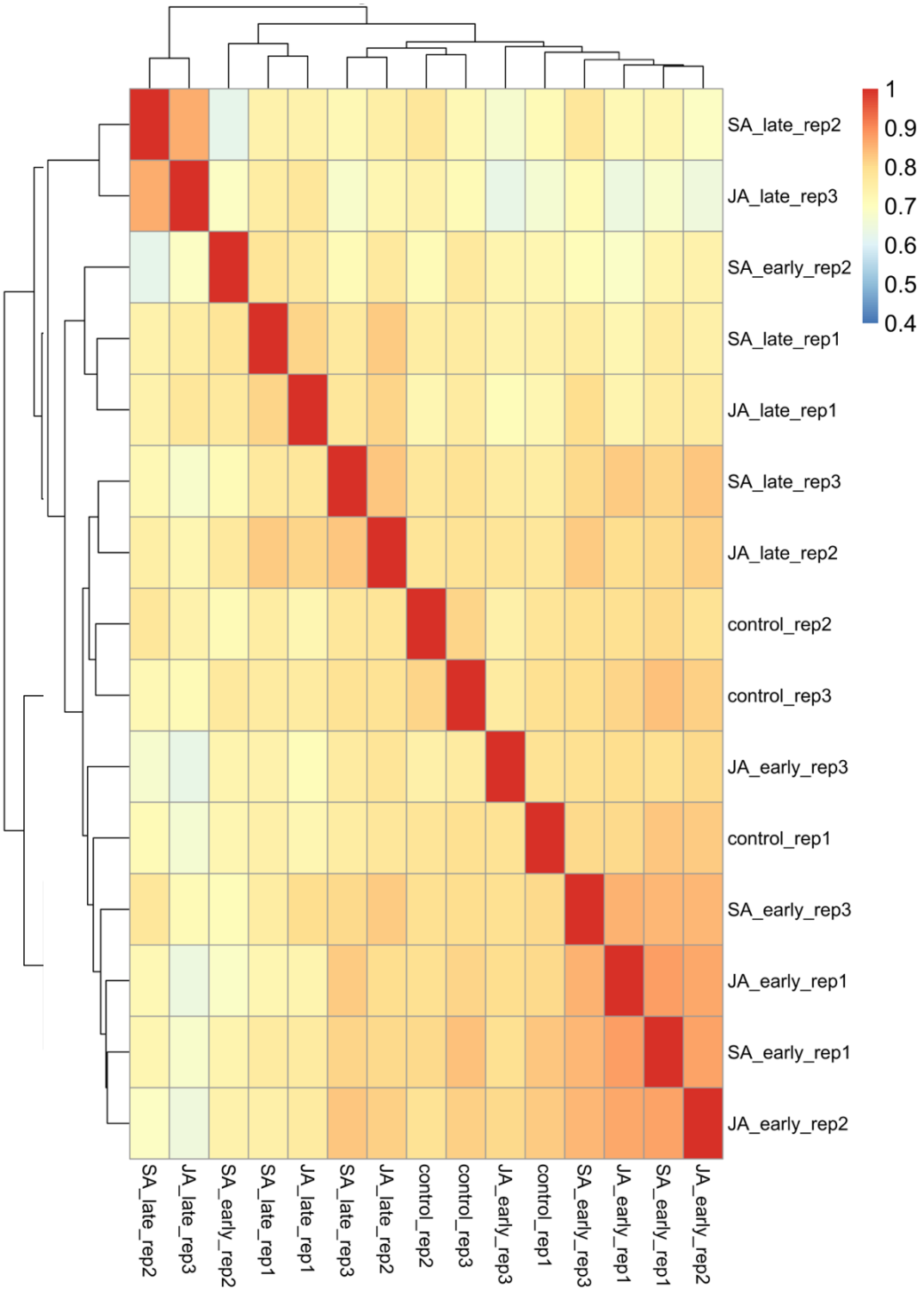
Supplemental Figure 3.5 MA Plots of gDEG Analyses

MA plots for each time point (0dpi – 22 dpi) in the alfalfa-whitefly treatment (Supplemental 3.5.A – 3.5.E

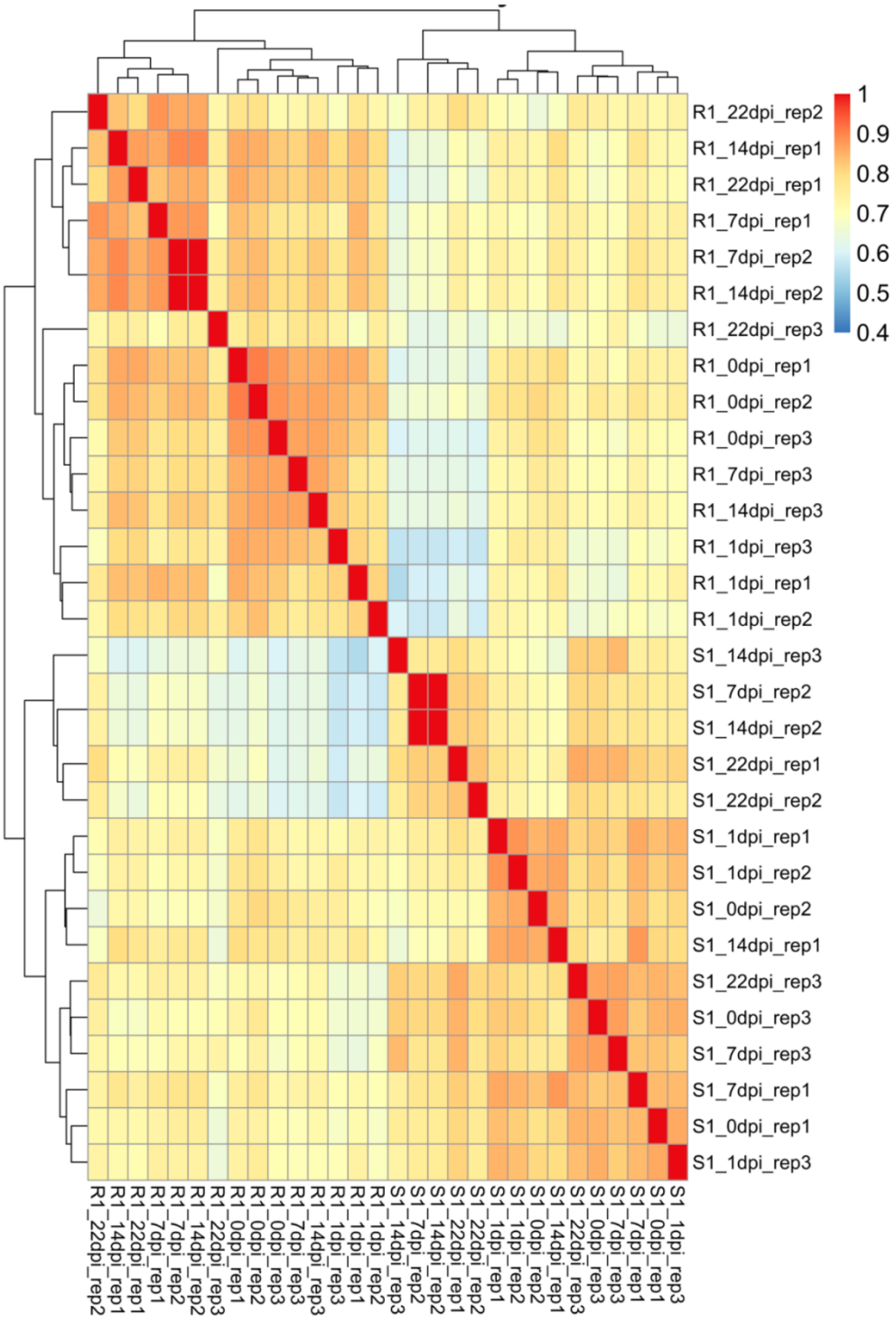


Supplemental Figure 3.6 MA Plots of tDEG Analyses

MA plots for each time point (1dpi – 22 dpi) in the alfalfa-whitefly treatment for resistant (Supplemental 3.6.A – 3.6.D) and susceptible (Supplemerntal 3.6.E – 3.6.H) alfalfa.



Supplemental Figure 3.7 Pearson correlation analysis of phytohormone libraries



Supplemental Figure 3.8 Pearson correlation analysis of alfalfa – whitefly libraries

Conclusion

The emergence of *Bemisia tabaci* MEAM1 as an invasive pest in California and North America has made management of this pest a high priority (Gill 1992; Gonzalez et al. 1992; Toscano et al. 1994; Naranjo and Ellsworth 2009). Best strategies to manage this pest center around integrated pest management (IPM) systems that prioritize host plant resistance (HPR). While HPR mechanisms for whiteflies have been identified in cotton, *Brassica*, melon, tomato, and multiple legume species (Butter and Vir 1989; Farnham and Elsey 1995; Simmons and Levi 2002; Rodriguez-Lopez et al. 2011; Firdaus et al. 2012; Cruz and Baldin 2017; Sari and Sulistyo 2018; Silva et al. 2019; dos Santos et al. 2021), the identification of additional whitefly HPR would go a long way in preventing the emergence of superabundant whitefly populations in complex cropping systems (Naranjo and Ellsworth 2009).

With this in mind, Teuber et al. (1997) identified a whitefly-resistance mechanism in alfalfa that is manifested as reduced adult populations and a corresponding reduction in honeydew secretions on resistant plants in the field. Highly-resistant lines were used to create a whitefly-resistant alfalfa germplasm (UC-356). Jiang et al. (2003) screened an elite subset of lines from this germplasm and found that nymph development is inhibited in the first-instar stage. While nymphs are able to reach the phloem on resistant alfalfa lines, phloem ingestion is reduced (Jiang and Walker 2007). The individuals screened by Jiang et al. (2003) were lost over time. However, the original germplasm was had been used by Larry Teuber (UC Davis) to create three populations of alfalfa: one population hypersusceptible to whiteflies (UC1872) and two populations resistant to whitefly (UC2933 and UC2845).

Because of alfalfa's high heterozygosity and polyploidy, there is no guarantee an individual line in a population will confer the desired phenotype (Li and Brummer 2012; Hawkins and Yu 2018). While these genetic properties make breeding for broad resistance against a wide clade of pathogens easier, it makes identifying a specific loci responsible for a trait more difficult (Comai 2005). Coupled with the limited availability of genomic resources for alfalfa, the genetic characterization of whitefly resistance in alfalfa has not been previously pursued. However, recent advances in next generation sequencing (NGS) and *de novo* transcriptome assembly have made it feasible to elucidate the mechanisms associated alfalfa's whitefly resistance.

In Chapter 1, we began an earnest pursuit to identify whitefly resistance in alfalfa. We propagated over >100 lines from the three populations (UC1872, UC2933, UC2845) and established a resistance assay, which would identify resistant lines that delayed *Bemisia tabaci* MEAM1 nymph development. While the screen was inspired by Jiang et al (2003), it was simplified to allow a higher throughput and to be less labor intensive. After screening 84 independent lines, we identified several lines, which were classified as one of five phenotypes: "highly susceptible", "susceptible", "moderately susceptible", "moderately resistant", and "highly resistant". We identified three highly resistant lines (R1, R2, and R3), which had 1%, 4%, and 6% of all nymphs advance past the first-instar stage. We investigated if the resistance conferred by R1, R2 and R3 had any other impacts on whitefly behaviors in MEAM1 and two other *B. tabaci* species.

We explored nymph development time, adult choice, oviposition, and adult longevity on R1, R2, R3, and a known susceptible line (S1) using MEAM1 s, the North American native species *B. tabaci* NW1, and another invasive species *B. tabaci* MED. When we explored nymph development with the other two species, NW1 nymphs were

unable to develop on any of our alfalfa lines indicating that alfalfa is a poor host for NW1 whiteflies. In addition, while MED whiteflies were able to develop on R1, R2 and R3 at a rate similar to S1, overall MED nymph development was delayed relative to MEAM1 on S1 plants. These data suggest that not only is alfalfa's ability to delay whitefly nymph development specific for MEAM1, but alfalfa is a suboptimal host for MED and NW1. Next, we wanted to explore the differences in oviposition between resistant and susceptible lines.

All three whitefly species had similar oviposition rates on resistant versus susceptible lines were compared there were no differences for MEAM1, MED or NW1. However, we did see some differences between the three resistant lines and MED and NW1. For example, higher rates of MED oviposition occurred on R2 compared to R3 and R1 plants and NW1 had higher rates of oviposition on R1 compared to R2. Differences in the response of the three whitefly species behaviors were also evidenced in adult-choice experiments. For example, MEAM1 adults preferred S1 over R1 plants but they did not discriminate between S1 vs R2 or S1 vs R3 plants. In contrast, MED whiteflies preferred susceptible S1 over R2 and R3. Finally, the adult-choice experiments confirmed that alfalfa is a non-host for NW1, as nearly all NW1 whiteflies tested died within the 24 h. The adult longevity studies also highlighted differences in the three species. Both MEAM1 and MED whiteflies had shorter survival times on particular resistant lines. Combined, these data allow us to conclude our whitefly resistance mechanism is species-specific antixenotic influence on MED and MEAM1 whiteflies and antibiotic influence on MEAM1 whiteflies.

With the well characterized resistant and susceptible lines in hand, we investigated the whitefly resistance mechanism(s) deployed in the alfalfa line R1.

MEAM1 infestations of R1 and S1 plants were performed and samples were collected at 0, 1, 7, 14, and 22 dpi; these times correlated with the significant points of whitefly development. While an alfalfa genome was not available at the time this project was initiated, a *de novo* transcriptome assembly enabled us to identify differentially expressed genes. A three major conclusions about our data set were made. First, Principal component analysis (PCA) of the infestation time courses indicated that whitefly resistance was mostly driven by genotype versus a temporal response. Of the 8202 gDEGs we identified, there were high levels of transcript reciprocity between S1 and R1. Furthermore, these expression trends were seen pre-infestation as well as at early and later times after infestation.

Second, analysis of DEGs showed that phytohormone signaling, specifically ethylene (ET) signaling, was at the core component of alfalfa's whitefly resistance response. Based on changes in transcript levels, R1 plants had suppressed JA, SA and ABA, as well as PTI, responses after whitefly infestation relative to S1 plants. These data were not anticipated. Based on the studies of *B. tabaci* MEAM1-Arabidopsis interactions, Zarate et al. (2007) showed that JA-SA crosstalk is an essential part of the basal immunity response in whitefly-susceptible Arabidopsis and JA-mediated defenses are required for slowing whitefly nymph development. The suppression of both JA and SA responses in whitefly-resistant R1 plants suggests a totally novel mechanism of resistance. In fact, the mechanisms deployed in R1 alfalfa are profoundly different that most hemipteran HPR mechanisms that rely on SA (Rodríguez-Álvarez et al. 2015), JA (Kamphuis et al. 2016), JA and ABA (Broekgaarden et al. 2018). In addition, the R1 mechanism resistance to MEAM1 is different that resistance to *Aleyrodes proletella* in Brassica, which is correlated with ABA (Broekgaarden et al. 2018) and *A. socialis* in

cassava, which is correlated with elevated ABA and suppressed SA (Garceau et al, in preparation).

Third, the cuticle and plant cell wall appears to play an important role in whitefly resistance in alfalfa. Among overrepresented GO terms among upregulated gDEGs expressed at all times after whitefly infestation ("constitutive DEGs") were associated with "suberin biosynthesis" and "very long chain fatty acid metabolism". Suberin and VLCFAs are associated with fortifying the cell wall and wax and cutin production, respectively. The accumulation of suberin has been linked to enhanced abiotic stress in *Arabidopsis* and increases in the waxes produced by CER1 (one of our upregulated gDEGs) results in a cuticle with reduced permeability (Bourdenx et al. 2011). It is plausible the constitutive upregulation of the suberin- and VLCFA-associated genes alters the physical barriers in R1 plants, resulting in a plant less susceptible to penetration by whitefly stylets and egg pedicels. Alternatively, the putative changes in the composition of the waxes of the cuticle and suberin in the cell wall, might generate new signals that elicit a robust defense response that deters whitefly development. Furthermore, changes in the cuticle composition could influence the phytochemicals imbedded in the cuticle and their access to whiteflies.

Finally, several gene encoding PRRs and their coreceptors, which are critical for recognizing microbial elicitors to induce MAMP/PAMP-triggered immunity were suppressed in R1 alfalfa. However, multiple chitin-responsive genes were upregulated in R1 alfalfa. Considering there is overlap between PTI and ETI, it might be reasonable to postulate the suppression of PTI is compensated by enhanced ETI in a mechanism coined called ETI-Mediating and PTI-Inhibited Sector (EMPIS) (Hatsugai et al. 2017; Chang et al. 2022; Martel et al. 2021; Yuan et al. 2021).Combined these data support

the hypothesis the spectrum of phenotypes observed among lines in Chapter 1 might be due to a multigenic whitefly resistance mechanism centered around the cuticle and ET-mediated defense responses.

Finally, while we garnered a better understanding of how whitefly resistance operates during *B. tabaci* infestation in R1 and S1 alfalfa in Chapter 2, there remained a deficit of knowledge pertaining to alfalfa's response to defense-associated phytohormones. Therefore, we elucidated the transcriptome responses of S1 alfalfa to SA and JA at 0, 1 and 8 h after treatment. The 1-h and 8-h responses to MeJA and SA were distinct. Larger numbers of up and downregulated DEGs were identified in the 8-h treatments. In addition, the GOs shared among DEGs induced by SA or JA at 1 h point to a defense response shared by both hormones . At 8-h, there were nearly no GOs associated with defense and many associated with metabolism. We also identified a large number of genes coregulated by both hormones and there was no crosstalk identified between hormones in alfalfa.

When alfalfa's responses to SA, JA and whitefly treatments were compared, few of the whitefly-regulated DEGs were SA or JA regulated. The paucity of gDEGs and tDEGs also identified as responsive to either SA or MeJA supported our hypothesis that ET signaling and other defense components play the defining role in alfalfa's whitefly resistance.

While significant advances towards understanding alfalfa's whitefly resistance mechanism has been made through my dissertation research, there are still numerous questions to be answered. While we focused on highly resistant individuals, it would be of interest to understand the differences between highly resistant plants like R1 and plants that are "moderately resistant" to whiteflies. We identified several "moderately

resistant" individuals in the UC2933 and UC2845 populations. It would be worth determining if these alfalfa lines cause whiteflies to exhibit the same behaviors related to nymph development in MED, oviposition, adult choice, and adult longevity in MEAM1, MED1, and NW1. Because we postulate alfalfa's whitefly resistance is multigenic, there is reason to believe "moderately resistant" lines might either possess antibiotic, antixenotic properties or both at weaker doses.

Another pending question is whether or not nymphs on a resistant genotype were delayed in development or if they had expired. Several staining techniques have been established and could be applied to resistant and susceptible alfalfa infested with MEAM1 whiteflies to answer that question. Additionally, following the whitefly transcriptome and transcriptomes of *B. tabaci's* endosymbionts during infestations of resistant and susceptible alfalfa may also provide clues to the reasons for nymph development delays.

Finally, the strong PCA analysis of our alfalfa-whitefly transcriptome pointed to whitefly resistance being a constitutive phenomenon. With this in mind, it might be worth analyzing the uninfested transcriptome of several lines from all three populations to see if the loci conferring resistance in R1 are the same within and across alfalfa populations. Given the differences in MEAM1, MED and NW1 performance on R1, R2, and R3 lines, it is expected that some elements of the resistance mechanism must be different; clearly the whiteflies species can discern differences in these three highly resistant lines.

Based on the data presented in this dissertation, I propose alfalfa's whitefly resistance is multigenic resulting in antibiosis (adult choice) and antixenosis (adult choice and longevity) towards MEAM1 whiteflies. There is also evidence for antixenosis and antibiosis (adult longevity) against MED1 whiteflies and that NW1 is incapable of

surviving on alfalfa. We also can conclude alfalfa's MEAM1 resistance maybe ET-dependent and is also reliant on decreased cuticle permeability, increased suberin deposition and/or suppression of PTI components. We can also conclude our alfalfa transcriptome has hormone signaling pathways distinct from whitefly-induced responses in Arabidopsis, as there are few, weak correlations between SA/JA responses and whitefly responses, with relation to gDEGs and tDEGs. The data presented in this dissertation have provided a foundation to further explore whitefly resistance in alfalfa, particularly the alfalfa populations established by Teuber et al. (1997).

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