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A Genomic Characterization of Whitefly Resistance and Defense Hormone Responses in Cassava

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ABSTRACT OF THE DISSERTATION

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and Defense Hormone Responses in Cassava

by

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Dr. Linda Walling, Chairperson

African whitefly *Bemisia tabaci* is a yield-limiting pest of the subsistence crop cassava through feeding and vectoring viral diseases. To address this issue, the African Cassava Whitefly Project is identifying whitefly resistance in South American cassava for selective breeding into African cassava. The collaborative efforts of project members at the International Center for Tropical Agriculture, UCR and the Royal Holloway University of London have focused on the genetic, transcriptomic and metabolic basis of resistance to Latin American whitefly *Aleurotrachelus socialis* in whitefly-resistant Ecuadorian cassava genotype ECU72. Here, the whitefly- and defense-hormone-responsive transcriptomes of ECU72 and its susceptible counterpart COL2246 have been defined for analysis and integration with these data sets.

To characterize the cassava-whitefly interaction, we identified a core transcriptome response of *Pathogenesis-related* (*PR*) gene families to whitefly infestation in four whitefly-

susceptible genotypes. Defense-hormone responses of COL2246 revealed that whitefly-responsive *PR* genes were mainly co-regulated by salicylic acid (SA) and jasmonic acid (JA). Cell-wall-related *PR* gene families were dominant in the responses to whiteflies and biotic stressors. *PR* gene phylogenies and biotic-stress-responsive transcriptomes revealed a possible selection in cassava for expansion of certain *PR* genes responsive to whiteflies and microbes.

More global characterization of the cassava-whitefly interaction compared whitefly-, SA-, JA-, ethylene-, and abscisic acid (ABA)-responsive transcriptomes in ECU72 and COL2246. SA responses were faster and more prolonged in ECU72, and SA-responsive genes showed reciprocity between genotypes, suggesting importance of crosstalk regulators. Hormone responses during infestation revealed evidence of ABA-mediated resistance in ECU72 and SA-mediated susceptibility in COL2246. Comparisons between cassava and Arabidopsis revealed divergent transcriptome responses to SA and JA. Enrichment analyses of whitefly- and hormone-regulated genes showed that early infestation prompted cell-wall-based defenses in ECU72, while late infestation invoked SA-signaling and immunity defenses in COL2246.

To identify whitefly defense genes, eQTL analysis was performed utilizing F_1 progeny generated from crosses of ECU72 with whitefly-susceptible COL2246 or 60444. Criteria applied to eQTLs identified immunity, defense-signaling and cell-wall-related processes as possibly important in defense against whiteflies. eQTL-identified genes involved in ABA responses, monolignol biosynthesis, and chitin perception/response are proposed as possible whitefly resistance factors for evaluation in transgenic cassava.

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Introduction

The tropical root crop cassava (*Manihot esculenta*) is a subsistence crop for East and Central African farmers that has suffered large yield losses, exceeding 70% in some regions, due to recent, dramatic increases in *Bemisia tabaci* whitefly populations (Howeler et al., 2013). As phloem-feeders, whiteflies deplete photosynthates and excrete fungal-growth-promoting honeydew, slowing cassava growth and production. Most damaging to cassava, whiteflies transmit viral diseases that are increasing in severity and range within Africa due to dense, “superabundant” whitefly populations (Bellotti and Arias, 2001, Legg et al., 2011). Many previous and current approaches to address whitefly superabundance including the use of insecticides and virus-resistant cassava varieties have proven to be ineffective, resulting in ongoing cassava viral pandemics (Legg et al., 2011).

To achieve food security for affected African farmers, an international team (the African Cassava Whitefly Project, ACWP), has recently formed to address this issue, with one solution being the production of whitefly- and virus-resistant cassava varieties (Zaidi et al., 2017). A major focus on the search for resistance was placed on the whitefly-resistant Ecuadorian cassava genotype ECU72 and for comparison the Colombian whitefly-susceptible cassava genotype COL2246. ECU72 possesses robust resistance to the African (*B. tabaci*) and Latin American (*Aleurotrachelus socialis*) whitefly, causing death of developing whitefly nymphs (Bellotti and Arias, 2001, Bohorquez et al., 2013, Omongo et al., 2012). ACWP teams at CIAT (International Center for Tropical Agriculture), UCR and RHUL (The Royal Holloway University of London) have collaborated to study whitefly resistance in ECU72 through QTL mapping, transcriptomics and metabolomics, respectively. At CIAT, restriction site-associated DNA (RAD) sequencing was used to map

whitefly (*A. socialis*)-resistance loci in ECU72. Whitefly (*A. socialis*) infestation experiments performed at CIAT have also been used to define the whitefly-responsive transcriptomes and metabolomes of ECU72 and COL2246 at UCR and RHUL, respectively.

Here, I describe my contribution to the ACWP effort by defining and analyzing the whitefly- and defense-hormone-responsive transcriptomes primarily in ECU72 and COL2246 in order to identify genes and pathways, which characterize the response of these whitefly-resistant and whitefly-susceptible cassava genotypes to whitefly infestation. Ultimately, such information, in addition to that provided by QTLs and metabolite markers, will be used to inform breeding/transformation efforts to introduce sources of whitefly-resistance into virus-resistant African cassava lines for eventual deployment in affected regions. In this Dissertation, a review of cassava's role in African agriculture, whitefly biology and methods of whitefly control will be provided as background information to elaborate on the cassava-whitefly issue. An overview of plant immunity will then be provided before summarizing what is currently known in the field of plant defense concerning defense against whiteflies and other Hemipteran pests. A summary of objectives of the Dissertation is lastly provided.

Cassava - the Hardy Staple of Africa:

Cassava is an incredibly important crop that feeds an ever-growing global population, providing the fourth highest source of calories in the world. While cassava appears in many South American dishes and is commonly used as raw material in Asian processed products, small shareholder farmers of sub-Saharan Africa often rely on their

cassava crop as a dietary staple to feed themselves and their families (FAO, 2018, Howeler et al., 2013). Cassava feeds over 500 million people daily in Africa, and provides not only invaluable calories but essential macro- and micro-nutrients. Cassava is mainly utilized for its starchy underground storage roots (also known as root tubers), which are a major source of starch, in addition to vitamin C, niacin and riboflavin. The leaves of cassava are also edible, providing a good source of protein, vitamin C, B vitamins, potassium, and calcium (FAO, 2018, Latif and Müller, 2015). However, cassava roots and especially leaves contain high enough levels of cyanogenic glucosides to harm humans if ingested without proper processing. Such compounds are broken down in response to wounding, such as through herbivory. The disruption of cell walls allows the release of the enzymes linamarase and hydroxynitrile lyase into intracellular spaces, where they breakdown cyanogenic glucosides to form the toxic compound hydrogen cyanide (HCN). To remove enough cyanogenic glucosides from cassava leaf/root tissue to be safe for consumption, the tissue must be ground and boiled, allowing the release and breakdown of HCN-forming enzymes resulting in reduced HCN content (Zagrobelny et al., 2004, Panghal et al., 2019).

In addition to being a good source of nutrition, cassava is a hardy crop with many characteristics that make it ideal for small shareholder African farmers. Cassava requires very few inputs, such as water or fertilizer, due to its drought tolerance and beneficial root associations with fungi, making cassava inexpensive to grow (FAO, 2018). Drought tolerance has become an increasingly necessary crop trait in the face of climate change. Average temperature increases alone in different regions of Africa by 2030 are expected to cause losses in yields of crops such as beans, maize and banana, but are not expected to negatively impact cassava yields (Ceballos et al., 2011, Jarvis et al., 2012). The growth

cycle of cassava is also amenable to flexible harvest dates. When stakes, also known as stem cuttings or “seeds”, taken from mature cassava are planted, tuberous roots can be harvested anywhere between six months to two years later. In this way, cassava acts as a calorie reservoir that can be available for long periods of time (FAO, 2018). However, long-term storage of unprocessed cassava is problematic, as roots quickly become oxidized and take on a brownish color in a process known as post-harvest physiological deterioration (PDD), making the root less palatable and reducing its nutritional value (Reilly et al., 2003).

Cassava has been a dependable staple crop to African farmers since its introduction from South America in the 19th century (FAO, 2018), but in recent years its yields have been diminished from whitefly infestation and whitefly-vectored viruses. The two major viruses impacting African cassava are *Cassava Mosaic Virus* (CMV) and *Cassava Brown Streak Virus* (CBSV), the causal agents of Cassava Mosaic Disease (CMD) and Cassava Brown Streak Disease (CBSD), respectively (Legg et al., 2011). Both viral diseases present with leaf chlorosis, while CBSD additionally causes necrosis of the roots and the appearance of brown streaks along the stems (Dixon et al., 2003, Legg et al., 2011). CMV and CBSV was detected in Northern or Eastern Africa as early as the 1920s. However, by the early 1990s, the status of CMD in Uganda shifted from epidemic to pandemic, coinciding with a surge in whitefly population levels deemed “superabundant”. From 2004 to 2005, CBSD outbreaks in Uganda also progressed to a pandemic status; again, associated with peak whitefly population levels. Correlations of expanded areas of disease-affected cassava with increased whitefly population levels have been noted throughout sub-Saharan Africa. By 2005, one third of cassava yield losses were attributable to whitefly-vectored diseases (Alicai et al., 2007, Legg et al.,

2011). Thus, the need for African cassava varieties resistant to both cassava viruses and whiteflies is apparent.

In the pursuit of whitefly-resistant cassava, the availability of diverse germplasm and genetic resources is required. As cassava originated and was domesticated in South America, later being brought to Africa, African cassava landraces of today have lost much of their genetic diversity (FAO, 2018). However, diversity still exists within South American germplasm and the thousands of South American cassava landraces from diverse climates that have been maintained at CIAT. Utilizing this resource, large-scale screening efforts have identified many whitefly-resistant and whitefly-susceptible South American cassava varieties (Bellotti and Arias, 2001, Bellotti et al., 1999). Cassava's genetic resources have also become more available, with the identification of genetic markers followed by the release of a sequenced reference genome of the Colombian cassava genotype AM560-2 (Bredeson et al., 2016, Prochnik et al., 2012).

Despite such resources, several factors can make performing studies in cassava difficult. Conducting replicated experiments can be challenging due to cassava's size and longevity, as it takes three months to grow two-foot-tall seedlings from stakes, with added time and space costs for experiments involving mature plants. Cassava is also a predominantly outcrossing species, making it difficult to acquire homozygous lines, requiring instead the maintenance of traits through clonal propagation (FAO, 2018). As a highly heterozygous tetraploid, cassava sequences can additionally be difficult to map in RNA-sequencing and other sequencing studies (Bredeson et al., 2016). In spite of this, numerous RNA-sequencing studies have been performed on different cassava tissues types (Wilson et al., 2017), as well as in cassava shoot tissues in response to

environmental stresses such as drought (Li et al., 2017), cold (An et al., 2012, Li et al., 2017) and shade (Ding et al., 2016) or biotic stresses such as bacteria (Muñoz-Bodnar et al., 2014), viruses (Allie et al., 2014a, Amuge et al., 2017, Anjanappa et al., 2017, Maruthi et al., 2014) and mealybugs (Rauwane et al., 2018). This Dissertation will now additionally define cassava's transcriptome in response to whitefly infestation (Irigoyen et al., 2020) and defense hormones, adding new genetic resources for the cassava community. Such novel resources will aid in the identification of the genetic mechanisms underlying resistance to whitefly and other agronomically important pests/pathogens in cassava.

Bemisia tabaci – A Threat to Cassava and Other Crops:

A major pest of cassava, causing up to 70% yield losses through feeding alone, are *Bemisa tabaci* species that populate Africa. Whiteflies, arthropod insects within the order Hemiptera, are able to devastate crop yields in part due to their long lifecycle, prolific reproduction and intimate and voracious feeding style. Adult whiteflies first choose a suitable plant host through a combination of visual cues like leaf "greenness", olfactory cues such as attracting or repelling plant volatiles, physical structures on the leaf surface such as trichome density, and biochemical properties of the leaf surface such as cuticle composition (Walling, 2008, Byrne and Bellows, 1991, Wang et al., 2017).

To identify a suitable feeding site, whiteflies also utilize their specialized mouthparts, known as stylets. Belonging to the sucking feeding guild of insects, whiteflies feed through extensive interaction of their stylets with various plant cell types (Freeman et al., 2001). Whitefly stylets are anchored to the epidermal surface by a gelling saliva, which also forms a sheath around the stylets as they travel between epidermal cells. These

stylets rarely puncture mesophyll cells while navigating the apoplast until reaching the phloem where photosynthates can be obtained (Kempema et al., 2007, Lei et al., 1998). In selecting a suitable feeding site, adults can use their stylets to taste the leaf surface or probe the phloem, salivating into the phloem and then tasting a saliva/phloem sap mixture (Walling, 2008, Miles, 1999). During feeding, whiteflies deplete photosynthates and also secrete honeydew, a sugar-rich substance that promotes the growth of sooty mold on lower leaves (Kamikawa et al., 2018). It is thought that effector proteins or small RNAs within the saliva, encoded by the whitefly or its endosymbionts (symbiotic bacteria), are secreted by whiteflies or present in whitefly honeydew and suppress plant defenses to allow for continued feeding (Kaloshian and Walling, 2016, van Kleeff et al., 2016, VanDoorn et al., 2015, Wang et al., 2019, Xu et al., 2019).

Adult whiteflies typically settle on the abaxial leaf surface due to a thinner cuticle, higher number of stomata, and closer proximity to the phloem. Once settled, adult females make a small incision into the leaf surface and deposit eggs equipped with pedicels, peg-like structures that anchor the egg to the leaf by inserting into stomata or epidermal cells (Byrne and Bellows, 1991). The pedicle is an important conduit for eggs to take in water from the leaf (Voigt et al., 2019, Buckner et al., 2002, Byrne et al., 1990). Eggs develop to adults in about 20-30 days through nymph stages called instars (Byrne and Bellows, 1991). After emergence from the egg, 1st instars called “crawlers” walk the phylloplane in search of a suitable feeding site. The nymph feeds at this site more or less continuously throughout the 2nd, 3rd, and 4th instar stages. At the time the 4th instar ceases feeding, it is called a pseudopupa and shortly thereafter it emerges as an adult. Altogether, a single adult female can lay hundreds of eggs in her approximately one-month-long lifespan,

meaning that with a suitable host, whitefly populations can quickly expand in the absence of adequate intervention (Byrne and Bellows, 1991).

The rate of whitefly population growth is dependent on a combination of several factors including the plant host species/variety, the whitefly species, what endosymbionts the whitefly harbors, and what whitefly-vectored viruses the whitefly and/or plant harbors (Legg et al., 2011, Li et al., 2014, Moreno-Delafuente et al., 2013, Su et al., 2015, Sun et al., 2017). Certain plant species or varieties may be more or less resistant to a whitefly species. Whitefly-vectored viruses or endosymbionts that a plant encounters can also alter a plant's response to whiteflies, and can even alter the behavior of the whitefly vector itself (Moreno-Delafuente et al., 2013, Pan et al., 2021). There are over 1,550 species of whiteflies belonging to 161 genera, and while some whitefly species thrive on a single or small number plant hosts to which they have adapted (specialists), other whiteflies can be highly polyphagous (Mound and Halsey, 1978, Malka et al., 2018). For example, *B. tabaci*, a cryptic species complex of 39-40 morphologically indistinguishable species with multiple biotypes (De Barro et al., 2011, Mugerwa et al., 2018), has a host range estimated at over 1,000 plant species including many crops. Indeed, production of crops around the world such as cassava, cotton, Solanaceous plants, legumes, and brassicas are affected by *B. tabaci* infestation (Abd-Rabou and Simmons, 2010). Several species of whitefly infest cassava, including members of the *B. tabaci* species complex, *B. tuberculate*, *A. socialis*, *Trialeurodes variabilis*, *Trialeurodes vaporariorum*, and *Aleurothrixus aepim* (Njoroge et al., 2016, Bellotti and Arias, 2001). This ubiquitous and prolific nature of whiteflies in agriculture necessitates intervention in the form of methods of whitefly control.

Methods of Whitefly Control:

In response to devasting crop losses, several strategies aiming to control whitefly population levels have been developed or employed with varying levels of success. Of these, the use of insecticides and virus-resistant cassava lines have proven to be ineffective (Alicai et al., 2007, He et al., 2013). Insecticides are generally ineffective at controlling whitefly population levels, as whiteflies are able to develop resistance to most insecticides rapidly (He et al., 2007). It can be additionally difficult to target whiteflies with pesticides applied via foliar spray, as most whiteflies reside on the abaxial leaf surface (He et al., 2013, Ellsworth and Martinez-Carrillo, 2001). Operators of such sprays in under-developed countries additionally may lack necessary safety equipment and suffer harmful health effects due to direct pesticide exposure. The use of insecticides also poses the risk of suppressing beneficial insect populations for biocontrol and for selecting for pesticide resistance in whiteflies, and should thus be avoided or employed in a strategic manner to minimize such risks (Roush and Tabashnik, 2012).

An alternative method of controlling whitefly populations is the use of biological control agents such as whitefly pathogens, parasitoids or predators. Spray/dip treatment of host plants with entomopathogenic fungi (typically from the *Verticillium*, *Isaria*, and *Aschersonia* genera) has been shown to cause whitefly mortality, particularly for early-stage nymphs (Sani et al., 2020). Whitefly parasitoids, belonging to the *Encarsia* and *Eretmocerus* genera within order Hymenoptera, can be found worldwide and lay their eggs beneath or within whitefly nymphs, later emerging from and killing late-stage nymphs (Liu et al., 2015a). Release of parasitoid *Eretmocerus mundus* into greenhouses in large-scale trials has been shown to be effective in controlling *B.tabaci* population levels, as has been

seen for example in tomato and pepper (Stansly et al., 2005). Release of whitefly predators in greenhouses or fields in the US southwest such as coleopterans (ladybugs), *Drapetis nr. divergens* (a predatory fly) and *Orius tristicolor* (minute flower bug) has also been found to effectively control *B. tabaci* population levels on cotton (Kheirodin et al., 2020). While biocontrol is promising, it is not yet sufficient to control whitefly populations in the field.

The deployment of virus-resistant cassava varieties in Africa was also unsuccessful. Cassava resistant to whitefly-vectored CMV remained susceptible to whitefly infestation, and in some cases were found to more whitefly-susceptible than CMV-susceptible lines (Macfadyen et al., 2018). Together, this resulted in the emergence of CBSV and new CMV strains (Alicai et al., 2007) and today African cassava yields continue to be affected by the CMD and CBSD pandemics (Legg et al., 2011, Legg et al., 2014). Other methods of control, while still under development and perhaps difficult to deploy, have shown promising results. One such strategy is utilizing transgenic cassava that produce dsRNAs to target whitefly genes essential for growth and/or fitness. In transgenic dsRNA-producing tobacco, the silencing of whitefly gene targets including *vATPase-a*, an essential metabolic gene, as well as *aquaporin* (*AQP*) and *alpha glucosidase* (*AGLU*), regulators of osmotic pressure necessary for feeding, resulted in mortality of whiteflies after feeding (Thakur et al., 2014, Raza et al., 2016). Expression of an insecticidal fern protein in transgenic cotton also conferred whitefly resistance (Shukla et al., 2016). While such results are promising, the acceptance of transgenic crops widely varies among African farmers and legislators. Eight of the 47 African countries currently allow the cultivation of genetically modified crops, with only South Africa allowing cultivation of a directly edible crop, maize. As more African countries take steps to adopt genetically

engineered crops, transgenic strategies to address whitefly superabundance remain promising (Turnbull et al., 2021). However, more easily implemented strategies are required to alleviate the immediate food insecurity crisis at hand.

As opposed to previously mentioned control methods, the selective breeding of whitefly-resistance traits into locally-adapted, farmer-preferred lines holds great promise as an easily deployable strategy to control whitefly populations infesting African cassava. Unfortunately, there are few known sources of whitefly- or Hemipteran-resistance, with many uncharacterized at the genetic/molecular level (Walling and Thompson, 2012, Wang et al., 2017, Alba et al., 2011, Kant and Schuurink, 2021). Before discussing what is known from the literature concerning mechanisms of plant resistance against whiteflies, and more broadly Hemipterans, a brief review of plant immunity is provided.

Plant Immunity:

Primary and Secondary Immune Responses:

Much of our knowledge of plant immunity is based on the plant-pathogenic microbe literature, of which the basics have been found to be broadly applicable to other biotic stressors, such as insects. From this literature, plant defense has been defined as consisting of primary and secondary immune responses (Jones and Dangl, 2006). The plant primary immune response (often called basal resistance) is triggered by the perception of a pathogen based on conserved molecular signatures specific to that class of pathogen. These signatures are called pathogen-associated molecular patterns (PAMPs) and are perceived by membrane-bound pathogen recognition receptors (PRRs).

This initial perception prompts the activation of a defense response termed PAMP-triggered immunity (PTI) (Schwessinger and Zipfel, 2008).

In a secondary response, pathogens that are well-adapted to their hosts secrete small molecules called effectors to suppress the initial PTI response. However, this process is subverted if the host possesses an NLR (nucleotide-binding leucine-rich repeat proteins) receptor cognate to the effector (Cui et al., 2015, Jones and Dangl, 2006). Upon effector recognition, NLRs signal a secondary immune response termed effector-triggered immunity (ETI) that mainly involves a faster and higher amplitude induction of primary immune responses to stop the spread of the pathogen (Cui et al., 2015, Yuan et al., 2021, Eitas and Dangl, 2010, Jacob et al., 2013, Jones and Dangl, 2006).

Pathogen/Pest Elicitor Perception:

In the process of PTI responses, plants can perceive not only pathogen-derived PAMPs, which are also referred to as MAMPs (microbe-associated molecular patterns), but also several other classes of molecular signatures more generally referred to as elicitors. While some groups use the terms “elicitor” and “effector” interchangeably, here elicitors and effectors explicitly refer to those molecules which trigger PTI and ETI, respectively. PAMP/MAMPs are elicitors derived from pathogens, including for example those derived from bacterial flagellin and fungal chitin (Schwessinger and Zipfel, 2008). Elicitors derived from herbivores such as piercing/sucking or chewing insects are instead referred to as herbivore-associated molecular patterns (HAMPs) (Malik et al., 2021, Acevedo et al., 2015). HAMPs may come into contact with plant cells as components of the insect’s eggs, saliva, excretions, or may even be derived from the insect’s endosymbionts. Well-known HAMPs include fatty acid amino acid conjugates, glucose

oxidases, inceptins (ATP synthase fragments), and caeliferins found in insect oral secretions (Alborn et al., 1997, Halitschke et al., 2003, Schmelz et al., 2007). Many HAMPs begin as plant compounds that have been ingested and modified within the insect before then coming into contact with plant cells. For example, many HAMPs are insect-modified versions of plant-cell-wall components like pectin, oligosaccharides and oligogalacturonide fragments (Basu et al., 2018).

Elicitors can also be compounds endogenous to the plant host but in a fragmented or otherwise modified state following damage, known as damage-associated molecular patterns (DAMPs) (Heil et al., 2012). HAMPs that are modified from plant compounds, such as plant cell wall fragments as previously mentioned, are sometimes also considered as DAMPs. Some DAMPs serve primary functions in healthy, undamaged tissue and only function as signals of danger when damaged during wounding or attack from a biotic stressor. These include extracellular ATP, sucrose and DNA, as the presence of such molecules outside of the cell indicates damage and cell rupture. On the other hand, some DAMPs' only purpose is to act as a danger signal, such as immunomodulatory peptides which are cleaved from their originating protein after distress such as wounding is sensed (Tanaka and Heil, 2021, Heil et al., 2012). While elicitors are thought of as signaling general responses, race-specific elicitors or combinations of DAMPs from specific plant hosts and HAMPs from specific attackers can allow the plant to respond with some degree of specificity (Duran-Flores and Heil, 2016).

Plant Defense Signaling:

Both PTI and ETI responses depend on the activation of downstream defense signaling. Plants activate distinct defense-signaling programs in response to their specific

type of pathogen attacker, broadly classified by the pathogen's feeding style. Biotrophic pathogens feed without killing host cells (e.g. bacteria, viruses, certain fungi), while necrotrophs kill host cells and therefore feed on dead tissue (e.g. certain fungi) (Glazebrook, 2005). These classifications can be extended to other pathogens, as well as insect pests. For instance, piercing/sucking insects are thought to elicit biotroph-like responses, while animal herbivores, chewing insects, or wounding are thought to elicit necrotroph-like responses in a plant host (Erb and Reymond, 2019, Zogli et al., 2020).

The defense hormones salicylic acid (SA) and jasmonic acid (JA)/ethylene (ET) primarily signal responses to biotrophic and necrotrophic pathogens, respectively (Glazebrook, 2005, Pieterse et al., 2012). These responses are additionally influenced by other hormones, such as ET and abscisic acid (ABA) with many examples of synergism or antagonism between hormone pathways (Robert-Seilaniantz et al., 2011, Mur et al., 2006). Interactions between the SA and JA pathways, in some cases manipulated by pathogen or insect attackers, are the most well-documented, with known regulatory hubs in Arabidopsis including *WRKY70*, *NPR1* and *GRX480* (Caarls et al., 2015, Li et al., 2019a). In addition to phytohormones, many other signals, often in concert with one another, can relay the activation of plant defense genes in response to an attacker including reactive oxygen species (ROS), calcium (Ca^{2+}), small peptides, and lipids (Gust et al., 2017, Noctor et al., 2018, Robert-Seilaniantz et al., 2011).

In the transcript-level characterization of plant defense-signaling responses, particularly in the case of non-model plants, oftentimes hormone responses of genes are inferred from known responses of orthologous genes in the model plant Arabidopsis (Teixeira et al., 2014, Tzin et al., 2015, Ziliotto et al., 2012). However, as hormone-

responsive transcriptomes become available for a wider array of plant species, it has been documented that hormone responsiveness of genes or even large gene sets does not always follow that of Arabidopsis (Irigoyen et al., 2020, Tamaoki et al., 2013, Zhu-Salzman et al., 2004). Indeed, it should be expected that plant species distinct from Arabidopsis (i.e., those with different ploidy levels, gene family expansions/contractions or monocots) may possess genes that have lost or taken on new functionalities. Genes involved in defense are particularly under the pressure of selection for altered functionality (Jones and Dangl, 2006).

Nevertheless, Arabidopsis hormone responses are still often referred to when characterizing the defense responses of other plant species. A prominent example is the use of *pathogenesis-related* (*PR*) genes as sentinels of a plant's activated hormone responses during pathogen/pest attack. *PR* genes are an arbitrary collection of pathogen-upregulated genes, originally defined based on the accumulation of their translated protein products following pathogen attack. With this limited criteria for definition, *PR* genes thus sometimes have unclear importance in immunity, and conversely, many genes not defined as *PR* genes have been shown to be important in plant immunity. *PR* genes are commonly used as markers of hormone pathway activation in Arabidopsis, however, when their expression is assessed in other species, *PR* genes do not always act as sentinels for the hormone pathways as delineated in Arabidopsis. For example, in Arabidopsis, *PR*-1 and *PR-2* induction are common signatures of SA-mediated responses to viruses and bacteria, whereas *PR-3* and *PR-4* induction is indicative of JA- or JA/ET-mediated responses to necrotrophic fungi and chewing insects or wounding (van Loon et al., 2006). However, *PR* genes known to be exclusively regulated by SA or JA in Arabidopsis are primarily regulated by ET but also influenced by SA and JA in the model dicot tomato (Puthoff et

al., 2010). Together, the need for accurate identification of gene hormone response networks for each plant species under study is clear.

Whitefly Resistance:

While whitefly resistance at the genetic level is poorly understood, several studies on plant-whitefly interactions have provided some insight. In plant-insect interactions, mechanisms of resistance are broadly classified as antibiotic or antixenotic. Antibiosis either delays insect development or promotes mortality via toxins or antifeedants. Antixenosis deters insect settling and establishment or attracts insect natural enemies (i.e., predators, parasitoids) via volatiles or physical structures (Walling and Thompson, 2012). Moderate resistance to whitefly has been phenotypically characterized in the wild relatives of some crops including: tomato, melon, cotton, cowpea, soybean, zucchini, melon, lettuce, and broccoli (Bellotti and Arias, 2001, Cruz et al., 2014, Li et al., 2016b, Walling and Thompson, 2012). On the other hand, strong, fast-acting antibiotic whitefly resistance has only been described in cassava, alfalfa and Brassica species (Nebreda et al., 2005, Bellotti and Arias, 2001, Jiang et al., 2003, Teuber et al., 1996). Death of first-instar nymphs has been reported in the Ecuadorian cassava genotype ECU72 and lines derived from a Californian alfalfa genotype (UC-256). Well-controlled screens for whitefly resistance in large germplasm collections have only been reported in these crops (Bellotti and Arias, 2001, Jiang and Walker, 2007) (Patrick Thomas, personal communication).

While several sources of plant whitefly resistance have been documented, only one whitefly-*R* gene has been cloned to date. The NLR *Mi1.2* in tomato confers broad-spectrum resistance to *B. tabaci* Middle-East Asia Minor 1 (MEAM1) and Mediterranean

1 (MED1) (formerly known as biotypes B and Q, respectively), the potato aphid, three species of nematode, and the potato psyllid (Casteel et al., 2007, Jiang et al., 2001, Kaloshian et al., 2000, Nombela et al., 2000, Nombela et al., 2001, Nombela et al., 2003, Roberts and Thomason, 1986). *Mi1.2*-mediated antibiotic resistance to *B. tabaci* is SA-mediated, localized to the apoplast of mature leaves and developmentally regulated (Jiang et al., 2001, Rodriguez-Alvarez et al., 2015, Rodriguez-Alvarez et al., 2017). Several whitefly-derived effectors have also been identified. The *B. tabaci* salivary effector protein Bt56 (later also identified as Bsp9) suppresses WRKY33, a positive regulator of immunity genes important for whitefly resistance in Arabidopsis (Wang et al., 2019) and activates SA responses in tobacco to suppress plant defenses (Xu et al., 2019). *B. tabaci* effector BtFer1, a salivary ferritin protein, suppresses ROS and JA signaling, as well as callose defenses to promote whitefly survival on tomato (Su et al., 2019). *BtPMaT1*, a plant gene acquired by *B. tabaci* through horizontal transfer, enables *B. tabaci* survival as BtPMaT1 detoxifies tomato phenolic glucosides (Xia et al., 2021).

Whitefly resistance in various plant species is also associated with plant physical characteristics or secondary metabolites. In response to whiteflies, broad spectrum plant defenses have been observed and often involve phenolic compounds, volatile terpenoids and acyl sugars (Wang et al., 2017). For example, several studies in wild tomato have found methyl ketones (Williams et al., 1980), acyl sugars (Escobar-Bravo et al., 2016, Oriani and Vendramim, 2010) and sesquiterpenoids (Bleeker et al., 2009, Bleeker et al., 2011) to be important for a broad-spectrum resistance to insects including whiteflies. Significantly, acyl-sugar-based resistance to whiteflies has been successfully transferred from wild to cultivated tomato (Escobar-Bravo et al., 2016). Leaf physical characteristics such as trichome density and type, cuticle thickness or composition and leaf coloration

have also been found to be associated with levels of basal resistance to whiteflies (Wang et al., 2017), with trichome characteristics associated with whitefly-specific resistance in tomato (Kortbeek et al., 2021). For cassava genotype ECU72, a metabolomics study conducted at RHUL found whitefly resistance involves cell wall fortification involving higher lignin levels compared to COL2246 (Perez-Fons et al., 2019).

Varying transcriptional responses to whitefly have also been described in species such as *A. thaliana*, lima bean, tobacco, tomato, and cotton. *B. tabaci* MEAM1 induces SA responses to repress effective JA responses in the whitefly-susceptible *A. thaliana* (Kempema et al., 2007, Zarate et al., 2007). JA responses are suppressed in *B. tabaci* and spider mite co-infested lima bean, likely through SA signaling independent of SA levels (Zhang et al., 2009). SA responses are induced in tobacco infested with *B. tabaci* MEAM1, promoting the systemic production of various phenolic compounds with roles in defense (Zhang et al., 2017). In contrast, the induction of JA/ET responses, transcription factors mitogen-activated protein kinases (MAPKs) and MAPKs, and WRKY-domain containing proteins (WRKYs) are required for resistance to *B. tabaci* MEAM1 in cotton (Li et al., 2016b). As a general response of susceptible tomato to *B. tabaci* MEAM1 and *Trialeurodes vaporariorum*, JA responses are similarly elicited (Puthoff et al., 2010). Recent studies in eggplant and tomato have additionally demonstrated the importance of JA and ABA responses in enhancing resistance to the whitefly *T. vaporariorum* (Esmaeily et al., 2020, Esmaeily et al., 2021). ROS may be another signal in whitefly responses; repression of ROS production has been associated with whitefly infestation in *A. thaliana* and cotton (Kempema et al., 2007, Li et al., 2016b) and in the resistant response of cassava, pepper and eggplant antioxidative enzymes show increased activity (Esmaeily et al., 2020, Mwila et al., 2017, Wu et al., 2019). Together, a variety of plant defense

signals to whitefly infestation have been reported and thus a common signature of a resistant response to whitefly remains unclear.

Hemipteran Resistance:

While whitefly resistance remains largely uncharacterized, some information can be gleaned from characterized resistance to other phloem-feeding Hemipterans (e.g. aphids, planthoppers, leafhoppers, etc.). Over 70 aphid *R*-genes have been identified in over a dozen crop species. Aphid-resistance is commonly conferred by a nucleotide-binding leucine-rich repeat protein (NLR)-mediated and resistance is usually phloem-localized (Smith and Chuang, 2014, Walling and Thompson, 2012). Two aphid *R*-genes have been cloned, *Mi1.2*, which confers antibiotic resistance to potato aphid in tomato, and *Vat*, which confers antibiotic and antixenotic resistance to cotton and melon aphids in melon (Anstead et al., 2010, Dogimont et al., 2008, Milligan et al., 1998, Li et al., 2006b, Walling and Thompson, 2012). *Mi1.2*-mediated resistance to aphids is phloem-localized and requires SA responses (Li et al., 2006b, Walling and Thompson, 2012). SA responses have also been observed in wheat, barley, and maize infested with aphids (Chaman et al., 2003, Tzin et al., 2015, Zhu-Salzman et al., 2004). The cloned brown planthopper (BPH) NLR *R* genes *Bph14* and *Bph9* in rice are also associated with SA responses (Du et al., 2009, Walling and Thompson, 2012, Zhao et al., 2016), while moderate BPH resistance in another rice variety (IR42) is induced by ABA-mediated callose deposition (Liu et al., 2017). In rice, many BPH and white-backed planthopper NLR and receptor kinase type *R* genes have been identified or cloned, many with phloem-localized resistance and some providing broad spectrum resistance (Sani Haliru et al., 2020, Walling and Thompson,

2012, Yuexiong et al., 2020, Du et al., 2009, Ji et al., 2016, Liu et al., 2015b, Tamura et al., 2014, Wang et al., 2015d, Zhao et al., 2016). *Vat*-mediated resistance to aphids instead requires JA/ET responses and is localized to the mesophyll/epidermis (Anstead et al., 2010). Phloem-localized aphid resistance in *Medicago truncatula* mediated by the NLR *AKR* also requires JA responses (Klingler et al., 2005, Walling and Thompson, 2012). Similarly, soybean *Rag1/2*-mediated resistance to aphids is JA-Ile-dependent (Yates-Stewart et al., 2020).

Many effectors have also been described in Hemipterans (in addition to previously mentioned whitefly effectors). Effectors found in the saliva of various species of aphids found to be important for aphid colonization, survival, development, or reproduction include AcDCXR, ACE1, ACE2, C002, Armet, Me47, Mp10, Mp42, and Mp55 (Bos et al., 2010, Elzinga et al., 2014, Kettles and Kaloshian, 2016, MacWilliams et al., 2020, Mutti et al., 2006, Wang et al., 2015a, Wang et al., 2015b). Brown planthopper salivary effector proteins NlSEF and NcSP75 have also been found to affect insect survival (Matsumoto and Hattori, 2018, Ye et al., 2017).

Of the dozens of studies that have documented plant responses to Hemipterans at the transcript level, eighteen have specifically assessed modulation of phytohormone-signaling pathways. Of these responses to Hemipterans, a mixture of SA (tomato, rice), JA (cotton, melon, soybean, and *M. truncatula*) and ABA (eggplant, tomato and rice) responses have been found to confer resistance, while mainly SA responses (*A. thaliana*, tobacco, lima bean, maize, wheat, barley) have been documented as responses of susceptible or tolerant plants (Anstead et al., 2010, Chaman et al., 2003, Du et al., 2009, Esmaeily et al., 2020, Esmaeily et al., 2021, Klingler et al., 2005, Li et al., 2016b, Li et al.,

2006b, Liu et al., 2017, Puthoff et al., 2010, Rodriguez-Alvarez et al., 2015, Tzin et al., 2015, Yates-Stewart et al., 2020, Zarate et al., 2007, Zhang et al., 2009, Zhang et al., 2017, Zhao et al., 2016, Zhu-Salzman et al., 2004). Hemipteran probing is thought to resemble cell wall stress caused by fungi/bacteria; although, aphids cause significantly more cellular damage and spend less time in contact with host cells than whiteflies (Kempema et al., 2007, Martinez de Ilarduya et al., 2003). Thus, it is tempting to speculate that responses to aphids resemble responses to necrotrophs (JA/ET) and that responses to whiteflies resemble responses to biotrophs (SA) (Walling and Thompson, 2012). However, with limited examples, and likely species-specific responses, a signature of aphid, whitefly, or Hemipteran-induced transcript-level responses in plants remains unclear.

Objectives of the Dissertation:

Whitefly superabundance is an ongoing source of food insecurity for small shareholder African cassava farmers (FAO, 2018). Methods of whitefly control employed or under development such as insecticides, virus-resistant cassava and transgenic cassava are either ineffective, lengthy, or not currently accepted by African governments (Alicai et al., 2007, He et al., 2013, Turnbull et al., 2021). Instead, the development of whitefly-resistant cassava varieties for deployment in affected regions holds promise as a more immediate solution. Identification of whitefly resistance and underlying resistance traits in South American cassava for transference to African cassava lines via breeding can achieve this goal. Also required is a better understanding of the mechanisms of whitefly resistance in cassava at the genetic level through analyzing the transcriptome

response of cassava to whitefly infestation. To identify whitefly-regulated genes that may have a role in defense, the defense hormone (SA, JA, ET, ABA) responses of such genes must also be assessed. The current understanding of defense-hormone responses in Arabidopsis is often not broadly applicable to other plant species for which such resources are unavailable, such as cassava. Thus, to better understand whitefly resistance in cassava at the genetic level, this Dissertation aims to define and compare the transcriptome responses of whitefly-resistant and whitefly-susceptible cassava to whitefly infestation as well as to defense hormone treatment.

Chapter 1: *Genome-wide analyses of cassava Pathogenesis-related (PR) gene families reveal core transcriptome responses to whitefly infestation, salicylic acid and jasmonic acid*

As a first characterization of the cassava-whitefly interaction at the transcriptome level, the responses of *PR* genes, common markers in defining pest/pathogen responses, to whitefly infestation and defense hormones was defined (Irigoyen et al., 2020). Four whitefly-susceptible cassava lines were utilized in this study, as resistance mechanisms were not yet fully studied or ready for dissemination. The full complement of cassava *PR* gene families were annotated and phylogenetic analyses were used to identify family expansion indicative of selection in cassava. Similar *PR* gene expression programs across cassava genotypes in response to infestation were identified to define a core *PR* gene transcriptome response of susceptible cassava to whiteflies. The responses of *PR* genes to the canonical defense hormones SA and JA were also assessed to define the hormone response of whitefly-regulated cassava genes. SA and JA response data sets were only available in genotype COL2246 for this analysis. Finally, *PR* gene responses to whitefly

and a variety of other biotic stressors were compared to determine, which characterized stresses most resemble whitefly infestation. The work presented in this Chapter is published and was equally contributed to by myself and Dr. Maria Irigoyen (UCR). Infestation and whitefly time course experiments as well as sample RNA extractions were performed by Dr. Adriana Bohorquez-Chaux (CIAT).

Chapter 2: *Integrative transcriptomics reveals association of salicylic acid, abscisic acid and lignin pathways with cassava whitefly resistance*

After a limited initial characterization of the cassava-whitefly interaction using sentinel *PR* genes, a broader transcriptome analysis was performed. The study reported in Chapter 2 sought to identify differences between the resistant and susceptible responses of cassava to whiteflies. In addition to SA and JA transcriptomes used in Chapter 1, ET- and ABA-responsive transcriptomes were used to allow a more comprehensive understanding of the complexity of cassava's defense hormone responses. The full transcriptomes of whitefly-resistant ECU72 and whitefly-susceptible COL2246 following whitefly infestation (Dr. Maria Irigoyen, UCR) and treatment with the defense hormones SA, JA, ET, and ABA were defined. Defense-hormone-responsive transcriptome timing, magnitude, and response directionality was compared between treatments and cassava genotypes. To place cassava's hormone responses within a broader context, hormone-responsive gene expression trends were compared between cassava and the model eudicot *Arabidopsis thaliana*; this included an in-depth analysis of hormone perception, biosynthesis, transport and response genes.

Comparative analyses of infestation-responsive genes between genotypes were additionally performed, with special attention to hormone pathway genes. Measurements

of identifiable defense hormones (SA, JA and ABA but not gaseous ET) were taken for infested samples (Dr. Laura Perez-Fons, RHUL) for comparison to transcriptome responses. Hormone and infestation transcriptome data sets were then combined in an integrative analysis to determine hormone regulatory status of whitefly-regulated genes. Up- or down-regulation by SA, JA, ET and ABA was used as an indicator of possible defense roles of whitefly-responsive gene sets. As such, these gene sets underwent enrichment analyses to identify processes possibly involved in resistance or susceptibility mechanisms. Pathways indicated as important for resistance or susceptibility to whitefly infestation from this analysis were further explored in this Chapter.

Chapter 3: *Identification of whitefly defense genes via eQTL analysis*

QTL mapping studies were performed at CIAT (Dr. Luis Augusto Becerra Lopez-Lavalle, Dr. Adriana Bohorquez-Chaux, Dr. Anestis Gkanogiannis, and Dr. Vianey Paola Barrera, CIAT) to define the genetic intervals critical for whitefly resistance. These studies used F_1 populations from crosses between the whitefly-resistant ECU72 with the whitefly-susceptible COL2246 or 60444 and defined several broad whitefly resistance loci. To narrow down important defense genes within such regions and to complement the QTL studies, an RNA-sequencing based approach to expression QTL (eQTL) mapping was utilized. Growth and RNA extraction of samples for sequencing was performed by Dr. Adriana Bohorquez-Chaux at CIAT. eQTLs have the added advantage of capturing regions of interest missed by QTL mapping due to the added association of expression with sequence data. RNA-sequencing of resistant and susceptible individuals from each F_1 population was used for eQTL analysis, with genotype and expression data derived from sequence reads. Genotype data was provided by Dr. Anestis Gkanogiannis at CIAT.

eQTLs identified for each population were filtered to better identify those with possible roles in defense against whiteflies using criteria such as status as an eQTL hotspot, parental differentially expressed gene (DEG), eQTL in both populations, or known defense gene as well as location within a QTL or eQTL physical cluster. eQTL-identified defense genes were assessed for strict correlation of parent and progeny expression with resistance phenotypes, or for previous evidence of roles in whitefly resistance. Genes meeting these criteria were highlighted as possible whitefly resistance factor candidates to be evaluated in transgenic cassava.

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Chapter 1

Genome-wide analyses of cassava *Pathogenesis-related* (*PR*) gene families reveal core transcriptome responses to whitefly infestation, salicylic acid and jasmonic acid

Abstract

Whiteflies are a threat to cassava (*Manihot esculenta*), an important staple food in many tropical/subtropical regions. Understanding the molecular mechanisms regulating cassava's responses against this pest is crucial for developing control strategies. Pathogenesis-related (PR) protein families are an integral part of plant immunity. With the availability of whole genome sequences, the annotation and expression programs of the full complement of *PR* genes in an organism can now be achieved. An understanding of the responses of the entire complement of *PR* genes during biotic stress and to the defense hormones, salicylic acid (SA) and jasmonic acid (JA), is lacking. Here, we analyze the responses of cassava *PR* genes to whiteflies, SA, JA, and other biotic aggressors.

The cassava genome possesses 14 of the 17 plant *PR* families, with a total of 447 *PR* genes. A cassava *PR* gene nomenclature is proposed. Phylogenetic relatedness of cassava PR proteins to each other and to homologs in poplar, rice and Arabidopsis identified cassava-specific *PR* gene family expansions. The temporal programs of *PR* gene expression in response to the whitefly (*Aleurotrachelus socialis*) in four whitefly-susceptible cassava genotypes showed that 167 of the 447 *PR* genes were regulated after whitefly infestation. While the timing of *PR* gene expression varied, over 37% of whitefly-regulated *PR* genes were downregulated in all four genotypes. Notably, whitefly-responsive *PR* genes were largely coordinately regulated by SA and JA. The analysis of

cassava *PR* gene expression in response to five other biotic stresses revealed a strong positive correlation between whitefly and *Xanthomonas axonopodis* and *Cassava Brown Streak Virus* responses and negative correlations between whitefly and *Cassava Mosaic Virus* responses. Finally, certain associations between *PR* genes in cassava expansions and response to biotic stresses were observed among *PR* families.

This study represents the first genome-wide characterization of *PR* genes in cassava. *PR* gene responses to six biotic stresses and to SA and JA are demonstrably different to other angiosperms. We propose that our approach could be applied in other species to fully understand *PR* gene regulation by pathogens, pests and the canonical defense hormones SA and JA.

Introduction

Cassava (*Manihot esculenta* Crantz) is grown by small shareholder farmers in more than 100 countries in tropical and subtropical areas, with a production close to 300 million tons (FAO, 2018). It is a tuberous crop consumed by nearly 800 million people worldwide, especially in Africa where it is a staple food for 500 million people. Cassava is well suited for meeting the challenges imposed by climate change (Ceballos et al., 2011, Jarvis et al., 2012), as cassava maintains nearly 50% of its photosynthetic rate under drought conditions (Ravi and Saravan, 2001) and is highly tolerant to acidic soils. However, cassava productivity is endangered by a variety of pests and diseases. Among these crop-damaging pests are whiteflies.

Aleurotrachelus socialis Bondar is the most damaging whitefly species in northern South America, particularly Colombia (Lundgren et al., 2013, Bellotti and Arias, 2001). Whiteflies cause direct damage to their hosts by voracious phloem feeding, honeydew production and subsequent sooty mold growth (Bellotti et al., 1999). In addition, whiteflies (*Bemisia tabaci*) are major vectors of the viruses *Cassava Mosaic Virus* and *Cassava Brown Streak Virus*, which devastate cassava in Eastern and Central Africa (Alicai et al., 2007, Colvin et al., 2004, Legg and Ogwal, 1998, Legg et al., 2014). Collectively, these attacks produce significant cassava yield losses (Fauquet and Fargette, 1990, Hillocks et al., 2001, Macfadyen et al., 2018). To reduce the impact of whiteflies on cassava, the identification of new resistance mechanisms and the use of novel transgenic strategies to improve cassava varieties has become increasingly important. A deeper understanding of the molecular basis controlling cassava's response to whitefly infestation will facilitate these strategies.

Plants have evolved a sophisticated immune system to defend themselves from pests and pathogens, as represented by the multilayered 'zig-zag' model (Jones and Dangl, 2006). In the first layer, plasma membrane-localized receptors (pattern-recognition receptors) recognize microbe- or pathogen- associated molecular patterns (PAMPs) inducing PAMP-triggered immunity (Monaghan and Zipfel, 2012). Damage-associated molecular patterns derived from the host after attack, as well as herbivory-associated molecular patterns, can also trigger PAMP-triggered immunity (Duran-Flores and Heil, 2016). The second layer involves intracellular receptors, belonging mainly to the nucleotide-binding leucine-rich-repeat (NLR) class, which recognize effectors released by the pathogen/pest to activate effector-triggered immunity (Cui et al., 2015). One of the outcomes of this initial recognition and the subsequent signaling cascades is the

expression of pathogenesis-related (PR) proteins. First reported in *Tobacco Mosaic Virus*-infected tobacco plants in the early 1970's (van Loon and van Kammen, 1970), PR proteins were later identified in many plant species after infection by a broad range of pathogens (Hou et al., 2012).

PR families are well characterized in Arabidopsis, tomato and potato (van Loon et al., 2006) and are composed of closely related homologs. Currently, there are 17 *PR* families encoding a broad spectrum of activities including glucanases, chitinases, peroxidases, thaumatin-like proteins, and proteases. With the advent of plant whole genome sequences, the complexity of *PR* gene families is beginning to emerge (Duplessis et al., 2009, Fister et al., 2016, Visser et al., 2018, Wanderley-Nogueira et al., 2012). To date, few studies have comprehensively examined expression of the entire complement of *PR* genes in response to multiple biotic stresses or defense hormones.

In this study, we defined the cassava *PR* families and propose a *PR* gene nomenclature. Using phylogenetic trees, we determined the evolutionary relatedness of cassava's PR proteins to each other and to PR proteins from a dicot (poplar, *Populus trichocarpa*) and a monocot (rice, *Oryza sativa*). To understand cassava's defense response to phloem-feeding whiteflies, we determined the expression of *PR* genes during whitefly (*Aleurotrachelus socialis*) infestation in four whitefly-susceptible cassava genotypes: COL2246 and COL1468, which are grown in South America, 60444 (one of the few cassava accessions amenable gene transformation technologies), and TME3, which is grown in Africa. Since *PR* genes are often used as markers of SA- and JA-defense responses (van Loon et al., 2006), changes in *PR* gene expression after SA and JA treatments were also determined and correlated with whitefly infestation. Lastly, *PR*

gene responses to whiteflies were compared to data sets in the literature that documented responses to five other aggressors: the cassava mealybug *Phenacoccus manihoti*; the bacterial blight pathogen *Xanthomonas axonopodis* pv. *manihotis*; the fungus causing cassava anthracnose disease *Colletotrichum gloeosporioides*, *Cassava Mosaic Virus* (CMV), and *Cassava Brown Streak Virus* (CBSV) (Allie et al., 2014a, Amuge et al., 2017, Anjanappa et al., 2017, Lopez et al., 2005, Maruthi et al., 2014, Muñoz-Bodnar et al., 2014, Rauwane et al., 2018, Utsumi et al., 2016). Together, our integrative analyses defined the core transcriptome response of susceptible cassava to whitefly infestation, and revealed key *PR* gene families (*PR-2*, *-5*, *-7* and *-9*) in the responses of cassava to whiteflies, SA, JA, and a variety of other biotic stresses.

Results

Cassava PR family composition is similar to poplar

Using founder PR proteins defined by van Loon et al. (2006) as queries, we identified 447 PR proteins (Table S1.1). Proteins within each cassava *PR* family were used to construct phylogenetic trees to establish *PR* gene nomenclature (see Methods). Fourteen of the 17 plant *PR* families were identified in cassava. The *PR-15* and *PR-16* (*PR-15/16* henceforth) families were consolidated because searches using PR-15 and PR-16 founder proteins identified the same set of proteins (Table 1.1). *PR* gene family annotations in cassava, rice, poplar, and Arabidopsis (Table 1.1) and visualization of *PR* gene phylogenies (Figures S1.1-S1.14) was performed by Dr. Maria Irigoyen.

Table 1.1. *PR* families of cassava, poplar, rice and Arabidopsis.

***PR*-gene family**[a]	**Function**	***Manihot esculenta***	***Populus trichocarpa***	***Oryza sativa***	***Arabidopsis thaliana***
PR-1	CAP/SCP superfamily	18	14	27	23
PR-2	β-1,3-glucanases	50	73	55	70
PR-3	Chitinases - Class I, II, IV, VI, VII	22	16	17	21
PR-4	Endochitinases	5	3	6	6
PR-5	Thaumatin-like	36	39	31	42
PR-6	Proteinase inhibitors	3	16	4	7
PR-7	Aspartic endoproteases	72	70	55	78
PR-8	Chitinases - Class III	10	11	26	1
PR-9	Lignin-forming peroxidases	110	88	113	97
PR-10	Ribonuclease-like	21	26	8	3
PR-11	Chitinases - Class V	5	7	2	9
PR-12	Defensins	0	0	2	13
PR-13	Thionins	0	0	2	4
PR-14	Lipid transfer proteins	30	19	20	23
PR-15/16	Oxalate oxidase/Germin-like	59	48	42	74
PR-17	unknown	6	7	4	8
Total		447	437	414	479

[a] Founder proteins used as query for each family can be found in Table S1.1

To ground our knowledge within the context of angiosperm evolution, we identified the PR proteins from poplar (*Populus trichocarpa*), rice (*Oryza sativa*) and *Arabidopsis thaliana* (see Methods) (Table 1.1). The total number of *PR* genes ranged from 414 in rice to 479 in Arabidopsis. Similar *PR* family composition was observed in cassava and poplar. For example, *PR-12* and *PR-13* families were absent in cassava and poplar but present in Arabidopsis and rice. Additionally, the PR-10 family was larger in both cassava (21 genes) and poplar (26 genes) relative to rice (8 genes) and Arabidopsis (3 genes) (Table 1.1).

Phylogenetic analysis and physical location of cassava PR genes

To investigate the evolution of cassava's *PR* families, we constructed phylogenetic trees for PR proteins of cassava, poplar, rice, and the founder PR protein(s) for each *PR* family (van Loon et al., 2006) (Figures S1.1-S1.14). We observed that for some *PR*

families (e.g., *PR-8* and *PR-14*), cassava PR proteins were more closely related to poplar than rice, suggesting a divergence between monocots and eudicots. In contrast, some *PR* families, like *PR-6* and *PR-17*, showed no clear monocot/eudicot divergence. Finally, cassava-specific *PR* gene family expansions were found; this involved a total of 132 *PR* genes belonging to one of ten different *PR* gene families.

In addition, physical clustering of over 50% of the genes in *PR* families *1*, *4*, *5*, *7*, *8*, *9*, *10*, and *15/16* was observed (Bredeson et al., 2016) (Figure 1.1; Table S1.2). Clustering was most prevalent in the *PR-15/16* family, where 29 of the 59 *PR-15/16* genes resided within three clusters on chromosome 8, with one cluster containing 20 genes. In contrast, all 50 *PR-2* family members were singletons, with no members belonging to a physical cluster (Figure 1.1; Table S1.2).

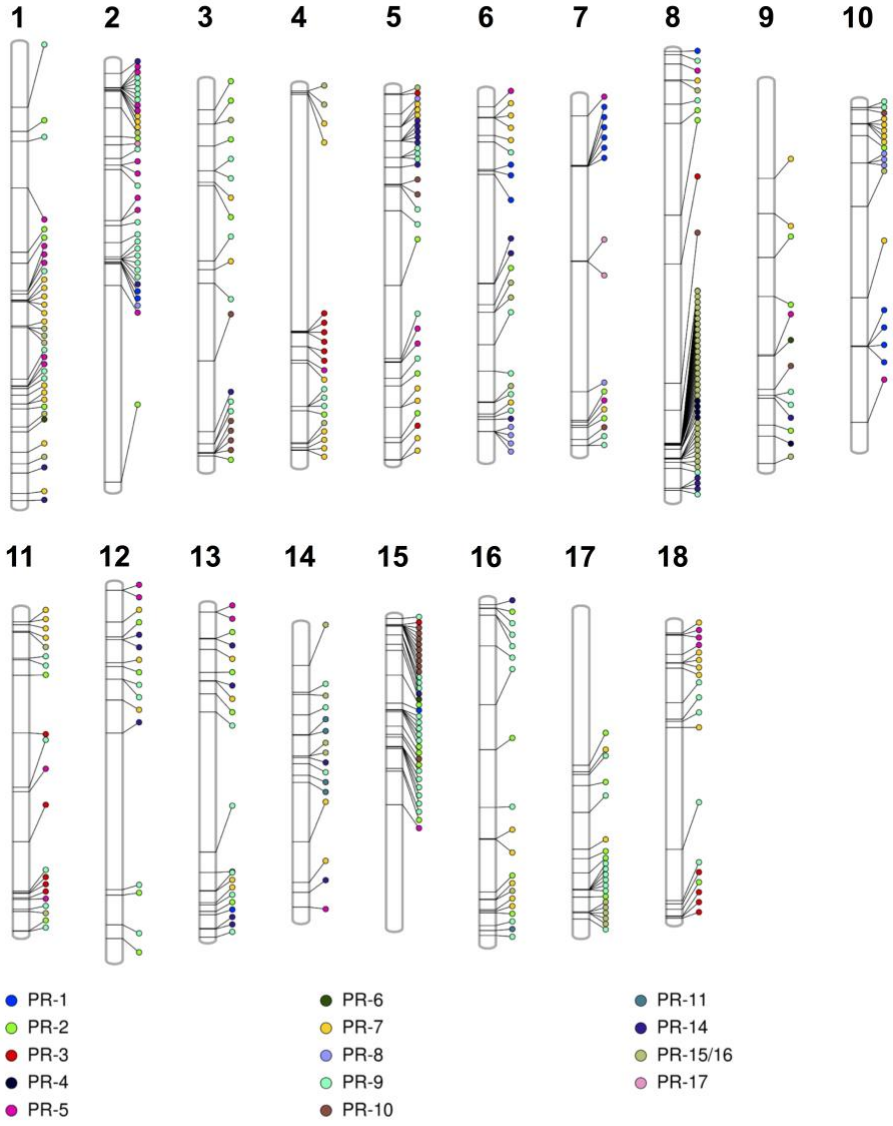

Figure 1.1. Physical locations of the 435 *PR* genes along cassava chromosomes.

PR families are color coded to reveal tandem arrays. Twelve *PR* genes have not been assigned to cassava chromosomes (Table S1.1).

Large PR families are downregulated after whitefly feeding

To characterize the response of *PR* genes to whitefly feeding, we analyzed the transcriptomes of four whitefly-susceptible cassava genotypes (COL2246, COL1468, 60444, and TME3) at 0, 1, 7, 14, and 22 days post-infestation (dpi) (Table S1.3-S1.6). We identified 167 *PR* genes that were differentially expressed (DEGs) during whitefly infestation in one or more genotypes at one or more time points (Table 1.2). RNA-sequencing, DEG analysis and data visualization for comparisons of the four whitefly-susceptible genotypes during infestation was performed by Dr. Maria Irigoyen (Figures 1.2-1.4; Table 1.2).

In the large *PR* families *2, 7* and *15/16* with 50, 72 and 59 genes, respectively (Table 1.1), DEGs were mainly downregulated in the four cassava genotypes (Table 1.2). For example, the number of downregulated *PR-2* DEGs in the four genotypes was 2.5- to 12-fold higher than upregulated DEGs; a similar trend was observed in the *PR-7* family. In contrast, fewer *PR-15/16* genes were whitefly responsive, ranging from three DEGs in TME3 to 13 DEGs in COL1468. Notably, 12 of the 13 *PR-15/16* DEGs in COL1468 were downregulated. The largest *PR* family, *PR-9* with 110 genes (Table 1.1), had variable expression profiles. For example, there were 1.6-fold more up- than downregulated *PR-9* DEGs in COL2246. While at the other end of the spectrum, 60444 had 2.3-fold more down- than up-regulated *PR-9* genes (Table 1.2). On the other hand, whitefly-upregulated DEGs were identified in most of the small *PR* families (*6*, *8*, *11*, and *17*, containing ten or fewer genes) but none were downregulated (Table 1.2).

Table 1.2. Number of differentially regulated *PR* genes in whitefly-susceptible genotypes.

PR gene family	COL2246		COL1468		60444		TME3		ALL		*PR* gene family size (# genes)
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	
1	3	3	1	4	2	1	3	1	0	0	18
2	4	10	2	21	2	19	1	12	1	8	50
3	5	2	4	3	6	4	2	1	2	0	22
4	1	0	0	0	1	0	0	1	0	0	5
5	5	4	2	12	1	6	2	5	0	4	36
6	0	0	0	0	1	0	0	0	0	0	3
7	5	11	3	15	1	16	3	7	0	2	72
8	3	0	0	0	2	0	1	0	0	0	10
9	10	6	6	7	3	7	6	6	2	1	110
10	7	1	4	3	5	3	4	0	2	0	21
11	1	0	2	0	0	0	1	0	0	0	5
14	1	2	1	0	1	0	2	1	0	0	30
15/16	1	4	1	12	2	7	1	2	1	1	59
17	3	0	1	0	2	0	2	0	1	0	6
Total Number of DEGs	49	43	27	77	29	63	28	36	9	16	

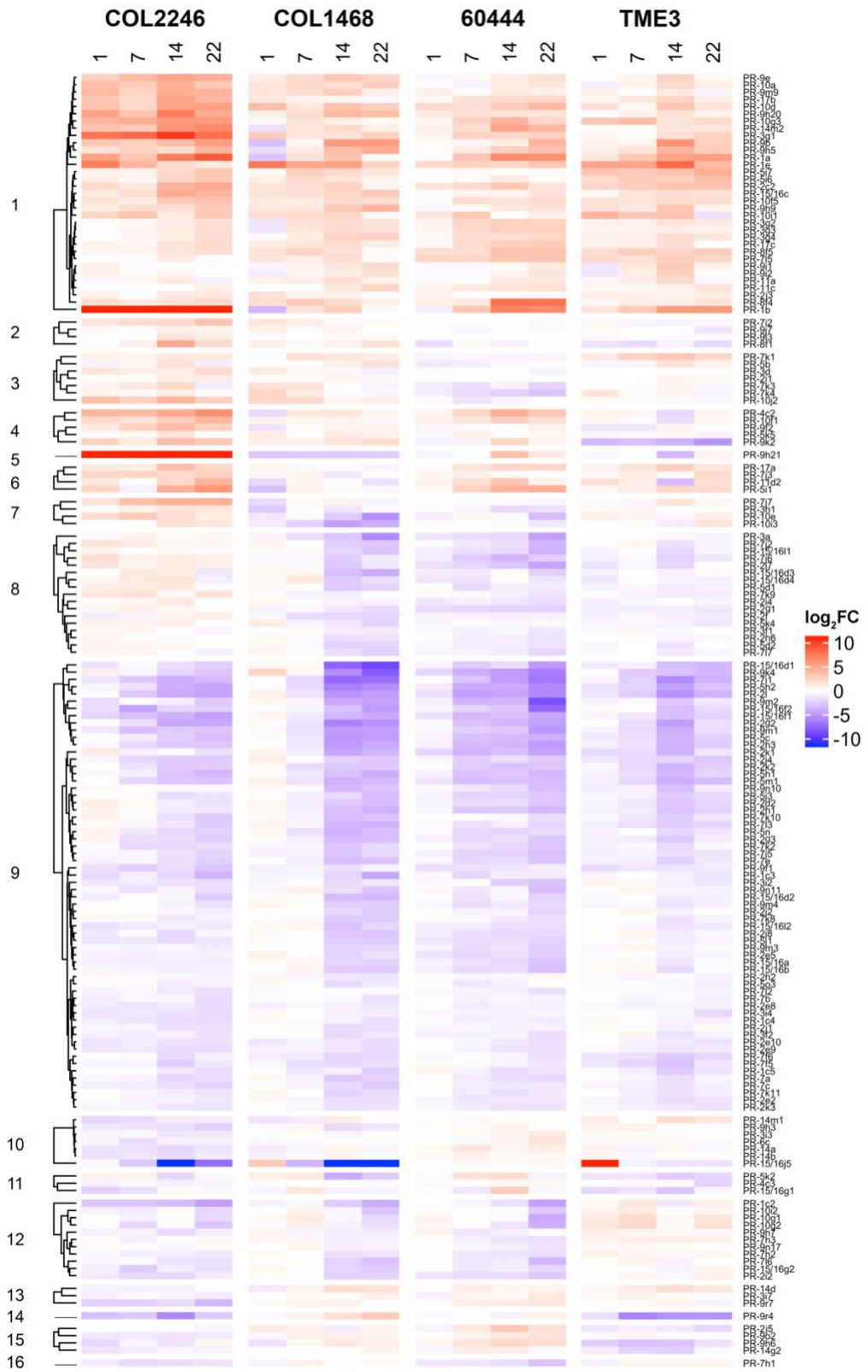
COL2246
COL1468
60444
TME3
1
7
14
22
log2FC
10
5
0
-5
-10

Figure 1.2. *PR* gene expression in whitefly-susceptible cassava genotypes during whitefly infestation.

Heatmaps display DEGs in COL2246, COL1468, 60444, and/or TME3 during whitefly infestation. *PR* genes are grouped along the y-axis by expression patterns across genotypes as defined in Table S1.15. Expression values are presented as log_2FC values in comparison to 0 dpi. These analyses were performed by Dr. Maria Irigoyen.

Timing of the response to whitefly varies among whitefly-susceptible genotypes

Heatmaps were used to define 16 temporal *PR* gene expression programs in response to whitefly feeding in the four genotypes (Figure 1.2); for cluster definitions, refer to Figure 1.4. Most striking, 57% of the 167 DEGs were similarly regulated among all genotypes, with 62 *PR* genes displaying negative trends (cluster 9) and 33 *PR* genes displaying positive trends (cluster 1) (Fig. 1.2; Table S1.15). Cluster 9 was dominated by four *PR* families: *PR-2* (19 DEGs), *PR-7* (14 DEGs), *PR-5* (8 DEGs), and *PR-9* (8 DEGs). Of the 62 cluster 9 genes, 31, 55, 39, and 28 were downregulated at one or more time points in COL2246, COL1468, 60444, and TME3, respectively (Table S1.15). A subset of these genes was downregulated in all four genotypes (16 DEGs) (Figure 1.3); eight of which were *PR-2* genes (Table 1.2). Of the 33 *PR* genes in cluster 1, only nine were upregulated in all four genotypes (Figure 1.4).

Cluster 1 and 9 genes displayed three temporal expression programs in response to whitefly infestation: early (1 and/or 7 dpi), late (14 and/or 22 dpi) and sustained (early and late). Few cluster 1 and 9 genes were differentially expressed at early time points. Only one early DEG in cluster 9 was identified (COL2246). For cluster 1, one early DEG was identified in COL2246 and 60444 and two early DEGs were found in COL1468. Finally, there are no early DEGs in either cluster 1 or 9 in TME3 (Figures 1.3 and 1.4 b-e).

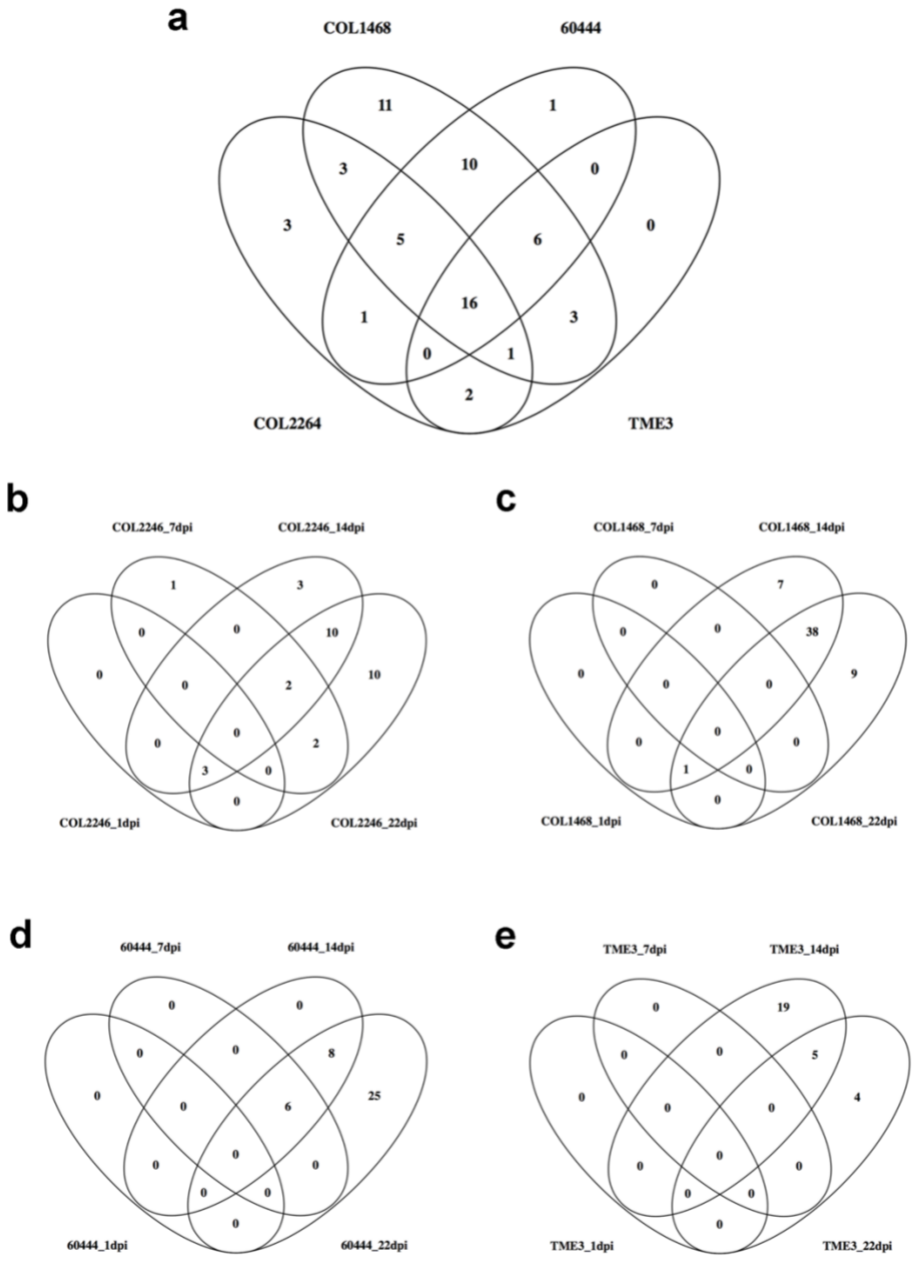

Figure 1.3. Cluster 9 *PR* gene regulation.

Venn diagrams comparing cluster 9 downregulated *PR* genes among four whitefly-susceptible cassava genotypes during whitefly infestation. **(a)** Comparison of cluster 9 DEGs in COL2246, COL1468, 60444, and TME3 during whitefly infestation. **(b)** Comparison of COL2 2 46 cluster 9 DEGs at 1–2 2 dpi. **(c)** Comparison of COL1468 cluster 9 DEGs at 1–2 2 dpi. **(d)** Comparison of 60444 cluster 9 DEGs at 1–2 2 dpi. **(e)** Com- parison of TME3 cluster 9 DEGs at 1–22 dpi. This analysis was performed by Dr. Maria Irigoyen.

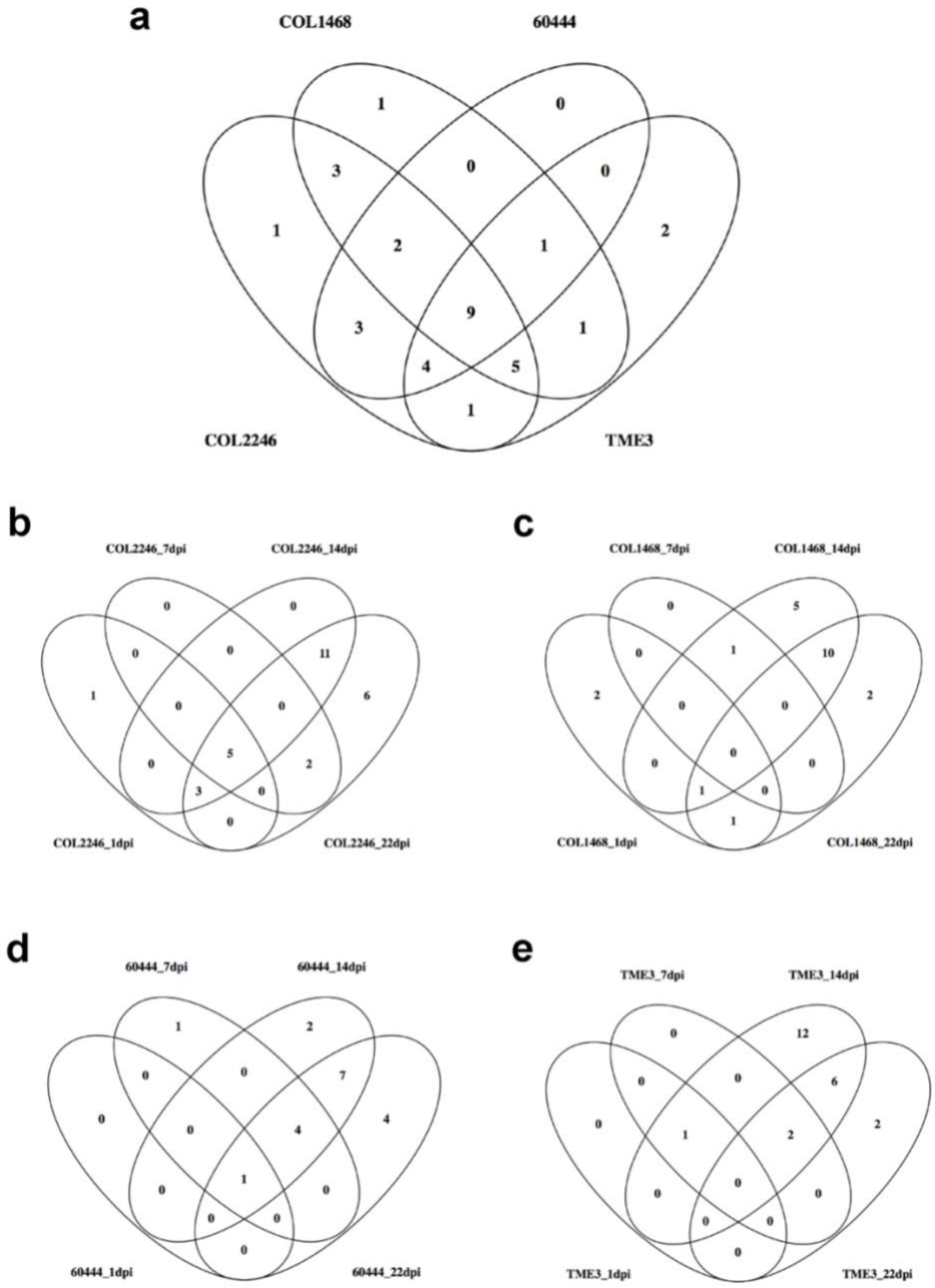

Figure 1.4. Cluster 1 *PR* gene regulation.

Venn diagrams comparing cluster 1 upregulated *PR* genes among four whitefly-susceptible cassava genotypes during whitefly infestation. **(a)** Comparison of cluster 1 DEGs in COL2246, COL1468, 60444, and TME3 during whitefly infestation. **(b)** Comparison of COL2246 cluster 1 DEGs at 1–22 dpi. **(c)** Comparison of COL1468 cluster 1 DEGs at 1–22 dpi. **(d)** Comparison of 60444 cluster 1 DEGs at 1–22 dpi. **(e)** Comparison of TME3 cluster 1 DEGs at 1–22 dpi. This analysis was performed by Dr. Maria Irigoyen.

A prominent late phase of gene expression emerged in all genotypes, which engaged most of the cluster 1 and 9 *PR* genes and corresponded to the time of 2nd and 3rd instar feeding (Figure 1.2). In all genotypes, most of the cluster 1 DEGs (39-78%) were first upregulated by 14 dpi (Figure 1.4). In contrast, the late phase of cluster 9 gene downregulation varied among the genotypes. For example, 42%, 82% and 86% of the cluster 9 *PR* genes were downregulated at 14 dpi in COL2446, COL1468 and TME3, respectively. In 60444, this down-regulatory phase was further delayed, beginning at 22 dpi when 64% of cluster 9 *PR* genes were repressed (Figure 1.3). The number of genes that displayed a sustained pattern of expression (DEGs at both early and late expression) varied among genotypes. While COL2246 and 60444 had 17 and 11 genes with sustained expression in cluster 1 or 9, respectively (Figures 1.2-1.4), fewer genes in COL1468 and TME3 (4 and 3 genes, respectively) were regulated at both early and late time points.

The remaining 43% of whitefly-responsive *PR* genes (72 genes) exhibited divergent temporal responses among the genotypes (clusters 2-8 and 10-16). For example, 17 *PR* genes in cluster 8 were upregulated in COL2246 and downregulated in the other cassava genotypes. Additionally cluster 12 genes were downregulated in COL2245, COL1468 and 60444. However, the timing of downregulation varied among genotypes, initiating later in COL1468 and 60444. Notably, ten of 60444's 17 DEGs were in this cluster. In contrast, TME3's cluster 12 genes had a slight positive trend (Figure 1.2, Table S1.15).

Cassava PR genes are predominantly co-regulated by SA and JA

To understand the roles of the two major plant-defense hormones (SA and JA) in regulating *PR* genes, we determined the transcriptomes of COL2246 at eight time points (0, 0.5, 1, 2, 4, 8, 12, and 24 h) after SA and JA treatments (Tables S1.7-S1.14). Hormone-responsive *PR* genes (103 DEGs out of the 447 *PR* genes) were organized into one of four hormone-expression programs: 1) SA-regulated (10 DEGs), 2) JA-regulated (42 DEGs), 3) co-regulated by SA and JA (49 DEGs), or 4) reciprocally regulated by SA and JA (2 DEGs) (Tables 1.3 and S1.17). *PR* families *2*, *5*, *7,* and *9* made up 65% of hormone-responsive DEGs and were mainly SA/JA co-regulated or JA-regulated. There was a very strong positive correlation ($R = 0.94$, $p = 2.2e^{-16}$) between SA and JA expression levels for the 49 DEGs defined as SA/JA co-regulated (Figure 1.5; Tables 1.3 and S1.17). Of the genes defined as solely SA- or JA-regulated, 81% exhibited similar expression levels in response to both treatments, but only met the statistical criteria to be designated as DEGs in one treatment (Table S1.17). Furthermore, while *PR* genes are useful markers to follow the activation of the SA (*PR-1*, *-2* and *-5*) and JA (*PR-3* and *-4*) pathways in Arabidopsis-pest/pathogen interactions (van Loon et al., 2006), we were unable to identify any *PR* gene that could distinguish activation of only the SA or JA pathway.

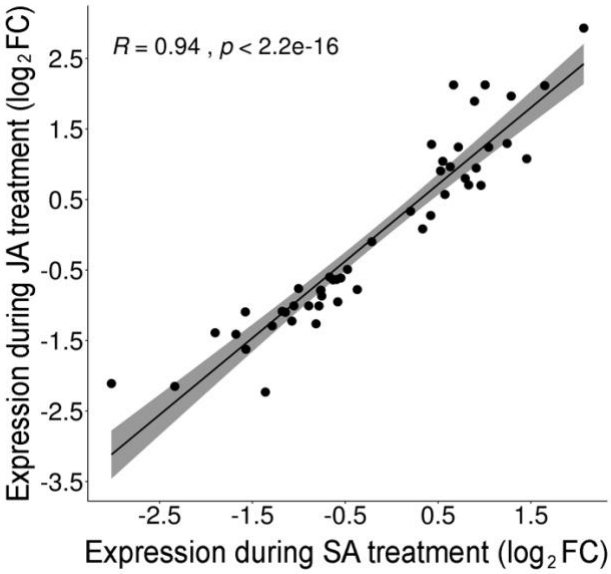

Figure 1.5. Correlation of SA/JA co-regulated *PR* genes.

Average log_2FC of DEGs in SA versus JA treatments for *PR* genes designated as SA/JA co-regulated (defined in Table S1.17). Pearson correlation value, *p*-value and a 95% confidence interval (grey) are provided.

To characterize the hormone regulation of whitefly-responsive *PR* genes in COL2246, we integrated whitefly, SA and JA transcriptome data (Figure 1.6). Among the 208 *PR* genes detected during whitefly infestation of COL2246, 152 genes were DEGs in whitefly, SA and/or JA treatments of COL2246 (Figure 1.6; Tables S1.3, S1.7 and S1.11). While plant defense typically enact a predominant SA or JA response in Arabidopsis (Mur et al., 2006, Robert-Seilaniantz et al., 2011), 122 (80%) of the 152 genes were co-expressed during SA and JA treatments (clusters 1, 2, 7, and 8). Notably, there were no whitefly-responsive *PR* genes that were solely detected after SA treatment (Figure 1.6).

In COL2246, these hormone-responsive *PR* genes displayed three temporal expression programs (early, late and sustained) after whitefly infestation (Figure 1.6). While only four whitefly-regulated DEGs followed an early expression program, 24

exhibited sustained regulation and 64 were late-regulated (Table S1.18). Genes with sustained regulation displayed more positive (40%) than negative (16%) expression trends in response to whiteflies, SA and JA (clusters 1 and 7, respectively) (Figure 1.6; Table S1.18). In contrast, for the late-regulated genes, negative expression trends were more frequent (31%) than positive (23%) trends in all three treatments (clusters 7 and 1, respectively) (Figure 1.6; Table S1.18).

Table 1.3. Hormone-regulated *PR* genes.

PR Gene Family	Number of normone-regulated DEGs				Number of hormone-responsive DEGs per *PR* family
	SA	JA	SA/JA co-regulated[b]	SA/JA reciprocally regulated	
1	0	1	2	0	3
2	1	4	10	1	16
3	2	3	0	0	5
4	0	2	0	0	2
5	1	4	5	0	10
6	1	0	1	0	2
7	2	8	14	0	24
8	0	0	1	0	1
9	1	7	8	1	17
10	1	4	2	0	7
11	0	2	1	0	3
14	0	0	2	0	2
15/16	1	6	1	0	8
17	0	1	2	0	3
Number of hormone-responsive DEGs across *PR* families	10	42	49	2	103

[a] For identities for hormone-regulated *PR* genes, see Table S1.18.
[b] SA and JA co-regulated genes are defined as genes whose RNAs are either up- or down-regulated by both hormones.

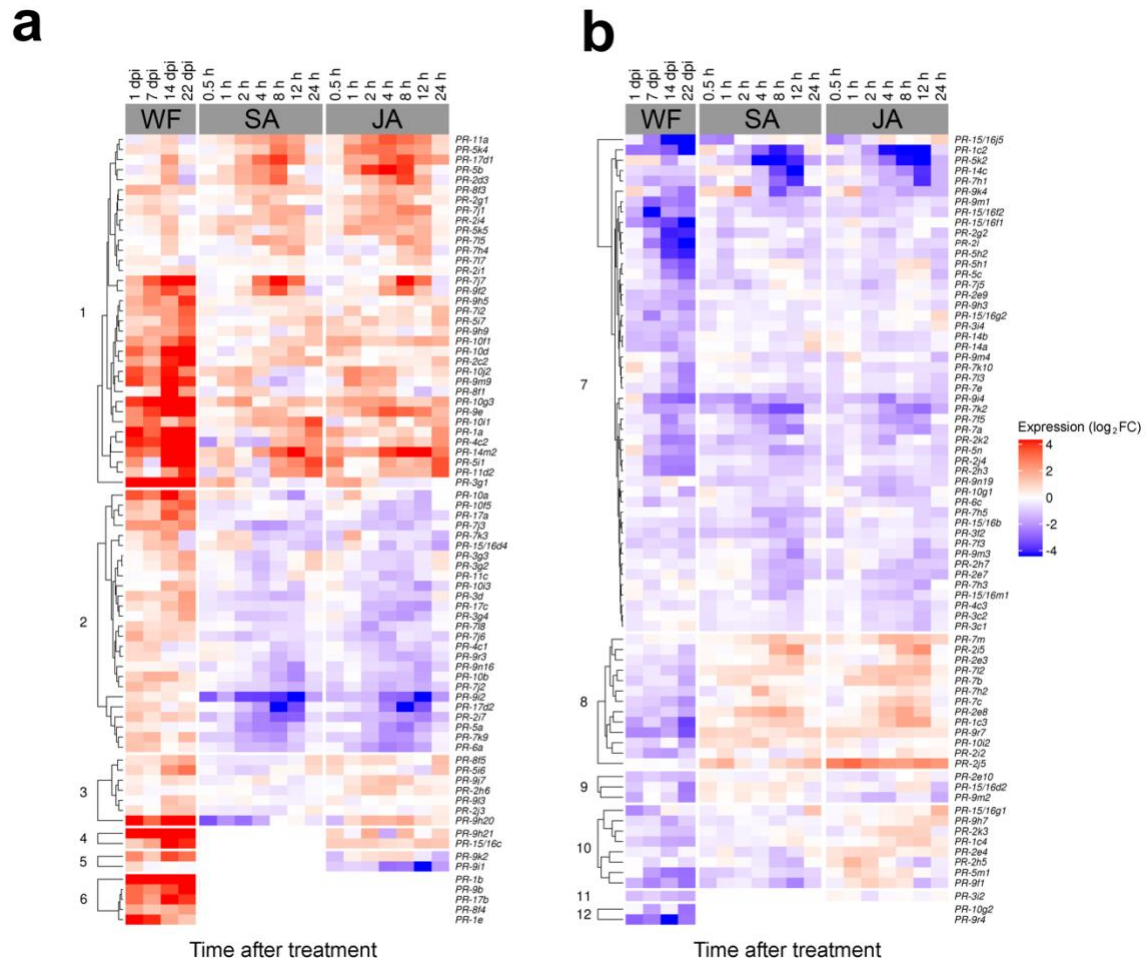

Figure 1.6. *PR* gene expression in COL2446 in response to whitefly infestation, SA and JA.

Heatmaps were organized along the y-axis to group *PR* genes with positive **(a)** or negative **(b)** expression values in whitefly (WF)-infested COL2246. Expression after SA and JA treatments are shown. log_2FC values relative to 0 dpi for whitefly-infested samples and 0 h for hormone treatments. Heatmap groups 1-12 are defined in Table S1.16.

qRT-PCR validation of RNA-sequencing data

To confirm expression values obtained *in silico*, transcript levels of selected whitefly- or hormone-responsive DEGs were assessed by qRT-PCR (Figure 1.7). Upregulation of *PR-3g4* and *PR-9e* and downregulation of *PR-7l3* at both 14 and 22 dpi after whitefly infestation was confirmed (Figure 1.7a). Similarly, *PR-9e* upregulation and *PR-7f5* downregulation after 4-h SA and JA treatments was confirmed (Figure 1.7b). In many cases, transcript fold-changes determined by qRT-PCR exceeded those measured by RNA-seq (Figure 1.7a and b). Nevertheless, expression values for *PR* genes obtained by qRT-PCR versus RNA-seq exhibited a strong positive correlation ($R = 0.73$; $p = 4.0E^{-06}$), validating our *in silico* expression values *in vivo* (Figure 1.7c).

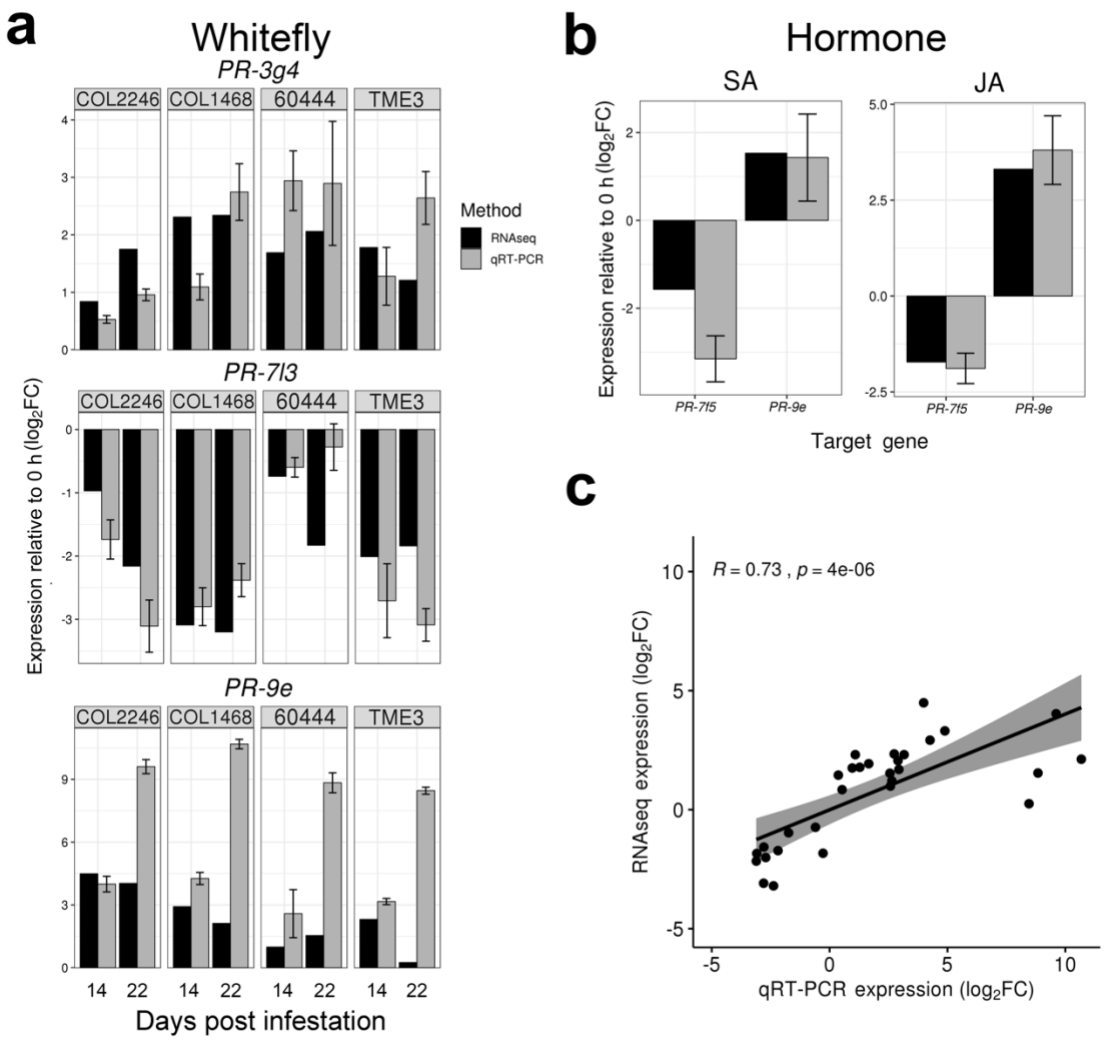

Figure 1.7. qRT-PCR validation of *PR* transcript levels.

(a-b) Relative transcript levels (log_2FC) of up- and down-regulated *PR* genes during whitefly infestation of COL2246, COL1468, 60444, and TME3 **(a)** and after SA and JA treatments of COL2246 **(b)** as determined by qRT-PCR and RNAseq. **(c)** Pearson correlation of transcript levels in **(a)** and **(b)** by qRT-PCR versus RNAseq. qRT-PCR validation of expression levels in response to whitefly infestation **(a)** was performed by Dr. Maria Irigoyen.

Comparison of PR family responses to a spectrum of biotic stressors

To more broadly define the responses of cassava's *PR* genes in pathogen and pest interactions, we compared *PR* gene expression programs to whiteflies (*A. socialis*) with five other pathogens/pests: cassava mealybugs (*Phenacoccus manihoti*), bacteria (*X. axonopodis*), fungi (*C. gloeosporioides*), and viruses (South African CMV and CBSV) (Rauwane et al., 2018, Muñoz-Bodnar et al., 2014, Utsumi et al., 2016, Allie et al., 2014a, Anjanappa et al., 2017) (Tables S1.1, S1.3, S1.7, S1.11, and S1.19). Each interaction elicited a different number of DEGs; therefore, to facilitate comparisons, the percent of DEGs from each *PR* family that responded to each biotic stress was determined (Figure 1.8a; Table 1.4). We found that *PR* families with roles in pathogen cell wall degradation (*PR-2*, *PR-5* and *PR-7*), as well as host cell wall fortification (*PR-9*) were most responsive to biotic stress, representing 10-26% of the *PR* genes responding to any of the examined stresses.

Table 1.4. *PR* gene family response (DEGs) to six biotic stresses.

PR Gene Family	Percent of response attributed to a *PR* gene family[b]						Percent of *PR* gene family that is responsive to one or more stresses
	whiteflies	mealybugs	bacteria	fungi	virus (CMV)	virus (CBSV)	
1	7	20	7	25	1	10	4
2	15	0	14	0	11	10	13
3	8	0	7	13	2	10	6
4	1	0	2	0	1	2	1
5	10	60	11	13	9	5	10
6	0	0	0	0	1	0	0
7	17	0	9	13	17	7	16
8	3	0	7	0	2	0	3
9	17	0	23	38	35	33	26
10	9	0	7	0	4	5	6
11	1	0	0	0	0	2	1
14	3	20	7	0	7	10	5
15/16	5	0	5	0	10	5	8
17	3	0	2	0	0	2	1
# of *PR* DEGs	92	5	44	8	135	42	326

[a] For identities *PR* genes differentially expressed during the biotic stresses, see Table S1.19. For study information, see Methods.

[b] Percents are rounded to the nearest integer.

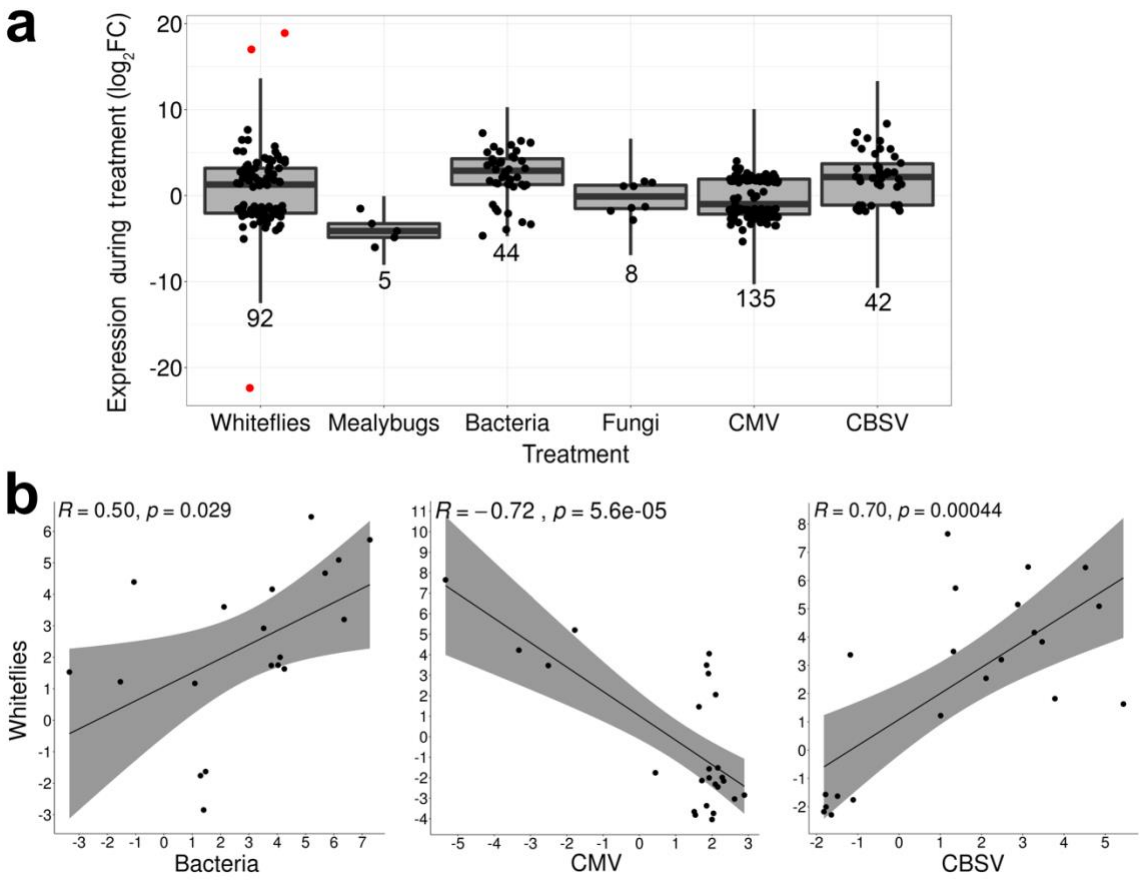

Figure 1.8. Correlation of cassava responses to whiteflies with other biotic stresses.

(a) Box-and-whisker plots overlaid with all data points displaying mean *PR* gene responses to treatments. Whiskers indicate the interquartile range multiplied by two. Total number of genes for each treatment is below each plot. Outliers (red) were identified and removed prior to correlation analyses in panel b. **(b)** Pearson correlation analyses of DEGs responding to whiteflies vs. bacteria (19 genes), CMV (25 genes) or CBSV (21 genes) were performed. Only four genes were identified in the whitefly-mealybug and whitefly-fungus interactions; therefore, these interactions were not included in these analyses.

In these interactions, 38-75% of differentially expressed *PR* genes responsive to whiteflies, bacteria or fungi were regulated by SA and/or JA (Figures S1.15-S1.28; Tables S1.20-S1.25) and a majority (57-67%) of these genes were in the *PR-5*, *PR-7* or *PR-9* families (Figures S1.19, S1.21 and S1.23; Tables S1.20, S1.22 and S1.23). In contrast, most genes (79–80%) regulated by mealybugs, CMV or CBSV were not responsive to either hormone (Figures S1.21-S1.28; Tables S1.21-S1.27).

Pearson correlation analyses were used to compare cassava's *PR* gene response to whiteflies, bacteria, CMV, and CBSV (see Methods) (Figure 1.8b). We identified a moderate positive correlation between whiteflies and bacteria ($R = 0.50$; $p = 2.9E^{-02}$) and a strong positive correlation between whiteflies and CBSV ($R = 0.70$; $p = 4.4E^{-04}$). In contrast, responses to whiteflies and CMV were dissimilar, showing a strong negative correlation ($R = -0.72$; $p = 5.6E^{-05}$) (Figure 1.8b). Correlations between whitefly and bacteria/CBSV and between whitefly and CMV were associated with 30 and 19 DEGs, respectively (Tables S1.26 and S1.27).

Integration of defense transcriptomes and cassava PR gene phylogenies

To visualize cassava *PR* family responses to biotic stress, SA and JA, *PR* clades identified in cassava *PR* phylogenetic trees were used to order heatmaps (Tables S1.20-S1.27). We integrated these data with the *PR* family expansions identified in the cassava, poplar and rice PR phylogenetic trees (Figures S1.1-S1.14). Of the 132 *PR* genes in cassava expansions, 43 were DEGs in response to at least one biotic interaction and/or defense hormone (Tables S1.20-S1.27).

Several expansions in *PR* families *2, 3, 9, 10, 14,* and *15/16* were associated with responsiveness to whiteflies. For example, in the *PR-2* gene family, all three genes (*PR-*

2e8, PR-2e9 and *PR-2e10*) in the *PR-2e* expansion were whitefly-downregulated (Figure S1.16). *PR-2e8* was also upregulated in response to SA and JA. The *PR-3g* expansion (*PR-3g2, PR-3g3* and *PR-3g4*) (Figure S1.17) was whitefly-upregulated. Although *PR-3g3* and *PR-3g4* were located in tandem on chromosome 11, they had distinct responses to other pathogens. *PR-3g4* was upregulated by whiteflies, fungi and bacteria, while *PR-3g3* and *PR-3g2* were strongly upregulated by whiteflies and CBSV (Figures S1.1-S1.14; Table S1.1).

While all genes in the *PR-2e* and *PR-3g* expansions were whitefly-responsive, within other cassava-specific expansions in *PR* families *9*, *10*, *14*, and *15/16* whitefly responsiveness was detected in a subset of the genes in the expansion. For example, in the 20 gene *PR-15/16j5* clade on chromosome 8, *PR-15/16j5* was the only gene regulated by any biotic stress and it was strongly downregulated by whiteflies (Figure S1.27).

Genes within 13 cassava expansions from *PR* families *1, 5, 7, 8,* and *9* did not contain whitefly-responsive DEGs (Figures S1.15, S1.19 and S1.21-S1.23). Strikingly, when cassava expansions were examined collectively numerous genes (34 genes) that responded to CMV and/or CBSV were identified and 16 of these genes belonged to the *PR-9* family.

These integrative analyses also highlighted that the *PR* families *1*, *14* and *15/16* had a high proportion (39-41%) of *PR-Like* genes, which were not differentially expressed in response to any stress or hormone (Table S1.1). In particular, the expansions of the *PR-1d*, *PR-14e*, *PR-15/16i*, and *PR-15/16j* clades were rich in *PR-Like* genes (Figures S1.15, S1.26 and S1.27), suggesting their recent evolution is not associated with the biotic stresses presented here. However, many of these genes are expressed in embryonic

structures, fibrous roots, or the root apical meristem (Table S1.28), suggesting they may function in different organs, during growth and development, or during biotic or abiotic interactions that were not included in our study.

Discussion

PR family composition and organization in cassava

In cassava and three other plant species, we showed that *PR* genes exist as multigene families, as previously described for cacao (*Theobroma cacao*), pine (*Pinus spp.*) and barrel medic (*Medicago truncatula*) (Fister et al., 2016, Visser et al., 2018, Wanderley-Nogueira et al., 2012). While *PR* family numbers differed from other published *PR* gene numbers due to our methods for gene identification (Fister et al., 2016), overall *PR* family sizes were similar in the four species analyzed, with a few exceptions. Similar to cacao (Fister et al., 2016), the *PR-10* family was expanded in both cassava and poplar, relative to rice and Arabidopsis. In addition, PR-12 and PR-13 proteins were not detected in the cassava or poplar genomes. PR-12 was also absent in two pine species (Visser et al., 2018) and PR-13 has been previously described only in monocots and in the Brassicaceae (Stec, 2006, Fister et al., 2016).

As described by Fister et al. (2016) for cacao, Arabidopsis*, Brachypodium distachyon,* rice, populus, and *Vitis vinifera*, many genes (51%) within each cassava *PR* family were clustered. As in cacao, *PR-10* and *PR-15/16* families had the largest gene clusters. In contrast, while *PR-2* and *PR-6* genes were clustered in the cacao genome (Fister et al., 2016), cassava's *PR-2* and *PR-6* genes occurred as singletons. Tandem

organization of *PR* genes has also been described for *PR-12* in Arabidopsis (Silverstein et al., 2005), *PR-7* in tomato (Jorda et al., 1999) and the *PR-10* family in grape (Lebel et al., 2010).

As plants and their attackers are continually coevolving, such evolutionary pressures commonly lead to the expansion and diversification of host-plant defense gene families (Jones and Dangl, 2006). This phenomenon is well-documented in resistance gene families, such as the NLRs, and can also be found in various defense gene families (Jacob et al., 2013, Yin et al., 2013). These principles also appear to apply to cassava's *PR* gene families. By integrating our transcriptomic and phylogenetic data sets, we observed multiple instances of cassava *PR* family expansions associated with responses to whiteflies and other biotic stresses that may indicate selection for new functions for these paralogs.

For example, expansions within the *PR-2e* and *PR-3g* clades were associated with whitefly downregulation and whitefly/microbe upregulation, respectively. As PR-2 (β-1,3-endoglucanases) and PR-3 (chitinases) proteins are commonly involved in responses to bacteria/fungi and have antimicrobial activities (van Loon et al., 2006, Gupta et al., 2013, Sridevi et al., 2008, Jongedijk et al., 1995), it is possible that whiteflies and microbes produce similar pressures for the evolution of paralogs in *PR* gene family expansions. Whitefly feeding produces little cellular damage similar to biotrophic bacteria/fungi, and whitefly stylet movement may be perceived as similar to bacterial spread or fungal hyphae movement through the apoplast (Walling, 2008). Alternatively, endosymbionts and/or their gene products present in whitefly saliva (Kaloshian and Walling, 2016) or chitin derived

from whitefly stylets or exoskeletons during molting may be perceived as bacteria/fungi-like triggers for regulation of *PR* and other defense genes (Wang et al., 2017).

Cassava's PR gene responses to whitefly and other pathogens

To date, there is limited information about cassava's *PR* gene regulation and function. In 2006, Antony and Palaniswami (2006) showed that PR activities (β-1,3-glucanase, peroxidase and chitinase) increased after *B. tabaci* infestation. In addition, using yeast two-hybrid assays, Román et al. (2014) constructed a PR protein-interaction network that is deployed during *Xanthomonas* infection. We discovered that 37% of the differentially expressed *PRs* were downregulated (62 genes in cluster 9, Figure 1.5). This large number of downregulated genes is a surprising result as it contrasts with the definition of *PR* genes as being upregulated after pathogen or pest attack (van Loon et al., 2006). Also, this large-scale downregulation of cassava *PR* genes after whitefly infestation does not align with previous studies in cacao in response to pathogens *Phytophthora palmivora* and *Colletotrichum theobromicola* (Fister et al., 2016).

Cassava's unique *PR* gene regulatory programs may be due to one or more factors. First, some of the cassava *PR* homologs identified in this study, while sharing sequence identity with other land plant PR proteins, may have been recruited for new functions to survive stressful environments or to play a role in growth and development. Second, as the size of the *PR* families increases, the resulting paralogs are functionally redundant and some can be recruited to new roles without compromising cassava's defense. Such gene family functional evolution may have occurred in cassava. In fact, the PR network established by Román et al. (2014) showed that many of the cassava proteins interacting with PRs were associated with abiotic stress or metabolic responses and were

not implicated in defense. Additionally, PR proteins regulate cell proliferation/differentiation in tobacco (Lee et al., 2003) or can rescue somatic embryos in carrot (Kragh et al., 1996). These new functional roles for PRs are also consistent with the expression of many cassava *PR* genes during somatic embryogenesis, as well as in shoots and roots.

Finally, the downregulation of *PR* genes could be due to whitefly effectors that actively suppress plant immunity (Walling, 2008, Kaloshian and Walling, 2016). Notably, in Arabidopsis, whiteflies promote SA-regulated *PR* gene expression, rather than suppress it (Kempema et al., 2007, Zarate et al., 2007). Alternatively, cassava's regulatory circuitry of *PR* genes may be significantly different than reported in other species to date. Indeed, our analysis of published data for five other cassava-pathogen interactions indicated that *PR* gene downregulation was common in these other cassava-pathosystems (Figure 1.8) (Rauwane et al., 2018, Lopez et al., 2005, Muñoz-Bodnar et al., 2014, Utsumi et al., 2016, Allie et al., 2014a, Amuge et al., 2017, Maruthi et al., 2014, Anjanappa et al., 2017).

Hormone regulation of whitefly-responsive PR genes

Plant defenses are commonly associated with hormone programs specific to the attacker and often controlled by SA or JA in response to biotrophs and necrotrophs/wounding, respectively (Glazebrook, 2005). In our analysis of cassava *PR* genes, coordinate SA and JA responses characterize over 90% of the interaction between whitefly-susceptible cassava and whiteflies. In the context of plant-hemipteran interactions, there are a few instances where SA and JA responses are activated concurrently, including the pepper-whitefly interaction (Park and Ryu, 2014). However

more generally, synergistic SA and JA responses have been found to be associated with resistance to certain biotic stressors (Pieterse et al., 2009).

While studies defining the hormone response of the full complement of *PR* families in other species have not been reported, transcriptome studies in Arabidopsis, rice and sorghum assessing response to SA and JA reported that 20–50% of all genes responsive to SA and/or JA exhibited coordinate responses (Schenk et al., 2000, Tamaoki et al., 2013, Zhu-Salzman et al., 2004). This variation in global SA and JA expression programs among plant species points to the need for species-specific definitions of *PR* genes used as defense markers.

Cassava PR families 2, 5, 7, and 9 are most responsive to biotic stress and hormones

Across the many cassava-pest/pathogen/hormone interactions examined in this study, *PR* families with roles in pathogen cell wall degradation (*PR-2*, *PR-5* and *PR-7*), as well as host cell wall fortification (*PR-9*) were the predominant families responding to whiteflies (*A. socialis*), SA, JA, and most other biotic stresses (mealybugs (*P. manihoti*), fungi (*C. gloeosporioides*), bacteria (*X. axonopodis*), CMV, and CBSV) (Rauwane et al., 2018, Muñoz-Bodnar et al., 2014, Utsumi et al., 2016, Allie et al., 2014a, Anjanappa et al., 2017). PR-2 and PR-5 proteins have been reported as β-1,3-endoglucanases and β-glucan-binding proteins, respectively, with roles in pathogen membrane degradation/permeabilization (Slusarenko et al., 2000, Abad et al., 1996). PR-7 proteins are endoproteinases and have proposed roles in aiding fungal cell wall degradation (Van Loon and Van Strien, 1999). PR-9s are lignin-forming that may help reinforce the cell wall

by catalyzing lignification and preventing pathogen penetration (Lagrimini et al., 1987, van Loon et al., 2006).

In other global *PR* family analyses, *PR* families *2*, *5* and *9* were also well-represented in the response of poplar to the fungus *Melampsora larici-populina* (Duplessis et al., 2009) and in cacao interactions with the oomycete *Phytophthora palmivora* and the fungus *Colletotrichum theobromicola* (Fister et al., 2016). Previous studies of plant responses to hemipterans have followed the regulation of only well-documented *PR* sentinel genes, or have not adequately analyzed *PR* gene expression profiles within transcriptome studies (Foyer et al., 2015). Among the most responsive *PR* families identified in our study (*PR-2*, *PR-5*, *PR-7*, and *PR-9*), Arabidopsis *PR-2* and *PR-5* (Zarate et al., 2007, Kempema et al., 2007) and tomato *PR-2* (Puthoff et al., 2010, Quintana-Camargo et al., 2015, Mayer RT et al., 1996) have been shown to be induced in response to whitefly infestation. Similarly, *PR-2* is induced after aphid feeding on sorghum and Arabidopsis (Moran et al., 2002, Zhu-Salzman et al., 2004).

Among the biotic stresses examined, a large portion (38-75%) of *PR* genes responsive to whiteflies (*A. socialis*), bacteria (*X. axonopodis*) or fungi (*C. gloeosporioides*) were regulated by SA and JA, while these hormones were unlikely to play a major role in regulating cassava's response to viruses. Other signals may be responsible for their regulation.

PR gene responses to whiteflies are more similar to CBSV than to CMV

Cassava's *PR* gene responses to whiteflies were positively correlated with its response to bacteria (*X. axonopodis*) and CBSV but negatively correlated with its responses to CMV (Muñoz-Bodnar et al., 2014, Anjanappa et al., 2017, Allie et al., 2014).

The distinct *PR* gene responses to CMV and CBSV could be due to the different mechanisms of inoculation used in each study (Allie et al., 2014, Anjanappa et al., 2017) or the different viral replication strategies employed by these two viruses (Ng and Falk, 2006). Alternatively, *PR* gene responses to the two viruses could be reflective of the different modes of CMV and CMSV acquisition and transmission by their whitefly vector (Maruthi et al., 2002, Maruthi et al., 2017, Ng and Falk, 2006). The biological significance and molecular mechanisms that underlie the distinct *PR* gene signatures to CBSV and CMV, and their correlations with whitefly *PR* gene expression programs remain to be discovered.

Materials and Methods

Plant growth

Shoot tips from *in vitro* grown *Manihot esculenta* genotypes (COL2246, COL1468 (CMC40), 60444 (TMS60444/NGA11), and TME3) in the CIAT culture collection were excised and grown in 17N rooting medium for 30 days. Plants were then sown in 2-L pots with sterile soil with a ratio of 1:3 sand to black soil (no clay topsoil). Plants were grown in a glasshouse with temperatures ranging from 24-28°C under a long-day light cycle (16-h light/ 8-h dark). Sixty days after sowing, plants were used for hormone treatments or whitefly infestation experiments.

Mass rearing of Aleurotrachelus socialis and whitefly bioassays

The *Aleurotrachelus socialis* colony was raised on *Manihot esculenta* var. COL1468 as previously described by Bellotti and Arias (Bellotti and Arias, 2001). For the

whitefly-infestation experiments, four whitefly-susceptible cassava genotypes (COL2246, COL1468, 60444, and TME3) were used (Parsa et al., 2015, Bohorquez et al., 2013). Each three-month-old plant was put into an individual mesh cage (1-m height x 30-cm diameter) in a glasshouse. Infestations were initiated by the release of 100 male and 100 female adults of *A. socialis* into each cage. When the adult whiteflies were removed at 3 dpi, the two youngest infested leaves, which are preferred by whiteflies for feeding and egg deposition, were tagged for future collection. Three biological replicates were used for each genotype. Infested plants were placed in a random design instructed by a factorial arrangement. In order to capture the effect of each life stage of the whitefly on the cassava plants, the sample collection time points were chosen to represent landmarks during the *A. socialis* life cycle (Bellotti and Arias, 2001). Samples were harvested at: 0 h post-infestation (hpi), 1 dpi (adult feeding and egg deposition), 7 dpi (eggs present), 14 dpi (1^{st} and 2^{nd} instar feeding), and 22 dpi (2^{nd} and 3^{rd} instar feeding). After collection, leaves were frozen in liquid nitrogen and stored at -80°C until use.

Hormone treatments

One day prior to hormone treatments, three-month-old COL2246 plants were moved from the glasshouse into growth chambers with a 16-h light/ 8-h dark cycle and a 24-28°C temperature range. Salicylic acid (200 μM SA, 0.1% EtOH, 0.01% Tween 20) and methyl jasmonate (7.5 mM MeJA, 0.1% EtOH, 0.01% Tween 20) treatments were performed in growth chambers in different rooms. The 0-h sample was collected at 9 AM. All leaves (4-6 per plant) were sprayed with SA or JA until saturation and were harvested at 0.5, 1, 2, 4, 8, 12, and 24 h post treatment by excising the leaf blade. Hormone treatment concentrations were based on similar values as used in tomato due to comparable

impermeability to foliar sprays in tomato and cassava (Li et al., 2013b). Tissue was frozen in liquid nitrogen and stored at -80°C until use. This experiment was conducted three times (three biological replicates).

PR protein phylogenetic trees, gene nomenclature and genome location

For the 17 PR protein families defined by van Loon et al. (2006), founder PR protein sequences were used as queries to identify cassava PR proteins. Cassava, poplar, rice, and Arabidopsis PR proteins were obtained from Phytozome (JGI) and Ensembl Plants using BLASTP and Hidden Markov Model searches (Finn et al., 2011), respectively. Percent identity and E-values of the cassava PR proteins are provided (Table S1.1). The Pfam database (Finn et al., 2016) was used to identify conserved protein domains that distinguish each PR family. The PR proteins that lacked canonical PR domains (20 of 447 proteins) were removed prior to alignment with ClustalW. The resulting alignments were manually curated and neighbor-joining phylogenetic trees were constructed using Geneious version 11.1.2 (Kearse et al., 2012). Bootstrapping was performed with 1000 replications and bootstrap values are shown only for branches with 50% or higher bootstrap support. Cassava-specific *PR* gene family expansions were defined as cassava clades that contained three or more cassava paralogs.

Proteins within each cassava *PR* family were used to construct family phylogenetic trees. The *PR* genes were named according to their phylogenetic relationships in *Manihot esculenta* (Tables S1.20-S1.27). Within a family, genes were assigned a letter indicating their clade (i.e., *PR-1d*). *PR* genes that were not differentially expressed during pest/pathogen treatment were designated as *PR-Like* genes (i.e., *PR-1dL*) (Table S1.19). *PR* and *PR-L* genes within a clade were numbered sequentially (Table S1.1). *PR* genes

assigned to cassava chromosomes 1 to 18 were visualized using the program PhenoGram (Wolfe et al., 2013).

RNA extraction and quality assessment

Total RNA was extracted using the methodology described by Behnam et al. (2019). RNA was quantified using a Nanodrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). RNA quality was assessed by absorbance ratios, denaturing agarose gels and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RIN values were above 6.00. RNAs were treated with 20 μL of RNAstable® (Biomatrica, San Diego, CA) and then dried using the Speedvac Concentrator™ (Eppendorf™) for 1 h at room temperature. RNA quantity and integrity was confirmed prior to RNA-seq library construction using the Nanodrop® ND-1000 spectrophotometer and 1% denaturing agarose gels, respectively.

cDNA library preparation, sequencing and bioinformatics analyses

Strand-specific cDNA libraries were prepared following the protocol of Wang et al. (2011) with two changes. The reverse transcriptase used was Superscript III (Invitrogen, Carlsbad, CA) and the high-fidelity DNA Polymerase was KAPA HiFi Hot start (KAPA Biosystems, Wilmington, MA). Universal and barcoded primers were purchased from Integrated DNA Technologies (Coralville, IA).

cDNA libraries generated from whitefly-infestation experiments were sequenced on the Illumina HiSeq2500 platform (single-end 50-bp reads) or on the Illumina NextSeq500 platform (single-end 75-bp reads) at the UCR Institute for Integrative Genome Biology Genomics Core. Seventy-five-bp reads were trimmed to 50 bp to allow

valid comparisons of all libraries. Libraries from SA and MeJA experiments were sequenced on the NextSeq500 platform (single-end 75-bp reads). For each infestation or treatment time point, the three biological replicates were used to construct libraries. Libraries were multiplexed (12-13 libraries/lane) and sequenced resulting in ~25-45 million reads per library. Using total read counts, Pearson correlation values ranging from 0.70-1.00 and from 0.89-0.99 were obtained among biological replicates for whitefly and hormone treatments, respectively, confirming their reproducibility (Figure S1.29). After trimming and filtering the fastq files, reads were aligned against the *Manihot esculenta* genome version 6.1 at Phytozome (Goodstein et al., 2012), using Bowtie2/2.2.5 and Tophat 2.0.14. The subsequent analyses of the sequences were made following the systemPipeR pipeline (Backman and Girke, 2016). Genes with an average of 20 reads or less across a treatment time course were not included in the DEG analysis. DESeq2 was used to determine DEGs, defined as having $|\log_2 FC| > 1$ and FDR $\leq$ 5%.

Heatmaps for whitefly infestation studies and hormone treatment studies were organized by defined expression programs (Tables S1.18-S1.19) and hierarchical clustering along the y-axis and were constructed using the R package ComplexHeatmap (Gu et al., 2016). Venn diagrams used to visualize DEGs were generated using the online program Venny (Oliveros, 2007). Raw data are provided in Table S1.2.

Data from published transcriptome studies investigating five additional cassava-pathogen/pest interactions were used for comparison to our transcriptome data sets for COL2246. In each of these interactions, we selected cassava genotypes susceptible to a pathogen/pest and time points similar to those used in the whitefly infestation studies presented here. These data included: the mealybug *P. manihoti* (P40/1; 24 and 72 hpi),

the bacteria *X. axonopodis* strain ORST4(*TALE1*$_{Xam}$) (MCOL1522; 5 and 7 dpi), the fungus *C. gloeosporioides* (HN; 24 and 72 hpi), and the viruses CMV (T200; 12 and 32 dpi) and CBSV (60444; 28 dpi) (Rauwane et al., 2018, Lopez et al., 2005, Muñoz-Bodnar et al., 2014, Utsumi et al., 2016, Allie et al., 2014a, Amuge et al., 2017, Maruthi et al., 2014, Anjanappa et al., 2017). Expression values from healthy cassava organs were also used (three-month-old TME204) (Wilson et al., 2017). Time-course expression data used in these studies, as well as our whitefly- and hormone-treatment studies in COL2246, were consolidated to facilitate comparisons. For each time course, we used all treatment time points that were differentially expressed relative to 0 h ($|\log_2 FC| > 1$ and FDR $\leq$ 5%) and calculated a mean $\log_2$FC value for each *PR* gene. For the dataset from Muñoz-Bodnar et al. (2014a), $\log_2$FC values were calculated as follows: $\log_2$(FPKM inoculated/FPKM mock). Heatmaps were constructed using the R package ComplexHeatmap (Gu et al., 2016) and organized along the y-axis according to *PR* phylogenetic clades.

qRT-PCR

For qRT-PCR, cDNA templates were synthesized using 5 ng of mRNA and the Improm II reverse transcriptase protocol (Promega, Madison, WI). We selected the control gene *UBQ* (Manes.10G122600) based on its low read count variation for all time points in each treatment. qRT-PCR was performed for selected *PR* genes and the *UBQ* control using gene-specific primers in the Bio-rad CFX Connects instrument using iQ SYBR Green Supermix (Bio-rad, Hercules, CA) (Table S1.29). Melting curve analyses were performed at the end of each cycle to confirm the specificity of the PCR product. Relative expression changes were calculated by the comparative Ct method; fold change was

calculated as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Three biological and technical replicates were used for these analyses. Fold-change values are displayed with standard error.

Pearson correlation analyses

Pearson correlation analyses were performed using the R package ggplot2 (Wickman, 2016). Correlation strength was defined according to Evans (1996) as very weak (|0.00-0.19|), weak (|0.20-0.39|), moderate (|0.40-0.59|), strong (|0.60-0.79|), or very strong (|0.80-1.00|). Prior to analyses of biotic stress, outliers were identified using the boxplot rule with a multiplicative constant of 2.0 and removed (Iglewicz and Hoaglin, 1993). Comparisons with fewer than ten DEGs in both treatments (e.g., whiteflies-fungi and whiteflies-mealybugs) did not undergo correlation analysis. No outliers were identified in the SA/JA co-regulation or qRT-PCR vs RNA-seq correlation analyses.

Supplemental Material

Supplemental Tables

Tables S1.1-1.2. Table S1.1. Cassava *PR* gene nomenclature. Founder protein(s) used to identify each PR family via BLASTP and HMM queries, a list of cassava *PR* genes and functions, and loci designations in the cassava genome are provided. E-values and % identity are also provided. **Table S1.2.** *PR* gene clusters in the cassava genome.

Tables S1.3-1.14. *PR* gene expression after whitefly, SA and JA treatments of cassava. **Table S1.3.** log_2FC and FDR values of DEGs identified in whitefly-susceptible genotypes (COL2246, COL1468, 60444, and TME3) after whitefly infestation. **Table S1.4.** log_2FC and FDR values for *PR* genes detected in whitefly-susceptible genotypes after whitefly infestation. **Table S1.5.** Mean RPKM values of *PR* genes during whitefly infestation (0–22 dpi) of four whitefly-susceptible genotypes. **Table S1.6.** Read counts of *PR* genes during whitefly infestation (0–22 dpi) of four whitefly-susceptible genotypes. **Table S1.7.** log_2FC and FDR values of DEGs in COL2246 after SA treatment. **Table S1.8.** log_2FC

and FDR values for *PR* genes detected in COL2246 after SA treatment. **Table S1.9.** Mean RPKM values for *PR* genes after SA treatment (0–24 h) of COL2246. **Table S1.10.** Read counts of *PR* genes after SA treatments (0–24 h) of COL2246. **Table S1.11.** log_2FC and FDR values of DEGs in COL2246 after JA treatment. **Table S1.12.** log_2FC and FDR values for *PR* genes detected in COL2246 after JA treatment. **Table S1.13.** Mean RPKM values for *PR* genes after JA treatment (0–24 h) of COL2246. **Table S1.14.** Read counts for *PR* genes after JA treatment (0–24 h) of COL2246.

Tables S1.15-S.1.16. Table S1.15. Expression profile clusters for *PR* responses to whitefly feeding. **Table S1.16.** Expression profile clusters for *PR* responses to whitefly, SA and JA.

Table S1.17. Mean log_2FC of SA and/or JA DEGs.

Table S1.18. Whitefly-responsive DEGs: Temporal expression programs and hormone-response clusters.

Table S1.19. *PR* gene expression values (log_2FC) during biotic stress. This table compiles DEGs identified in response to: SA, JA and whitefly (this study); *Xanthomonas* (bacteria) (Lopez et al., 2005, Muñoz-Bodnar et al., 2014); *C. gloeosporioides* (fungi) (Utsumi et al., 2016); and the viruses South African CMV (Allie et al., 2014b) and CBSV (Amuge et al., 2017, Anjanappa et al., 2017, Maruthi et al., 2014).

Table S1.20-S1.25. Hormone regulation of stress-responsive *PR* genes. Numbers and percentages of stress-regulated genes belonging to each hormone-expression program are provided. **Table S1.20.** Hormone regulation of whitefly-responsive *PR* genes. **Table S1.21.** Hormone regulation of mealybug-responsive *PR* genes. **Table S1.22.** Hormone regulation of bacteria-responsive *PR* genes. **Table S1.23.** Hormone regulation of fungi-responsive *PR* genes. **Table S1.24.** Hormone regulation of CMV-responsive *PR* genes. **Table S1.25.** Hormone regulation of CBSV-responsive *PR* genes.

Table S1.26-S1.27. Table S1.26. *PR* genes associated with correlations between whitefly and bacteria and/or CBSV responses. **Table S1.27.** *PR* genes associated with correlation between whitefly and CMV responses.

Table S1.28. *PR* gene expression in TME2 04 shoots, roots and embryonic callus. Loci (cassava genome v6), *PR* gene names and expression values from Wilson et al. (2017a) are provided. These data are used in Tables S1.20-S1.27.

Table S1.29. qRT-PCR primers.

Supplemental Figures

Figures S1.1-S1.14. Neighbor-joining phylogenetic trees of cassava PR families. **Figure S1.1.** *PR-1*. **Figure S1.2.** *PR-2*. **Figure S1.3**. *PR-3*. **Figure S1.4.** *PR-4*. **Figure S1.5.** *PR-5*. **Figure S1.6.** *PR-6*. **Figure S1.7.** *PR-7*. **Figure S1.8.** *PR-8*. **Figure S1.9.** *PR-9*. **Figure S1.10.** *PR-10*. **Figure S1.11.** *PR-11*. **Figure S1.12.** *PR- 14*. **Figure S1.13.** *PR-15/16*. **Figure S1.14.** *PR-17*. Founder (green), cassava (pink), poplar (blue), and rice (black) PR proteins are indicated. Branches with bootstrap values of 50% or higher are shown.

Figures S1.15-S1.17. *PR-1*, *PR-2* and *PR-3* family member phylogenies and consolidated gene expression heatmaps are displayed. Genes within a clade are designated by a letter and color bars in the circular phylogenetic trees and heatmaps. Information about physical clustering and cassava-specific expansions are provided beside the heatmaps, which provide gene expression changes during biotic stresses or hormone treatments (SA and JA) and in shoots and storage roots. Recent *PR* family expansions are shown in red in the circular trees and expansion column; other genes (light grey) in the expansion column are not part of cassava-specific *PR* family expansions (see Methods). Genes belonging to the same physical cluster are denoted with the same color in the cluster column; genes that do not belong to a cluster are in light grey. Genes displayed as dark grey do not have an assigned chromosomal position in the cassava genome version 6. **Figure S1.15.** *PR-1*. **Figure S1.16.** *PR-2*. **Figure S1.17.** *PR-3*.

Figures S1.18-S1.20. *PR-4*, *PR-5* and *PR-6* family member phylogenies and consolidated gene expression heatmaps are displayed. The *PR-6* family phylogenetic tree is not displayed due to its small size. **Figure S1.28.** *PR-4*. **Figure S1.19.** *PR-5*. **Figure S1.20.** *PR-6*.

Figures S1.21-S1.22. *PR-7* and *PR-8* family member phylogenies and consolidated gene expression heatmaps are displayed. **Figure S1.21.** *PR-7*. **Figure S1.22.** *PR-8*.

Figures S1.23-S1.25. *PR-9*, *PR-10* and *PR-11* family member phylogenies and consolidated gene expression heatmaps are displayed. **Figure S1.23.** *PR-9*. **Figure S1.24.** *PR-10*. **Figure S1.25.** *PR-11*.

Figures S1.26-S1.28. *PR-14*, *PR-15/16* and *PR-17* family member phylogenies and consolidated gene expression heatmaps are displayed. **Figure S1.26.** *PR-14*. **Figure S1.27.** *PR-15/16*. **Figure S1.28.** *PR-17*.

Figure S1.29. Pearson correlations of count values obtained for three biological replicates for all whitefly infestation and hormone treatments. (a) Correlations for SA and JA treatments (0, 0.5, 1, 2 , 4, 8, 12 , 24 h). (b) Correlations for whitefly infestations (0, 1, 7, 14, and 22 d) for COL2246, COL1468, 60444, and TME3.

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Chapter 2

Integrative transcriptomics reveals association of salicylic acid, abscisic acid and lignin pathways with cassava whitefly resistance

Abstract

Outbreaks of superabundant whitefly populations throughout Eastern and Central Africa in recent years have dramatically increased the pressures of whitefly feeding and virus transmission on their host, cassava (*Manihot esculenta*). Whitefly-transmitted viral diseases threaten the food security of millions of African farmers, evidencing the need for whitefly-resistant cassava lines. However, basic knowledge of the defense programs in cassava is lacking, limiting the characterization of whitefly-resistance mechanisms. Here, we define defense-hormone-responsive and whitefly-responsive transcriptomes in cassava utilizing RNA-sequencing, characterizing the whitefly infestation, salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) responses of whitefly-susceptible (COL2246) and whitefly-resistant (ECU72) cassava genotypes. Strikingly, we found that SA responses are largely reciprocal between ECU72 and COL2246, and we suggest several known regulators of crosstalk between the SA and other hormone pathways (WRKY70, NPR1 and GRX480) as candidate regulators. An association of SA with COL2246's susceptibility and ABA with ECU72's resistance to whitefly was evidenced by expression of genes within the SA and ABA pathways and SA and ABA levels during infestation, which additionally showed evidence of SA-ABA antagonism in ECU72. Gene enrichment analyses of whitefly- and hormone-responsive genes additionally suggest the importance of fast-enacted, non-hormone regulated cell wall defenses (e.g., elicitor recognition, lignin biosynthesis) during egg deposition and early nymph stages in ECU72.

Instead, COL2246 mounted a surge in ineffective immune and SA responses in response to late-stage nymphs. Additionally, in comparison with the commonly referenced model, Arabidopsis, cassava's hormone-responsive genes showed striking divergence in expression, demonstrating that defense programs in Arabidopsis may not always mirror those in crop species. Our results shed light on possible mechanisms whitefly resistance in cassava and more broadly provide a baseline for characterizing the resistance responses of cassava to other yield-limiting pathogens and pests.

Introduction

The tropical tuber crop cassava (*Manihot esculenta*) is a starchy staple grown in over 100 countries that feeds 800 million people worldwide (FAO, 2019). Smallholder sub-Saharan African farmers grow cassava as an inexpensive source of calories that is resilient to drought, poor soils and a changing climate (Jarvis et al., 2012, Manyong, 2000). However, in the 1990's, superabundant whitefly (*Bemisia tabaci*) populations devastated cassava yields in Africa (Hillocks et al., 2001, Macfadyen et al., 2018). As phloem-feeders, whiteflies deplete photosynthates thereby slowing cassava growth and root production and deposit their sugar-rich honeydew that supports sooty mold infection (Byrne, 1991). Most damaging, whiteflies transmit viral diseases that have increased in severity and range within Africa due to whitefly superabundance (Alicai et al., 2007, Legg et al., 2014, Macfadyen et al., 2018, Seal, 2006). As a practical control strategy, the development of whitefly- and virus-resistant cassava is highly promising (Ally et al., 2019). To date, whitefly resistance in cassava has primarily been studied in South American varieties identified through mass screening against the South American whitefly *Aleurotrachelus*

socialis (Bellotti and Arias, 2001). Similar to *B. tabaci*, *A. socialis* feeding has been shown to significantly reduce yields, by as much as 60-80%, in South American cassava fields (Bellotti, 1983, Gold et al., 1991). Among identified varieties, the Ecuadorian genotype ECU72 possesses robust resistance to five species of whitefly (*A. socialis*, *Bemisia tuberculata* and three species within the *B. tabaci* species complex (SSA1-SG1, SSA1-SG2 and SSA1-SG3) (Atim, 2021)), causing death of immature whiteflies before they can emerge as adults (Barilli, 2019, Omongo et al., 2012, Bellotti and Arias, 2001). To achieve food security for affected farmers, a better understanding of the genetic basis of this resistance is needed to enable breeding of the whitefly resistance traits into the cassava of sub-Saharan Africa.

At the genetic/molecular level, plant defense against biotic stressors follows a primary and secondary immune response (Jones and Dangl, 2006, Lolle et al., 2020). In the primary response, generic molecular signatures derived from pathogens (pathogen-associated molecular patterns, PAMPs), insect herbivores (herbivore-associated molecular patterns, HAMPs), or from 'debris' from damaged host cells (damage-associated molecular patterns, DAMPs) are recognized by extracellular receptors known as pattern-recognition receptors (PRRs) (Duran-Flores and Heil, 2016, Kanyuka and Rudd, 2019). This recognition event, termed PAMP-triggered immunity (PTI), activates an intracellular signaling cascade, regulating defense genes and prompting a defense response. When the attacker is well-adapted to its host, it secretes small molecules known as effectors to suppress PTI responses to promote colonization. However, well-adapted hosts possess cognate resistance genes capable of recognizing their attacker's effectors to trigger a secondary immune response termed effector-triggered immunity (ETI). ETI is an amplified PTI response resulting in resistance to the attacker (Jones and Dangl, 2006,

Alhoraibi et al., 2019). Resistance involves slowing or stopping the spread of the attacker, which sometimes occurs through a form of programmed cell death (PCD), known as the hypersensitive response (HR) (Hofius et al., 2007).

The defense signals involved in such immune responses are numerous and can be associated with specific attacker types. Generally, biotrophs (e.g. bacteria, viruses, some fungi), which feed without killing host cells, elicit salicylic acid (SA)-dependent responses, that effectively control these pathogens. In contrast, necrotrophs (e.g. pathogens or fungi that inflict wounding), which kill host cells to feed on dead tissue, elicit jasmonic acid (JA)-regulated defense responses for control (Glazebrook, 2005b). While SA and JA are the two major plant defense hormones, ET and ABA have also emerged as critical defense signals (Robert-Seilaniantz et al., 2011). Other defense signals include reactive oxygen species (ROS), Ca^{2+}, extracellular ATP (eATP), small peptides, and lipids. Oftentimes interaction among multiple defense hormones and/or signals is necessary for successful orchestration of a defense response (Gust et al., 2017, Noctor et al., 2018, Robert-Seilaniantz et al., 2011).

Defense-signaling pathways that have been elucidated via studies of microbial pathogens and defense-hormone treatments also play an important role in plant resistance to insect herbivores (Howe and Jander, 2008). Insect resistance is broadly categorized as antibiotic or antixenotic. Antibiosis delays insect development or promotes mortality via toxins or antifeedants, while antixenosis deters insect settling via volatiles, physical structures or repellant phytochemicals (Walling, 2000, Smith and Chuang, 2014). While whiteflies affect the yields of numerous crops (Alon et al., 2012), moderate resistance has only been identified in a few crops or their wild relatives (Broekgaarden et

al., 2018, Bellotti and Arias, 2001, Cruz et al., 2014, Hondelmann et al., 2020, Khan, 2018, Li et al., 2016b, Walker and Natwick, 2006, Walling and Thompson, 2012). Strong, fast-acting resistance to specific to whiteflies has only been identified in cassava, alfalfa and Brassica species (Nebreda et al., 2005, Bellotti and Arias, 2001, Jiang et al., 2003, Teuber et al., 1996). To date, molecular mechanisms underlying resistance remain largely uncharacterized (Walling and Thompson, 2012).

Previous studies characterizing whitefly responses have provided some insight on the plant-defense signals elicited during infestation (Foyer et al., 2015, Walling and Thompson, 2012). In whitefly-resistant lines of tomato, *Medicago truncatula,* cotton, and cabbage, SA, JA, JA/ET and ABA responses, respectively, are primarily elicited by whiteflies (Rodriguez-Alvarez et al., 2015, Gao et al., 2007, Li et al., 2016b, Broekgaarden et al., 2018). Resistance to whiteflies is enhanced in JA-overexpressing Arabidopsis (Zarate et al., 2007), JA-overexpressing tomato (Sun et al., 2017) and JA- and/or ABA-treated eggplant and tomato (Esmaeily et al., 2020, Esmaeily et al., 2021). Furthermore, whitefly infestation of whitefly-susceptible lima bean and tobacco resulted in increased SA levels (Zhang et al., 2009, Zhang et al., 2015a) and increased SA and JA/ET signaling in pepper (Park and Ryu, 2014). ROS may also act as signals in plant defense against whiteflies as the resistant responses of cassava, eggplant and pepper include increased activity of antioxidative enzymes (Esmaeily et al., 2020, Mwila et al., 2017, Wu et al., 2019). Heightened activity of such enzymes during infestation has also been observed in whitefly-susceptible cabbage and cassava (Antony and Palaniswami, 2006, Zhang et al., 2013b).

Secondary metabolites, such as phenolic compounds, volatile terpenoids and acyl sugars, are commonly involved in plant responses to whiteflies (Wang et al., 2017). Phenolic compounds increase in abundance in whitefly-susceptible tobacco during infestation (Zhang et al., 2017) and are associated with JA/ABA-induced resistance in eggplant (Esmaeily et al., 2020). Phenolics vary in abundance among whitefly-resistant and -susceptible cassava lines (Mwila et al., 2018) and are associated with resistance to whitefly in cashew and tomato (Goiana et al., 2020, Yao et al., 2019). Volatile terpenoids emitted by wild tomato or tobacco have been shown to provide whitefly resistance (Bleeker et al., 2009, Luan et al., 2013), and to attract parasitoids to whitefly-infested Arabidopsis (Zhang et al., 2013a). Acyl sugars and methyl ketones found in glandular trichome exudates also provide whitefly resistance in wild tomato and backcrossed tomato lines (Escobar-Bravo et al., 2016, Muigai et al., 2002, Walling and Thompson, 2012).

Prior genome-wide studies in cassava have begun to unravel the cassava-whitefly interaction at the molecular level (Irigoyen et al., 2020, Perez-Fons et al., 2019). Our previous analysis of *Pathogenesis-related* (*PR*) genes in the whitefly-responsive transcriptomes of four susceptible cassava genotypes suggested that *PR* genes involved in cell wall processes are involved in defense against whiteflies, and that most whitefly-regulated *PR* genes were coordinately regulated by SA and JA (Irigoyen et al., 2020). The metabolic profiles of whitefly-resistant ECU72 and whitefly-susceptible COL2246 during whitefly infestation were determined by Perez-Fons et al. (2019). In this study, ECU72's whitefly resistance was suggested to be constitutive and involved cell wall reinforcement evidenced by differential abundance of cell wall-related phenolic metabolites (Perez-Fons et al., 2019).

To obtain a global understanding of cassava's responses to whiteflies, we define the transcriptomes of whitefly-resistant (ECU72) and whitefly-susceptible (COL2246) cassava genotypes in response to whitefly infestation and treatment with the plant defense hormones SA, JA, ET, and ABA. To compare hormone and infestation responses in ECU72 and COL2246, we assessed response timing and amplitude, and used clustering and enrichment analyses to identify processes associated with differential responses between genotypes. In order to place cassava's hormone responses within a broader context, we compared cassava and Arabidopsis hormone-responsive transcriptomes with a focus on defense-hormone pathway genes. Whitefly- and hormone-responsive gene sets were integrated for identification of differential responses between genotypes during whitefly and hormone treatments. Further enrichment analyses of such gene sets were used to identify processes in the resistant or susceptible response to whitefly associated with hormone regulation.

Together, our integrative transcriptomics approach identified genotype differences in global SA programming and gene responsiveness to whitefly in the SA and ABA pathways, supported by metabolomics quantification of differences in SA and ABA levels during infestation. Observed enrichment of lignin biosynthetic genes induced in whitefly-resistant ECU72 versus SA pathway genes induced in whitefly-susceptible COL2246 additionally suggests such processes may be important in the whitefly resistance and susceptibility mechanisms of these cassava genotypes, respectively.

Results

ECU72 is primed for a fast, considerable transcript-level response to SA

To define the defense hormone-responsive transcriptomes of cassava, we profiled the response of cassava genotypes ECU72 (whitefly-resistant) and COL2246 (whitefly-susceptible) to the defense hormones SA, JA, ET, and ABA at 0, 0.5, 1, 2, 4, 8, 12, and 24 h post treatment (hpt) (Figure 2.1). Treatment experiments and RNA extractions were performed by Dr. Adriana Bohorquez-Chaux at CIAT. DEGs identified by temporal comparisons within a genotype ("temporal" DEGs) were identified (Figure 2.1a). The magnitude of transcriptome responses varied substantially, ranging from 4,727 DEGs in ECU72's SA response to 8,071 DEGs in COL2246's JA response. For each treatment, the number of DEGs elicited was higher in COL2246 than in ECU72 (Figure 2.1b-c; Table S2.1), but the timing of responses was similar after JA, ET or ABA treatments (Figure 2.1; Table S2.2).

In contrast, ECU72 and COL2246 had distinct temporal responses to SA treatments (Figure 2.1b-c; Table S2.2). Similar to its response to other hormones, COL2246's response to SA was depicted by a small number of DEGs at 0.5 hpt, a steady rise in DEGs peaking at 8-12 hpt and a sharp decline in DEGs by 24 hpt. In contrast, ECU72 had a swift transcriptome response to SA at 0.5 and 1 hpt; for example, by 0.5 hpt ECU72 had 5.2 to 75.3-fold more up- and down-regulated DEGs than COL2246 (Table S2.2). ECU72's DEG count rose until a sharp drop at 8 hpt, which was followed by a robust response at 12 hpt, which continued to 24 hpt when there were 34- and 69-fold more up- and down-regulated genes in ECU72, respectively (Figure 2.1b,c; Table S2.2).

ECU72 and COL2246 transcriptomes were also compared at each time point after hormone treatments to identify “genotype” DEGs (Figure 2.1d; Table S2.2). While the overall magnitude of JA- (6,779 DEGs), ET- (4,088 DEGs) and ABA- (4,312 DEGs) responsive genotype DEGs was variable, their temporal profiles were similar; each elicited similar numbers of up- and down-regulated DEGs at each time point. Additionally, at each time the number of downregulated DEGs exceeded the number of upregulated DEGs (Figure 2.1d; Tables S2.1 and S2.2). In contrast, SA provoked a larger transcriptome response with 10,299 genotype DEGs and unique regulatory and timing trends. At all time points, similar numbers of up- and down-regulated genotype DEGs were elicited in ECU72. Unlike JA, ABA and ET, large numbers of genotype DEGs were identified at 0.5 to 1 hpt and from 4 to 12 hpt (Figure 2.1d; Table S2.2).

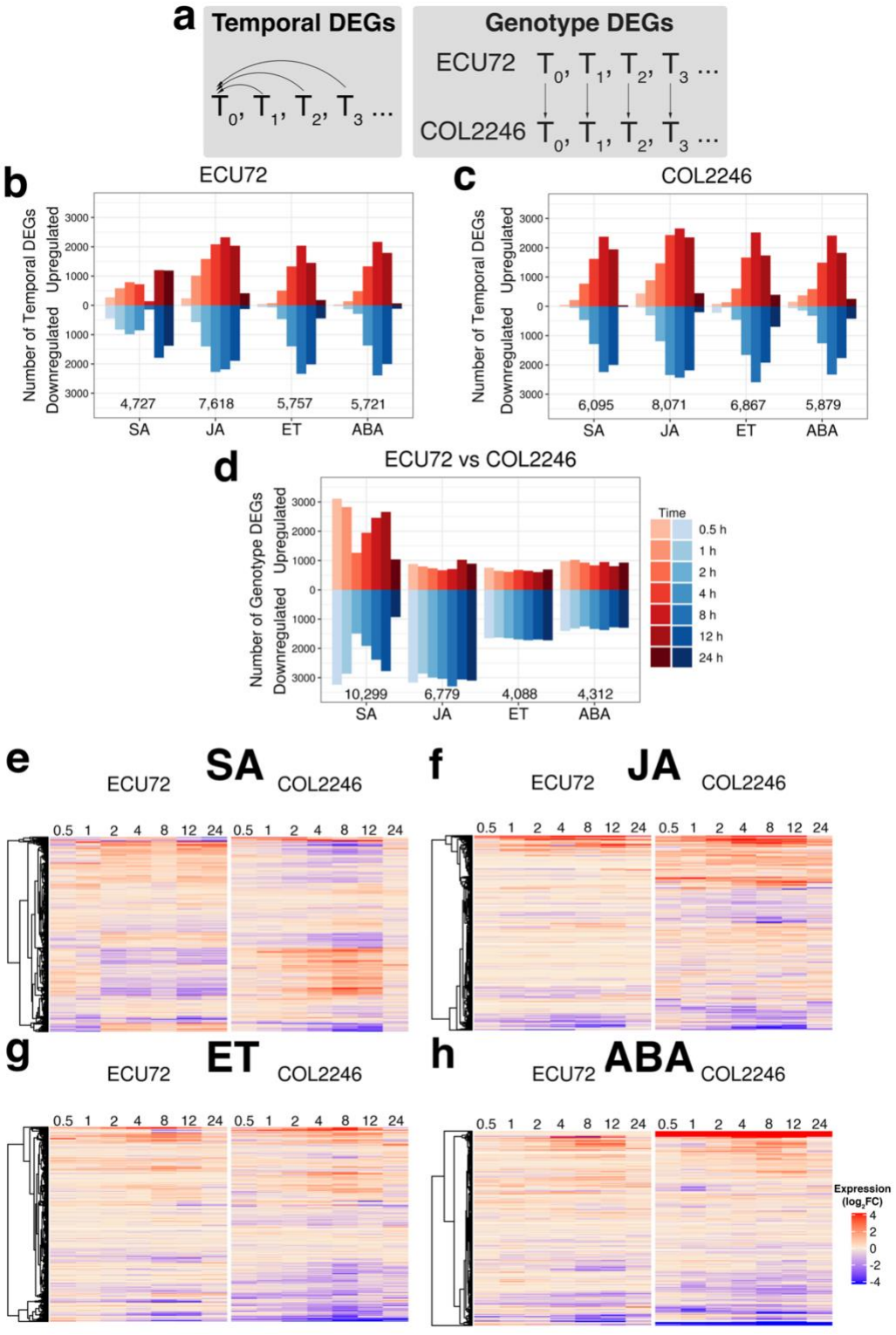

a
Temporal DEGs
T0, T1, T2, T3 ...
Genotype DEGs
ECU72 T0, T1, T2, T3 ...
COL2246 T0, T1, T2, T3 ...
b
ECU72
Number of Temporal DEGs
Upregulated
Downregulated
4,727
7,618
5,757
5,721
SA
JA
ET
ABA
c
COL2246
6,095
8,071
6,867
5,879
d
ECU72 vs COL2246
Number of Genotype DEGs
10,299
6,779
4,088
4,312
Time
0.5 h
1 h
2 h
4 h
8 h
12 h
24 h
e
ECU72 SA COL2246
f
ECU72 JA COL2246
g
ECU72 ET COL2246
h
ECU72 ABA COL2246
0.5 1 2 4 8 12 24
Expression (log2FC)
4
2
0
-2
-4

Figure 2.1. Transcriptome profiles of ECU72 and COL2246 DEGs in response to SA, JA, ET, and ABA treatments.

(a) Visual definition of temporal and genotype DEGs. Temporal DEGs were identified by comparisons of each time point to 0 hpt. Genotype DEGs were identified by comparisons of ECU72 and COL2246 at each time point.

(b-c) Temporal DEG counts in ECU72 and COL 2246 during SA, JA, ET, and ABA treatments (0, 0.5, 1, 2, 4, 8, 12, and 24 hpt). Early activation and prolonged expression genes of SA-regulated genes was seen in ECU72.

(d) Genotype DEG counts in ECU72 versus COL2246 during SA, JA, ET, and ABA treatments. The magnitude of SA-responsive genotype DEGs varied temporally, whereas other genotype response differences to other hormones remained stable over time.

Treatment experiments and RNA extractions were performed by Dr. Adriana Bohorquez-Chaux at CIAT. DEG expression values are provided in Table S2.1. Number of up- and down-regulated genes (red and blue, respectively) are displayed and total number of DEGs at each timepoint are provided. DEGs had $|\log_2 FC| \geq 1$ and FDR ≤ 5%.

(e-h) Heatmaps displaying genotype DEGs in ECU72 and COL2246 in response to SA, JA, ET, and ABA treatments. Genotypes responded similarly to JA, ET and ABA but reciprocally to SA treatments. Expression is displayed as $\log_2 FC$ values comparing 0 hour post treatment (hpt) to 0.5-24 hpt. Genotype DEGs were identified by comparisons of transcript levels in ECU72 versus COL2246 during treatments and had $|\log_2 FC| \geq 1$ and FDR ≤ 5%.

To further elucidate the timing of transcriptome changes, principal component analyses (PCAs) were performed with genes detected in SA, JA, ET, and ABA time courses (Figure 2.2). Clear separation of genotype responses to SA and ET along PC2 (25-31%) was observed; a similar separation along PC2 (24%) is seen in ABA responses, although with less distinct grouping of genotypes due to replicate variability in COL2246. In contrast, genotype responses to JA were resolved along PC1 (41%). Temporally, early (0.5 – 2 hpt) and late (4 – 12 hpt) time points were clustered in both genotypes in response to JA, ET and ABA. Notably, for these treatments, 24-h samples clustered with early time points. The PCA analyses also revealed a marked shift in timing between genotypes in response to SA (Figure 2.2). In COL2246, the late SA response began at 4 h, continued until 12 h, and returned to a basal state at 24 h. In contrast, ECU72's late SA response initiated earlier and was more prolonged, beginning at 2 h and continuing to 24 h. Finally, there was a reciprocity in the ECU72 and COL2246 responses to SA. For example, the early ECU72 samples (0-1 hpt) were most similar the late SA response of COL2246 (4 – 12 hpt) and vice versa (Figure 2.2a).

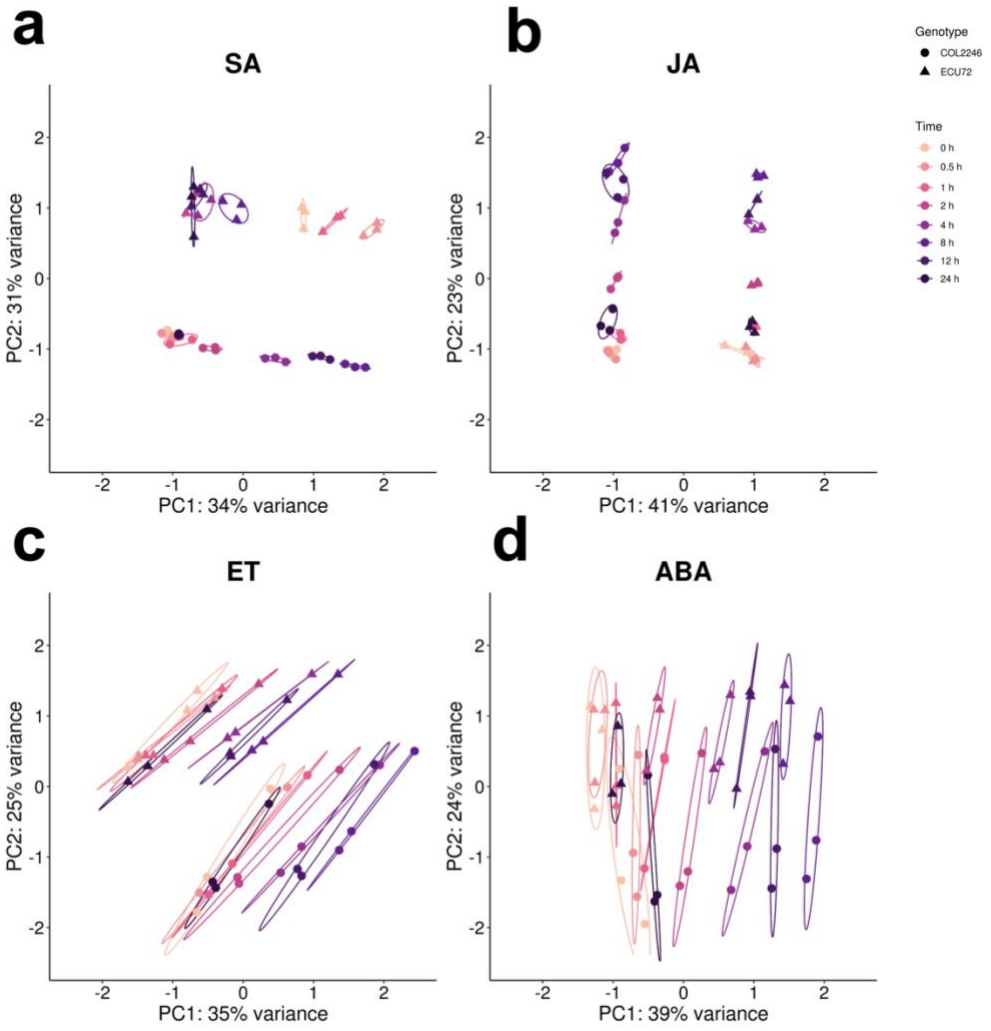


Figure 2.2. Hormone response timing in whitefly-resistant (ECU72) and -susceptible (COL2246) cassava.

(a-d) PCAs of detected gene expression prior to and after SA, JA, ET, and ABA treatments (0, 0.5, 1, 2, 4, 8, 12, 24 hpt) in ECU72 and COL2246. Clustering of time points defined early and late response phases of responses to treatments. In JA, ET and ABA treatments of ECU72 and COL2246, early (0.5-2 hpt) and late (4-12 hpt) responses had similar timing. In contrast, SA responses in ECU72 and COL2246 were dissimilar. COL2246 early and late SA phases were similar to responses to JA, ET and ABA (0.5-2 hpt and 4-12 hpt, respectively). In ECU72 the early phase was shorter (0.5-1 hpt) and late phase prolonged (2-24 hpt). Detected genes were defined as having an average of 20 reads or more across a hormone-treatment time course. Read count values for three biological replicates are shown per time point. Time points and genotypes are labeled by color and shape, respectively.

Transcriptome responses of ECU72 and COL2246 to SA are largely reciprocal

The temporal patterns of genotype DEG expression in ECU72 and COL2246 following SA, JA, ET, and ABA treatments were visualized using heatmaps (Figure 2.1; Table S2.1). Most strikingly, after SA treatments, most genotype DEGs were reciprocally regulated (Figure 2.1e). In contrast, most genes responding to JA, ET and ABA treatments had similar positive or negative expression trends in each genotype (Figure 2.3f-h); although some notable differences were observed (Figure 2.3f-h).

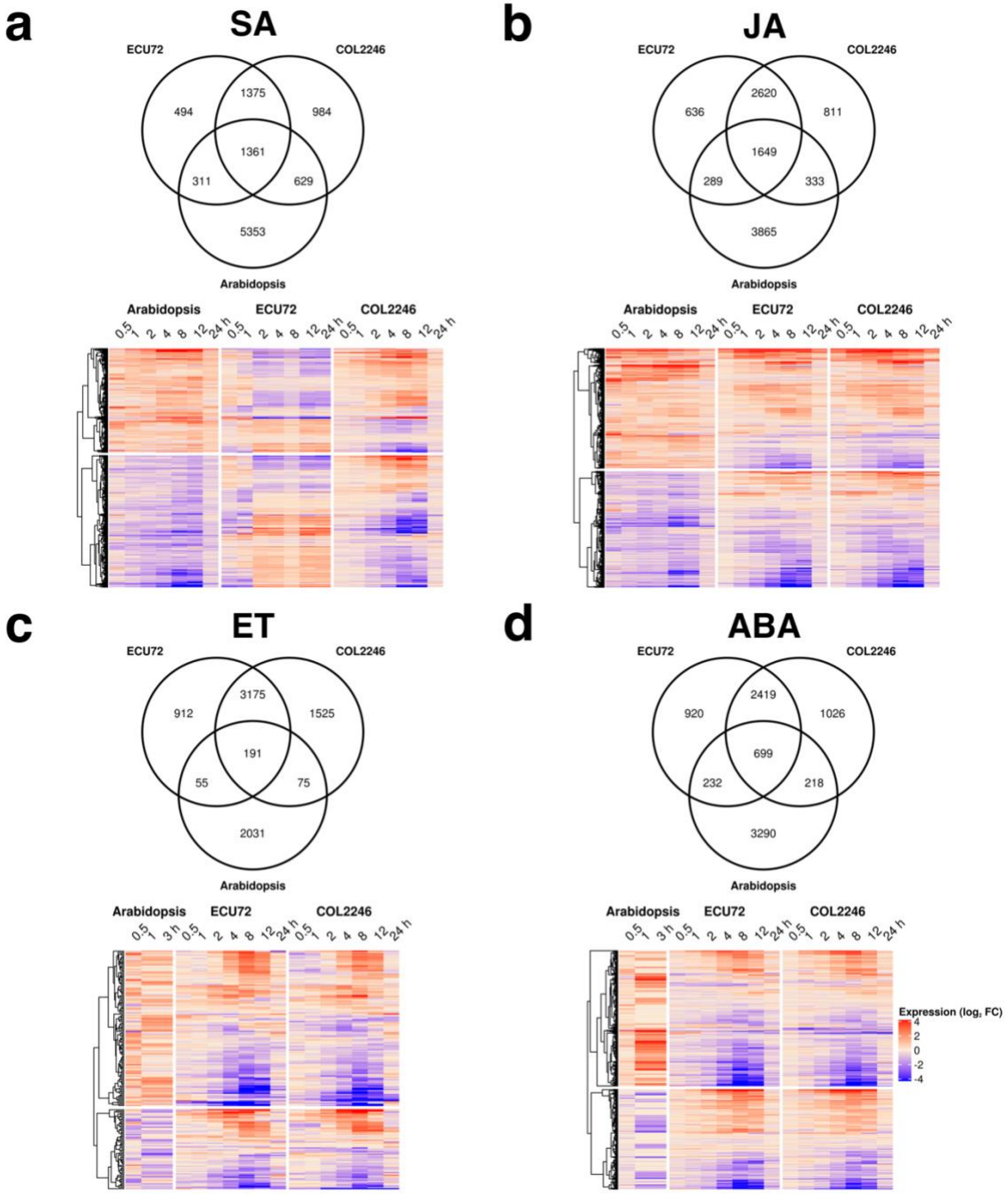

a
SA
ECU72
COL2246
494
1375
984
1361
311
629
5353
Arabidopsis
Arabidopsis
ECU72
COL2246
0.5 1 2 4 8 12 24 h
b
JA
ECU72
COL2246
636
2620
811
1649
289
333
3865
Arabidopsis
Arabidopsis
ECU72
COL2246
0.5 1 2 4 8 12 24 h
c
ET
ECU72
COL2246
912
3175
1525
191
55
75
2031
Arabidopsis
Arabidopsis
ECU72
COL2246
0.5 1 3 h
0.5 1 2 4 8 12 24 h
d
ABA
ECU72
COL2246
920
2419
1026
699
232
218
3290
Arabidopsis
Arabidopsis
ECU72
COL2246
0.5 1 3 h
0.5 1 2 4 8 12 24 h
Expression (log2 FC)
4
2
0
-2
-4

Figure 2.3. Comparison of Arabidopsis and cassava hormone responses.

(a-d) Venn diagrams and heatmaps comparing temporal DEGs in Arabidopsis and cassava (COL2246 and ECU72) identified during SA, JA, ET, and ABA treatments, respectively. Less than 40% of Arabidopsis DEGs had similar hormone responses with cassava. There are substantial differences in the regulatory programs of these DEGs in Arabidopsis, ECU72 and/or COL2246.

Arabidopsis SA and JA DEGs were identified in this study. Arabidopsis ET DEGs at 0.5, 1 and 3 hpt were retrieved from Goda et al. (2008) and 24 hpt from Schenk et al. (2000). ABA DEGs at 0.5, 1 and 3 hpt were from Goda et al. (2008) and 6 and 24 hpt were reported by Huang et al. (2007). Expression is displayed as $\log_2$FC values comparing 0 hpt to 0.5-24 hpt. Cassava temporal DEGs for all hormone treatments and Arabidopsis SA and JA treatments were identified by comparisons of 0 hpt to 0.5-24 hpt samples and had $|\log_2 FC| \geq 1$ and FDR ≤ 5%.

To further elucidate genotypic differences among the four hormone responses, Pearson correlation analyses were used to compare early- and late-phase treatment DEG expression (Figure 2.4; Table S2.3). Notably, the late SA response of ECU72 had a moderate to very strong negative correlation with early and late phases of all other hormone responses (Figure 2.2e). The early SA response of ECU72 on the other hand displayed a moderate positive correlation with late but not early JA, ET and ABA responses. All other hormone responses of ECU72 (early and late JA, ET and ABA), as well as all hormone responses in COL2246 (early and late SA, JA, ET, and ABA), displayed weak to very strong positive correlation with one another (Figure 2.4; Table 2.S3).

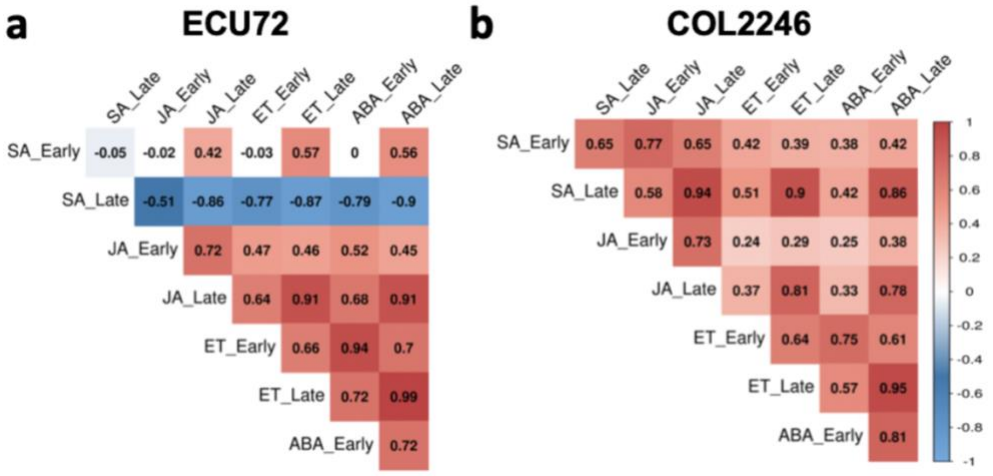


Figure 2.4. Correlation of SA, JA, ET, and ABA responses in ECU72 and COL2246.

(a, b) A correlation matrix of early and late SA, JA, ET, and ABA responses in ECU72 **(a)** and COL2246 **(b)**. Early and late responses to JA, ET and ABA corresponded to times 0.5-2 hpt and 4-12 hpt in ECU72 and COL2246; a similar temporal program was seen in response to SA in COL2246. In contrast, the timing of ECU72's SA response was different with the early phase spanning 0.5-1 hpt and late phase spanning 2-24 hpt. Response phases were defined in Figure 2.1. ECU72's late response to SA was negatively correlated with all other hormone treatments. In contrast, COL2246's hormone responses were positively correlated with all other hormone responses. Correlation values are based on average $\log_2$FC values of ECU72's and COL2246's temporal DEGs and are shaded according to the scale of R-values provided in **(b)**. Non-significant correlation values ($p>0.05$) are not shaded. R- and p-values are provided in Table S2.3.

To uncover the biological processes associated with reciprocal SA responses between the genotypes, clustering and functional enrichment analyses were performed. Genotype DEGs during SA treatments were grouped into k-means clusters. RPKM means and categories of enriched GO terms are displayed in order of the significance of their enrichment. (Figures 2.5-2.8; Tables S2.1 and S2.4). Two temporal expression programs in response to SA were observed in ECU72. Cluster 1 and Cluster 2 genes peaked after 2 h, while COL2246 responses were reciprocal. These clusters were enriched for GO term categories including ion transport, response to stimulus and phenylpropanoid metabolic process. In contrast, Cluster 3, Cluster 4 and Cluster 5 genes were expressed at higher levels from 0-1 hpt in ECU72 and then declined. Again, the opposite regulatory program was observed in COL2246. Genes in Clusters 3 to 5 were involved in processes like polysaccharide, nucleic acid and glucosinolate metabolism, auxin signaling, and immune system processes. Higher overall transcript levels were associated with the smaller Clusters 1 and 3 as opposed to the larger clusters (Figure 2.5; Table S2.4).

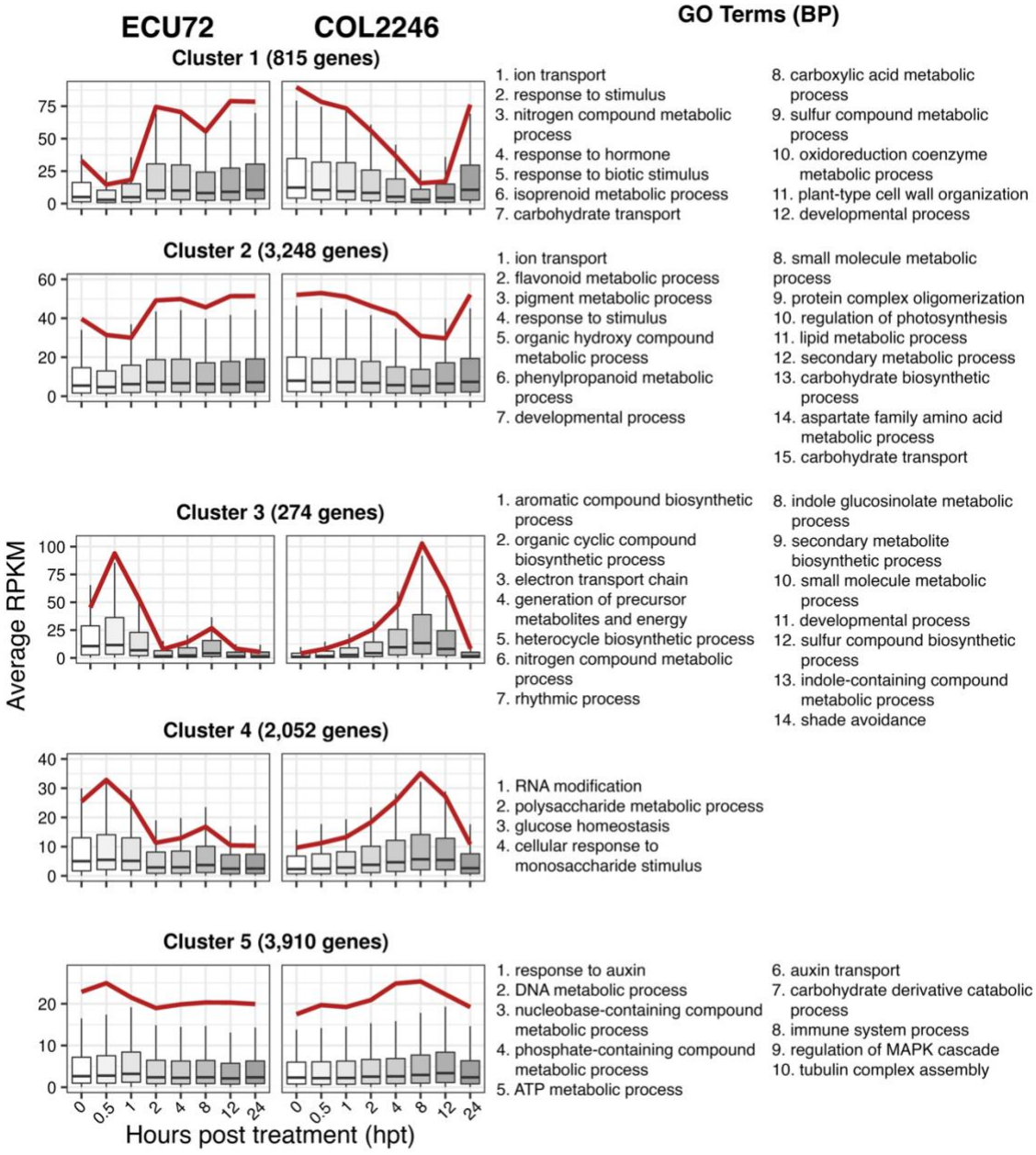

ECU72
COL2246
GO Terms (BP)
Cluster 1 (815 genes)
1. ion transport
2. response to stimulus
3. nitrogen compound metabolic process
4. response to hormone
5. response to biotic stimulus
6. isoprenoid metabolic process
7. carbohydrate transport
8. carboxylic acid metabolic process
9. sulfur compound metabolic process
10. oxidoreduction coenzyme metabolic process
11. plant-type cell wall organization
12. developmental process
Cluster 2 (3,248 genes)
1. ion transport
2. flavonoid metabolic process
3. pigment metabolic process
4. response to stimulus
5. organic hydroxy compound metabolic process
6. phenylpropanoid metabolic process
7. developmental process
8. small molecule metabolic process
9. protein complex oligomerization
10. regulation of photosynthesis
11. lipid metabolic process
12. secondary metabolic process
13. carbohydrate biosynthetic process
14. aspartate family amino acid metabolic process
15. carbohydrate transport
Cluster 3 (274 genes)
1. aromatic compound biosynthetic process
2. organic cyclic compound biosynthetic process
3. electron transport chain
4. generation of precursor metabolites and energy
5. heterocycle biosynthetic process
6. nitrogen compound metabolic process
7. rhythmic process
8. indole glucosinolate metabolic process
9. secondary metabolite biosynthetic process
10. small molecule metabolic process
11. developmental process
12. sulfur compound biosynthetic process
13. indole-containing compound metabolic process
14. shade avoidance
Average RPKM
Cluster 4 (2,052 genes)
1. RNA modification
2. polysaccharide metabolic process
3. glucose homeostasis
4. cellular response to monosaccharide stimulus
Cluster 5 (3,910 genes)
1. response to auxin
2. DNA metabolic process
3. nucleobase-containing compound metabolic process
4. phosphate-containing compound metabolic process
5. ATP metabolic process
6. auxin transport
7. carbohydrate derivative catabolic process
8. immune system process
9. regulation of MAPK cascade
10. tubulin complex assembly
0 0.5 1 2 4 8 12 24
Hours post treatment (hpt)

Figure 2.5. Clustering and functional enrichment of genes differentially expressed in ECU72 versus COL2246 during SA treatment.

K-means expression clusters of genotype DEGs in ECU72 versus COL2246 during SA treatment. Clusters display reciprocity of ECU72 and COL2246 responses, as well as the temporal trends of DEGs within clusters and their association with several GO terms related to stress/defense responses. Categories of significantly enriched ($p \leq 0.05$) GO terms ranked by p-value are provided for each of the five clusters (Table S2.4). Genotype DEGs were identified by comparisons of transcript levels in ECU72 versus COL2246 during SA treatments and had $|\log_2 FC| \geq 1$ and FDR ≤ 5%. Boxplot whiskers represent values within 1.5 x IQR, and box values represent the first quartile, median, and third quartile values. Outliers (points beyond whiskers) are not displayed. Lines display mean RPKM values at 0 to 24 hpt.

In contrast to the reciprocity of responses to SA in ECU72 versus COL2246, JA, ET and ABA treatments had similar temporal responses between genotypes. Gene clusters with similar trends in expression between genotypes were enriched for many defense-related terms, including JA Clusters 1 and 2 (response to JA/SA, response to wounding/fungus/insect), ET Cluster 2 (response to fungus) and ABA Cluster 4 (cell death, immune system process, response to stimulus) (Figures 2.6-2.8). Divergent genotype responses were however observed in ET Cluster 5 and ABA Cluster 3. Genes in these clusters were more highly expressed in COL2246 constitutively, but markedly declined following infestation, and were induced at 0.5 hpt before declining in ECU72. Such genes were enriched for terms such as response to mechanical stimulus and cell wall organization/modification (Figures 2.7 and 2.8).

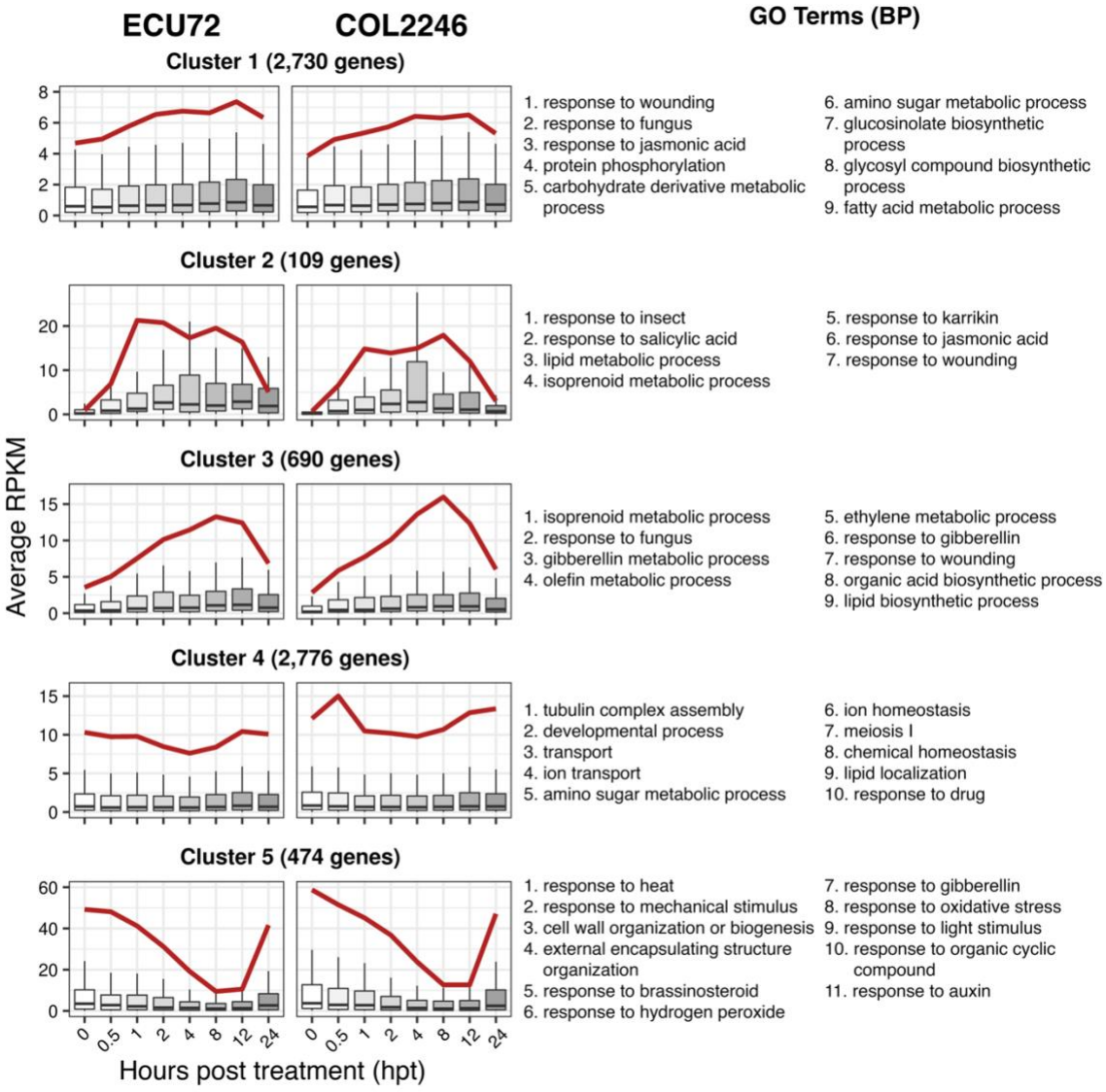

ECU72
COL2246
GO Terms (BP)
Cluster 1 (2,730 genes)
1. response to wounding
2. response to fungus
3. response to jasmonic acid
4. protein phosphorylation
5. carbohydrate derivative metabolic process
6. amino sugar metabolic process
7. glucosinolate biosynthetic process
8. glycosyl compound biosynthetic process
9. fatty acid metabolic process
Cluster 2 (109 genes)
1. response to insect
2. response to salicylic acid
3. lipid metabolic process
4. isoprenoid metabolic process
5. response to karrikin
6. response to jasmonic acid
7. response to wounding
Cluster 3 (690 genes)
1. isoprenoid metabolic process
2. response to fungus
3. gibberellin metabolic process
4. olefin metabolic process
5. ethylene metabolic process
6. response to gibberellin
7. response to wounding
8. organic acid biosynthetic process
9. lipid biosynthetic process
Cluster 4 (2,776 genes)
1. tubulin complex assembly
2. developmental process
3. transport
4. ion transport
5. amino sugar metabolic process
6. ion homeostasis
7. meiosis I
8. chemical homeostasis
9. lipid localization
10. response to drug
Cluster 5 (474 genes)
1. response to heat
2. response to mechanical stimulus
3. cell wall organization or biogenesis
4. external encapsulating structure organization
5. response to brassinosteroid
6. response to hydrogen peroxide
7. response to gibberellin
8. response to oxidative stress
9. response to light stimulus
10. response to organic cyclic compound
11. response to auxin
Average RPKM
0 0.5 1 2 4 8 12 24
Hours post treatment (hpt)

Figure 2.6. Clustering and functional enrichment of genes differentially expressed in ECU72 versus COL2246 during JA treatment.

K-means expression clusters of genotype DEGs in ECU72 versus COL2246 during JA treatment. Clusters display trends in the timing and magnitude of JA responses and association with several GO terms related to stress/defense responses. Categories of significantly enriched GO terms ($p \leq 0.05$) ranked by p-value are provided for each of the five clusters (Table S2.4). Genotype DEGs were identified by comparisons of transcript levels in ECU72 versus COL2246 during SA treatments and had $|\log_2 FC| \geq 1$ and FDR ≤ 5%. Boxplot whiskers represent values within 1.5 x IQR, and box values represent the first quartile, median, and third quartile values. Outliers (points beyond whiskers) are not displayed. Lines display mean RPKM values of genes at each time point.

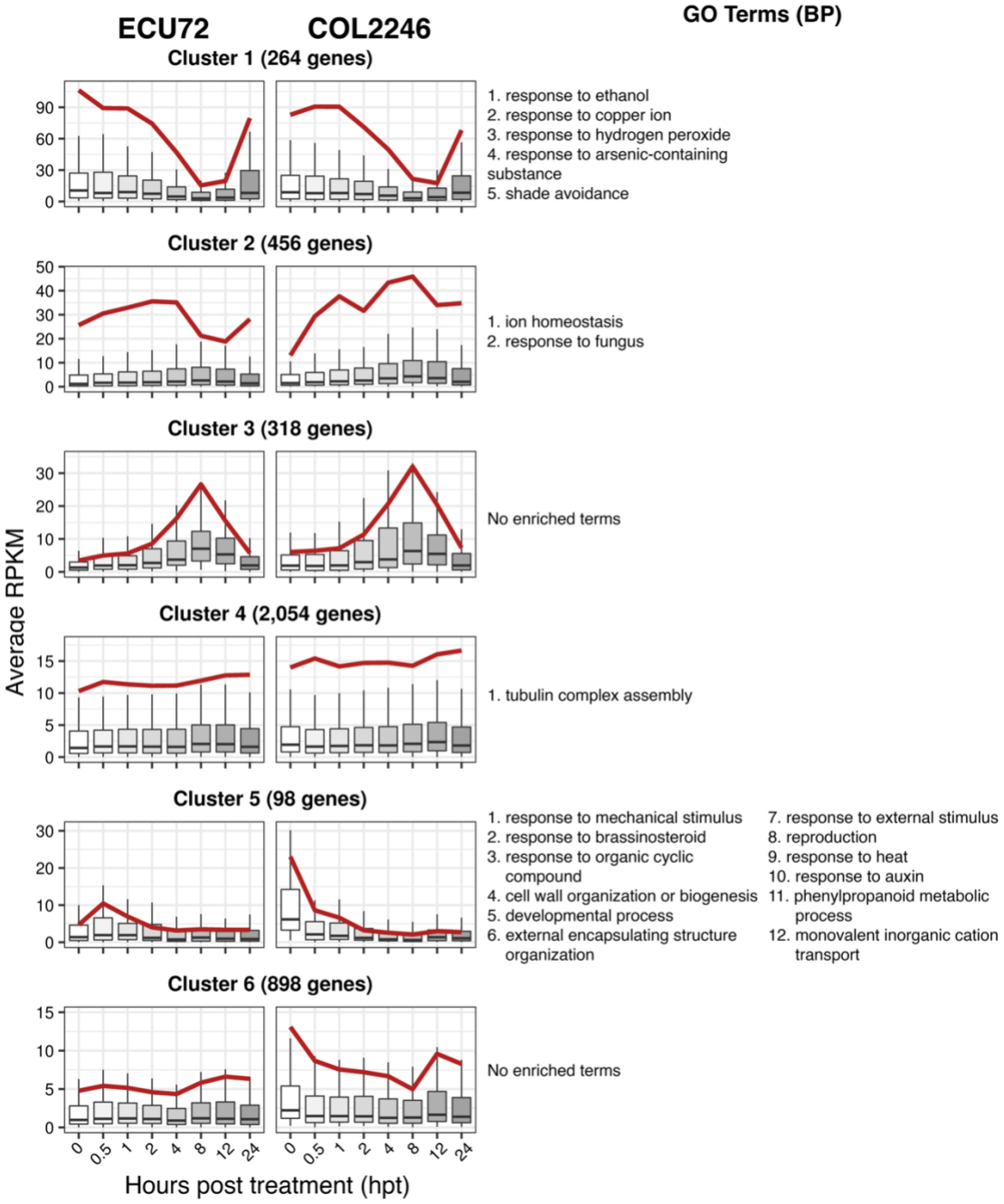

ECU72
COL2246
GO Terms (BP)
Cluster 1 (264 genes)
1. response to ethanol
2. response to copper ion
3. response to hydrogen peroxide
4. response to arsenic-containing substance
5. shade avoidance
Cluster 2 (456 genes)
1. ion homeostasis
2. response to fungus
Cluster 3 (318 genes)
No enriched terms
Cluster 4 (2,054 genes)
1. tubulin complex assembly
Cluster 5 (98 genes)
1. response to mechanical stimulus
2. response to brassinosteroid
3. response to organic cyclic compound
4. cell wall organization or biogenesis
5. developmental process
6. external encapsulating structure organization
7. response to external stimulus
8. reproduction
9. response to heat
10. response to auxin
11. phenylpropanoid metabolic process
12. monovalent inorganic cation transport
Cluster 6 (898 genes)
No enriched terms
Average RPKM
Hours post treatment (hpt)

Figure 2.7. Clustering and functional enrichment of genes differentially expressed in ECU72 versus COL2246 during SA treatment.

K-means expression clusters of genotype DEGs in ECU72 versus COL2246 during SA treatment. Clusters display reciprocity of ECU72 and COL2246 responses, as well as the temporal trends of DEGs within clusters and their association with several GO terms related to stress/defense responses. Categories of significantly enriched ($p \leq 0.05$) GO terms ranked by p-value are provided for each of the five clusters (Table S2.4). Genotype DEGs were identified by comparisons of transcript levels in ECU72 versus COL2246 during SA treatments and had $|\log_2 FC| \geq 1$ and FDR ≤ 5%. Boxplot whiskers represent values within 1.5 x IQR, and box values represent the first quartile, median, and third quartile values. Outliers (points beyond whiskers) are not displayed. Lines display mean RPKM values at 0 to 24 hpt.

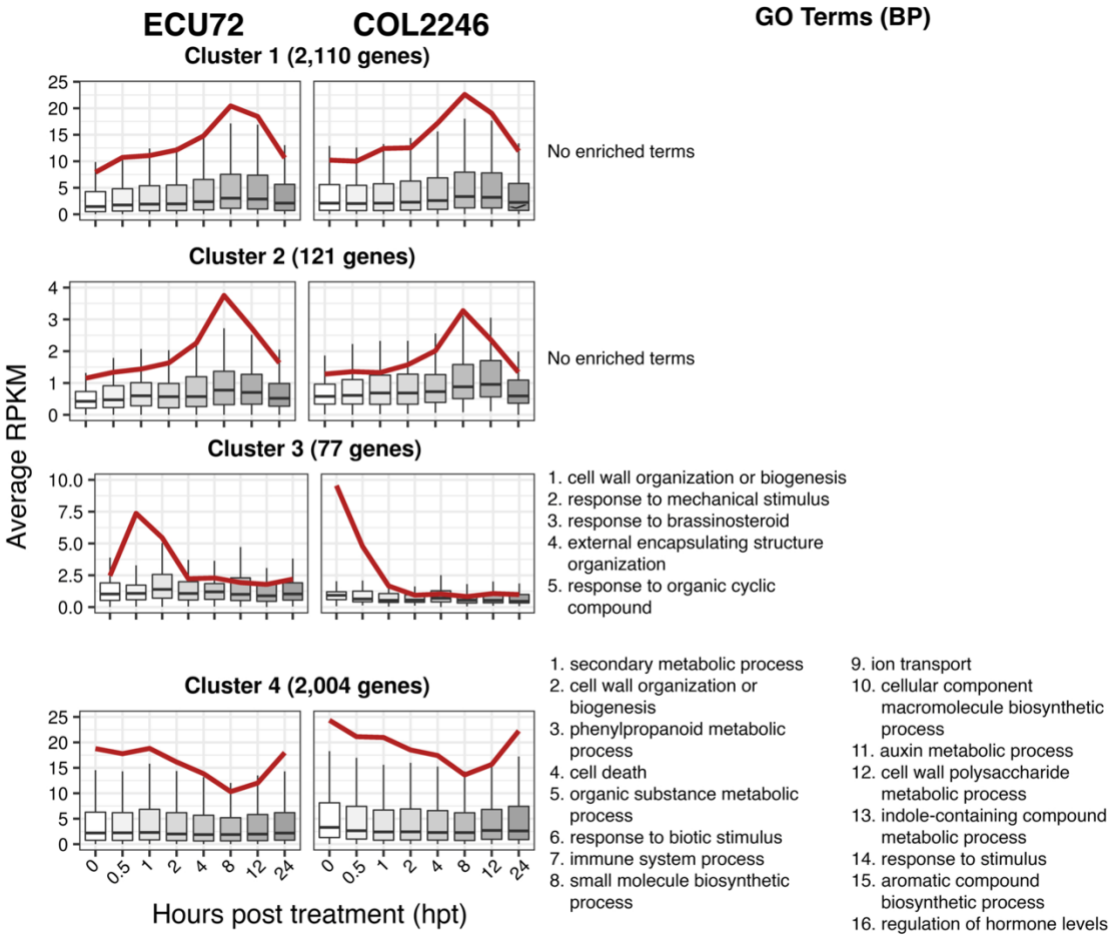

ECU72
COL2246
GO Terms (BP)
Cluster 1 (2,110 genes)
No enriched terms
Cluster 2 (121 genes)
No enriched terms
Cluster 3 (77 genes)
1. cell wall organization or biogenesis
2. response to mechanical stimulus
3. response to brassinosteroid
4. external encapsulating structure organization
5. response to organic cyclic compound
Cluster 4 (2,004 genes)
1. secondary metabolic process
2. cell wall organization or biogenesis
3. phenylpropanoid metabolic process
4. cell death
5. organic substance metabolic process
6. response to biotic stimulus
7. immune system process
8. small molecule biosynthetic process
9. ion transport
10. cellular component macromolecule biosynthetic process
11. auxin metabolic process
12. cell wall polysaccharide metabolic process
13. indole-containing compound metabolic process
14. response to stimulus
15. aromatic compound biosynthetic process
16. regulation of hormone levels
Average RPKM
Hours post treatment (hpt)

Figure 2.8. Clustering and functional enrichment of genes differentially expressed in ECU72 versus COL2246 during JA treatment.

K-means expression clusters of genotype DEGs in ECU72 versus COL2246 during JA treatment. Clusters display trends in the timing and magnitude of JA responses and association with several GO terms related to stress/defense responses. Categories of significantly enriched GO terms ($p \leq 0.05$) ranked by p-value are provided for each of the five clusters (Table S2.4). Genotype DEGs were identified by comparisons of transcript levels in ECU72 versus COL2246 during SA treatments and had $|\log_2 FC| \geq 1$ and FDR ≤ 5%. Boxplot whiskers represent values within 1.5 x IQR, and box values represent the first quartile, median, and third quartile values. Outliers (points beyond whiskers) are not displayed. Lines display mean RPKM values of genes at each time point.

Cassava's hormone responses are divergent from those of Arabidopsis

The unanticipated reciprocity in the SA-regulated DEGs for ECU72 and COL2246 suggested a major transcriptome reprogramming in these genotypes. To place cassava's hormone responses within the context of hormone responses from a well-characterized model plant, the SA- and JA-dependent transcriptomes of cassava were compared to those of *Arabidopsis thaliana*. Previous microarray or RNA-seq studies profiling SA and JA responses in Arabidopsis utilized various hormone concentrations and plants of different ages, resulting in marked differences in DEGs identified in each study (Figure 2.9a,b; Table S2.5) (Hickman et al., 2017, Pauwels et al., 2008, Sawant et al., 2009, Singh et al., 2015, Thibaud-Nissen et al., 2006, Yang et al., 2017). Therefore, RNA-seq analyses of SA- and JA- treatment time courses (0, 0.5, 1, 2, 4, 8, 12, and 24 hpt) in Arabidopsis were performed for comparison to cassava time-course data (Table S2.6). We identified 7,654 and 6,136 SA and JA temporal DEGs in Arabidopsis, respectively, and 21% and 41% of these DEGs were identified in one or more previous Arabidopsis SA- or JA-treatment transcriptome studies (Figure 2.9c,d).

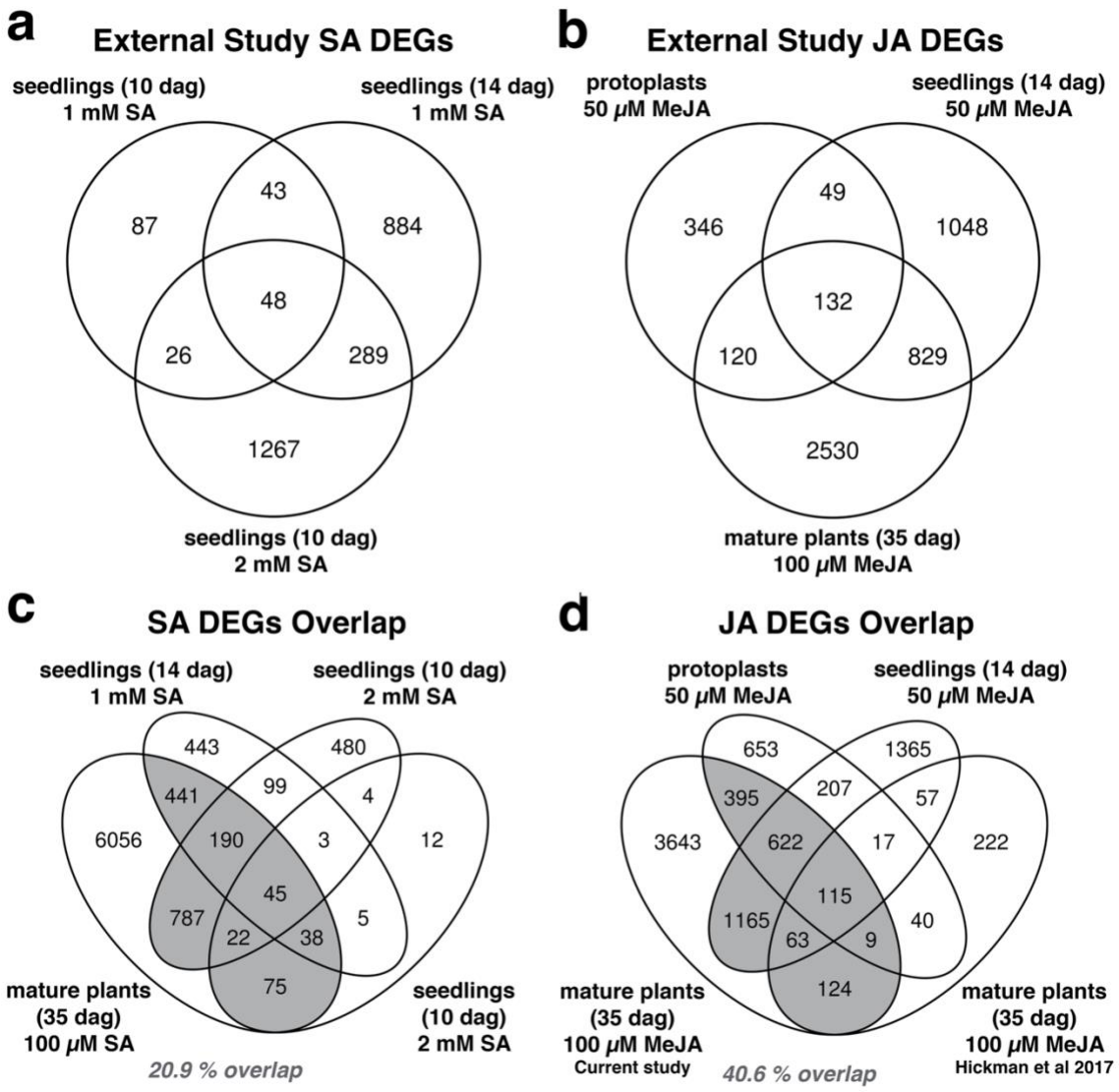


Figure 2.9. Overlap of SA and JA temporal DEGs identified by this and external studies.

(a-b) Venn diagrams comparing SA- and JA-responsive DEGs, respectively, identified by previous studies that used plants of different ages and different hormone concentrations (Hickman et al., 2017, Pauwels et al., 2008, Sawant et al., 2009, Singh et al., 2015, Thibaud-Nissen et al., 2006, Yang et al., 2017). A surprisingly small number of DEGs were identified by all SA or JA studies.

(c-d) Venn diagrams comparing Arabidopsis SA- and JA-responsive DEGs, respectively, identified by this and previous studies. DEGs identified in our study and by other studies are shaded in grey.

As we performed Arabidopsis and cassava SA and JA treatments with similar experimental designs, the timing of Arabidopsis SA- and JA-dependent transcriptome responses were compared using PCA analyses (Figures 2.2 and 2.10). In response to SA, Arabidopsis and COL2246 exhibited similar temporal phases; early (0.5 to 2 hpt), 0-hpt and 24-hpt samples grouped together and distinctly from late time point samples (4-12 hpt). In addition, similar JA expression phases were observed in Arabidopsis and both cassava genotypes. However, more variability among biological replicates was observed in Arabidopsis. In particular, much of this variation was observed 1 and 2 h after SA treatment and 1 and 4 h after JA treatment, suggesting that these may be times of active transcriptome reprogramming in Arabidopsis (Figures 2.2 and 2.10).

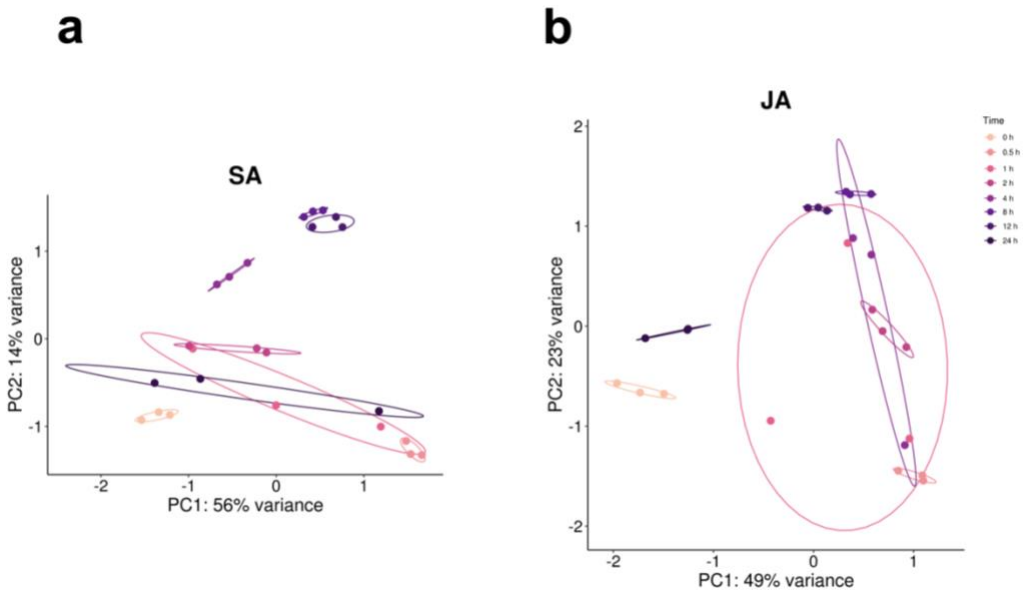


Figure 2.10. PCA analyses of the timing of SA and JA responses in Arabidopsis.

(a-b) PCA analyses were used to examine the temporal responses of Arabidopsis to SA **(a)** and JA **(b)** treatments. Timing of Arabidopsis's early (0.5-2 hpt) and late (4-12 hpt) SA response phases was more distinct while JA samples showed more variability and less distinct grouping. Based on the clustering of 0 and 24 hpt samples, Arabidopsis SA and JA responses returned to the basal state (0 hpt) at 24 hpt.

Detected genes were defined as having an average of 20 reads or more across a hormone treatment time course. Read count values for three biological replicates are shown per time point. Time points and genotypes are labeled by color and shape, respectively.

Comparison of number of temporal DEGs in Arabidopsis versus cassava also revealed faster SA and JA responses in Arabidopsis (Tables S2.2 and S2.6). In response to SA and JA, Arabidopsis elicited 1,610 and 2,210 DEGs by 0.5 hpt, respectively; whereas, in cassava SA and JA temporal DEGs did not increase to these levels until 1 to 2 hpt (Figures 2.1 and 2.11). Finally, the SA responses of both species and the JA response of both cassava genotypes peaked at about 1,000 to 2,000 up- and down-regulated DEGs by 8 or 12 hpt (Figures 2.1 and 2.11). This is in marked contrast with Arabidopsis' transcriptome response to JA, where the numbers of up- and down-regulated DEGs hovered around 1,000 from 0.5 to 12 hpt and then declined at 24 hpt (Figure 2.11; Table S2.2).

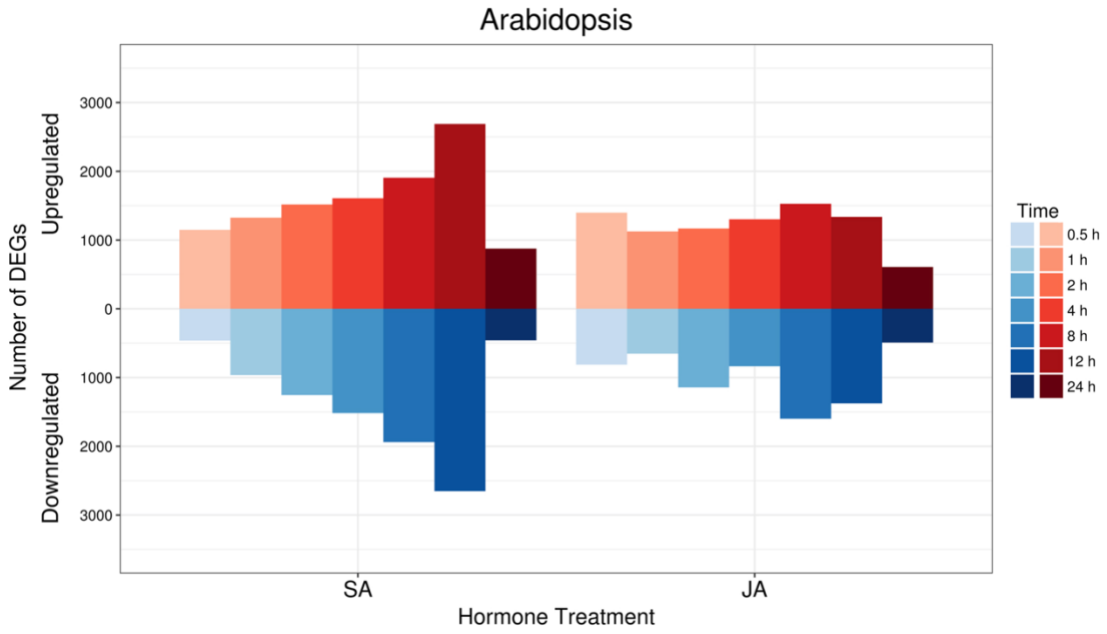


Figure 2.11. Temporal DEG during SA and JA treatments of Arabidopsis.

Arabidopsis temporal DEG during SA and JA treatments are shown. The magnitude and timing of DEG expression programs in response to SA and JA are distinct. The largest number of DEGs in response to SA was detected at 12 hpt, while the response to JA engages a similar number of genes at all time points, with a decline at 24 h (see Table S2.2). Number of up- and down-regulated genes are displayed in red and blue, respectively. Treatment DEGs were identified by comparisons of 0 hpt and 0.5-24 hpt and had $|\log_2 FC| \geq 1$ and FDR ≤ 5%.

To compare expression of temporal DEGs expressed in both species, heatmaps were used to display SA, JA, ET, and ABA-responsive DEGs in Arabidopsis and both cassava genotypes (Figure 2.3). Previous microarray studies documenting the response of Arabidopsis to ET (0.5, 1, 3, and 24 hpt) (Goda et al., 2008, Schenk et al., 2000) and ABA (0.5, 1, 3, 6, and 24 hpt) (Goda et al., 2008, Huang et al., 2007) were used to identify ET (2,352 genes) and ABA (4,439 genes) temporal DEGs (Figure 2.3 and Table S2.7). Among the four hormones, differences in the SA response between the species/genotypes were most striking. Of the 2,736 genes regulated by SA in both cassava genotypes, only

50% (1,361 genes) were also SA-regulated in Arabidopsis (Figure 2.3). For such genes, reciprocity in gene expression was clearly seen between ECU72 and COL2246. Furthermore, Arabidopsis' SA-regulatory programs did not align exclusively with one cassava genotype. For example, among Arabidopsis SA-upregulated DEGs, approximately one-third displayed similar trends in ECU72, while the other two-thirds had similarity with COL2246. Additionally, while Arabidopsis and COL2246 SA-responsive DEGs exhibited generally either positive or negative regulation, the same genes often had more complex regulation in ECU72 (Figure 2.3). For temporal DEGs identified in both ECU72 and COL2246 during JA (4,269 genes), ET (3,366 genes) or ABA (3,118 genes) treatments, 39%, 6% and 22% were also regulated by the corresponding hormone treatment in Arabidopsis (Figure 2.3). Although these genes were similarly regulated in the two cassava genotypes, similar to SA, there were substantial differences in their patterns of expression of between Arabidopsis and cassava (Figure 2.3).

Given the marked differences in the transcriptome responses of Arabidopsis and cassava to these defense hormones, we compared the numbers and expression programs of genes central to SA, JA, ET, and ABA accumulation and perception in the two species (Figures 2.12-2.15; Table S2.9). To this end, 154 Arabidopsis genes involved in defense hormone biosynthesis, modification, transport, or signaling/response were identified from the literature (Finkelstein, 2013, Seyfferth and Tsuda, 2014, Stepanova and Alonso, 2009, Wasternack and Strnad, 2016). Cassava orthologs to these genes were identified using the online program eggNOG (Huerta-Cepas et al., 2017, Huerta-Cepas et al., 2019) (Tables S2.8 and S2.10). Sixty-seven of these core Arabidopsis defense-hormone pathway genes had more than one ortholog in cassava, while only six genes had fewer orthologs in cassava (Table S2.8).

Notably among SA biosynthesis genes, cassava possessed only one *ICS* gene (*MeICS1*), which was equally related to *AtICS1* and *AtICS2*, and five genes related to *AtPAL1* and *AtPAL2* (*MePAL1a-e*) (Figure 2.12; Tables S2.8 and S2.10). Although it remains unknown, observed gene responsiveness to SA suggests that *PAL* and not *ICS* genes are predominantly important for SA biosynthesis in the defense response of cassava. *AtICS1* was strongly induced by SA within 8 hpt, whereas *MeICS1* weakly responded to SA. Three of the *MePAL1* genes had different expression programs in the two cassava genotypes. While *AtPAL1*and*AtPAL2* and *MePAL1a-c* and *MePAL1e* were repressed in Arabidopsis and COL2246, respectively, these *MePAL* genes were induced in ECU72 following 2 h of SA treatment. Another notable difference between these species is that three key genes involved in regulating SA biosynthesis in Arabidopsis (*AtCBP60g*, *AtEPS1* and *AtPBS3*) were not identified in cassava (Dempsey et al., 2011). Finally, 15 of the 20 Arabidopsis SA-signaling/response genes were positively regulated following SA treatment. In contrast, the cassava orthologs were not regulated in a coordinate fashion by SA (Figure 2.12). Many of these and other SA-pathway genes (i.e. *MeSMTb*-c, *MeGRX480a*, *MeNPR1*, *MeSARD1a*, *MeTGA2a-c*, *MeTGA8a*, *MeWRKY70a-b, and MeEDS5a-c*) displayed opposite expression trends in ECU72 versus COL2246 (Figure 2.12).

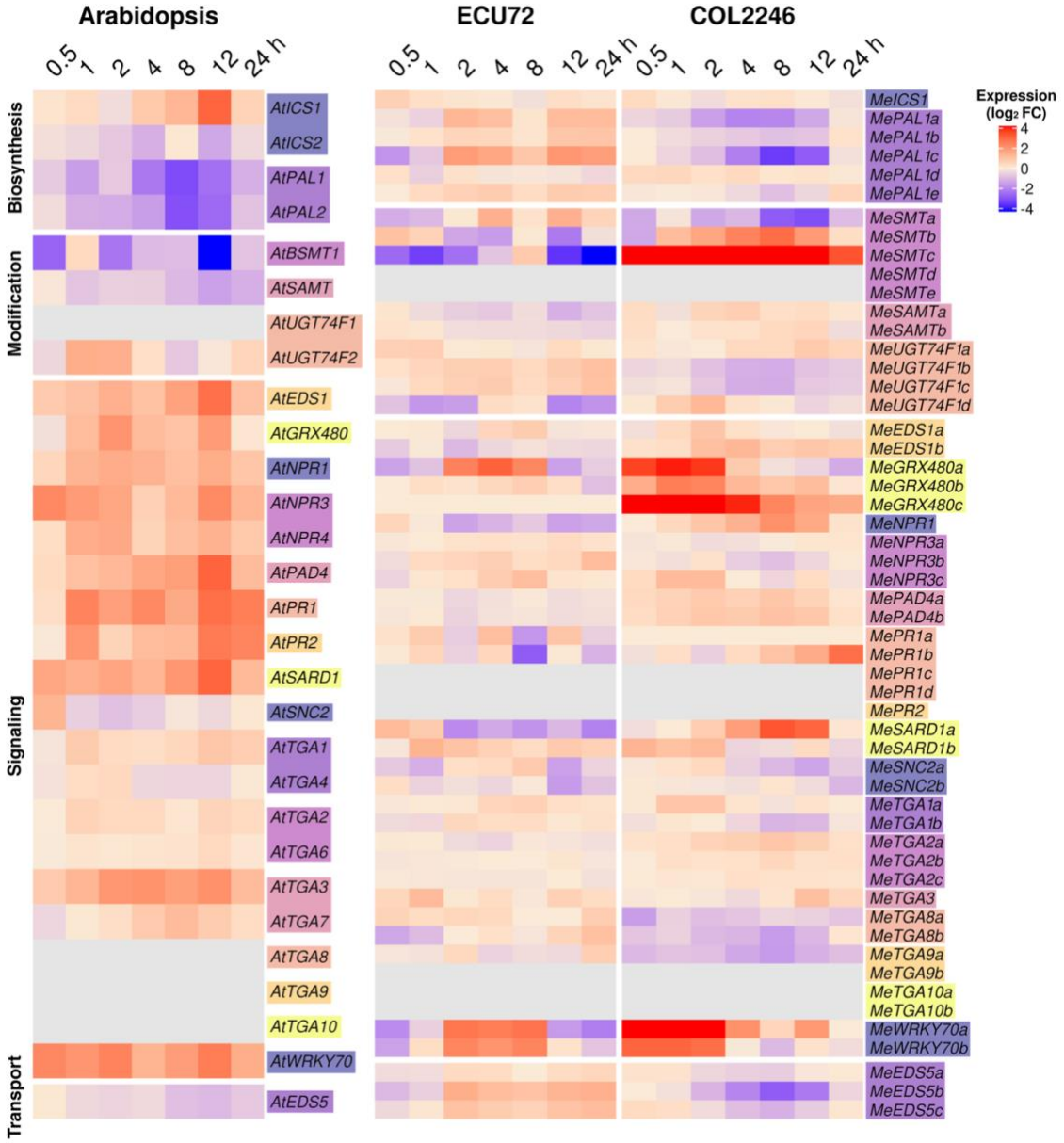

Arabidopsis
ECU72
COL2246
0.5
1
2
4
8
12
24 h
Biosynthesis
Modification
Signaling
Transport
AtICS1
AtICS2
AtPAL1
AtPAL2
AtBSMT1
AtSAMT
AtUGT74F1
AtUGT74F2
AtEDS1
AtGRX480
AtNPR1
AtNPR3
AtNPR4
AtPAD4
AtPR1
AtPR2
AtSARD1
AtSNC2
AtTGA1
AtTGA4
AtTGA2
AtTGA6
AtTGA3
AtTGA7
AtTGA8
AtTGA9
AtTGA10
AtWRKY70
AtEDS5
MeICS1
MePAL1a
MePAL1b
MePAL1c
MePAL1d
MePAL1e
MeSMTa
MeSMTb
MeSMTc
MeSMTd
MeSMTe
MeSAMTa
MeSAMTb
MeUGT74F1a
MeUGT74F1b
MeUGT74F1c
MeUGT74F1d
MeEDS1a
MeEDS1b
MeGRX480a
MeGRX480b
MeGRX480c
MeNPR1
MeNPR3a
MeNPR3b
MeNPR3c
MePAD4a
MePAD4b
MePR1a
MePR1b
MePR1c
MePR1d
MePR2
MeSARD1a
MeSARD1b
MeSNC2a
MeSNC2b
MeTGA1a
MeTGA1b
MeTGA2a
MeTGA2b
MeTGA2c
MeTGA3
MeTGA8a
MeTGA8b
MeTGA9a
MeTGA9b
MeTGA10a
MeTGA10b
MeWRKY70a
MeWRKY70b
MeEDS5a
MeEDS5b
MeEDS5c
Expression
(log2 FC)
4
2
0
-2
-4

Figure 2.12. SA-pathway gene expression in Arabidopsis and two cassava genotypes.

Genes involved in SA biosynthesis, modification, and transport and in transducing or responding to SA (signaling) were identified from the literature and cassava orthologs identified. Expression of SA-pathway genes are presented as log_2FC values during SA treatment in Arabidopsis, ECU72 and COL2246. Cassava genes show varying expression trends in response to SA as compared to Arabidopsis. Several genes show reciprocal SA regulation between cassava genotypes. Biosynthetic genes are ordered by their approximate step in the pathway, while other pathway genes are ordered alphabetically. To enable identification of orthologous genes in Arabidopsis and cassava, orthologous genes in each category are denoted by box color.

In response to JA, the biosynthesis, modification and signaling/response genes of Arabidopsis and cassava had more similar responses (Figure 2.13). A striking distinction between these species was the regulation of *LOX2* and *VSP* orthologs. The single-copy JA biosynthetic gene *AtLOX2,* with established roles in JA biosynthesis (Wasternack, 2014), had eight orthologs with variable expression programs in cassava (Table S2.8). While *AtLOX2* and *MeLOX2a* are strongly induced by JA, *MeLOX2c-h* are repressed in one or both cassava genotypes; with *MeLOX2f-h* displaying a genotype-dependent response. Like *AtLOX2*, the JA-response genes *AtVSP1* and *AtVSP2* are strongly induced by JA, while their ortholog *MeVSP1* shows no or a minimal response to JA treatments in both cassava genotypes (Figure 2.13).

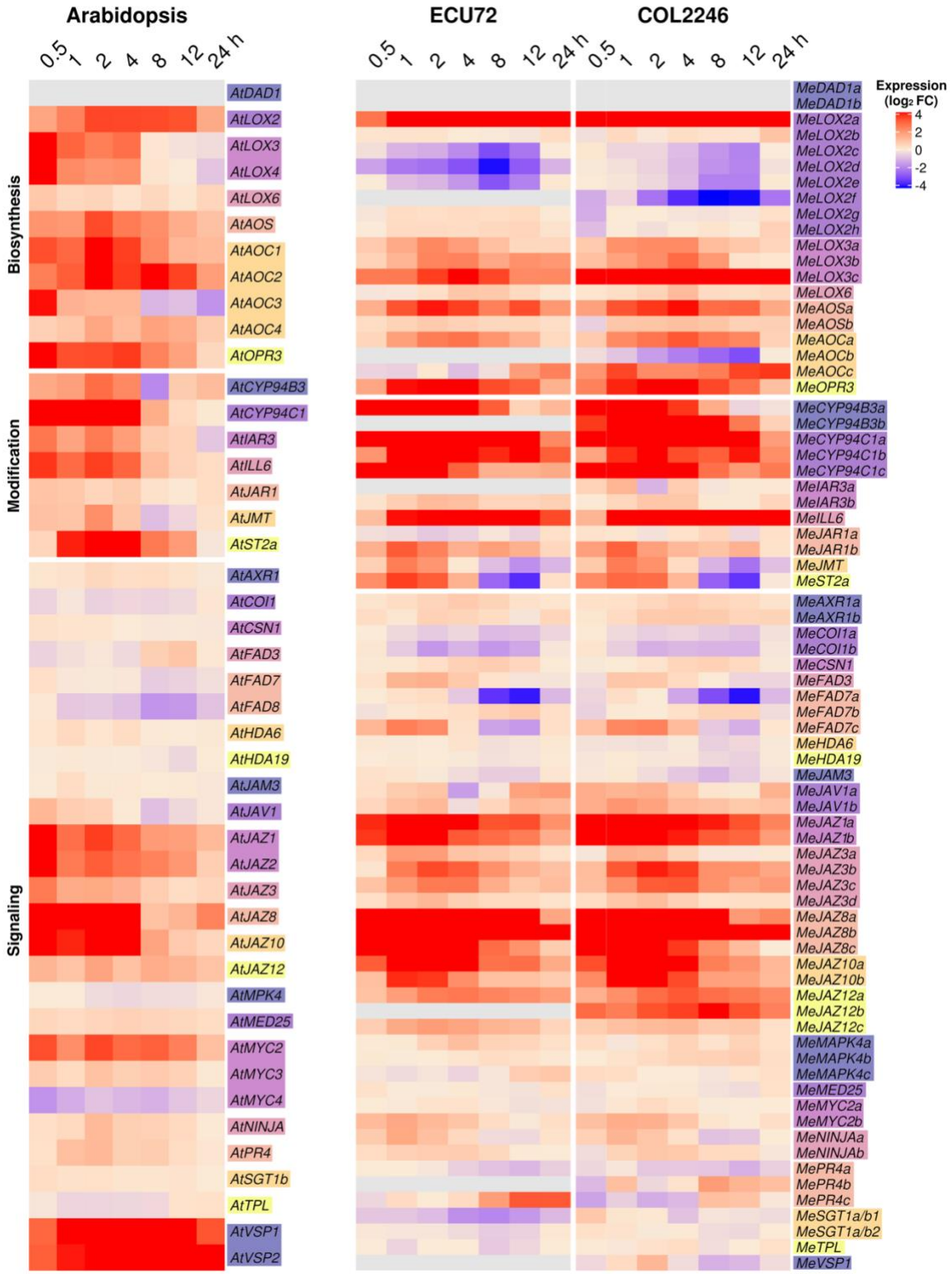

Arabidopsis
ECU72
COL2246
0.5
1
2
4
8
12
24 h
Biosynthesis
Modification
Signaling
AtDAD1
AtLOX2
AtLOX3
AtLOX4
AtLOX6
AtAOS
AtAOC1
AtAOC2
AtAOC3
AtAOC4
AtOPR3
AtCYP94B3
AtCYP94C1
AtIAR3
AtILL6
AtJAR1
AtJMT
AtST2a
AtAXR1
AtCOI1
AtCSN1
AtFAD3
AtFAD7
AtFAD8
AtHDA6
AtHDA19
AtJAM3
AtJAV1
AtJAZ1
AtJAZ2
AtJAZ3
AtJAZ8
AtJAZ10
AtJAZ12
AtMPK4
AtMED25
AtMYC2
AtMYC3
AtMYC4
AtNINJA
AtPR4
AtSGT1b
AtTPL
AtVSP1
AtVSP2
MeDAD1a
MeDAD1b
MeLOX2a
MeLOX2b
MeLOX2c
MeLOX2d
MeLOX2e
MeLOX2f
MeLOX2g
MeLOX2h
MeLOX3a
MeLOX3b
MeLOX3c
MeLOX6
MeAOSa
MeAOSb
MeAOCa
MeAOCb
MeAOCc
MeOPR3
MeCYP94B3a
MeCYP94B3b
MeCYP94C1a
MeCYP94C1b
MeCYP94C1c
MeIAR3a
MeIAR3b
MeILL6
MeJAR1a
MeJAR1b
MeJMT
MeST2a
MeAXR1a
MeAXR1b
MeCOI1a
MeCOI1b
MeCSN1
MeFAD3
MeFAD7a
MeFAD7b
MeFAD7c
MeHDA6
MeHDA19
MeJAM3
MeJAV1a
MeJAV1b
MeJAZ1a
MeJAZ1b
MeJAZ3a
MeJAZ3b
MeJAZ3c
MeJAZ3d
MeJAZ8a
MeJAZ8b
MeJAZ8c
MeJAZ10a
MeJAZ10b
MeJAZ12a
MeJAZ12b
MeJAZ12c
MeMAPK4a
MeMAPK4b
MeMAPK4c
MeMED25
MeMYC2a
MeMYC2b
MeNINJAa
MeNINJAb
MePR4a
MePR4b
MePR4c
MeSGT1a/b1
MeSGT1a/b2
MeTPL
MeVSP1
Expression
(log2 FC)
4
2
0
-2
-4

Figure 2.13. JA-pathway gene expression in Arabidopsis and in two cassava genotypes.

Genes involved in JA biosynthesis, modification, and transport and in transducing or responding to JA (signaling) were identified from the literature and cassava orthologs identified. Expression of JA-pathway genes are presented as log_2FC values during JA treatment in Arabidopsis, ECU72 and COL2246. Cassava and Arabidopsis genes generally have similar expression trends in response to JA.. Biosynthetic genes are ordered by their approximate step in the pathway, while other pathway genes are ordered alphabetically. Orthologous genes are denoted by box color.

To compare the Arabidopsis and cassava ET biosynthesis, modification and signaling/response pathways, ET data sets were extracted from the literature (Goda et al., 2008) (Table S2.7). Heatmaps revealed that the expression programs of ET-pathway genes were substantially different between the two species (Figure 2.14). For example, six Arabidopsis *ACS* genes involved in ET biosynthesis were responsive to ET; in sharp contrast, with one exception the cassava *ACS* orthologs were not regulated by ET; only *MeACS6b* was ET responsive and it was negatively regulated similar to *AtACS6* (Figure 2.14). The ET-biosynthetic *ACO* gene family was expanded in cassava with *AtACO1, 4* and *5* having one, three and two orthologs in cassava, respectively. Of these only *AtACO4* and cassava's *MeACO4a-c* genes had similar expression trends at early response times. Divergent expression was also observed among ET-signaling/response genes expanded in cassava. The single-copy Arabidopsis genes *AtCTR1*, *AtERF1*, *AtETR1*, and *AtPR3* had four, four, three, and fifteen orthologs (Figure 2.14) (Irigoyen et al., 2020) in cassava, respectively, and these genes had varied ET responses between the species. Among these genes, the majority responded similarly in the cassava whitefly-resistant and susceptible genotypes, however six genes (*MeCTR1d*, *MeERF1a*, *MeERF1c*, *MeETR1a, MeETR1c*, and *MePR3o*) displayed more complex expression patterns in ECU72 versus predominant repression or induction in COL2246 (Figure 2.14; Table S2.8).

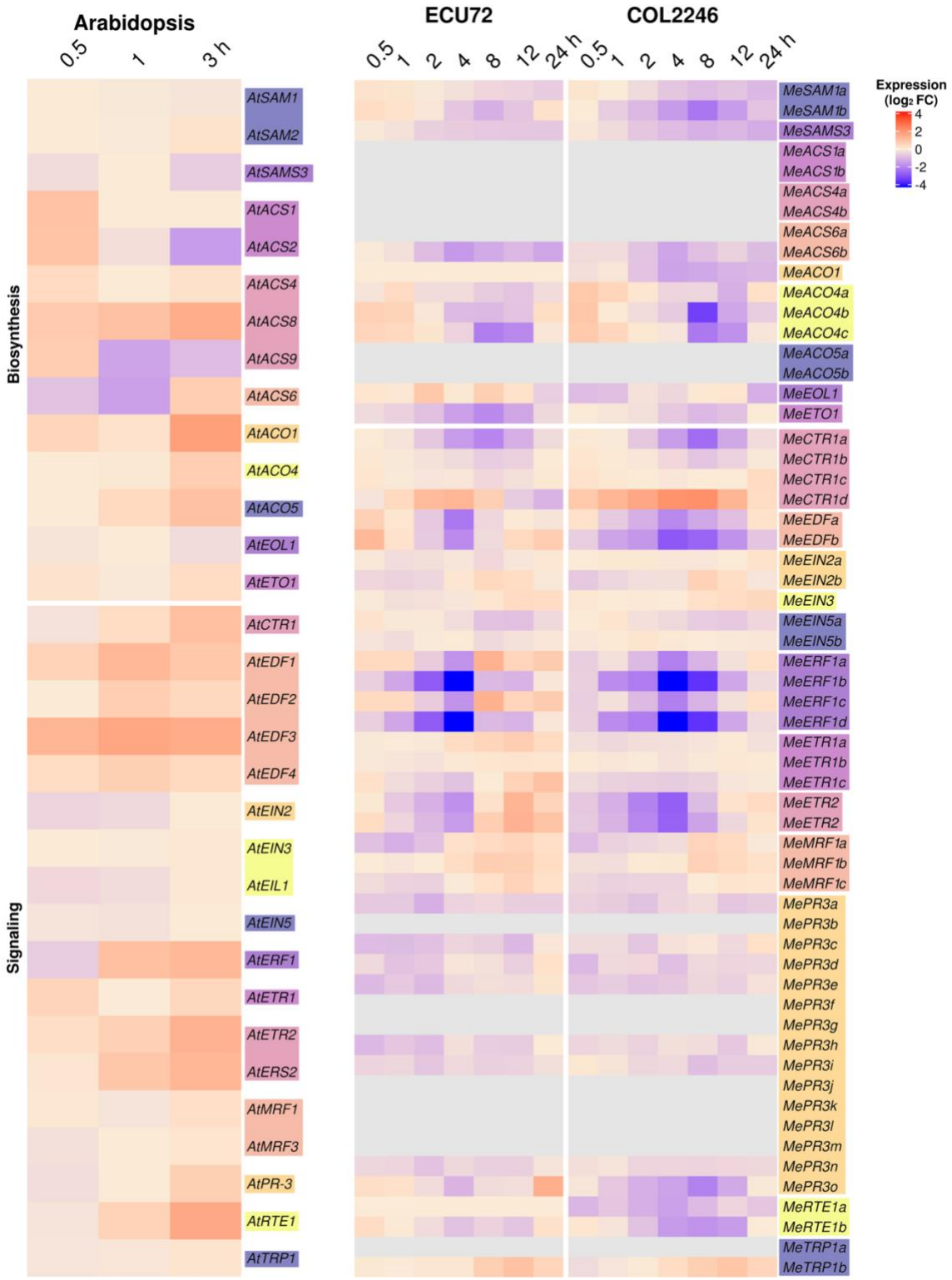

Arabidopsis
0.5
1
3 h
ECU72
0.5
1
2
4
8
12
24 h
COL2246
0.5
1
2
4
8
12
24 h
Expression (log2 FC)
4
2
0
-2
-4
Biosynthesis
Signaling
AtSAM1
AtSAM2
AtSAMS3
AtACS1
AtACS2
AtACS4
AtACS8
AtACS9
AtACS6
AtACO1
AtACO4
AtACO5
AtEOL1
AtETO1
AtCTR1
AtEDF1
AtEDF2
AtEDF3
AtEDF4
AtEIN2
AtEIN3
AtEIL1
AtEIN5
AtERF1
AtETR1
AtETR2
AtERS2
AtMRF1
AtMRF3
AtPR-3
AtRTE1
AtTRP1
MeSAM1a
MeSAM1b
MeSAMS3
MeACS1a
MeACS1b
MeACS4a
MeACS4b
MeACS6a
MeACS6b
MeACO1
MeACO4a
MeACO4b
MeACO4c
MeACO5a
MeACO5b
MeEOL1
MeETO1
MeCTR1a
MeCTR1b
MeCTR1c
MeCTR1d
MeEDFa
MeEDFb
MeEIN2a
MeEIN2b
MeEIN3
MeEIN5a
MeEIN5b
MeERF1a
MeERF1b
MeERF1c
MeERF1d
MeETR1a
MeETR1b
MeETR1c
MeETR2
MeETR2
MeMRF1a
MeMRF1b
MeMRF1c
MePR3a
MePR3b
MePR3c
MePR3d
MePR3e
MePR3f
MePR3g
MePR3h
MePR3i
MePR3j
MePR3k
MePR3l
MePR3m
MePR3n
MePR3o
MeRTE1a
MeRTE1b
MeTRP1a
MeTRP1b

Figure 2.14. ET pathway gene expression in Arabidopsis and in two cassava genotypes.

Genes involved in ET biosynthesis and in transducing or responding to ET (signaling) were identified from the literature and cassava orthologs identified. Expression of ET-pathway genes are presented as log_2FC values during ET treatment in Arabidopsis, ECU72 and COL2246. Cassava genes show varying expression trends in response to ET as compared to Arabidopsis. Biosynthetic genes are ordered by their approximate step in the pathway, while other pathway genes are ordered alphabetically. Orthologous genes in Arabidopsis and cassava are denoted by box color. Expression values for Arabidopsis genes were obtained from the Goda et al 2008 study.

While 56 ABA-pathway genes were identified in Arabidopsis, there were significant expansions in genes associated with ABA-signaling/response (eg., *OST1*, *PYL*s, *ANAC83*) and transport (*ABCG40* and *AIT*) in cassava (Table S2.8). For example, *AtOST1* had five cassava orthologs and was undetected in Arabidopsis but induced in cassava during ABA treatment (Figure 2.15; Table S2.8). Additionally, ABA transporters (*ABCG25*, *ABCG40* and *AIT*) had two-to-eight orthologs in cassava showing divergent expression trends between species. Expression programs of cassava and Arabidopsis orthologs after ABA treatments were revealed in heatmaps (Figure 2.15). ABA-pathway gene expression programs, established by Goda et al. (2008), showed induction of most Arabidopsis genes (excluding four *AtPYL* genes) after ABA treatments or no ABA response from 0.5 to 3 hpt. Cassava's ABA-pathway gene expression programs diverged substantially from Arabidopsis, such as *MePDS*, *MeNCED3a*, *MeNCED3b, CYP707A1a,* and *CYP707A1b,* which were oppositely expressed in ECU72 versus COL2246 (Figure 2.15).

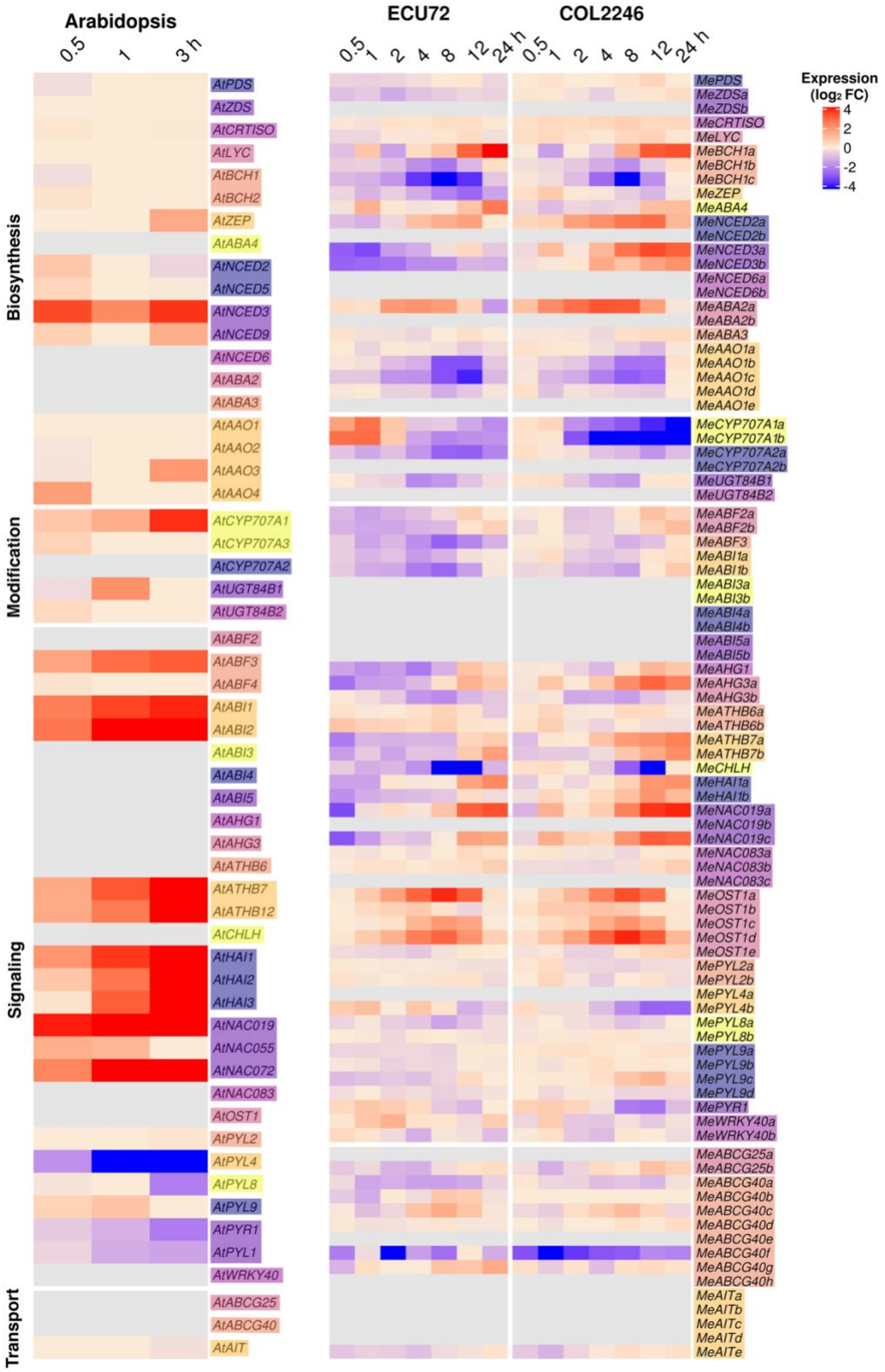

Arabidopsis
ECU72
COL2246
0.5
1
3 h
2
4
8
12
24 h
Expression
(log2 FC)
4
2
0
-2
-4
Biosynthesis
Modification
Signaling
Transport
AtPDS
AtZDS
AtCRTISO
AtLYC
AtBCH1
AtBCH2
AtZEP
AtABA4
AtNCED2
AtNCED5
AtNCED3
AtNCED9
AtNCED6
AtABA2
AtABA3
AtAAO1
AtAAO2
AtAAO3
AtAAO4
AtCYP707A1
AtCYP707A3
AtCYP707A2
AtUGT84B1
AtUGT84B2
AtABF2
AtABF3
AtABF4
AtABI1
AtABI2
AtABI3
AtABI4
AtABI5
AtAHG1
AtAHG3
AtATHB6
AtATHB7
AtATHB12
AtCHLH
AtHAI1
AtHAI2
AtHAI3
AtNAC019
AtNAC055
AtNAC072
AtNAC083
AtOST1
AtPYL2
AtPYL4
AtPYL8
AtPYL9
AtPYR1
AtPYL1
AtWRKY40
AtABCG25
AtABCG40
AtAIT
MePDS
MeZDSa
MeZDSb
MeCRTISO
MeLYC
MeBCH1a
MeBCH1b
MeBCH1c
MeZEP
MeABA4
MeNCED2a
MeNCED2b
MeNCED3a
MeNCED3b
MeNCED6a
MeNCED6b
MeABA2a
MeABA2b
MeABA3
MeAAO1a
MeAAO1b
MeAAO1c
MeAAO1d
MeAAO1e
MeCYP707A1a
MeCYP707A1b
MeCYP707A2a
MeCYP707A2b
MeUGT84B1
MeUGT84B2
MeABF2a
MeABF2b
MeABF3
MeABI1a
MeABI1b
MeABI3a
MeABI3b
MeABI4a
MeABI4b
MeABI5a
MeABI5b
MeAHG1
MeAHG3a
MeAHG3b
MeATHB6a
MeATHB6b
MeATHB7a
MeATHB7b
MeCHLH
MeHAI1a
MeHAI1b
MeNAC019a
MeNAC019b
MeNAC019c
MeNAC083a
MeNAC083b
MeNAC083c
MeOST1a
MeOST1b
MeOST1c
MeOST1d
MeOST1e
MePYL2a
MePYL2b
MePYL4a
MePYL4b
MePYL8a
MePYL8b
MePYL9a
MePYL9b
MePYL9c
MePYL9d
MePYR1
MeWRKY40a
MeWRKY40b
MeABCG25a
MeABCG25b
MeABCG40a
MeABCG40b
MeABCG40c
MeABCG40d
MeABCG40e
MeABCG40f
MeABCG40g
MeABCG40h
MeAITa
MeAITb
MeAITc
MeAITd
MeAITe

Figure 2.15. ABA-pathway gene expression in Arabidopsis and in two cassava genotypes.

Genes involved in ABA biosynthesis, modifications, signaling, and were identified from the literature and cassava orthologs identified. Expression of ABA-pathway genes are presented as log_2FC values during ABA treatment in Arabidopsis, ECU72 and COL2246. Cassava genes show varying expression trends in response to ABA as compared to Arabidopsis. Biosynthetic genes are ordered by their approximate step in the pathway, while other pathway genes are ordered alphabetically. Orthologous genes in Arabidopsis and cassava are denoted by box color. Expression values for Arabidopsis were obtained from the Goda et al 2008 study.

Genotypic differences in the whitefly-infestation responses of ECU72 vs COL2246

To define cassava's response to whiteflies, we analyzed the transcriptomes of whitefly-resistant ECU72 and whitefly-susceptible COL2246 in response to infestation by the Latin American whitefly *Aleurotrachelus socialis.* Infestation experiments and RNA extractions were performed by Dr. Adriana Bohorquez-Chaux at CIAT. RNA-sequencing of whitefly-infested samples was performed by Dr. Maria Irigoyen at UCR. During this infestation time course (0, 1, 7, 14, and 22 days post infestation (dpi)), we identified 7,574 and 5,590 temporal DEGs in ECU72 and COL2246, respectively (Figure 2.16; Table S2.1). The temporal responses of these genotypes were distinct. At 1 dpi, when adults are feeding and eggs are being deposited, ECU72 had a limited response while COL2246 had 4- and 61-fold more genes that were up- and down-regulated, respectively (Table S2.2). By 7 dpi, when adults are absent and eggs are present, ECU72's transcriptome response ramped up and reached a similar magnitude as COL2246. However, by 14 to 22 dpi when first to third instar nymphs are feeding, the magnitude of ECU72's response exceeded that of COL2246; for example, ECU72 repressed $\geq$ 2-fold more genes at these later times (Figure 2.16a,b; Table S2.2).

Prior to the onset of nymph feeding at 14 dpi, genotype DEGs revealed that ECU72 and COL2246 had different transcriptomes at 0-1 dpi (adult feeding) (Figure 2.16c). A clear shift from early (1-7 dpi) to late (14-22 dpi) phase infestation was also observed when comparing genotype infestation responses at each time point. The total number of genotype DEGs peaked at 14 dpi. Equivalent numbers of up- and down-regulated genotype DEGs were identified at early times (0, 1 and 7 dpi), while somewhat larger numbers of downregulated genes in ECU72 were identified at 14 and 22 dpi (Figure 2.16c;

Tables S2.1 and S2.2). PCA analyses of the infestation time course further revealed genotype differences. ECU72 displayed a distinct constitutive and early (0-1 dpi) phase and later infestation response phases (7-22 dpi). In contrast, COL2246 had no clear delineation of infestation phases, as well as more variation among replicates at each time point (Figure 2.16d).

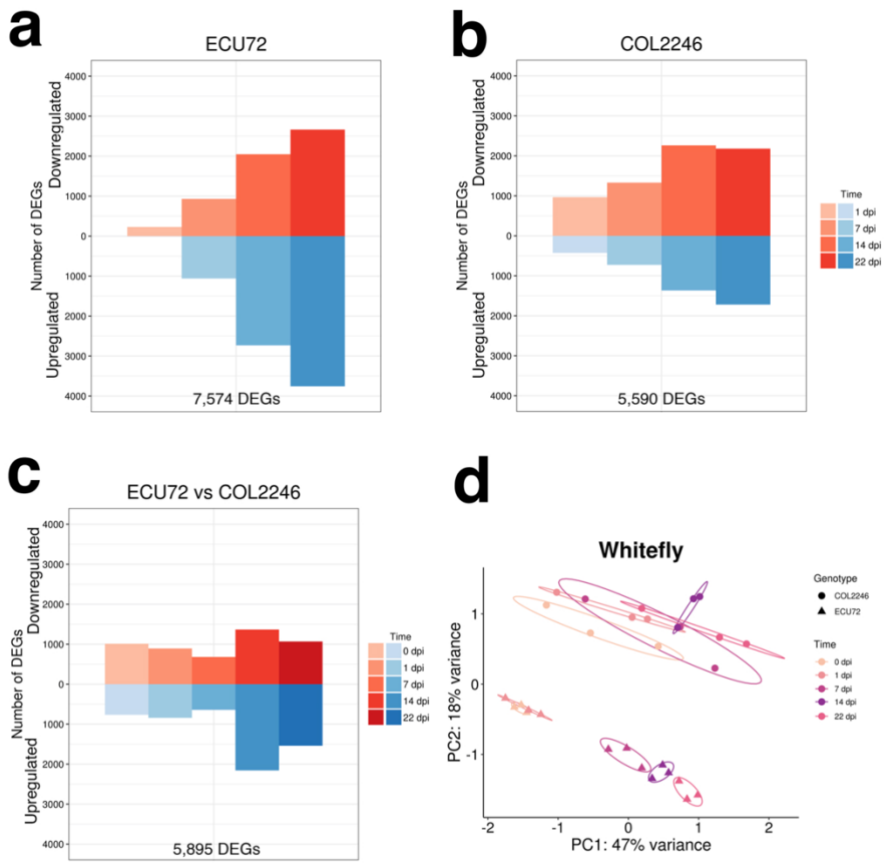

a
ECU72
Number of DEGs
Downregulated
Upregulated
7,574 DEGs
b
COL2246
5,590 DEGs
Time
1 dpi
7 dpi
14 dpi
22 dpi
c
ECU72 vs COL2246
5,895 DEGs
0 dpi
1 dpi
7 dpi
14 dpi
22 dpi
d
Whitefly
Genotype
COL2246
ECU72
PC2: 18% variance
PC1: 47% variance

Figure 2.16. DEG and PCA analyses of whitefly infestation DEGs in ECU72 and COL2246.

(a-b) Numbers of temporal DEGs in ECU72 and COL2246 during whitefly infestation. Temporal DEGs were identified by comparisons of 0 hpt to 0.5-24 hpt in ECU72 **(a)** and COL2246 **(b)**. While the magnitude of the COL2246 transcriptome response at 1 dpi surpassed ECU72, by 14-22 dpi more DEGs were identified in ECU72. The numbers of DEGs at each time point are provided in Table S2.2.

(c) Numbers of genotype DEGs in ECU72 versus COL2246 during whitefly infestation. Genotype DEGs were identified by comparisons of ECU72 and COL2246 at each time point. Total genotype DEG counts peaked between 14 and 22 dpi.

Infestation experiments and RNA extractions were performed by Dr. Adriana Bohorquez-Chaux at CIAT. RNA-sequencing of whitefly-infested samples was performed by Dr. Maria Irigoyen at UCR. Number of up- and down-regulated genes in ECU72 relative to COL2246 are in red and blue, respectively. DEGs had $|\log_2 FC| \geq 1$ and FDR ≤ 5%. The numbers of DEGs at each time point are provided in Table S2.

(d) PCA analyses of detected genes expressed during whitefly infestation (0, 1, 7, 14 and 22 dpi) in ECU72 and COL2246. The genotypes were well separated based on PC2. While PC1 separated ECU72 responses into early and late infestation phases, this phase distinction was not detected in COL2246. Detected genes were defined as having an average of 20 reads or more across a the infestation time course. Read count values for three biological replicates are shown per time point. Time points and genotypes are labeled by color and shape, respectively.

To associate the genotype transcriptome differences with biological processes, k-means clustering and GO-term enrichment of genotype DEGs was performed (Figure 2.17; Table S2.1). The genes in Cluster 1 and Cluster 2 were expressed at higher levels in ECU72 at 0 and 1 dpi than in COL2246 (Figure 2.17; Table S2.10). For ECU72, the Cluster 1 and 2 gene transcript levels declined across the 22-day infestation and COL2246's Cluster 2 genes followed the same pattern of expression. While Cluster 1 had no enriched GO terms, Cluster 2 was associated with stimulus response and many cell wall- and secondary metabolism-related processes (i.e. cell wall organization or biogenesis, cell wall polysaccharide metabolism, phenylpropanoid metabolism, and flavonoid biosynthesis). Cluster 2 also included genes involved in secondary cell wall formation, cell wall loosening and the production of cell wall elicitors. Unlike genes in Cluster 1, 2, 4 and 5, genes in Cluster 3 had high basal levels of expression (0 dpi) and throughout the time course. In particular, Cluster 3 genes had higher mean RPKM levels in ECU72 versus COL2246 at 14 dpi and were enriched for terms such as ion transport, response to stimulus or hormone and regulation of signaling. In contrast, genes in Cluster 4 and Cluster 5 were more strongly induced at the time of nymph feeding (14-22 dpi) in COL2246 than in ECU72. Such genes were associated with responses to hormone, stimulus, biotic stimulus, and immune responses (Figure 2.17; Table S2.10).

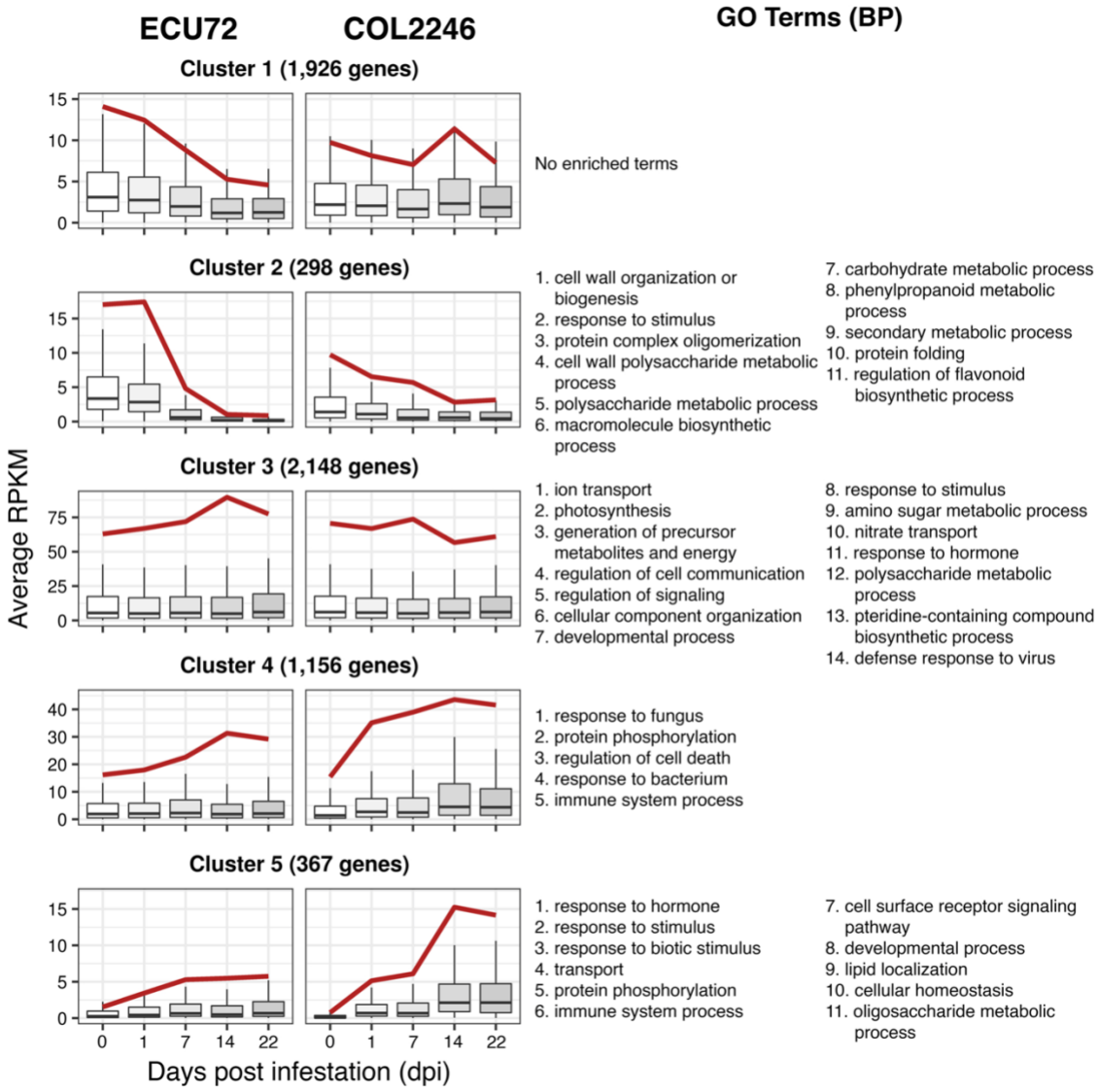

ECU72
COL2246
GO Terms (BP)
Cluster 1 (1,926 genes)
No enriched terms
Cluster 2 (298 genes)
1. cell wall organization or biogenesis
2. response to stimulus
3. protein complex oligomerization
4. cell wall polysaccharide metabolic process
5. polysaccharide metabolic process
6. macromolecule biosynthetic process
7. carbohydrate metabolic process
8. phenylpropanoid metabolic process
9. secondary metabolic process
10. protein folding
11. regulation of flavonoid biosynthetic process
Cluster 3 (2,148 genes)
1. ion transport
2. photosynthesis
3. generation of precursor metabolites and energy
4. regulation of cell communication
5. regulation of signaling
6. cellular component organization
7. developmental process
8. response to stimulus
9. amino sugar metabolic process
10. nitrate transport
11. response to hormone
12. polysaccharide metabolic process
13. pteridine-containing compound biosynthetic process
14. defense response to virus
Cluster 4 (1,156 genes)
1. response to fungus
2. protein phosphorylation
3. regulation of cell death
4. response to bacterium
5. immune system process
Cluster 5 (367 genes)
1. response to hormone
2. response to stimulus
3. response to biotic stimulus
4. transport
5. protein phosphorylation
6. immune system process
7. cell surface receptor signaling pathway
8. developmental process
9. lipid localization
10. cellular homeostasis
11. oligosaccharide metabolic process
Average RPKM
Days post infestation (dpi)

Figure 2.17. Clustering and functional enrichment of genes differentially expressed in ECU72 versus COL2246 during whitefly infestation.

K-means expression clusters of genotype DEGs in ECU72 versus COL2246 during SA treatment. Clusters display differences in the timing and magnitude of responses in ECU72 and COL2246 and were associated with GO terms related to stress/defense responses. Categories of significantly enriched GO terms ($p \leq 0.05$) ranked by p-value are provided for each of the five clusters (Table S2.10). Genotype DEGs were identified by comparisons of transcript levels in ECU72 versus COL2246 during SA treatments and had $|\log_2 FC| \geq 1$ and FDR ≤ 5%. Boxplot whiskers represent values within 1.5 x IQR, and box values represent the first quartile, median, and third quartile values. Outliers (points beyond whiskers) are not displayed. Lines displayed mean RPKM values at 0 to 24 hpt. Infestation experiments and RNA extractions were performed by Dr. Adriana Bohorquez-Chaux at CIAT. RNA-sequencing of whitefly-infested samples was performed by Dr. Maria Irigoyen at UCR.

qRT-PCR validation of RNA-seq transcript levels

RNA-seq expression values were confirmed using qRT-PCR for hormone-biosynthetic genes *MePAL1c*, *MeLOX3a*, *MeACS6b*, and *MeAAO1c* in cassava genotypes ECU72 and COL2246 following SA, JA, ET, and ABA treatments, respectively. In addition, the hormone biosynthesis genes *AtPAL1* and *AtLOX3* were assessed in Arabidopsis following SA and JA treatments, respectively (Figure 2.18a,c). qRT-PCR was also used to confirm the whitefly induction of *MePR-9e* in ECU72 (Figure 2.18b); *MePR-9e* RNAs were previously assessed in COL2246 by Irigoyen et al. (2020). Pearson correlation analysis of the relative expression values for these genes confirmed a significant and very strong positive correlation between transcript levels determined *in silico* and *in vivo* (Figure 2.18d). qRT-PCR validation was performed by Diana Medina-Yerena and Danielle Garceau at UCR.

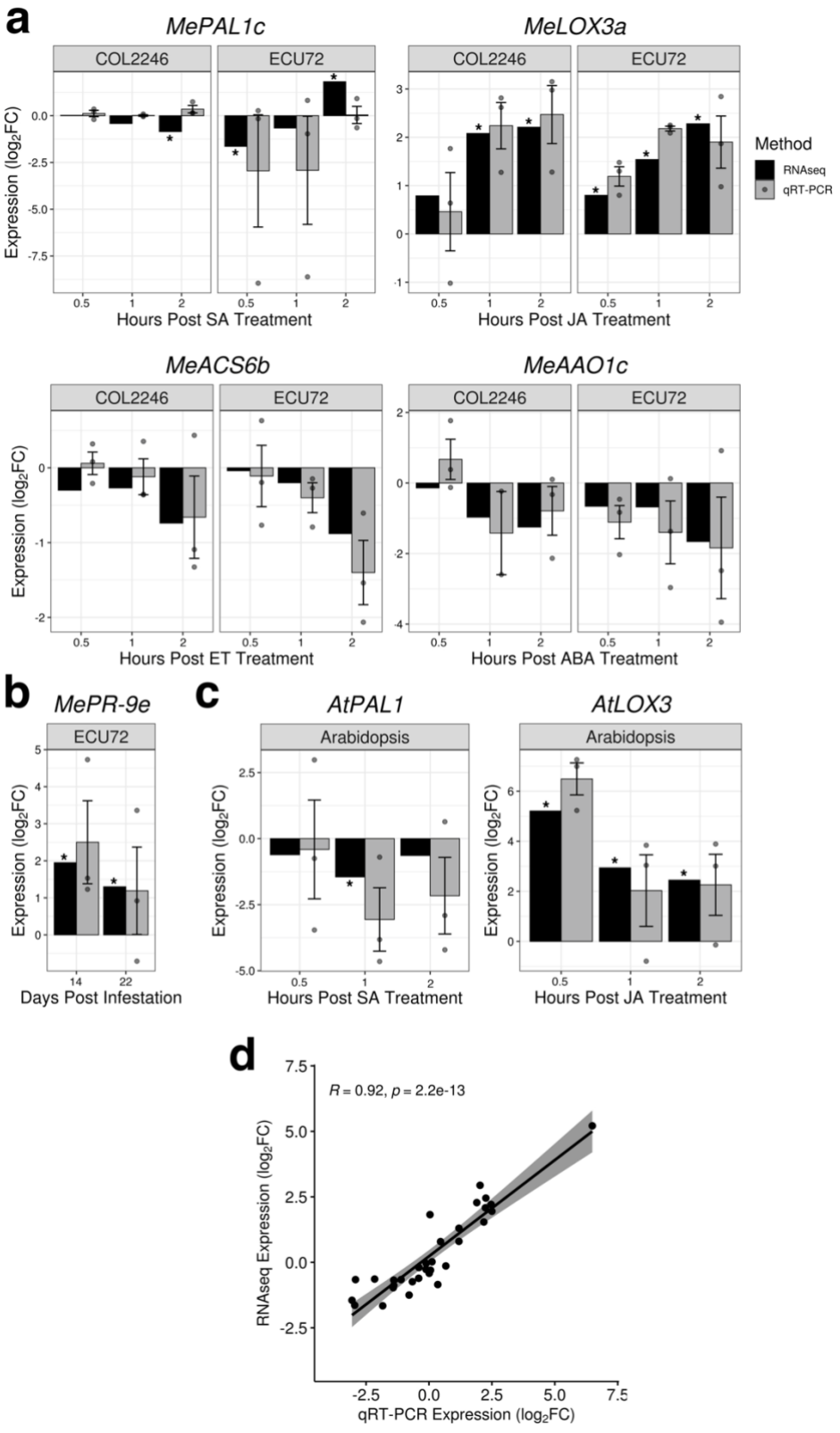

a
MePAL1c
MeLOX3a
COL2246
ECU72
Expression (log2FC)
Hours Post SA Treatment
Hours Post JA Treatment
Method
RNAseq
qRT-PCR
MeACS6b
MeAAO1c
Hours Post ET Treatment
Hours Post ABA Treatment
b
MePR-9e
Days Post Infestation
c
AtPAL1
AtLOX3
Arabidopsis
d
R = 0.92, p = 2.2e-13
RNAseq Expression (log2FC)
qRT-PCR Expression (log2FC)

Figure 2.18. qRT-PCR validation of RNA-seq expression values.

All expression values are normalized to a control gene (*MeUBQ* in cassava, *AtACT7* in Arabidopsis) and are relative to the treatment's 0-h time point. qRT-PCR values are displayed as the average and SE of three biological replicates (bar graph), as well as individual biological replicate values (overlayed points). qRT-PCR validation was performed by Diana Medina-Yerena and Danielle Garceau at UCR.

(a) The *in silico* relative expression values of hormone-biosynthetic genes *MePAL1c*, *MeLOX3a*, *MeACS6b*, and *MeAAO1c* following SA, JA, ET, and ABA treatments, respectively, were confirmed in ECU72 and COL2246 at 0.5, 1 and 2 hpt by qRT-PCR. For *MeAAO1c* in COL2246 at 1 hpt, only two biological replicates are provided.

(b) The *in silico* relative expression value of sentinel *PR* gene *MePR-9e* following whitefly infestation in ECU72 was confirmed at 14 and 22 dpi in vivo. Expression of *MePR-9e* in COL2246 was previously determined (Irigoyen et al 2020).

(c) The *in silico* relative expression values of *AtPAL1* and *AtLOX3* following SA and JA treatments, respectively, were confirmed in *Arabidopsis thaliana* at 0.5, 1 and 2 hpt in vivo.

(d) A scatter plot with Pearson correlations demonstrates the relative expression determined by qRT-PCR and RNA-seq show a strong and significant positive correlation for all biological replicates displayed in panels a-c.

Hormone-pathway gene expression during whitefly infestation in ECU72 versus COL2246

To identify associations between cassava's genotype-specific whitefly and hormone responses, we analyzed the expression of SA, JA, ET, and ABA hormone-pathway genes in ECU72 and COL2246 during whitefly infestation and hormone treatments (Figure 2.19). Of the 154 genes highlighted as being involved in SA, JA, ET, or ABA biosynthesis, modification, signaling/response, or transport, 77 were identified as genotype DEGs during whitefly infestation and expression programs were visualized by heatmaps. Clustering by infestation expression trends in ECU72 and COL2246 revealed several associations between whitefly and hormone responses. In Cluster 1, 32 genes were induced in both genotypes during infestation, many of which displayed stronger induction by 14 dpi in COL2246. A subset of these genes were upregulated by JA in both genotypes, but many showed no clear hormone regulation. However, Cluster 1 genes more strongly induced by infestation in COL2246 showed a more complex temporal program in ECU72 than in COL2246 in response to SA. These genes included six positive regulators of SA signaling (*MeNPR1*, *MeSARD1a-b*, *MeWRKY70a-b*, and *MeGRX480c*) and the SA response gene *MePR1b*, two ABA transporter genes (*MeABCG40f-g*), one JA-biosynthetic gene (*MeOPR3*), and three JA-signaling repressors (*MeJAZ1a* and *MeJAZ3a-b*). *MeWRKY70a-b* and *MeJAZ3a* were additionally downregulated by ET and ABA in both genotypes, whereas *MePR1b* and *MeABCG40f-g* were only downregulated by ET in COL2246. The JA-biosynthetic gene *MeOPR3*, pathogen-responsive gene *MeWRKY40*, JA-signaling repressor *MeJAZ1a*, and positive regulator of JA signaling through 12OH-JA-Ile production *MeCYP94B3a* had stronger whitefly induction in COL2246 and showed strong JA induction in both genotypes (Figure 2.19).

In Cluster 2, 16 genes were repressed in both cassava genotypes during infestation. This included *MePAL1e* and four JA-biosynthetic genes (*MeAOSb* and *MeLOX2b*, *g* and *h*), which were weakly induced at 1 dpi then repressed in ECU72 but repressed at all infestation times in COL2246; these genes were not clearly hormone-regulated (Figure 2.19).

Genes in Clusters 3 (23 genes) and 4 (6 genes) displayed opposite infestation expression trends in ECU72 versus COL2246, with Cluster 3 genes induced and Cluster 4 genes repressed in ECU72 (Figure 2.19). Strikingly, 18 of the 23 genes in Cluster 3 showed an association between whitefly and SA responses; these genes were induced by infestation and SA in ECU72, while being suppressed by these treatments in COL2246. In general, the Cluster 3 genes were also negatively regulated in both genotypes by JA, ABA and ET. These genes included five ABA co-receptors that act as negative regulators of ABA signaling (*MeAHG1*, *MeAHG3b*, *HAI1a-b*, and *MeABI1a*), three ABA-response genes (*MeABF2a-b* and *MeABF3*), four ET-response genes (*MePR3a*, *c*, *i* and *n*), and two SA-modification genes, which convert SA to an inactive form (*UGT74F1b-c*). The hormone expression pattern of Cluster 3 was also seen in several genes within Cluster 1, where ECU72 and COL2246 displayed reciprocal SA responses but similar responses to all other hormones. These Cluster 1 genes included: the ET-biosynthetic gene *MeACO4c* and JA-biosynthetic gene *MeLOX2c-d* that were induced by SA in ECU72 (similar to Cluster 3 genes) and the positive regulator of ABA signaling *MeOST1a*, and the positive regulators of SA signaling *MeSARD1a* and *MeNPR1,* which were repressed by SA in ECU72.

In Cluster 4, genes were mainly repressed in ECU72 and induced in COL2246 during infestation. Of the six genes in this cluster, four were upregulated at 1 dpi then strongly

repressed in ECU72 versus upregulated in COL2246 at most times when eggs and nymphs are present. Such genes included the JA-biosynthetic gene *MeLOX3c*, the JA-signaling repressor *MeJAZ10a*, and the SA-methylation gene *MeSMTa*. *MeJAZ10a* was strongly induced by JA in both genotypes, while *MeLOX3c* was strongly repressed versus induced by infestation and SA in ECU72 versus COL2246, respectively. *MeSMTa* showed strong repression by ET and ABA in COL2246 and strong JA induction in both genotypes (Figure 2.19).

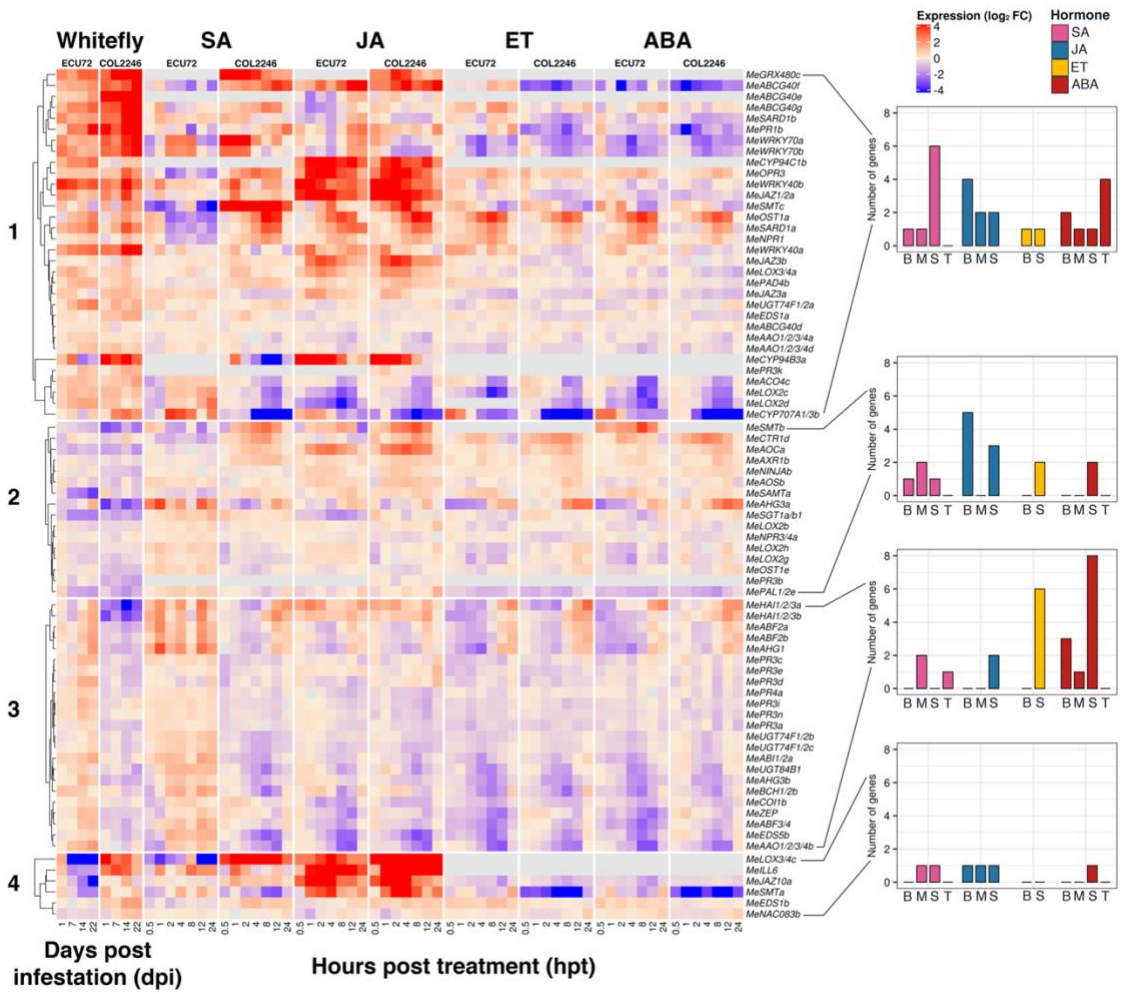


FIGURE 2.19. CASSAVA HORMONE-PATHWAY GENE EXPRESSION DURING WHITEFLY AND HORMONE TREATMENTS.

Hormone-pathway genes that are genotype DEGs during whitefly infestation are displayed. Gene expression values are presented as log_2FC values during whitefly infestation (1,7,14 and 22 dpi) and SA, JA, ET and ABA treatments (0.5, 1, 2, 4, 8, 12, 24 hpt). Genes are clustered hierarchically. The number of DEGs associated with biosynthesis (B), modification (M), signaling (S), transport (T)) for each hormone is provided.

Hormone responses in ECU72 and COL2246 during whitefly infestation

To gain a more global understanding of the association of hormone-responsive genes with whitefly infestation, we classified ECU72's and COL2246's whitefly-regulated DEGs by their responsiveness to a single or multiple defense hormones (Figure 2.20.a-f; Table S2.11). A large proportion (between 32-46%) of the temporal DEGs in ECU72 and COL2246 responded to signals independent of SA, JA, ET, and/or ABA (Figure 2.20a,b,d,e). This analysis also showed that many of the remaining whitefly-regulated DEGs were responsive to SA, JA, both SA and JA, or all four hormones. Each of the other hormone-responsive classes (single hormone or in combination with others) constituted less than 6.0% of the total DEGs; collectively these smaller groups included between 19.6-25.3% of the temporal DEGs in cassava.

In ECU72 and COL2246, temporal DEGs regulated by all hormones formed the largest hormone-regulated class, ranging from 11.0 (ECU72 1 dpi) to 20.2% (COL2246 14 dpi) (Figure 2.20d,e; Table S2.11). JA-responsive genes made a more substantial contribution to ECU72's whitefly response (ranging from 13.3-21.6%) than COL2246's response (9.0-10.7%) (Figure 2.20d,e). In addition, ABA appears to be an important regulator in ECU72's response to whitefly infestation at 14 to 22 dpi (Table S2.11); this was evidenced by the fact that the number of ABA-, SA/ABA-, JA/ABA-, and JA/ET/ABA-responsive genes was 1.4 to 2.5-fold higher in ECU72 than in COL2246. In contrast, SA/JA-responsive genes were more prevalent in the infestation response of COL2246 (7.3-10.1%) compared to ECU72 (3.5-8.1%). At early infestation times (1-7 dpi), ET may regulate COL2246's response, as the number of genes regulated by ET, SA/ET, JA/ET, ET/ABA, SA/JA/ET, SA/ET/ABA, and JA/ET/ABA at these times was 1.4 to 26.5-fold higher in COL2246 than in ECU72 (Figure 2.20a,b; Table S2.11).

To determine if genotypic differences in responses to whiteflies and hormone treatments are associated, we identified the whitefly-responsive genotype DEGs that also respond to one or more hormone treatments (Figure 2.20c,f; Table S2.11). The contribution of classes of hormone-responsive genes in genotype-specific responses differed from the temporal DEGs analysis. We found that 6.1-24.0% and 25.2-49.6% of the whitefly-responsive genotype DEGs are responsive to SA or all hormones, respectively, at all infestation times (Figure 2.20c,f; Table S2.11). It is important to note that a substantial number of genotype DEGs during infestation (11.0-20.2%) were not responsive to any of the four hormones (Figure 2.20d-f; Table S2.11).

The levels of SA, JA, ABA, and their derivatives during whitefly infestation (0, 0.5, 1, 7, 14, and 22 dpi) of ECU72 and COL2246 were extracted from an untargeted metabolomics data set by Dr. Laura Perez-Fons at RHUL (Perez-Fons et al., 2019) (Figure 2.20g; Table S2.12). JA and JA-Ile were not detected and gaseous ET could not be measured by this approach (Table S2.12). Overall trends showed opposing levels of SA as compared to ABA and SAG throughout infestation, with ABA and SAG higher in ECU72 and SA higher in COL2246 (Figure 2.20g). Statistical differences were observed in the levels of SA, salicylic acid-glucoside (SAG), 12-oxo-phytodienoic acid (12-OPDA), and ABA at specific times. At 0 dpi, SAG levels were higher in ECU72. At 1 dpi, 12-OPDA levels were higher in COL2246, with similar yet non-significant trends in MeJA levels. By 0.5 dpi, ABA levels were approximately 2-fold higher in ECU72 than in COL2246, with higher ABA levels additionally seen in ECU72 7-22 dpi. Non-significant differences in the levels of PA mirror such trends in ABA. In contrast, SA levels were higher in COL2246 at 7 and 22 dpi.

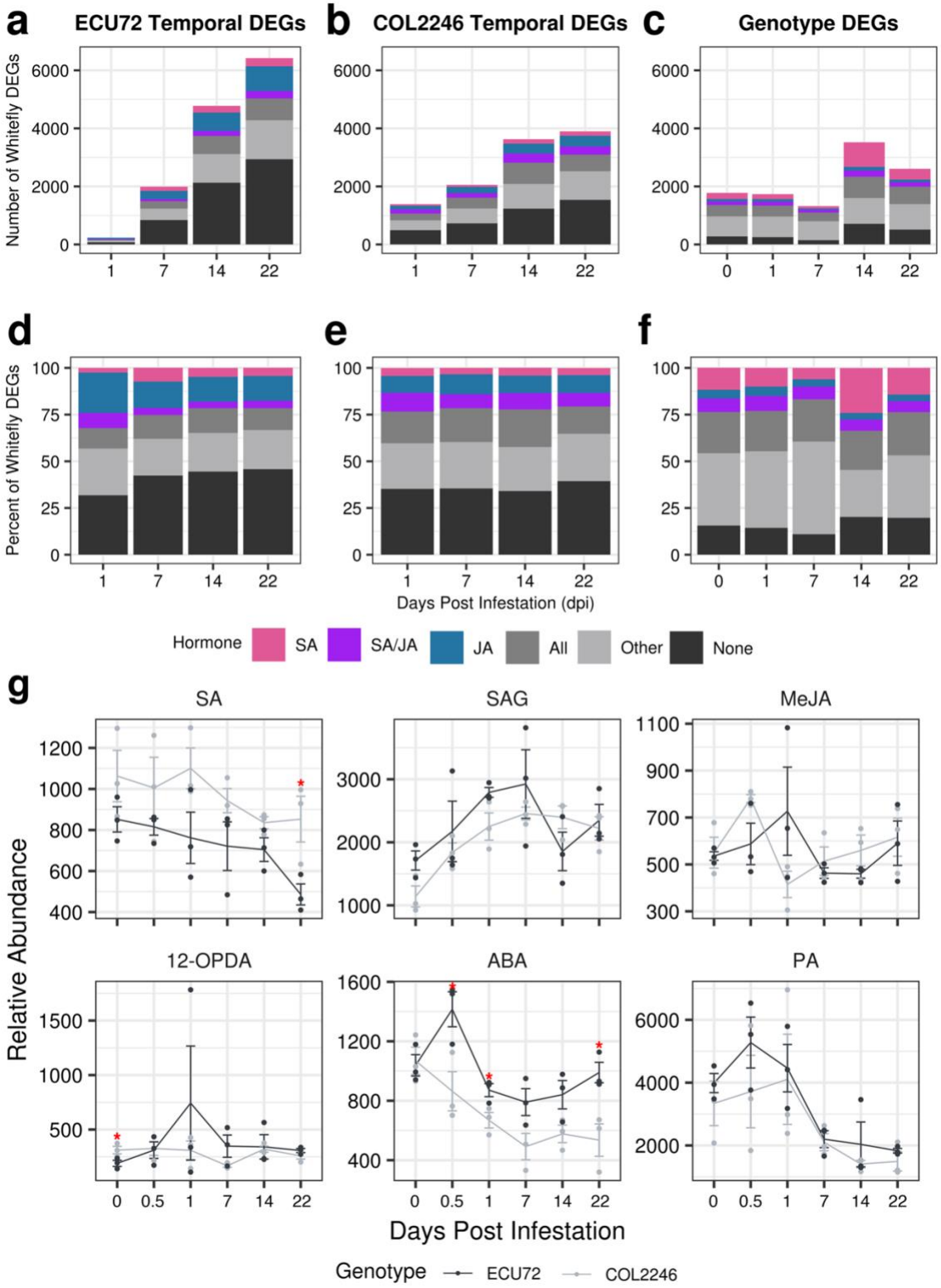

a
ECU72 Temporal DEGs
b
COL2246 Temporal DEGs
c
Genotype DEGs
Number of Whitefly DEGs
6000
4000
2000
0
1
7
14
22
0
d
e
f
Percent of Whitefly DEGs
100
75
50
25
0
Days Post Infestation (dpi)
Hormone
SA
SA/JA
JA
All
Other
None
g
SA
SAG
MeJA
12-OPDA
ABA
PA
Relative Abundance
0.5
Days Post Infestation
Genotype
ECU72
COL2246

Figure 2.20. Hormone regulation categories of whitefly-regulated DEGs and hormone levels in whitefly-infested cassava.

(a-b) Bar graphs displaying number of whitefly-regulated temporal DEGs in ECU72 **(a)** and COL2246 **(b)** categorized by their hormone-response class. JA-responsive DEGs make up more of the response to whitefly in ECU72 than COL2246. Number of DEGs in each class are provided in Table S2.11.

(c) Bar graphs displaying number of whitefly-regulated DEGs differentially expressed in the ECU72 versus COL2246 genotypes. These genotype DEGs were categorized by their hormone-response class. Many DEGs were differentially expressed between genotypes during SA or all hormone treatments. DEG count values are provided in Table S2.11.

(d-f) Bar graphs displaying the % of whitefly-regulated temporal DEGs in ECU72 **(d)** and COL2246 **(e)** and genotype DEGs **(f)** belonging to each hormone-response class. Hormone-response categories included responses to single or multiple defense hormones. Hormone categories that contributed to more than 10% of the whitefly-infestation response are shown. The hormone-response category "All" reflects the ability of a DEG to independently respond to SA, JA, ET and ABA. The "None" category indicates that DEGs responded to whitefly infestation but none of the defense hormones tested. The hormone category "Other" includes all single or multiple hormone-response categories that constitute less than 10% of whitefly-responsive genes at a time point.

(g) Levels of detected defense hormones during infestation in ECU72 and COL2246. Hormone levels were extracted from an untargeted metabolomics data set by Dr. Laura Perez-Fons at RHUL. Asterisks indicate significant difference in hormone level between genotypes as identified by Student's t-test (* = $p \leq 0.05$; ** = $p \leq 0.01$). Complete list of detected hormones and p-values is provided in Table S2.12. SA = salicylic acid, SAG = salicylic acid glucoside, MeJA = methyl jasmonate, 12-OPDA = 12-oxo-phytodienoic acid; ABA = abscisic acid; PA = phaseic acid.

We correlated changes in metabolite levels with their hormone-pathway transcripts during infestation (Figure 2.21). Correlations were performed using the software MOCA with the assistance of Dr. Manhoi Hur. SA, ABA, SAG, and PA were strongly correlated ($R \geq 0.70$) with 482, 591, 127, and 2,281 transcripts. In contrast, MeJA and 12-OPDA had no strong transcript correlations. Several transcripts in the SA (*MeUGT74F1c* and *MeNPR3a*) and ABA (*MeOST1e*, *MeAAO1d*, *MeABCG40d*, *MeHAI1b*, *MeABI1a*, *MeABF2a*, *MeAHG3b*, and *MeAAO1b*) pathways strongly correlated with the changes in SA or ABA levels during infestation, respectively (Figure 2.21).

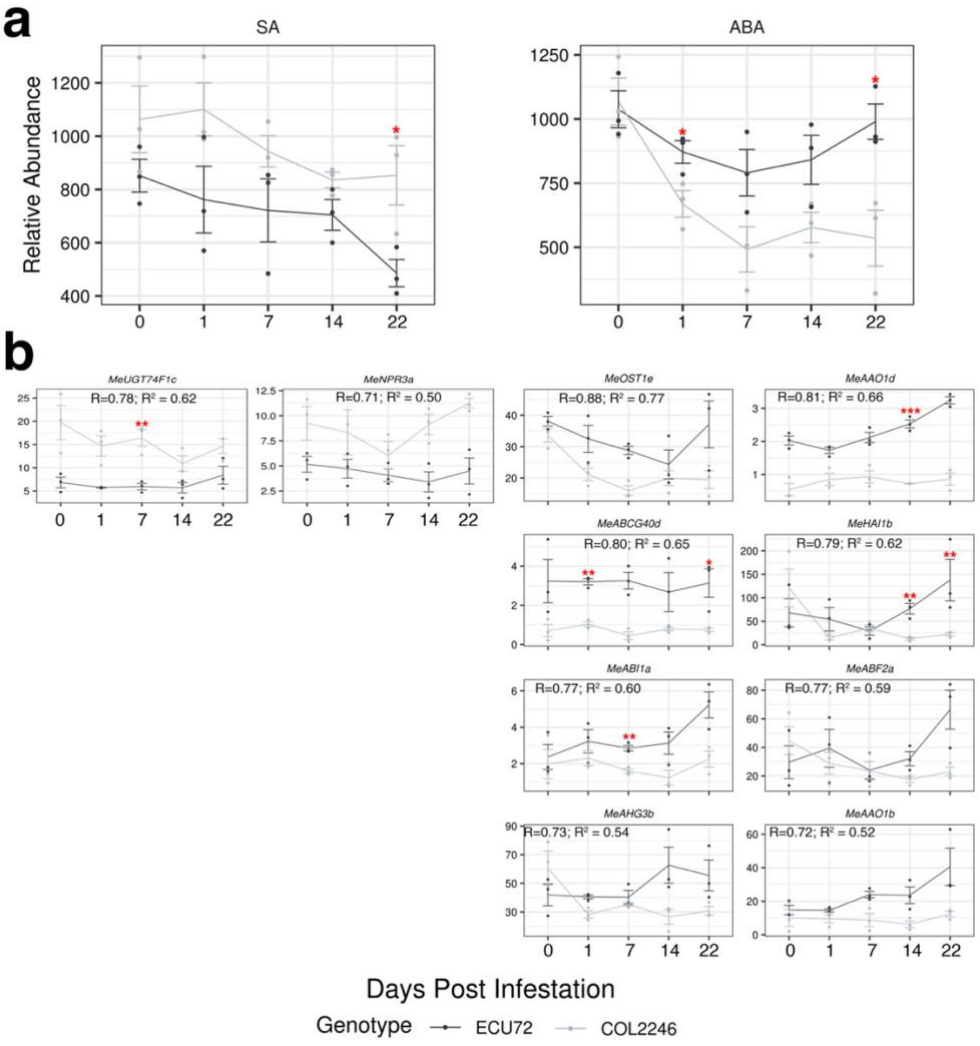


Figure 2.21. Strong metabolite-transcript correlations in the SA and ABA pathways.

Correlations of SA or ABA with cassava hormone pathway transcripts expressed during whitefly infestation were identified using in-house MOCA program. Only strong correlations with R ≥ 0.70 are displayed. The mean and individual biological replicate values are displayed with error bars representing SEM. Hormone levels were extracted from an untargeted metabolomics data set by Dr. Laura Perez-Fons at RHUL. Correlations were performed using the software MOCA with the assistance of Dr. Manhoi Hur.

(a) Relative abundance, measured as EIC area, of SA and ABA during whitefly infestation. Asterisks indicate significant difference in hormone level between genotypes as identified by Student's t-test (* = p ≤ 0.05; ** = p ≤ 0.01). **(b)** RPKM levels of hormone-pathway genes correlated with SA or ABA levels.

Early infestation prompts multiple biochemical defense responses in ECU72

To assign biological functions to the genotype DEGs identified in both whitefly and hormone treatments, GO term-enrichment analyses were performed (Figures 2.22 and 2.23; Table S2.14). Genotype DEGs were grouped by infestation time point, hormone-response categories and up- or down-regulation in ECU72 versus COL2246 during whitefly infestation. The number of DEGs associated with each enriched GO term and their hormone responsiveness are categorized as defense-related (Figure 2.22; Table S2.14) or associated with other processes (Figure 2.23; Table S2.14).

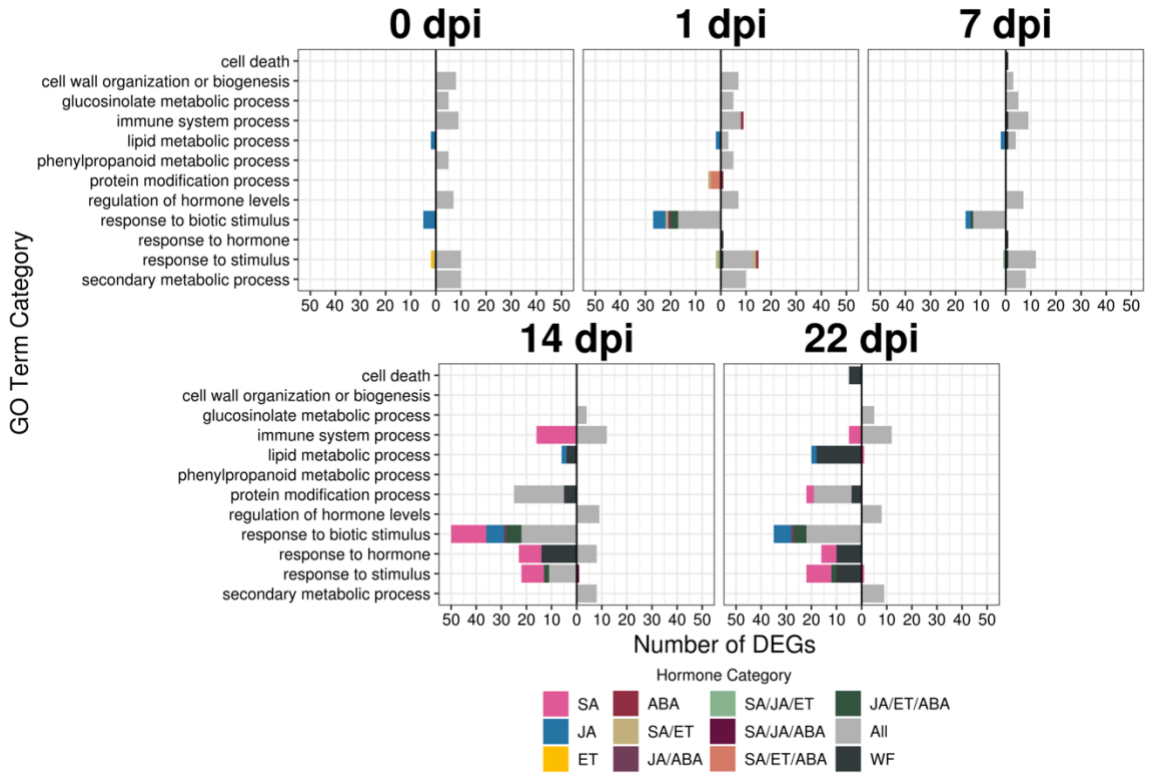


Figure 2.22. Functional enrichment of cassava whitefly- and hormone-regulated DEGs involved in defense processes.

The enriched GO terms associated with whitefly-infestation genotype DEGs and that were hormone responsive are shown. DEGs with GO terms linked to plant defense are shown. Several constitutive and induced defenses were associated with response to all hormones in ECU72, while many defenses induced late were associated with SA responses in COL2246. Hormone categories and DEGs are defined in Figure 2.20 and presented in Table S2.11. Genes up- and down-regulated in ECU72 relative to COL2246 at each time point are displayed on the right and left sides of the y-axis, respectively. GO term categories were ordered alphabetically as listed in Table S2.14. For complete list of enriched GO terms, see Figure 2.23 and Table S2.14.

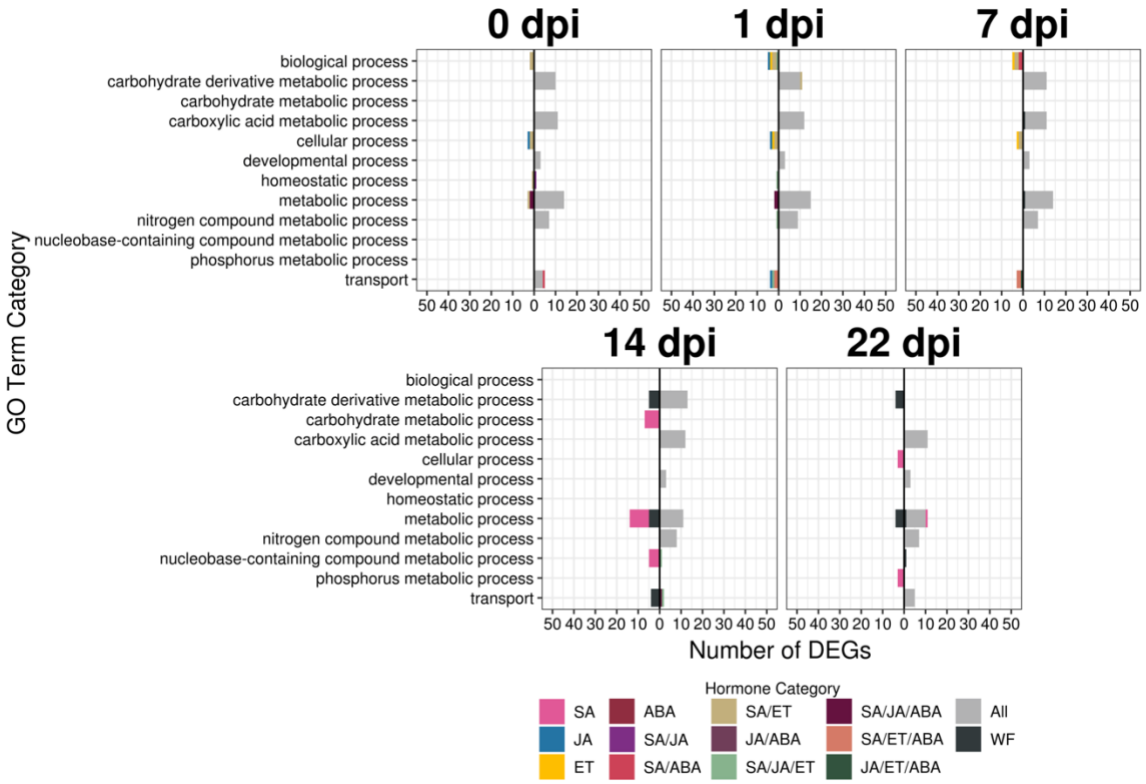


Figure 2.23. Functional enrichment of cassava whitefly- and hormone-regulated DEGs.

Genotype DEGs after whitefly infestation (0, 1, 7, 14, and 22 dpi) were classified by their responses to single or multiple defense hormones (SA, JA, ET, and/or ABA) or as hormone-independent (as described in Figure 2.20 and Table S2.11). Enriched GO term categories were identified and numbers of genes in each category and responses to defense hormones are shown. DEGs with higher or lower expression in ECU72 versus COL2246 during infestation are displayed on the right and left sides of the y-axis, respectively. The top twelve GO term categories are presented and ordered alphabetically. The full data set can be found in Table S2.11.

Among genotype DEGs associated with defense and up-regulated in ECU72 at early infestation times (0, 1 and 7 dpi), 80-100% responded to all four hormones. These genes were associated with cell wall-related and immune system processes, glucosinolate, phenylpropanoid and lignin metabolism, and hormone/stimulus responses. Genes in the response to biotic stimulus category were down-regulated in ECU72 relative to COL2246 at these times and corresponded with mainly differential regulation by JA or all four hormones.

Visualizing the RPKM expression trends of individual genes within enriched GO categories using line graphs showcased associations between infestation and hormone regulation (Figure 2.24). Gene names were obtained from Arabidopsis orthologs available through Phytozome (JGI) (Goodstein et al., 2012) unless previously annotated (Table S2.9). Genes involved in cell wall-related processes such as cell wall remodeling (*MeXTH23*) or response to fungal cell wall elicitors (*MeCAD8*) (Trezzini et al., 1993) and lignin biosynthesis (*MeCOMTf*, *MeCCOAMTa*, *MeMYB63*, and *MeLAC4*) were more highly expressed in ECU72 versus COL2246 constitutively (0 dpi) and at most times during infestation and hormone treatments. With the exceptions of *MeXTH23* and *MeMYB63,* which increased in expression throughout the infestation, the expression of the remaining genes was reduced during the periods of nymph feeding (14 and 22 dpi).

The vitamin C biosynthetic gene *MeGULLO3*, associated with the small molecule biosynthesis GO term (Table S2.14), is involved in protection against oxidative stress and was more highly expressed in ECU72 in non-infested leaves and during whitefly and hormone treatments. Similar expression trends were observed for the lipid metabolic gene *MePIP5K1*, involved in phosphoinositide signaling. *MeHNL*, involved in HCN production

(a notable trait in cassava), was more highly expressed in ECU72 at 1 dpi and during early SA treatment but expressed at lower levels during late infestation and other hormone-treatment timepoints. This gene was also more strongly induced by JA in COL2246 than in ECU72. Notably, eight genes involved in immune system processes were more highly expressed at 0 or 1 dpi in ECU72 versus COL2246. Seven of these genes were more highly expressed in control (0 h) ECU72 leaves and during whitefly and hormone treatments, including pathogenesis-related osmotin (*MeOSM34a-b*) and chitinase (*MePR3e-g*) genes, G3P-signaling gene *MePEPCK*, and ETI/PTI-signaling gene *MeRPM1*.

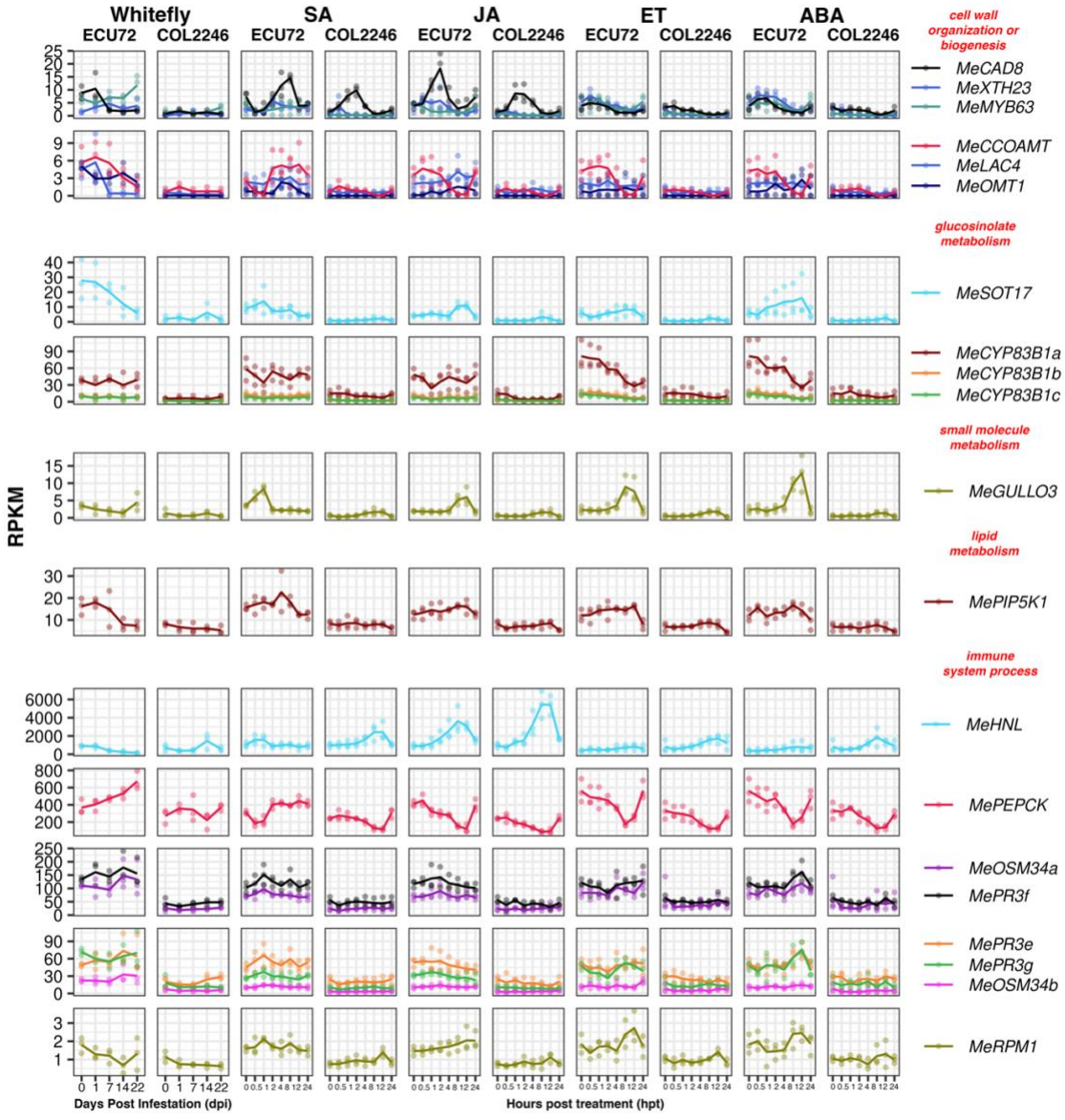
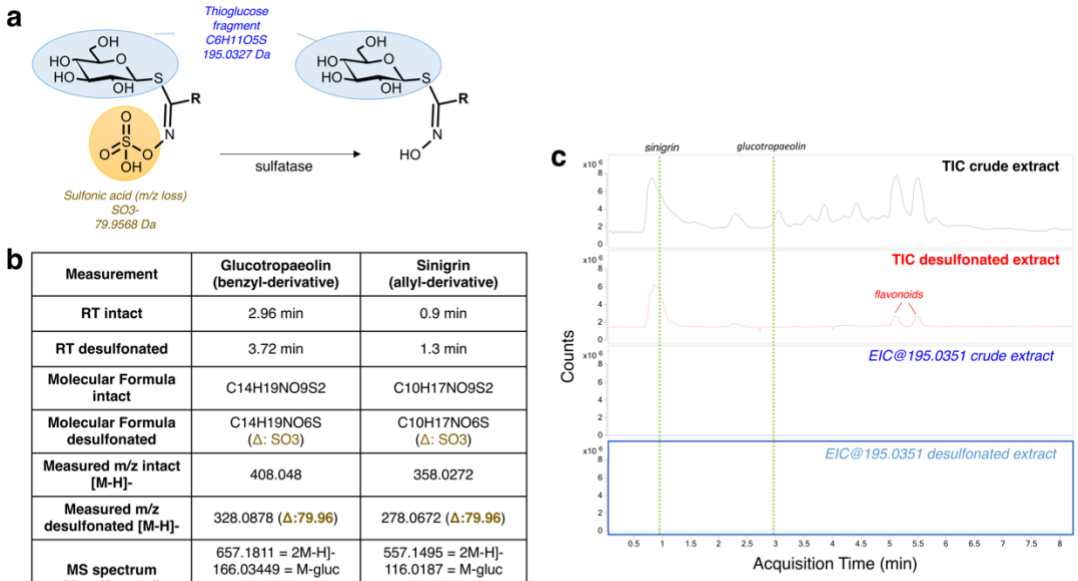


Figure 2.24. Whitefly- and hormone-regulated genotype DEG expression from selected enriched GO-term categories in ECU72 versus COL2246.

Expression of genotype DEGs associated with the selected GO-term categories enriched in ECU72 versus COL2246 are displayed in Figure 2.22. Five enriched GO term categories are shown including: cell wall organization or biogenesis, glucosinolate metabolism, small molecule metabolism, lipid metabolism, and immune system process. For these categories, higher levels of gene expression during infestation was associated with higher expression in response to all hormones in ECU72. Expression values are displayed as average RPKM values (lines) with individual biological replicates shown (circles). Genes are grouped by similar RPKM values and denoted in different colors.

Four genes categorized as glucosinolate metabolism genes (*MeSOT16i*, *MeCYP83B1b*, *h* and *s*) were additionally more highly expressed in ECU72 constitutively, during infestation and during hormone treatments. Given that Arabidopsis AtCYP83A1 and AtCYP83B1 catalyze the conversion of valine, isoleucine and phenylalanine aldoximes into their corresponding glucosinolates (Naur et al., 2003), we attempted to detect and identify the presence of glucosinolates in cassava leaves. Glucosinolate analyses were performed by Dr. Laura Perez-Fons at RHUL. Using the standard metabolomics analytical methodologies for detecting glucosinolates in plant tissue (Crocoll et al., 2016), we were unable to detect the presence of desulfonated glucosinolates (Figure 2.25a,c), the thioglucose moieties characteristic of glucosinolate structures (Figure 2.25a,c), or molecular ions corresponding to glucosinolates derived from either valine, isoleucine or phenylalanine (Figure 2.25c). Previous evidence of glucosinolate composition in cassava is scarce and only a limited number of publications report the ability of cassava genes to produce glucosinolates when expressed in yeast or Arabidopsis (Andersen et al., 2000, Mikkelsen and Halkier, 2003). Our results suggest that glucosinolates may be at undetectable levels or that cassava's *MeCYP83B1* genes are acting in alterative pathways in infested leaves.



b

Measurement	Glucotropaeolin (benzyl-derivative)	Sinigrin (allyl-derivative)
RT intact	2.96 min	0.9 min
RT desulfonated	3.72 min	1.3 min
Molecular Formula intact	C14H19NO9S2	C10H17NO9S2
Molecular Formula desulfonated	C14H19NO6S (Δ: SO3)	C10H17NO6S (Δ: SO3)
Measured m/z intact [M-H]-	408.048	358.0272
Measured m/z desulfonated [M-H]-	328.0878 (Δ:79.96)	278.0672 (Δ:79.96)
MS spectrum (desulfonated)	657.1811 = 2M-H]- 166.03449 = M-gluc **195.0351 = thioglucose**	557.1495 = 2M-H]- 116.0187 = M-gluc **195.0351 = thioglucose**

Figure 2.25. Analysis of glucosinolates in cassava leaves.

Glucosinolate detection analyses was performed by Dr. Laura Perez-Fons at RHUL.

(a) Scheme of sulfatase reaction and its effect on glucosinolate structure

(b) LC-MS measurements of glucosinolates standards solution treated with sulfatase

(c) Total ion chromatograms (TIC) of leaf extract before (crude) and after (desulfonated) enzyme reaction and extracted ion chromatogram (EIC) of thioglucose fragment as indicator of presence of glucosinolated structures.

As several genes involved in lignin biosynthesis (*MeCOMTf*, *MeCCOAMTa*, *MeMYB63*, and *MeLAC4*) were identified as up-regulated genotype DEGs in the whitefly-resistant ECU72, we identified cassava orthologs in these metabolic pathways using eggNOG (Huerta-Cepas et al., 2017, Huerta-Cepas et al., 2019) (Table S2.9). Among these genes, hormone- or whitefly-responsive DEGs were identified and visualized adjacent to this pathway (Figure 2.26). Of the eleven DEGs in the lignin biosynthetic pathway, nine were more highly expressed in ECU72 during infestation at one or more time points (Figure 2.26). Among these, four (*MeHCTe*, *MeCCoAOMTa, MeCCoAOMTb,* and *MeCAD6*) were more highly expressed in ECU72 during SA treatment, while *MePAL1e* and *MeC3H* were not differentially regulated by hormones between genotypes , and *MeCOMT1* and *MeCAD8i* showed differential responses to multiple hormones. Notably, *MeCOMTf* showed strong upregulation in ECU72 versus COL2246 at all infestation and the majority of hormone-treatment time points. Only two genes were more highly expressed in COL2246 during infestation, *MeCCoAOMTd* at 14-22 dpi and *MeCAD1* at 0 dpi (Figure 2.26).

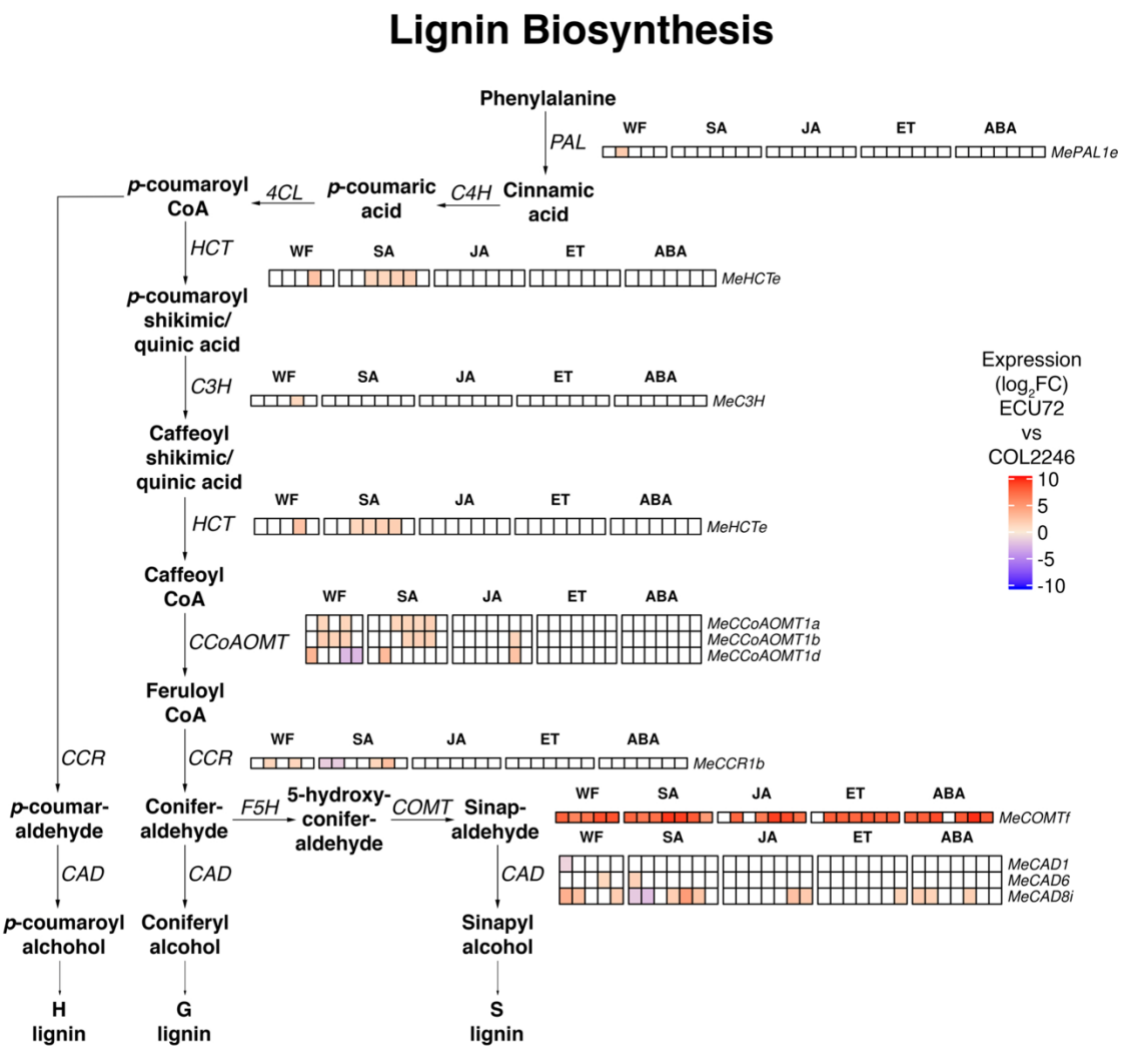


Figure 2.26. Cassava's lignin biosynthetic pathway.

Expression values for genotype DEGs during whitefly infestation are displayed as log_2FC values during whitefly and hormone treatment in ECU72 versus COL2246. Time points from left to right during infestation are 0, 1, 7, 14, and 22 dpi and during hormone treatments are 0.5, 1, 2, 4, 8, 12, and 24 hpt. Time points with genotype DEGs ($|log_2FC| \geq 1$ and FDR ≤ 5%) are shown and DEGs with higher transcript levels in ECU72 (red) or COL2246 (blue) are shown. Time points where gene expression was not significantly different between the genotypes are shown in white.

Late infestation invokes a surge in immune signaling in COL2246

At the time of nymph feeding (14-22 dpi), there was a major shift in the enriched GO terms associated with defense. Most noticeably, genes enriched in seven defense-related categories (cell death, immune system process, lipid metabolic process, protein modification process, response to biotic stimulus, response to hormone, and response to stimulus) were upregulated in the whitefly-susceptible COL2246 versus whitefly-resistant ECU72. Genes within these GO term categories were mainly regulated by SA, all hormones, or were defense-hormone independent (Figure 2.22). Visualization of individual gene transcript levels exemplified such expression trends.

The GO immune system process category, which was enriched in COL2246 versus ECU72 at 14-22 dpi, contained several PTI/ETI-signaling genes (Figure 2.27). Basal defense genes *MeFMO1* and *MeMOS2* were most highly expressed at 0 dpi following a decline and plateau for other infestation times. These genes were additionally more highly expressed in COL2246 during all hormone treatments. All other genes in this category peaked in expression at 14-22 dpi in COL2246 and showed lower, more stable expression in ECU72 during infestation. While more highly expressed during infestation in COL2246, four genes associated with basal immunity (*MeNDR1*, *BIR1a, BIR1e*, and *PEPR1*) showed more complex expression programs in response to hormone treatments in both ECU72 and COL226.

MePERK1a-c proteins detect cell wall perturbations and were more highly expressed during infestation and all hormone treatments in COL2246 versus ECU72. In addition, two genes involved in response to oxidative stress (*MePA2* and *MePDI)* and four starch catabolism genes (*MeDPE2*, *MeSBE2.2*, *MeISA3*, and *MeSEX1)* had rapid SA

responses in ECU72 (1 hpt) but were induced substantially later in COL2246 (~8 hpt). Lastly, four sesquiterpenoid biosynthetic genes (*MeTPS6* and *MeTPS21a-c*) were more highly expressed in COL2246 after whitefly infestation, particularly at 14-22 dpi (the times of nymph feeding) and these genes were regulated in a hormone-independent manner (Figure 2.27). Collectively the GO-term enrichment analyses showed that genes associated with ETI/PTI signaling, immune system process and cell death are expressed constitutively or early (1-7 dpi) in ECU72, when eggs and feeding adults reside on leaves, while in COL2246 these GO terms were enriched at the time of nymph feeding (14-22 dpi) (Figure 2.22; Table S2.13). Interestingly, for genes enriched in processes other than defense (i.e. metabolism, carbohydrate metabolism, transport), a similar shift in enriched terms from 0-7 versus 14-22 dpi was also observed (Figure 2.23; Table S2.13).

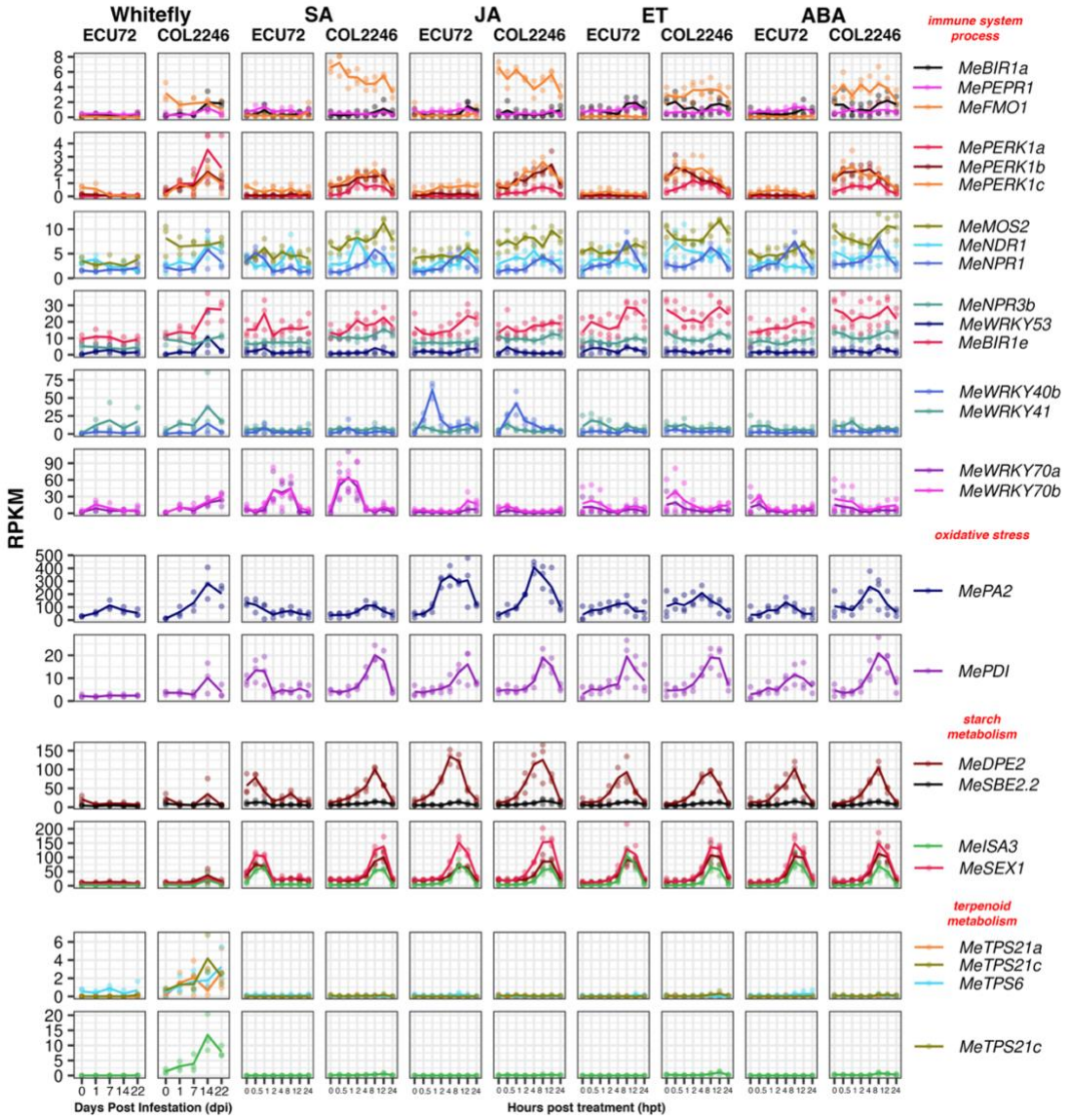
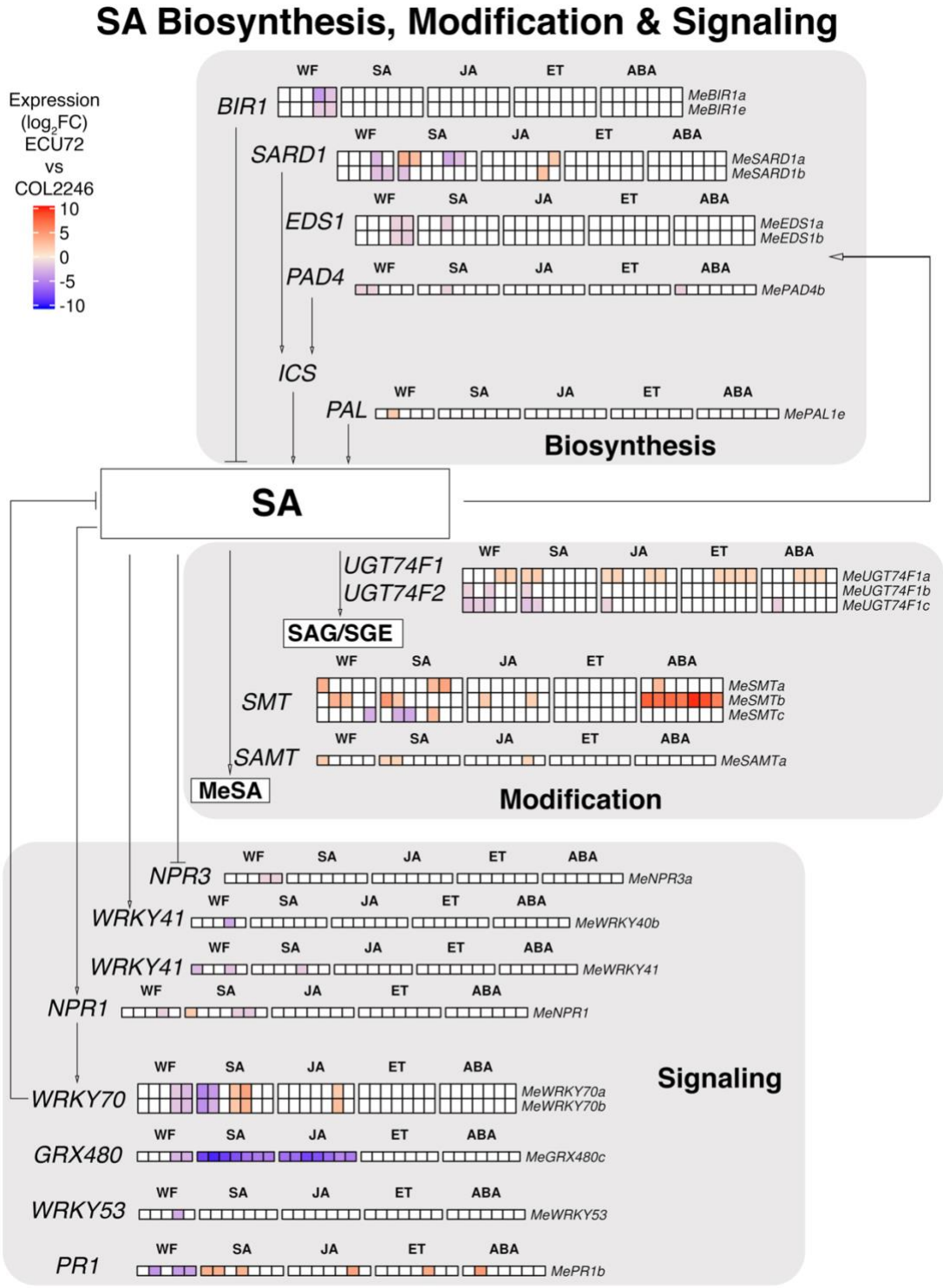


Figure 2.27. Expression of whitefly- and hormone-regulated DEGs from selected enriched GO-term categories in COL2246 versus ECU72.

Expression of genotype DEGs associated with the selected GO-term categories enriched in COL2246 versus ECU72 are displayed in Figure 2.22. Four enriched GO term categories are shown including: immune system process, oxidative stress, starch metabolism, and terpenoid metabolism. For these categories, higher levels of gene expression during infestation was associated with higher expression in response to all hormones in ECU72. Expression values are displayed as average RPKM values (lines) with individual biological replicates shown (circles). Genes are grouped by similar RPKM values and denoted in different colors.

Given the reciprocal regulation of SA-responsive genes in ECU72 and COL2246 (Figure 2.3) and the presence of many SA-pathway and -response genes in the enriched GO-term categories, their expression was graphically illustrated emphasizing their roles in biosynthesis, modification and signaling (Figure 2.28; Table S2.9). With one exception (*MePAL1e*), all of the 15 genotype DEGs identified after whitefly infestation that are involved in SA biosynthesis or signaling were more highly expressed in COL2246 versus ECU72 between 14 and 22 dpi. Notably, three (*MeEDS1a*, *MeWRKY41*, and *MeNPR1*) and five (*MeSARD1a* and *b*, *MeWRKY70a* and *b* and *MeGRX480c*) were genotype DEGs in response to SA or both SA and JA, respectively. In contrast, *MePAD4b* responded to SA and ABA, *MePR1b* responded to all hormones, and six genes (*MeBIR1a*, *MeBIR1e*, *MeEDS1b*, *MeNPR3a*, *MeWRKY40*, and *MeWRKY53*) showed no potential for hormone regulation.

In contrast, SA-modification genes that were genotype DEGs after whitefly infestation had more complex temporal patterns of expression during whitefly infestation in ECU72 and COL2246. At early infestation times (0-7dpi), *MeSMTa, MeSMTb* and *MeSAMTa,* enzymes that convert SA into the mobile SAR signal MeSA, were more highly expressed in ECU72. These genes were additionally more highly expressed in ECU72 during SA treatment and, in some cases, in JA or ABA treatments. In contrast, *MeUGT74F1b* and *MeUGT74F1*c, enzymes which convert SA into its storage forms SAG or SGE, were more highly expressed in COL2246 at 0-7 dpi. In contrast, *MeUGT74F1a* was more highly expressed in ECU72 at 14-22 dpi (Figure 2.28).


SA Biosynthesis, Modification & Signaling
Expression
(log2FC)
ECU72
vs
COL2246
10
5
0
-5
-10
WF
SA
JA
ET
ABA
BIR1
MeBIR1a
MeBIR1e
SARD1
MeSARD1a
MeSARD1b
EDS1
MeEDS1a
MeEDS1b
PAD4
MePAD4b
ICS
PAL
MePAL1e
Biosynthesis
SA
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MeUGT74F1a
MeUGT74F1b
MeUGT74F1c
SAG/SGE
SMT
MeSMTa
MeSMTb
MeSMTc
SAMT
MeSAMTa
MeSA
Modification
NPR3
MeNPR3a
WRKY41
MeWRKY40b
WRKY41
MeWRKY41
NPR1
MeNPR1
WRKY70
MeWRKY70a
MeWRKY70b
GRX480
MeGRX480c
WRKY53
MeWRKY53
PR1
MePR1b
Signaling

Figure 2.28. Cassava's SA biosynthesis, modification and signaling pathway.

Expression values for genotype DEGs during whitefly infestation are displayed as log_2FC values during whitefly and hormone treatment in ECU72 versus COL2246. Time points from left to right during infestation are 0, 1, 7, 14, and 22 dpi and during hormone treatments are 0.5, 1, 2, 4, 8, 12, and 24 hpt. Time points with genotype DEGs ($|log_2FC| \geq 1$ and FDR ≤ 5%) are shown and DEGs with higher transcript levels in ECU72 (red) or COL2246 (blue) are shown. Time points where gene expression was not significantly different between the genotypes are shown in white.

Discussion

Hormone responses of whitefly-resistant (ECU72) and -susceptible (COL2246) cassava

Identifying active defense signals is essential to understanding and characterizing a plant's specific response to different biotic stressors. To this end, we defined the temporal transcriptome response of whitefly-resistant (ECU72) and whitefly-susceptible (COL2246) cassava genotypes to four defense hormones: SA, JA, ET, and ABA. Surprisingly, we discovered that while both genotypes displayed similar JA, ET and ABA responses, responses to SA were profoundly different. Temporal DEG counts showed that ECU72's response to SA was faster, more prolonged and more dynamic than that of COL2246. While few prior studies have examined global SA responses within a species, similar variation in SA responses has been observed in Arabidopsis ecotypes. Using microarrays to monitor SA responses in seven Arabidopsis ecotypes, van Leeuwen et al. (2007) reported that ecotypes varied in the number of DEGs, as well as the timing and directionality of regulation.

The differences in the temporal programming of SA and other hormone responses in ECU72 versus COL2246 as revealed by heatmaps were striking. In addition, Pearson's correlation analyses showed that while COL2246 responded similarly to all hormones, ECU72's late SA response was inversely related to JA, ET and ABA treatments (Figure 2.4). SA antagonism with other hormones, most commonly JA but also ET and ABA, is documented (Robert-Seilaniantz et al., 2011). However, intraspecies variation in such hormone crosstalk is seldom assessed. In a study of over 300 Arabidopsis accessions, variation in the degree of SA antagonism/cooperation with JA was seen (Proietti et al., 2018). Another study across plant clades identified that SA-JA antagonism was not

present in all species; for example, in *Asclepias* both the presence and absence of such antagonism among species of the same genera was found (Thaler et al., 2012). While analyses of genome-scale SA-ET or SA-ABA antagonism are currently lacking, similar variation within or among species may be possible.

Genes, including many defense-related genes, regulated by SA in ECU72 and COL2246 displayed a high degree of reciprocity. Given the wide range of possible SA responses observed among Arabidopsis accessions (Proietti et al., 2018, van Leeuwen et al., 2007), such intraspecies reciprocity may be common but understudied. Another example is seen in cacao, where a fungus-resistant and -susceptible genotype display reciprocity in about one quarter of their SA-responsive DEGs (Fister et al., 2015). The observed differences in SA responses between ECU72 and COL2246 may reflect hormonal fine tuning specific to the suites of pests, pathogens or environmental conditions to which each genotype has adapted.

Among reciprocal SA-responsive DEGs in ECU72, heatmaps revealed that many displayed complex and dynamic temporal regulation that was correlated with the transition from the early to late phases of the SA response. It is tempting to speculate that a master transcriptional regulator acts as a molecular switch to facilitate this phase shift. From our analyses and what is known from the Arabidopsis literature, several possible candidates arose. In our analysis of cassava, *MeNPR1*, *MeWRKY70a, MeWRKY70b*, and *MeGRX480a* stood out as SA-signaling genes, which displayed shifts from up- to down-regulation or vice versa during SA treatment in ECU72 as opposed to solely induction in COL2246 (Figure 2.12). In Arabidopsis, *AtNPR1*, *AtWRKY70* and *AtGRX480* are well-known regulators that promote SA signaling, while suppressing JA and JA/ET signaling

(Caarls et al., 2015, Li et al., 2019a), and *AtWRKY70* is also known to suppress ABA-induced stomatal closure (Li et al., 2013a). Antagonism between SA as compared to JA, ET and ABA responses was also found by Pearson correlation analysis which showed a negative correlation between ECU72's late SA- and overall JA, ET and ABA-responsive transcriptomes (Figure 2.4). It may be possible that one or more of these candidate SA-signaling genes may facilitate such antagonism and transcriptome reprogramming in ECU72 as seen in Arabidopsis.

Divergent hormone responses in cassava versus Arabidopsis

In comparing the SA-, JA-, ET-, and ABA-responsive transcriptomes of cassava and Arabidopsis, several similarities and distinctions were revealed. PCA analyses revealed similar groupings of time points into early versus late phases of response to SA and JA between species, excluding ECU72's SA response. Heatmaps of SA and JA transcriptome responses revealed striking temporal differences in the mode of regulation (e.g., up- vs down-regulation) between the species. For example, of the hormone-responsive genes expressed in both Arabidopsis and the two cassava genotypes, approximately 50% displayed different expression programs between the two species. While few studies have compared Arabidopsis' hormone responses to those of other species on a global scale, such interspecies divergence in hormone responses may be more common than currently understood, as it occurs even within species (Proietti et al., 2018, Broekgaarden et al., 2010).

Ortholog identification and heatmap analyses of genes involved in the biosynthesis, modification, transport, signaling, and responses to SA, JA, ET, and ABA showed that many of these gene families were expanded in cassava relative to Arabidopsis. Notably

MePAL1 (SA-biosynthetic gene), *MeLOX2* (JA-biosynthetic gene), *MePR3* (ET-response gene), and *MeOST1* (ABA-signaling gene) had 5, 8, 15, and 5 paralogs in cassava, respectively. Furthermore, many of these genes displayed differing hormone responses between species. As the occurrence of neofunctionalization is observed in many diploid, triploid and tetraploid species (Greenham et al., 2020), expansions of cassava hormone-pathway orthologs may also suggest adapted functionalities in defense. Expansions associated with stress responsivity of the chitinase *PR3* gene family in cassava and other species relative to Arabidopsis have also been observed (Cao and Tan, 2019, Irigoyen et al., 2020, Tobias et al., 2017), suggesting a possible adaptation for response to insect/pathogen-derived chitin (van Loon et al., 2006). Absence of several *PYL* family ABA receptors (*PYL3*, *5*, *6*, *7*, and *10*) accompanied by expansion of others (*PYL2*, *4*, *8*, and *9*) was also identified in cassava. As PYL receptors allow for cell type- and condition-specific responses to ABA (Finkelstein, 2013), cassava's suite of PYLs may represent a divergence from Arabidopsis' mechanisms for specifying ABA responses. More generally, it is also important to note that some canonical markers of SA, JA, ET, or ABA signaling in Arabidopsis, such as many of the classical Arabidopsis *PR* genes (Irigoyen et al., 2020) as well as the JA-induced Arabidopsis marker *AtVSP1*, are not indicative of and should not be used as markers for such signaling in cassava.

Possible ABA-mediated whitefly-resistance and SA-mediated whitefly-susceptibility mechanisms in cassava and candidate regulators

Integration of whitefly- and hormone-responsive DEGs and quantification of hormone levels during whitefly infestation highlighted the association of SA and ABA with whitefly susceptibility and resistance in COL2246 and ECU72, respectively. Early infestation is characterized by higher ABA and SAG levels but lower SA levels in ECU72 versus

COL2246 (Figure 2.20g). Additionally, the higher SAG levels observed during early stages of whitefly infestation in ECU72 is correlated with lower free SA levels; these data suggest that vacuolar storage of SA's inactive form (SAG) may be a means of lowering active SA levels during stages of whitefly infestation in whitefly-resistant cassava (Thompson et al., 2017).

Trends of higher ABA levels and lower SA levels in ECU72 versus COL2246 also corresponded with hormone-pathway transcript levels during infestation. Heatmaps demonstrate that at the time of nymph feeding (14-22 dpi) ,many SA-signaling genes are SA-responsive and strongly upregulated in COL2246. Whereas, in ECU72, these genes are downregulated or display a more complex temporal pattern of expression (Figure 2.19). In contrast, the percent of whitefly-responsive DEGs expressed that were ABA responsive was higher in ECU72 versus COL2246 (Table S2.11). Heatmaps also showed that at these late infestation times five negative regulators of ABA signaling and three ABA-response genes involved in osmotic stress response are upregulated in ECU72 but downregulated in COL2246 during SA treatment and late whitefly infestation (Figure 2.19). This set of ABA-signaling genes is positively regulated by SA but repressed by ABA in ECU72. While induction of some negative regulators of ABA signaling at the time of rising ABA levels is not well-correlated, it still marks response to ABA and may indicate the presence of negative feedback to control ABA responses. While SA-induction of ABA repressors further supports the presence of SA-ABA antagonism, SA also induces some ABA-response genes, suggesting SA-ABA antagonism may apply to subsets of these pathway genes in ECU72. Together, our transcriptomics and metabolomics results suggest the presence of SA-ABA antagonism in whitefly-infested ECU72; similar trends

have been previously described in Arabidopsis in pathogen interactions (Robert-Seilaniantz et al., 2011).

Further interaction between the SA and ABA pathways was additionally indicated during early infestation. ECU72's early responses to whitefly infestation involved a rise in ABA levels at 0.5 dpi (Figure 2.20g). Intriguingly, an increase in ABA is an early response to PAMPs and regulates stomatal closure to interfere with pathogen access to a leaf's interior spaces (Cao et al., 2011). This process is linked to SA signaling, as NPR1 (an SA receptor) positively regulates the ABA-induced *OST1*, which is positive regulator of ABA signaling and a key regulator of stomatal closing. Importantly, we observe co-expression of *MeNPR1* and *MeOST1a* in response to infestation and SA treatment. Coupled with the identification of whitefly-induction of a cell wall modifying enzyme and cell wall elicitor-response gene in ECU72 (Figure 2.24), our results may indicate that ECU72 also enacts defense against whiteflies to close stomata in response to cell wall-derived DAMPs or possibly HAMPs derived from whiteflies' chitinous stylets. Importantly, plants' successful perception of DAMPs/HAMPs has been shown to trigger fast immune responses conferring resistance to pests/pathogens (Duran-Flores and Heil, 2016). A study of the tomato-caterpillar interaction suggested that stomatal closure may be a means of preventing the emission of some herbivore-induced plant volatiles to dampen the plants defenses (Lin et al., 2021). It is also possible that this reduced transpiration through stomatal closure is a means of impairing sap ingestion by phloem-feeders, however the connection between plant transpiration rate and feeding efficiency of phloem-feeders remains understudied and unclear (Shannag, 2007). While the overall role of ABA in defense against insects remains unclear, several studies have shown either ABA or osmotic stress responses to be important for plant resistance to whiteflies (Broekgaarden

et al., 2018, Esmaeily et al., 2020, Esmaeily et al., 2021) and other pests (Nguyen et al., 2016).

Infestation expression trends of candidate genes (*MeNPR1*, *MeWRKY70a, MeWRKY70b*, *MeGRX480a*, and *MeGRX480c*) previously discussed as possible regulators of transcriptome reprogramming between hormone pathways suggest that these genes may additionally serve this function during infestation. These genes, excluding *MeGRX480a*, were whitefly-induced in both genotypes, with stronger induction by 14 dpi in COL2246 (Figure 2.19); another *GRX480* gene, *MeGRX480c*, additionally showed these infestation trends with SA induction in COL2246 alone (Figures 2.12 and 2.19). It has been shown that *AtNPR1* and *AtWRKY70* generally promote SA responses important for resistance to biotrophic pathogens, while suppressing JA/ET responses important for resistance to insect herbivores (like whiteflies and caterpillars) and necrotrophic pathogens (Derksen et al., 2013, Li et al., 2006a, Onkokesung et al., 2016, Zarate et al., 2007). If the functionalities of their Arabidopsis orthologs are present in cassava, it may be possible that one or more of these candidate genes (*MeNPR1*, *MeWRKY70a, MeWRKY70b*, *MeGRX480a*, and *MeGRX480c*) act as positive regulators of the SA pathway and negative regulators of the JA, ET, and/or ABA pathways during infestation in cassava. SA-induction and stronger whitefly-induction of these genes in addition to higher SA levels in COL2246 may cause activation of an ineffective, biotrophic SA response negatively associated with whitefly resistance. In contrast, a more fine-tined regulation by SA and weaker whitefly-induction of these genes coupled with lower SA levels in ECU72 may allow for a reduction in SA's antagonistic effect on ABA, allowing for the activation of ABA responses and higher ABA levels positively associated with whitefly resistance. Additional genetic testing such as infestation studies using RNA-silencing in

cassava transformants, a lengthy process made difficult by gene expansions in a tetraploid, non-model plant, is necessary to determine a possible role of these genes in defense against whiteflies and/or other biotic stressors.

Previous studies have also shown the involvement of SA and ABA in plant-hemipteran interactions. The role of SA in such interactions has been shown to depend on the plant and pest species (Walling and Thompson, 2012). As in COL2246, SA responses mounted during whitefly infestation are ineffective in whitefly-susceptible Arabidopsis, tobacco and lima bean (Zarate et al., 2007, Zhang et al., 2009, Zhang et al., 2017). However, SA responses were effective in apoplast-mediated resistance to whitefly in whitefly-resistant tomato (Rodriguez-Alvarez et al., 2015). SA is also associated with the phloem-mediated resistance responses of tomato and rice to aphids and brown planthoppers (Li et al., 2006b, Zhao et al., 2016), respectively, but are associated with response of aphid-susceptible wheat to the greenbug aphid (Zhu-Salzman et al., 2004). In contrast, ABA responses in plant-whitefly interactions have had limited assessment, but they have been shown to be associated with resistance to whitefly in cabbage and eggplant (Broekgaarden et al., 2018, Esmaeily et al., 2020). The importance of SA antagonism with other hormones has also been observed in the Arabidopsis-whitefly interaction, in which whiteflies activate ineffective SA responses in order to suppress effective JA responses (Zarate et al., 2007, Zhang et al., 2013a).

While SA and ABA appear to be important signals in cassava whitefly resistance/susceptibility, it should also be noted that a large portion of whitefly-responsive genes displayed differential regulation in response to all four hormones or were hormone-nonresponsive (Figure 2.20f). A possible explanation is that these genes are regulated by

an unidentified signal outside of the tested hormones, such as reactive oxygen species (ROS). Known to regulate defense signaling, ROS have the ability to reprogram the transcriptome through redox-regulated transcription factors and have been implicated in controlling crosstalk (Li and Loake, 2016). For example, *GRX480* has been shown to regulate crosstalk between hormone-signaling pathways during aphid-plant interactions (Foyer et al., 2015). SA-signaling components, such as GRX480 and NPR1, are known to be regulated by cellular redox status (Herrera-Vasquez et al., 2015, Noctor et al., 2018). As such, it may be possible that differences in ROS signals and redox status in subcellular compartments in ECU72 and COL2246 could underlie differential SA levels and responses. Further studies are required to identify additional unknown defense signals/hormones important for regulating cassava's response to whitefly infestation.

Other possible mechanisms of whitefly resistance and susceptibility in cassava

Several genotype-dependent biological processes in ECU72 and COL2246 during infestation were identified by GO-term enrichment and hormone-pathway gene analyses (Figure 2.22). As shown in PCA analyses and numbers of DEGs (Figure 2.16), such processes showed clear shifts from early infestation, when whitefly adults are feeding (0-3 dpi) and eggs are in intimate contact with the leaf (1-7 dpi) versus the times of voracious phloem processing by first to third instar nymphs (14-22 dpi). Similar shifts in plant transcriptome responses to hemipterans have also been reported in maize and tomato (Delano-Frier and Estrada-Hernandez, 2009, Tzin et al., 2015) and through using sentinel gene expression in tomato (Puthoff et al., 2010, van de Ven et al., 2000).

During early stages of infestation, GO-term enrichment analysis showed that while few defenses were mounted in COL2246, in ECU72, which is known to cause nymph mortality,

several possible mechanisms of resistance emerged (Figure 2.22). Cell wall processes were active at 0 to 7 dpi in ECU72 and were generally regulated by all hormones (Figure 2.22). More specifically, many lignin-biosynthetic genes were more highly expressed in ECU72 during these early times of infestation and were regulated by all hormones or SA alone (Figure 2.26). Universal hormone regulation of some of these genes may suggest regulation by ROS, as it is known to be essential for lignin formation at the cell wall (O'Brien et al., 2012). These findings are supported by discovery of higher basal and whitefly-induced leaf lignin levels in ECU72 versus COL2246 by Perez-Fons et al. (2019), as well as observed cell wall-based defenses in cotton responses to whiteflies (Ibrahim, 2016, Li et al., 2016b, Li et al., 2019a).

In addition to lignin formation, genes associated with cell wall remodeling (*MeXTH23*) and response to fungal cell wall elicitors (*MeCAD8i*) were more highly expressed at 0 and 1 dpi in ECU72 (Figure 2.24). These data suggest cell wall fortification against probing whitefly stylets through lignin biosynthesis and the perception of damage through cell wall elicitors such as DAMPs or possibly insect-derived HAMPs may be important components of ECU72's defense during early stages of infestation that include 3 days of adult feeding and 7 days of interactions with eggs (Bertea et al., 2020, Gouhier-Darimont et al., 2019, Oates et al., 2021).

A marked shift in the cassava transcriptome was observed at 14-22 dpi, when several defense processes emerged in whitefly-infested COL2246 (Figure 2.22). Most notably, many SA signaling genes, as well as genes involved in immune signaling and responses to hormone/stimulus, were more highly expressed in COL2246 versus ECU72 during infestation and in response to SA, all hormones, or no hormone treatments (Figures 2.22

and 2.27). At 14-22 dpi, the susceptible plant is experiencing a heavy load of second and third-instar nymphs, while the resistant ECU72 has a lower nymph load, as most small, first-instar nymphs have ceased development or perished. This reduces the quantity of effectors/elicitors being delivered to the plant at these later times of infestation in resistant plants and may explain the burst in defensive responses in COL2246 and its absence in ECU72. Additionally, PCA analyses reveal that the 22 dpi samples have more variation in COL2246, which could reflect the higher density of nymphs and their asynchronous transition to later instars (Figure 2.16d). In addition, it is possible that the more variable defense responses are correlated with different signals provided by the more mature nymphs, as it is known that an insect's developmental stage influences plant responses at the transcriptome and metabolome level (Irigoyen et al., 2020, Perez-Fons et al., 2019, Tzin et al., 2015).

Higher expression of starch catabolism genes in COL2246 was also observed at 14-22 dpi and in response to all hormones (Figure 2.27). As observed in other plants, the breakdown of starch may be a strategy to mobilize stored energy to compensate for photosynthate depletion due to insect infestation (Zhou et al., 2015). Sesquiterpenoid biosynthetic genes were also more highly expressed in COL2246 at 14-22 dpi but were not hormone responsive (Figure 2.27). Emission of terpenoid volatiles has been previously found in response to aphids and whiteflies among other insects, but their purpose can be varied, including attracting or deterring insects or their natural predators or signaling responses in nearby plants (Kaloshian and Walling, 2005, Aljbory and Chen, 2018, Zhang et al., 2019).

Together, it appears that COL2246's late infestation response to copious quantities of large, late-stage nymphs that have developed in the absence of effective early control strategies results in symptoms of heavy infestation and ineffective, SA-mediated defenses. In contrast, ECU72's faster response to eggs and first-instar nymphs via ABA-mediated responses and lignin-based cell wall defenses may underlie it's resistance to whitefly infestation.

Materials and Methods

Plant growth

In vitro-grown cassava (*Manihot esculenta*) genotypes ECU72 (whitefly-resistant) and COL2246 (whitefly-susceptible) (Bohorquez et al., 2013, Parsa et al., 2015) from the CIAT collection were grown as described in Irigoyen et al. (2020). Shoot tips were placed in rooting medium for 30 days then moved to soil. Potted plants were grown in a greenhouse under long-day light conditions at 24-28°C for 60 days before use in whitefly-infestation and hormone-treatment experiments.

Arabidopsis thaliana Col-0 seeds (sterilized with chlorine gas and cold-treated for 2 days) were sown on ½ MS 1% sucrose agar plates (Murashige and Skoog, 1962) and kept at room temperature under constant light. One week after plating, seedlings were moved to soil (autoclaved Sunshine Mix (Sun Gro Horticulture, Agawam, MA) supplemented with 2% Osmocote (w/w) (The Scotts Company, Marysville, OH) in 2.5" x 2.5" cells. Plants were grown in a Percival I-36LL growth chamber under incandescent and fluorescent lights (180 $\mu E\ m^{-2}\ s^{-1}$) under a short-day light cycle (6-h light/18-h dark) at

24°C for 27 days, then adjusted to a long-day light cycle (16-h light/8-h dark) for one day before use in hormone-treatment experiments.

Whitefly rearing and infestation experiments

The *Aleurotrachelus socialis* Bondar colony used for cassava infestation experiments were maintained at CIAT as described by Bellotti and Arias (2001a). Whitefly infestations of 3-month-old cassava genotypes ECU72 and COL2246 were performed in a greenhouse using mesh cages as described by Irigoyen et al. (2020). Leaf tissue was collected at 0, 1, 7, 14, and 22 days post infestation and stored at -80°C until use. Experiments were repeated to obtain a total of three biological replicates.

Plant hormone treatments

Hormone treatments of 3-month-old cassava genotypes ECU72 and COL2246 were performed in growth chambers under long-day light conditions at 24-28°C in separate rooms as described by Irigoyen et al. (2020). Cassava leaves were sprayed to saturation with salicylic acid (200 µM SA, 0.1% EtOH, 0.01% Tween 20), methyl jasmonate (7.5 mM MeJA, 0.1% EtOH, 0.01% Tween 20), 1-aminocyclopropane-1-carboxylic acid (200 µM ACC, 0.1% EtOH, 0.01% Tween 20), or abscisic acid (200 µM ABA, 0.1% EtOH, 0.01% Tween 20). Hormone treatment concentrations were based on similar values as used in tomato or wheat due to comparable impermeability to foliar sprays in tomato, wheat and cassava (Li et al., 2013b, Chen et al., 2013). Leaf tissue was collected at 0, 0.5, 1, 2, 4, 8, 12, and 24 hours post treatment and stored at -80°C until use. Experiments were repeated to obtain a total of three biological replicates.

SA and JA treatments of 5-week-old Arabidopsis plants were performed in separate rooms at 22-27°C under incandescent lights (180 µE m^{-2} s^{-1}) under a long-day

light cycle (16-h light/8-h dark). Rosettes were sprayed until saturation with SA (100 μM SA, 0.1% EtOH, 0.01% Tween 20) or MeJA (100 μM MeJA, 0.1% EtOH, 0.01% Tween 20), with treatments beginning at 6AM. Leaf tissue was collected at 0, 0.5, 1, 2, 4, 8, 12, and 24 hours post treatment and stored at -80°C until use. Experiments were repeated to obtain a total of three biological replicates.

RNA extraction, cDNA library preparation, sequencing, and data processing

Cassava RNA extraction was performed as described by Behnam et al. (2019) and RNA quality was assessed as described by Irigoyen et al. (2020). cDNA library preparation and RNA-sequencing for cassava and Arabidopsis samples were performed as according to Irigoyen et al. (2020), with sequencing carried out at the UCR Institute for Integrative Genome Biology Genomics Core. Libraries were prepared for the three biological replicates of each time point in the whitefly-infestation and hormone-treatment experiments. For libraries from whitefly-infestation experiments, the Illumina NextSeq500 and Illumina HiSeq2500 platforms were used to sequence single-end 75-bp reads (trimmed to 50-bp) and 50-bp reads, respectively. For libraries from Arabidopsis and cassava hormone-treatment experiments, the Illumina NextSeq500 platform was used to sequence single-end 75-bp reads. Twelve to fifteen libraries were multiplexed per lane to obtain an average of ~30-50, ~13-52 and ~20-38 million reads among the three biological replicates per time point for Arabidopsis hormone treatments, cassava hormone treatments and cassava whitefly infestations, respectively. Pearson correlation values ranging from 0.85-1.00, 0.73-0.99 and 0.69-0.98 were obtained among biological replicates for Arabidopsis hormone treatments, cassava hormone treatments and cassava whitefly infestations, respectively, confirming their reproducibility (Figure S2.1).

Following read trimming and filtering, alignment using Bowtie2/2.2.5 and Tophat 2.0.14 and DEG calling using DESeq2 were performed using systemPipeR (Backman and Girke, 2016). Detected genes were defined as having an average of 20 reads or less across a treatment time course. Temporal and genotype DEGs were identified by comparisons of 0.5-24 hpt to 0 hpt and transcript levels in ECU72 versus COL2246 during treatment, respectively, and had $|\log_2 FC| > 1$ and FDR ≤ 5%.

Ortholog identification

Hormone-, glucosinolate- and lignin-pathway genes in *Arabidopsis thaliana* as annotated in TAIR version 10 (Berardini et al., 2015) were assigned orthologs in the *Manihot esculenta* genome version 6.1 obtained from Phytozome (JGI) (Goodstein et al., 2012) using the online program eggNOG-mapper version 4.5 (Huerta-Cepas et al., 2017, Huerta-Cepas et al., 2019). The cassava proteome was submitted as query to eggNOG-mapper using a DIAMOND (Double Index Alignment of Next-generation sequencing Data)-sensitive search to match input sequences to a protein with the most similar sequence (termed a “seed ortholog”, supported by an e-value and a score) within the eggNOG database. eggNOG-mapper also assigned query sequences to eggNOG orthologous groups (OGs) based on precomputed phylogenies. The locations of cassava seed orthologs relative to Arabidopsis genes within an OG phylogenetic tree was ultimately used to assign cassava orthologs and inform cassava gene nomenclature. In the case of Arabidopsis genes equally distant from one or more cassava orthologs, the lower numbered Arabidopsis gene was used for naming. For example, as *AtICS1* and *AtICS2* are equally distant from the cassava seed ortholog POPTR_0012s07180.1 in the virNOG group 1EKG5 phylogenetic tree, the cassava gene corresponding to this seed ortholog is named *MeICS1* (Table S2.9). Plant-specific OGs (virNOGs) were used in

naming cassava orthologs over database-wide OGs (NOGs). All other cassava gene names were obtained from Arabidopsis orthologs annotated for the *Manihot esculenta* genome version 6.1 on Phytozome (JGI) (Goodstein et al., 2012).

qRT-PCR

RNA levels for selected differentially expressed genes identified using RNAseq were confirmed by qRT-PCR using iQ SYBR Green Supermix (Bio-rad, Hercules, CA). cDNA templates were synthesized using 5 ng of mRNA following the Improm II reverse transcriptase protocol (Promega, Madison, WI). The control genes *MeUBQ* (Manes.10G122600) and *AtACT7* (AT5G09810) were selected based on their low variation in RPKM values across experimental time points. qRT-PCR was performed for selected genes using gene-specific primers (Table S2.15) and run on the Bio-rad CFX Connects instrument. Melting curve analyses were performed to confirm PCR product specificity. Relative expression changes were calculated by the comparative Ct method; fold change was calculated as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Three biological and technical replicates were used. Fold-change values are displayed with standard error.

Principal component and correlation analyses

Principal component analyses were performed using the R packages ggplot2 and DESeq2 (Love et al., 2014, Wickman, 2016) using count values for all detected genes. For Pearson correlation analyses comparing biological replicates following hormone or whitefly treatments in cassava or Arabidopsis, the R package ggplot2 was used. Pearson correlation for the comparison of cassava early and late phase hormone responses was performed using the R package corrplot (Wei and Simko, 2017). Average $\log_2$FC values were calculated across time points for each treatment phase. Phases were defined using

Figure 2.2. COL2246's early and late phases for all hormone treatments were 0.5-2 and 4-12 hpt, respectively. ECU72's phases were identical to those of COL2246 for JA, ET and ABA treatments, but its SA early and late phases were 0.5-1 and 2-24 hpt, respectively. The strength of correlation R-values was defined according to Evans (1996) as very weak (|0.00–0.19|), weak (|0.20–0.39|), moderate (|0.40–0.59|), strong (|0.60–0.79|), or very strong (|0.80–1.00|). PCA time points were labeled using color scales from the R package viridis (Garnier, 2018).

Gene expression clustering and visualization

Ordering of gene expression values by k-means or hierarchical clustering was performed using the base R stats package v3.6.2 and the R package ComplexHeatmap (Gu et al., 2016), respectively. Heatmaps displaying gene expression values (mean RPKM or $\log_2$FC) clustered by either method were constructed using the R package ComplexHeatmap. Other visualizations of gene expression data in boxplot or line graphs were constructed using the R package ggplot2, with scale colors for DEG count bar graphs from the R package RColorBrewer (Neuwirth, 2014). Boxplot whiskers represent values within 1.5 x IQR, and box values represent the first quartile, median, and third quartile values. Arabidopsis ET and ABA microarray data sets (0.5, 1, and 3 hpt) visualized in heatmaps comparing hormone responses of Arabidopsis and cassava hormone-pathway genes were acquired from Goda et al. (2008). To better represent the full spectrum of temporal responses to ET and ABA, these data sets were combined with those acquired from an ET treatment performed in Schenk et al. (2000) (24 hpt) and an ABA treatment performed in Huang et al. (2007) (6 and 24 hpt) for comparison of Arabidopsis and cassava hormone-responsive DEG counts. The R package VennDiagram was used to construct Venn diagrams. Gene expression data is available in Tables S2.1 and S2.6.

Metabolite quantification and transcript correlation

Metabolite levels of hormones were obtained from untargeted metabolomics on cassava samples prepared by Perez-Fons et al. (2019). Detection and characterization of putative glucosinolates in a pool containing equal parts of infested ECU72 and COL2246 samples was attempted using collection methods stated in Perez-Fons et al. (2019) and the standard methodology described in Crocoll et al. (2016) and Clark (2010). 30 mg of dried powder was dissolved in 1 ml of 85% methanol and shake for 4 min at room temperature, centrifuged at 20 000 g for 5 min and pellet discarded. An aliquot of 100 µl was used as crude extract and the remaining solution used for in-column sulfatase treatment. Sulfatase (Sigma-Aldrich) solution and DEAE-Sephadex A25 column (Sigma-Aldrich) were prepared as detailed in Crocoll et al. (2016). Briefly, a 200 µl column solution in potassium acetate 20 mM pH 5 was loaded into 1 ml glass pipette tip with glass wool and remaining crude extract (900 µl) loaded in. The column was washed twice with 70% ethanol and water (100 µl each).Then 20 µl of sulfatase solution was added and reaction left overnight at room temperature. Reaction products (desulfonated glucosinolates) were collected by eluting with 200 µl of water and stored at -20° C until LC-MS analysis. The procedure was repeated using a solution of sinigrin and glucotropaeolin (0.5 mg/ml, 200 µl) as references of aliphatic and phenyl derivatives of glucosinolates as positive control, and a blank solution (70% ethanol) as negative control. A separate experiment was prepared by extracting plant material at 100° C for 4 min to inactivate any residual myrosinase activity and both experiments compared. No differences in composition were observed.

For the analysis of the crude extracts and collected fractions, an Agilent's 1290 UPLC and a 6560 Ion mobility Q-TOF mass spectrometer equipped with an Agilent Jet Stream

(AJS) electrospray source was used in negative mode. Compounds were separated in a Zorbax RRHD Eclipse Plus C18 2.1x50 mm, 1.8 µm using a two solvents gradient consisting of (A) 2.5% acetonitrile in water and (B) acetonitrile, both solvents containing 0.03% vol. formic acid. Gradient started at 2% B for 1 min, increase to 30% B over 5 min, stay isocratic for 1 min followed by an increase to 90% B in two minutes and stay isocratic for another two minutes. Initial conditions were restored and re-equilibration lasted 3 minutes. The total runtime per sample was 15 min and flowrate was set at 0.3 ml/min. Nebulizer and sheath gas temperatures were 325° and 275° C respectively; flowrate of drying and sheath gas (nitrogen) were 5 and 12 L/min respectively and nebulizer pressure 35 psi. Capillary VCap, nozzle and fragmentor voltages were set at 4000, 500 and 400 V respectively. A reference mass solution was continuously infused to ensure mass accuracy calibration at 24-25 K resolution. Injection volume was 1 µl. A mix solution of commercial standards of glucosinolates (sinigrin and glucotropaeolin) representing allyl and benzyl-derivatives were used as reference material and for methodology validation purposes (Figure 2.25b).

The UCR in-house program MOCA v0.9.6 (Hur and Kirkwood, 2019) was used to identify hormone-pathway transcripts with expression levels strongly correlated ($R \geq 0.70$) to levels of their corresponding metabolite to confirm metabolite classification.

GO term enrichment

The R package ClusterProfiler (Yu et al., 2012) was used to perform GO-term enrichment analyses of cassava whitefly- and/or hormone-responsive DEG gene sets. Results of enrichment analyses display only those terms within the “biological process”

GO category. Terms were additionally grouped into categories based on shared ancestral GO terms. Significant GO terms are defined as those with p-values ≤ 0.05.

Supplemental Material

Supplemental Tables

Table S2.1. Log_2FC and FDR values for whitefly- and hormone-regulated cassava DEGs.

Table S2.2. Counts of temporal and genotype DEGs identified in cassava after whitefly or hormone treatments.

Table S2.3. Pearson correlation R and p-values for cassava hormone correlations.

Table S2.4. Enriched GO term IDs, descriptions, adjusted p-values, and associated loci for cassava hormone expression clusters.

Table S2.5. Expression values for external study Arabidopsis hormone treatment DEGs.

Table S2.6. Expression values for Arabidopsis hormone treatment DEGs.

Table S2.7. log2FC and p-values or signal ratio values of DEGs in Arabidopsis after ET treatment.

Table S2.8. Hormone and lignin pathway nomenclature definitions in cassava.

Table S2.9. Hormone pathway gene counts in Arabidopsis vs cassava.

Table S2.10. Enriched GO term IDs, descriptions, adjusted p-values, and associated loci for cassava infestation expression clusters.

Table S2.11. Counts and percents of cassava hormone- and whitefly-regulated treatment and genotype DEGs.

Table S2.12. Identifying information and statistical values for hormones measured via untargeted metabolomics.

Table S2.13. Enriched GO term IDs, descriptions, adjusted p-values, and associated loci for whitefly- and hormone-regulated cassava genotype DEGs.

Table S2.14. Counts of genes associated with enriched GO term categories in ECU72 versus COL2246 or vice versa during whitefly and hormone treatments.

Table S2.15. qRT-PCR primers.

Supplemental Figures

Figure S2.1. Correlations for cassava and Arabidopsis treatment samples.

(a,b) Pearson correlation R and p-values between biological replicates for genes detected during whitefly infestation in ECU72 and COL2246, respectively.

(c-f) Pearson correlation R and p-values between biological replicates for genes detected during SA, JA, ET, and ABA treatments, respectively, in ECU72.

(g-j) Pearson correlation R and p-values between biological replicates for genes detected during SA, JA, ET, and ABA treatments, respectively, in COL2246.

(k,l) Pearson correlation R and p-values between biological replicates for genes detected during SA and JA treatments, respectively, in Arabidopsis.

Detected genes were defined as having an average of 20 reads or more across a hormone treatment time course. Read count values for three biological replicates are shown per time point. Count values for each time point are labeled by color.

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Chapter 3

Identification of whitefly defense genes via eQTL analysis

Abstract

Cassava, an important staple crop for small-shareholder African farmers, has suffered devastating yield losses due whitefly feeding and whitefly-vectored viral diseases. In an effort to better understand the genetic basis of resistance found in South American sources, whitefly-resistance loci were identified by Quantitative Trait Loci (QTL) mapping at CIAT. Two mapping populations were generated through crosses of whitefly-resistant South American genotype ECU72 with whitefly-susceptible South American genotype COL2246 (generating the CM population), or between ECU72 and whitefly-susceptible African genotype 60444 (generating the GM population). As an additional means of identifying genes important for defenses against whiteflies, here we utilize RNA sequencing on a selection of resistant and susceptible progeny from each population to identify eQTLs (expression QTLs). Several criteria to better identify eQTL-identified genes important for defense against whiteflies were applied including: location within a known whitefly-resistance QTL, location within a cluster of eQTLs, status as an eQTL hotspot, status as a differentially expressed gene (DEG) at 0 d post infestation (dpi) between parental lines, known role in plant defense, or status as an eQTL in both CM and GM populations. We identified more eQTLs and eQTL-associated genes, including those with known roles in defense, in the GM as compared to the CM population. In both populations, among defense-associated eQTL-identified genes, many were involved in immunity, defense signaling and cell wall processes. While no eQTL hotspots were identified in the CM population, a GM population hotspot identified *MeSOAR1*, a master negative regulator

of ABA signaling, which likely suppresses effective ABA responses in 60444 but not in ECU72. Such ABA responses included stomatal regulation or callose deposition. A set of eQTL-identified genes that are proposed whitefly-resistance factors are suggested as possible candidates for further testing in transgenic cassava.

Introduction

Crucial for the subsistence of small farmers in Africa, the staple crop cassava has faced and continues to suffer from substantial yield losses due to whiteflies, specifically the *Bemisia tabaci* species of sub-Saharan Africa (FAO, 2018). However, breeding efforts to improve whitefly resistance to combat such losses are stifled by a lack of genetic diversity within the African cassava germplasm (Bredeson et al., 2016). A more diverse spectrum of traits, including whitefly resistance, is instead found closer to cassava's center of origin (the Amazon) in South American cassava genotypes (Bellotti et al., 1999). With this resource, the International Center for Tropical Agriculture (CIAT, Cali, Colombia) has utilized whitefly-resistant South American, as well as whitefly-susceptible South American and African cassava genotypes, to better understand resistance. These efforts include quantitative trait loci (QTL) mapping and conducting infestations using the Latin American whitefly *Aleurotrachelus socialis* for transcriptomic and metabolomic analyses as previously reported (Chapter 2) (Irigoyen et al., 2020, Perez-Fons et al., 2019). Such work has resulted in the delineation of several QTL regions and a better understanding of cassava's transcriptome response to whitefly.

Expression QTLs (eQTLs) are defined as genomic markers (such as single nucleotide polymorphisms, SNPs) associated with changes in the expression of one or

more local (*cis*-eQTLs) or distal (*trans*-eQTLs) target genes. When an eQTL has many target genes, it is called a “hotspot”, and suggests that the hotspot eQTL may act as major transcriptional regulator. As they can affect the expression of genes anywhere in the genome, *trans*-eQTLs have the possibility of affecting the expression of more genes and are thus more likely than *cis*-eQTLs to be major eQTL hotspots (Breitling et al., 2008). Together, the valuable associative information provided by eQTLs, especially when used in conjunction with other data sets (QTLs, transcriptomes, epigenomes, etc.), can aid in identifying genes or processes underlying desired traits (Gilad et al., 2008). In this study, we utilized eQTLs to identify genes with known roles in plant defense. Plant defense can be distinguished by two phases (Jones and Dangl, 2006). First, plants recognize conserved molecular signatures (also called elicitors), which can be divided into pathogen-associated molecular patterns (PAMPs), herbivore-associated molecular patterns (HAMPs) or damage-associated molecular patterns (DAMPs). This recognition triggers an initial immune response called PAMP-triggered immunity (PTI) (Schwessinger and Zipfel, 2008). If the attacker is well-adapted to its plant host, it will then secrete effectors to suppress PTI. However, plant hosts that possess cognate resistance (*R)* genes (such as nucleotide-binding leucine-rich repeat proteins, NLRs) are able to recognize specific effectors and then activate defenses similar to but stronger than PTI called effector-triggered immunity (ETI). ETI results in resistance and the activation of downstream defense signals and defense-response genes (Cui et al., 2015).

Here, we identify eQTLs in the parents and F_1 progeny of two mapping populations to expedite the identification of cassava genes associated with whitefly resistance. These F_1 mapping populations were generated to map genes associated with whitefly resistance using QTLs. An Ecuadorian genotype (ECU72) showing pronounced and fast-acting

whitefly resistance (Omongo et al., 2012, Bellotti and Arias, 2001) was selected as the resistant parent in crosses with the South American whitefly-susceptible genotype COL2246 (the CM population) and the whitefly-susceptible African transformation line 60444 (the GM population). We sequenced the transcriptomes of selected resistant and susceptible F_1 progeny of CM and GM populations to identify single nucleotide polymorphisms (SNPs) and transcript levels for eQTL analysis. For mapping purposes, traditionally, F_2 populations are used, however, as cassava is a highly heterozygous tetraploid, sufficient genetic diversity among offspring can be generated in F_1 progeny for mapping. Several criteria were used to identify eQTLs that may play an important role in whitefly resistance or susceptibility: location within a known whitefly-resistance QTL, location within a cluster of eQTLs, status as an eQTL hotspot, status as a differentially expressed gene (DEG) at 0 dpi between parental lines, known role in plant defense, or status as an eQTL in both CM and GM populations. Using these criteria, genes involved in ABA-, redox-, cell-wall-, and other defense-related processes were identified as possible regulators or signatures of whitefly resistance in ECU72.

Results

eQTL analysis approach

In an effort to delineate the location of whitefly resistance genes, QTL regions were mapped using F_1 progeny of crosses between the whitefly-resistant ECU72 and the whitefly-susceptible COL2246 or 60444 (Dr. Luis Augusto Becerra Lopez-Lavalle, Dr. Adriana Bohorquez-Chaux, Dr. Anestis Gkanogiannis, and Dr. Vianey Paola Barrera, CIAT) (Bohorquez et al., 2013). The cross between ECU72 and COL2246 resulted in 238

F_1 progeny (the CM population) and the ECU72 and 60444 cross generated 196 F_1 progeny (the GM population) (Figure 3.1). The resistance/susceptibility phenotypes of these plants were assessed by counting the number of nymphs three weeks after *A. socialis* infestation (Dr. Adriana Bohorquez-Chaux, CIAT). A spectrum of whitefly-susceptible to whitefly-resistant phenotypes was observed in the progeny, showing that whitefly resistance is a quantitative trait (Young, 1996). Many progeny exceeded the resistance level of parent ECU72 in both populations. While several F_1 progeny were more susceptible than the susceptible parent 60444 in the GM population, this was not seen in the CM population. These phenomena indicate the presence of transgressive segregation (de Los Reyes, 2019). One example was seen with the resistance traits present in the susceptible parent (60444 or COL2246) that are passed to F_1 progeny to enhance the resistance mechanisms inherited from the resistant parent (ECU72) (Figure 3.1). The second example is the loci associated with susceptibility to whiteflies in the ECU72 genome that enhanced 60444's susceptibility. We additionally acknowledge that although the resistance/susceptibility levels of the selected progeny exist on a continuum, for the purposes of simplifying this study, progeny are referred to strictly as resistant or susceptible.

QTL mapping performed at CIAT identified seven and two QTL regions in the CM and GM populations, respectively (Dr. Luis Augusto Becerra Lopez-Lavalle, Dr. Adriana Bohorquez-Chaux, Dr. Anestis Gkanogiannis, and Dr. Vianey Paola Barrera, CIAT). Currently, the QTL regions are large with over 1,000 genes; refinement of these regions will be made after QTL mapping of F_2 populations, which are now being prepared for analysis (Dr. Luis Augusto Becerra Lopez-Lavalle and Dr. Adriana Bohorquez-Chaux, personal communication). To expedite the identification of cassava genes associated with

whitefly resistance, RNA sequencing of RNA samples provided by Dr. Adriana Bohorquez-Chaux was performed on selected resistant and susceptible individuals of the F_1 CM (11 resistant and 13 susceptible progeny) and GM (16 resistant and 17 susceptible progeny) populations for the purpose of eQTL identification (Figure 3.1). In addition to eQTLs that reside within the F_1 population QTL regions, additional eQTLs located in other genomic regions may capture a distinct set of genes associated with whitefly resistance that would be otherwise missed by QTL mapping alone.

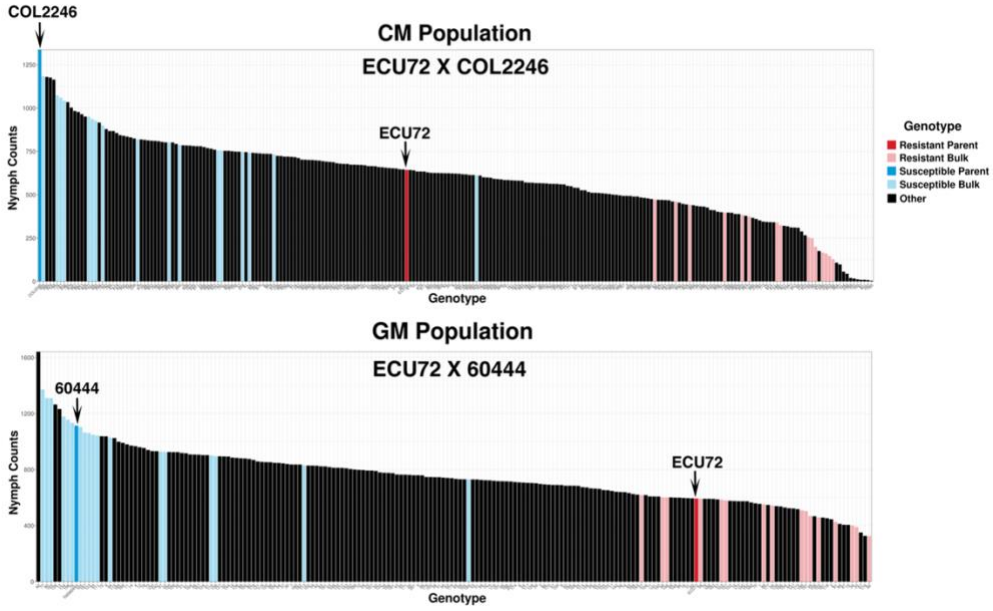

Figure 3.1. Spectrum of whitefly resistance phenotypes in the CM and GM populations.

Numbers of nymphs following *A. socialis* infestation of ECU72 (red), COL2246 (blue), 60444 (blue), and F_1 progeny of the CM population and GM populations are shown. The whitefly-resistance F1 progeny (light red) and whitefly-susceptible F_1 progeny (light blue) selected for eQTL analyses are shown. Progeny not included in the eQTL analyses are shown in black. Plant growth and phenotype analysis was performed by Dr. Adriana Bohorquez-Chaux at CIAT.

For use in eQTL mapping, SNPs were first identified in comparisons of the cassava reference genome (Colombian cassava genotype AM560-2) (Bredeson et al., 2016) with the parental genotypes (ECU72, COL2246 and 60444) and the CM and GM population F_1 progeny. Filtering was performed to obtain 121,778 high-quality SNPs (see Methods). SNP calling and initial filtering was performed by Dr. Anestis Gkanogiannis at CIAT. Further filtering was performed to remove SNPs with differing genotype calls across a parent's 15 samples (5 infestation time points, 3 replicates), as well as SNPs with no genotype calls among the progeny (Figure 3.2). To analyze only eQTLs representing genotypically distinct loci between the parents, SNP calls identical in both parental lines (ECU72 and COL2246 or ECU72 and 60444) were removed; in addition, if a SNP was called in one parent but the genotype at that position could not be determined in the other parent, it was removed (Figure 3.2).

Next, SNPs were separated based on the type of inheritance they displayed before further filtering. Four inheritance classes are defined and abbreviated henceforth as follows: (1) R→R: SNPs in the resistant parent inherited by the resistant progeny; (2) R→S: SNPs in the resistant parent inherited by the susceptible progeny; (3) S→S: SNPs in the susceptible parent inherited by the susceptible progeny; and (4) S→R: SNPs in the susceptible parent inherited by the resistant progeny. We next sought to reduce the complexity of the dataset. As whitefly resistance was shown to be quantitative (Figure 3.1), it was not expected that all resistant progeny should have all alleles required for resistance. To account for this, for each inheritance class, a SNP was retained if most F_1 progeny ($\geq$2/3 of the individuals) matching each parental allele displayed the same (R→R or S→S classes) or opposite (R→S or S→R classes) phenotype as the parent. In these

cases, at least three individuals of the F_1 progeny must display the parental phenotypes (Figure 3.2).

After SNP filtering, eQTL analysis was performed, with eQTLs meeting $p \leq 0.05$ and FDR ≤ 0.05 criteria considered to be significant (Figure 3.2). eQTLs were then filtered to examine only those eQTLs that were dominantly inherited, as such traits may be more easily introduced through future breeding efforts for whitefly resistance in cassava. As we are using F_1 progeny in these eQTL analyses, separation of F_1 phenotypes resulting from dominant traits can only be observed when at a locus the resistant parental genotype is heterozygous and the susceptible parent is homozygous (or vice versa) (Figure 3.2).

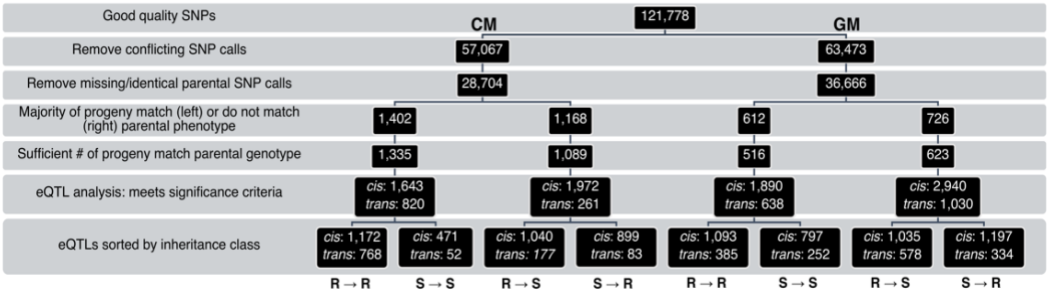

Figure 3.2. Analysis workflow of SNP filtering and eQTL calling.

SNPs identified for ECU72, COL2246, and the F_1 progeny of the CM and GM populations were filtered as follows: (1) SNPs with differing genotype calls across a parent's 15 samples were removed; (2) SNPs with no genotype calls among the progeny or parents, or SNPs identical for both parents were removed; (3) for SNPs shared by one parent and several progeny, at least 2/3 of progeny must *match* the parental phenotype (R→R and S→S inheritance classes) or must *not match* the parental phenotype (R→S and S→R inheritance classes); (4) for SNPs shared by one parent and several progeny, the number of progeny matching the parental phenotype must be at least three; (5) after SNP filtering, an eQTL analysis was performed, with significant eQTLs ($p \leq 0.05$ & FDR ≤ 0.05) retained; (6) resulting eQTLs were separated by inheritance class (R→R, S→S, R→S, or S→R). The number of SNPs or eQTL for the CM and GM populations are shown following each aforementioned step. SNP calling and initial filtering to obtain good quality SNPs was performed by Dr. Anestis Gkanogiannis at CIAT.

Expression data was also filtered prior to eQTL analysis to remove genes that did not meet our criteria for detection. Beginning with 33,033 genes in the cassava genome, genes with an average RPKM < 1 in both parental genotypes or in all F_1 progeny were removed. Finally, eQTLs of each inheritance class (R→R, R→S, S→S, and S→R) were categorized as *cis*- or *trans*-eQTLs. A total of 4,662 (3,582 *cis* and 1,080 *trans*) and 5,671 (4,122 *cis* and 1,549 *trans*) eQTLs were identified in the CM and GM populations, respectively (Figure 3.2; Table S3.1). A total of 309 and 768 gene targets of eQTLs in the CM and GM populations were identified, respectively (Table S3.2).

Table 3.1. eQTL-identified defense gene annotations.

Criteria Met[a]	Population	Cassava Locus	Cassava Gene Name[b]	Defense Role in Arabidopsis	Arabidopsis Ortholog	Arabidopsis Full Name	# of *cis*-eQTLs[c]	# of *trans*-eQTLs[c]	Reference
QTL	CM	Manes.03G127100	*MeMOS2a*	PTI/ETI responses	AT1G33520	*MODIFIER OF SNC1, 2*	38	0	Wu et al 2013
	CM	Manes.03G127200	*MeMOS2b*				1	0	Zhang et al 2005
	GM	Manes.18G111800	*MeDRP2B*	Response to bacterial PAMP flg22	AT1G59610	*DYNAMIN RELATED PROTEIN 2B*	3	0	Ekanayake et al 2021
	GM	Manes.18G124300	*MeEFR*	EF-TU bacterial PAMP receptor	AT5G20480	*EF-TU RECEPTOR*	0	1	Zipfel et al 2006
	GM	Manes.18G125500	*MeHAK1*	Probable HAMP/DAMP-perceiving polysaccharide receptor	AT1G06840	*HDS-ASSOCIATED RLK1*	1	0	Uemura et al 2020
	GM	Manes.18G112100	TNL *R* gene	ETI, specific role unknown	AT5G17680	TIR-type nucleotide-binding leucine-rich repeat protein	2	0	TAIR
Cluster	CM	Manes.10G023200	*MeCAR1*	ETI receptor of bacterial effectors	AT1G50180	*CEL-ACTIVATED RESISTANCE 1*	21	0	Laflamme et al 2020
	CM	Manes.14G097400	*MeSOG1*	Positive regulator of chitin response genes; DNA damage/immune response crosstalk	AT1G25580	*SUPPRESSOR OF GAMMA RADIATION 1*	29	0	Yoshiyama et al 2020
	CM	Manes.04G115600	*MeSUT1*	Mutant shows resistance to root knot nematode	AT1G22710	*SUCROSE TRANSPORTER 1*	39	0	Zhao et al 2018
	CM	Manes.04G016700	*MeTCP9*	Positive regulator of SA biosynthetic gene *ICS1*	AT2G45680	*TCP DOMAIN PROTEIN 9*	42	24	Wang et al 2015
	GM	Manes.13G094300	LRR Kinase	LRR kinase of unknown function	AT5G10290	leucin-rich repeat kinase	13	0	TAIR
	GM	Manes.03G092300	*MeLIK1*	RLK involved in chitin perception	AT3G14840	*LYSM RLK1 INTERACTING KINASE 1*	13	0	Le et al 2014

Hotspot - Target Gene	GM	Manes.11G133300	*MeADF4*	Positive regulator of callose-mediated ETI to bacteria *Pseudomonas syringae*	AT5G59890	*ACTIN DEPOLYMERIZING FACTOR 4*	0	1	Tian et al 2009
	GM	Manes.02G108700	*MeASK2*	Raises ABA sensitivity	AT5G42190	*ARABIDOPSIS SKP-LIKE 2*	5	50	Li et al 2012
	GM	Manes.14G027300	*MeATI-2*	Negative regulator of ABA signaling	AT5G52200	*INHIBITOR-2*	1	0	Hou et al 2016
	GM	Manes.14G031900	*MeATS3B*	Confers resistance to tobacco cutworm	AT5G62200	*EMBRYO-SPECIFIC PROTEIN 3B*	2	0	Savadogo et al 2021
	GM	Manes.18G020500	*MeBBD1*	Positive regulator of ABA-mediated callose deposition resulting in resistance to fungus *Botrytis cinerea*	AT1G75380	*BIFUNCTIONAL NUCLEASE IN BASAL DEFENSE RESPONSE 1*	4	1	You et al 2010
	GM	Manes.03G116500	*MeCcdA*	Regulator of chloroplast redox state	AT5G54290	Cytochrome c-type biogenesis CCDA-like chloroplastic protein	0	1	Motohashi and Hisabori 2010
	GM	Manes.03G006000	*MeCOL4*	Lowers ABA sensitivity	AT5G24930	*CONSTANS-LIKE 4*	6	0	Min et al 2015
	GM	Manes.09G103400	*MeEAP3*	Confers resistance to fungus *Fusarium oxysporum* via PEN3 trafficking	AT3G09030	*ENDOPLASMIC RETICULUM-ARRESTED PEN3*	3	0	Mao et al 2017
	GM	Manes.08G055000	*MeERD15*	Negative regulator of ABA signaling; regulator of stomatal pore adjustment	AT2G41430	*EARLY RESPONSIVE TO DEHYDRATION 15*	0	1	Aalto et al 2012
	GM	Manes.06G106400	*MeGRXC1*	Redox sensor	AT5G63030	*GLUTAREDOXIN C1*	15	1	Riondet et al 2012
	GM	Manes.09G063500	*MeHD2C*	Lowers ABA sensitivity	AT5G03740	*HISTONE DEACETYLASE 2C*	7	0	Luo et al 2012
	GM	Manes.03G026700	*MeHXK1*	Positive regulator of cell death	AT4G29130	*HEXOKINASE 1*	19	1	Bruggeman et al 2015
	GM	Manes.07G012100	*MeLRRAC1*	Confers resistance to the fungus *Golovinomyces orontii* and the bacteria *Pseudomonas syringae*	AT3G14460	*LEUCINE-RICH REPEAT (LRR) PROTEIN 1*	4	0	Bianchet et al 2019
	GM	Manes.11G118000	*MeMIK2a*	Cell wall damage sensing and *Fusarium* spp. fungal elicitor perception	AT4G08850	*MDIS1-INTERACTING RECEPTOR LIKE KINASE2*	29	0	Coleman et al 2021
	GM	Manes.11G118800	*MeMIK2b*				35	0	
	GM	Manes.11G119100	*MeMIK2c*				15	0	
	GM	Manes.11G119000	*MeMIK2d*				8	0	
	GM	Manes.14G026800	*MeMSBP2*	Regulator of monolignol biosynthesis as a scaffold for monolignol P450 monoxygenases	AT3G48890	*MEMBRANE STEROID BINDING PROTEIN 2*	1	0	Gou et al 2018
	GM	Manes.03G016800	*MeOCP3*	Negative regulator of JA/ABA-mediated callose deposition required for defense against fungi *Botrytis cinerea* and *Plectosphaerella cucumerina*	AT5G11270	*OVEREXPRESSOR OF CATIONIC PEROXIDASE 3*	10	1	Garcia-Andrade et al 2011
	GM	Manes.09G105200	*MePDX1*	Confers Vitamin B6-mediated resistance to bacteria *Pseudomonas syringae* and fungus *Botrytis cinerea*	AT5G01410	*PYRIDOXINE BIOSYNTHESIS 1*	4	0	Zhang et al 2015
	GM	Manes.S053100	*MePERK14a*	Receptor kinase of unknown function	AT4G32710	*PROLINE-RICH EXTENSIN-LIKE RECEPTOR KINASE 14*	0	45	TAIR
	GM	Manes.S103400	*MePERK14b*				0	41	
	GM	Manes.14G034600	*MePMEI*	Cell wall modification via inhibition of pectin methyl esterases	AT5G62350	Plant invertase/pectin methylesterase inhibitor superfamily protein	2	0	Jolie et al 2010
	GM	Manes.02G105400	*MePR-5b*	Pathogen and SA responsive gene	AT4G38660	Pathogenesis-related thaumatin superfamily protein	0	1	Irigoyen et al 2020
	GM	Manes.18G018400	*MeRD19*	Confers *RRI*-mediated resistance to bacteria *Ralstonia solanacearum*	AT4G39090	*RESPONSIVE TO DEHYDRATION 19*	22	0	Bernoux et al 2008
	GM	Manes.06G102200	*MeROP10*	Negative regulator of ABA signaling	AT3G48040	*RHO-RELATED PROTEIN FROM PLANTS 10*	4	0	Li et al 2012
	GM	Manes.15G135000	*MeRR2*	Positive regulator of ROS-mediated stomatal closure	AT4G16110	*RESPONSE REGULATOR 2*	0	1	Wang et al 2020
	GM	Manes.03G015400	*MeSHN3*	ET-responsive transcription factor	AT5G25390	*SHINE3*	51	0	Dejemal et al 2015
	GM	Manes.03G041400	*MeSSL4*	Susceptibility factor against bacteria *Ralstonia solanacearum* at elevated temperature	AT3G51420	*STRICTOSIDINE SYNTHASE-LIKE 4*	4	0	Aoun et al 2017
	GM	Manes.03G123800	*MeTHI*	Positive regulator of ABA-mediated stomatal closure	AT5G54770	*THIAMINE4*	0	1	Li et al 2016
	GM	Manes.02G005400	*MeTRE1*	Positive regulator of ABA-mediated stomatal closure	AT4G24040	*TREHALASE 1*	0	1	Van Houtte et al 2013
	GM	Manes.06G111400	*MeWR3*	Wounding-responsive gene	AT5G50200	*WOUND-RESPONSIVE 3*	14	0	Leon et al 1998
	GM	Manes.07G008700	NLR	ETI, specific role unknown	AT3G14470	NB-ARC domain-containing disease resistance protein	6	0	TAIR
	GM	Manes.07G044300	NLR				3	0	
	GM	Manes.07G044700	NLR				2	0	
	GM	Manes.07G045400	NLR				3	0	

Hotspot - Target Gene & SNP	GM	Manes.03G065400	*MeSOAR1*	Master negative regulator of ABA signaling	AT5G11310	*SUPPRESSOR OF THE ABAR OVEREXPRESSOR 1*	2	0	Ma et al 2020
	GM	Manes.11G119900	*MeVEP1a*	Wounding-responsive gene	AT4G24220	*VEIN PATTERNING 1*	21	0	Yang et al 1997
	GM	Manes.11G120000	*MeVEP1b*				30	6	
Hotspot - SNP	GM	Manes.09G069000	*MeAIRP3*	Negative regulator of ABA signaling	AT3G09770	*ABA INSENSITIVE RING PROTEIN 3*	NA	NA	Pan et al 2020
	GM	Manes.03G015100	*MeEBF1*	Negative regulator of ET signaling	AT2G25490	*EIN3-BINDING F BOX PROTEIN 1*	NA	NA	An et al 2010
	GM	Manes.09G063600	*MeEIN2*	Positive regulator of ET signaling	AT5G03280	*ETHYLENE INSENSITIVE 2*	NA	NA	Ju et al 2012
	GM	Manes.06G106000	*MeEX2*	Positive regulator of singlet oxygen-mediated cell death in response to fungal toxin; Involved in retrograde signaling	AT1G27510	*EXECUTER 2*	NA	NA	Chen et al 2015
	GM	Manes.11G117500	*MeGPX2*	Redox sensor	AT2G31570	*GLUTATHIONE PEROXIDASE 2*	NA	NA	Passaia and Margis-Pinheiro 2015
	GM	Manes.03G011800	*MeKNAT3*	Positive regulator of monolignol biosynthesis	AT5G25220	*KNOTTED1-LIKE HOMEOBOX GENE 3*	NA	NA	Qin et al 2020
DEG*	CM	Manes.17G080400	Cell wall modification gene	Mutant contributes to cell-wall-based resistance to oomycete *Hyaloperonospora arabidopsidis*	AT1G23170	NA	1	0	TAIR
	CM	Manes.11G049900	*MeABCG40*	ABA importer	AT1G15520	*ATP-BINDING CASSETTE G40*	1	0	Finkelstein 2013
	GM	Manes.13G068600	*MeCSLB5*	Cellulose biosynthetic gene	AT4G15290	*CELLULOSE SYNTHASE LIKE 5*	11	0	Richmond and Somerville 2000
	GM	Manes.03G007700	*MeGPX1*	Redox sensor	AT2G25080	*GLUTATHIONE PEROXIDASE 1*	1	0	Passaia and Margis-Pinheiro 2015
	GM	Manes.09G071300	*MeMOS11*	mRNA exporter involved in ETI	AT5G02770	*MODIFIER OF SNC1, 11*	2	0	Dong et al 2016
	GM	Manes.11G104100	*MeWAK2*	Receptor that senses pathogen/wounding-derived pectin fragments	AT1G21270	*WALL-ASSOCIATED KINASE 2*	1	0	Kohorn et al 2014
Shared	CM & GM	Manes.02G108700	*MeASK2*	Raises ABA sensitivity	AT5G42190	*ARABIDOPSIS SKP-LIKE 2*	5	50	Liu et al 2011
	CM & GM	Manes.11G097800	*MeEEN*	Epigenetic regulator of *EIN2*	AT4G38495	*ENHANCER OF ETHYLENE INSENSITIVITY*	40	0	Zander et al 2019
	CM & GM	Manes.04G047000	*MeLRK10L1.2*	Confers resistance to leaf rust fungus	AT1G18390	*LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR-LIKE PROTEIN KINASE-LIKE 1.2*	3	0	Shin et al 2015
	CM & GM	Manes.07G038300	*MeSPF2*	Activates whitefly defense gene *WRKY33* via sumolyation	AT4G33620	*SUMO PROTEASE RELATED TO FERTILITY2*	5	1	Verma et al 2021
Defense	CM	Manes.14G033200	*MeENO2*	Positive regulator of ABA responses	AT2G36530	*ENOLASE 2*	1	0	Kang et al 2013
	GM	Manes.14G155900	*MeNPR4*	SA receptor and negative regulator of SA signaling	AT4G19660	*NPR1-LIKE PROTEIN 4*	2	0	Liu et al 2020
	GM	Manes.17G094700	*MeOST1*	Positive regulator of ABA-mediated and ABA-indepdent stomatal closure	AT4G33950	*OPEN STOMATA 1*	1	0	Yoshida et al 2006
	GM	Manes.11G106100	*MePR-3d*	ET-inducible gene	AT3G12500	*PATHOGENESIS-RELATED 3*	10	0	Irigoyen et al 2020

[a] Criteria abbreviations are as follows: QTL - eQTL-associated genes residing within a whitefly-resistance QTL; Cluster - eQTL-associated genes residing within a cluster of eQTLs; Hotspot - eQTLs defined as a hotspot (SNP: cassava gene listed contains a SNP associated with a hotspot,Target Gene: cassava gene listed is affected by a hotspot, SNP& Target Gene: cassava gene listed both contains a SNP associated with a hostpot and is affected by a hotspot);
DEG - eQTL-associated gene that is differentially expressed between parental lines at 0 dpi; Defense: eQTL with known role in defense not meeting other criteria; Shared: eQTL identified in both CM and GM populations.
Note that Hotspot SNPs or target genes with no known roles in defense are not listed here.
The following eQTL-identified genes met multiple criteria: MeASK2: Hotspot - Target Gene, CM DEG and Shared; MeSPF2: Hotspot - Target Gene and Shared; and MeSOAR1: GM DEG and Hotspot - Target Gene, SNP.
* Note that MeASK2 and MeSOAR1 are additionaly GM population 0 dpi genotype DEGs but not listed in the DEG category.

[b] Cassava genes are named based on the gene name of their ortholog in Arabidopsis, if Arabidopsis gene name is unavaillble, the cassava name is based on the available description for its ortholog in TAIR (Berardini et al 2015). Multiple cassava orthologs of the same gene in Arabidopsis are named with additional letters in order of genomic locus. *MeASK2* appears twice as it was identified as a hotspot and as a shared eQTL.

[c] eQTLs are listed in Table S3.1.

To summarize the processes eQTL target genes were involved in, target genes of each population were assigned to Mercator functional bins (Schwacke et al., 2019) (Figure 3.3; Table S3.2). While most genes were not assigned a bin, many genes in both populations fell into bin categories for RNA biosynthesis and processing, as well as protein biosynthesis, modification and homeostasis and enzyme classification. However, a striking difference was observed in the number of genes associated with external stimuli response bin, with 112 genes in the GM population but only 11 genes in the CM population (Figure 3.3). Annotations or functions assigned to cassava genes were based primarily on functions present in Arabidopsis orthologs. This approach has limitations, as sequence similarities used to identify orthologs do not always accurately predict conserved functions, especially in comparisons of two species with different ploidy levels (Arabidopsis is diploid, whereas cassava is tetraploid) where gene expansions/contractions can be found.

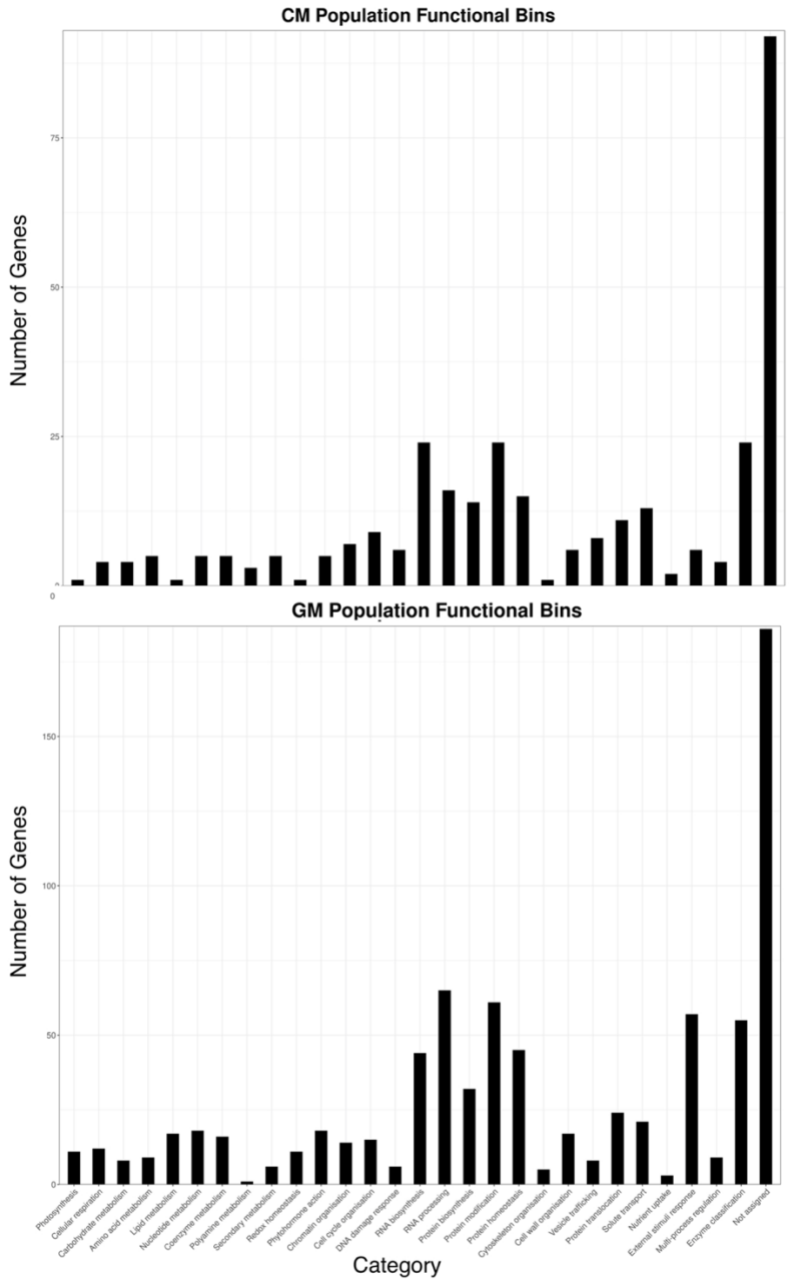

Figure 3.3. Functional bins of eQTL target genes identified in the CM and GM populations.

Functional bins were identified using the online program Mercator for the CM and GM populations. Bins appear in order of primary bin number. Bin assignments are provided in Table S3.2.

eQTLs imbedded in QTL regions

To determine which eQTLs correlated with QTLs, we identified eQTL SNPs or their target genes that were located within QTLs of the CM and GM populations (Figures 3.4-3.5; Table S3.3). QTLs were identified at CIAT by Dr. Luis Augusto Becerra Lopez-Lavalle, Dr. Adriana Bohorquez-Chaux, Dr. Anestis Gkanogiannis, and Dr. Vianey Paola Barrera. For the CM population, two QTLs were identified on chromosome 3, one on chromosome 5, one on chromosome 7, one on chromosome 10, and two on chromosome 18 (Figure 3.4). For the GM population, one QTL was identified on chromosome 1 and two QTLs were found on chromosome 18 (Figure 3.5). A total of 24 and 16 eQTL target genes were found to reside within QTL regions in the CM and GM populations, respectively (Table S3.3), of which, two and four genes had defense-related functions, respectively (Table 3.1).

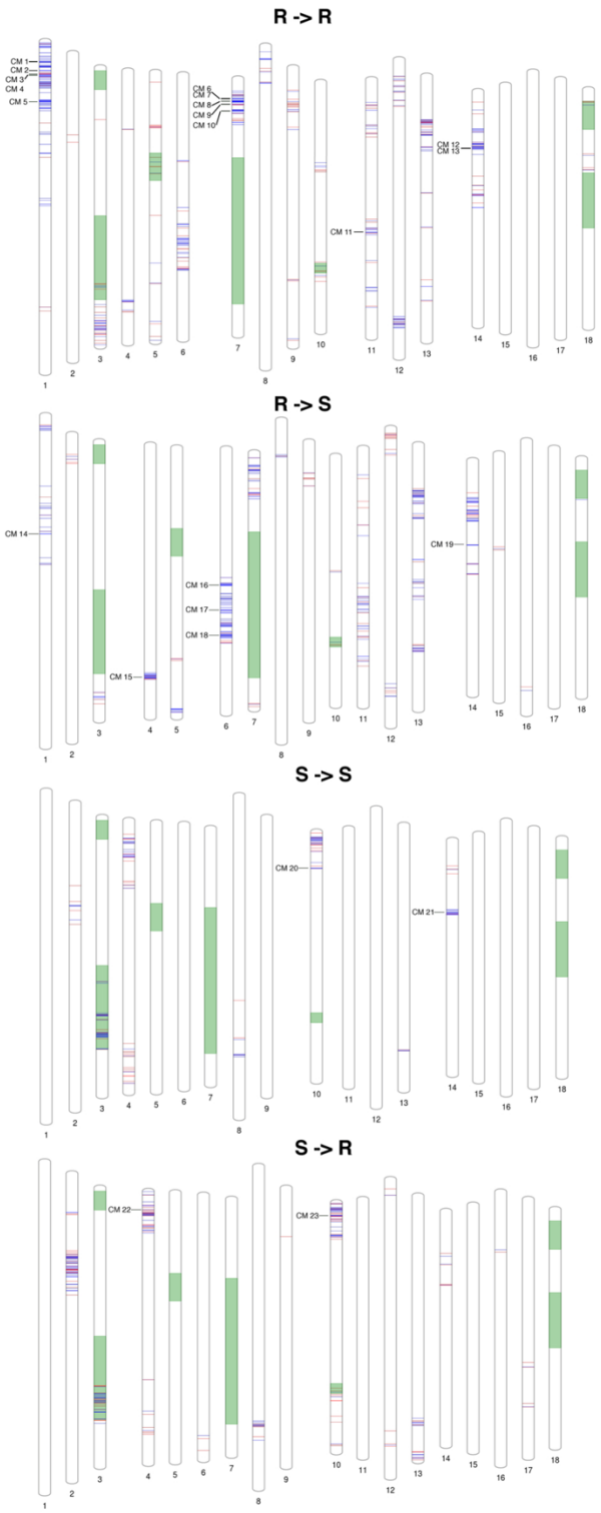
R -> R
CM 1
CM 2
CM 3
CM 4
CM 5
CM 6
CM 7
CM 8
CM 9
CM 10
CM 11
CM 12
CM 13
R -> S
CM 14
CM 15
CM 16
CM 17
CM 18
CM 19
S -> S
CM 20
CM 21
S -> R
CM 22
CM 23

Figure 3.4. Physical locations of CM population eQTLs.

Chromosome map displays locations of eQTL SNPs (blue lines) and their target genes (red lines) as well as previously identified whitefly-resistance QTLs (green-shaded regions). Locations of clusters of eQTLs are additionally annotated, with two or more clusters in very close proximity denoted with a single line. Twelve SNPs and four genes that have not been assigned to cassava chromosomes are not displayed (Table S3.1). QTLs were identified at CIAT by Dr. Luis Augusto Becerra Lopez-Lavalle, Dr. Adriana Bohorquez-Chaux, Dr. Anestis Gkanogiannis, and Dr. Vianey Paola Barrera

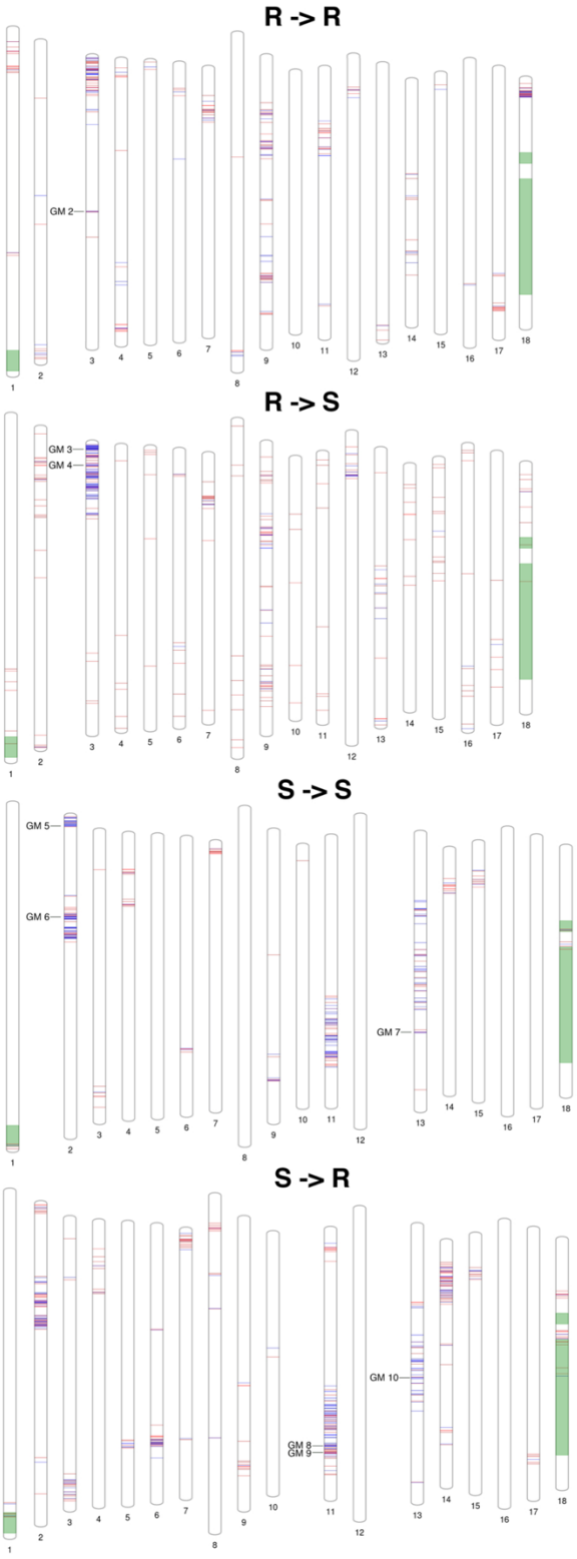
R -> R
GM 2
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
R -> S
GM 3
GM 4
S -> S
GM 5
GM 6
GM 7
S -> R
GM 8
GM 9
GM 10

Figure 3.5. Physical locations of GM population eQTLs.

Chromosome map displays locations of eQTL SNPs (blue lines) and their target genes (red lines) as well as previously identified whitefly-resistance QTLs (green-shaded regions). Locations of clusters of eQTLs are additionally annotated, with two or more clusters in very close proximity denoted with a single line. The locations of 12 SNPs and seven genes that have not been assigned to cassava chromosomes are not displayed (Table S3.1). QTLs were identified at CIAT by Dr. Luis Augusto Becerra Lopez-Lavalle, Dr. Adriana Bohorquez-Chaux, Dr. Anestis Gkanogiannis, and Dr. Vianey Paola Barrera

To better understand the effect of eQTL SNPs on the expression of these defense genes in resistant and susceptible parents and their F_1 progeny, we visualized transcript levels in non-infested leaves according to the genotype of the parents and their progeny (violin plots), as well as expression throughout a 22-d whitefly infestation of parental lines (bar graphs) (Figure 3.6). Bar graphs do not indicate significant expression differences unless specifically stated in the text. Only 0-dpi genotype DEGs are explicitly called out in this Chapter, as the 0-htimepoint is comparable among parental lines and F_1 progeny. However, some eQTLs and target genes were identified as genotype and/or temporal DEGs during the whitefly infestation time course experiments with ECU72 and COL2246 as described in Chapter 2; these data can be found in Chapter 2's Table S2.1. Plants were binned into three genotype classes: Bin 0 (homozygous for the reference genome allele), Bin 1 (heterozygous), and Bin 2 (homozygous for the alternate allele). While only the most significant *cis*- or *trans*-eQTLs were visualized, all significant eQTLs are provided in Table S3.1 and numbers of significant eQTLs identifying defense-related genes are provided in Table 3.1.

In these visualizations, we identify eQTLs that are strictly correlated with resistance or susceptibility to whitefly, as well as others, which instead provide insights into general defenses against whiteflies. We define eQTLs showing strict correlation with resistance/susceptibility as those of R→R or S→S inheritance classes in which the expression level of the progeny and parent of the same genotype is similar, and in which the expression level of the resistant progeny and parent is distinct from that of the susceptible progeny and parent. This contrasts with the eQTLs of the R→S or S→R inheritance classes. In these cases, while the progeny and parent of the same genotype

may show comparable expression levels, we cannot determine whether the trait is important for resistance or susceptibility. Additionally, variability at the expression level is expected, as F_1 population phenotypes indicated whitefly resistance is quantitative in both populations (Figure 3.1). Thus, target gene expression may vary based on the genetic background of each parent or progeny member, or, due to slight differences in environmental conditions, as tissue collection occurred at different times for parents and progeny.

For the CM population, only R→R type eQTLs were found in the chromosome 5 and 18 QTLs, while R→R, R→S and S→R type eQTLs were found in the chromosome 10 QTL (Figures 3.4 and 3.6a; Tables 3.1 and S3.3). Three CM population *cis*-eQTLs were located in the lower QTL on chromosome 3 and were transmitted from COL2246 to susceptible (S→S) or resistant (S→R) F_1 progeny. These SNPs affected the expression of two *MeMOS2* genes, which encode for RNA-binding proteins important for innate immunity and *R*-gene-mediated resistance (Wu et al., 2013, Zhang et al., 2005) (Figure 3.6a;Table 3.1). During whitefly infestation, *MOS2a* and *MOS2b* RNAs were present at all time points and accumulated to higher levels in COL2246 than in ECU72. However, visualization of F_1 progeny alongside parent transcript levels revealed that resistant progeny displayed higher expression than susceptible progeny. In cases such as these, while progeny and parental genotypes match, expression levels did not strictly correlate with resistance or susceptibility, making the role of the affected target genes *MOS2a* and *MOS2b* in whitefly resistance/susceptibility unclear (Figure 3.6a). Other QTL regions on chromosomes 3 and 7 did not contained eQTL-identified defense genes.

GM population eQTLs of the R→S, S→S and S→R classes were identified within chromosome 1 and 18 QTL regions (Figures 3.5 and 3.6b; Tables 3.1 and S3.3). Among these 16 eQTLs, four defense genes were identified including three immune receptors (*MeHAK1*, *MeEFR* and a TIR-type NLR, TNL) and a dynamin-related protein (*MeDRP2b*) with an established role in immunity (Table 3.1). All four of these genes were located on chromosome 18, and were found in QTL regions identified in both the GM and CM populations (Figures 3.4 and 3.5). During whitefly infestations, all four transcripts displayed a similar trend of having higher levels in 60444 versus ECU72. A S→R *cis*-eQTL identified *MeHAK1*, a probable HAMP/DAMP-perceiving polysaccharide receptor important for ET-dependent herbivory resistance (Uemura et al., 2020). At 0-7 dpi, *MeHAK1* was more highly expressed in 60444 than in ECU72. By 22 dpi, *MeHAK1* reached higher expression in ECU72 (Figure 3.6b).

A *trans*-eQTL in this Chr 18 region additionally implicated PAMP perception as a potential important trait donated from 60444 to whitefly resistant F_1 progeny (S→R) (Figure 3.6b). *MeEFR*, a receptor that recognizes the EF-TU bacterial PAMP (Zipfel et al., 2006) to trigger PAMP-triggered immunity was not expressed in ECU72 and was detected at all times after WF infestation in 60444. Similar to *MeEFR*, the TNL *R* gene Manes.18G112100, which was identified by a S→R *cis*-eQTL, had markedly higher transcript levels in 60444 versus ECU72 throughout the entire whitefly infestation (0-22 dpi). To date the ligand for this TNL has not been identified, so its exact role in defense is unknown. For these three genes (*MeHAK1*, *MeEFR* and TNL Manes.18G112100), 60444 and resistant progeny showed higher transcript levels than ECU72 and susceptible progeny. Therefore, it is difficult to determine the role of these genes in resistance or susceptibility to whitefly infestation. It is to be noted however that for *MeEFR*, a high

percentage of the resistant and susceptible F_1 progeny shared the parental resistant and susceptible alleles, respectively (Figure 3.6b).

In contrast, one (Chromosome18_9868040) of the two *cis*-eQTLs identifying *MeDRP2B* was strictly correlated with susceptibility. *MeDRP2B* is a dynamin-related protein important for response to the bacterial PAMP flg22 via endocytosis of the flg22 receptor FLS2 (Ekanayake et al., 2021). This gene was more highly expressed in 60444 versus ECU72 at 0-1 dpi and at equivalent levels at all later timepoints (Figure 3.6b).

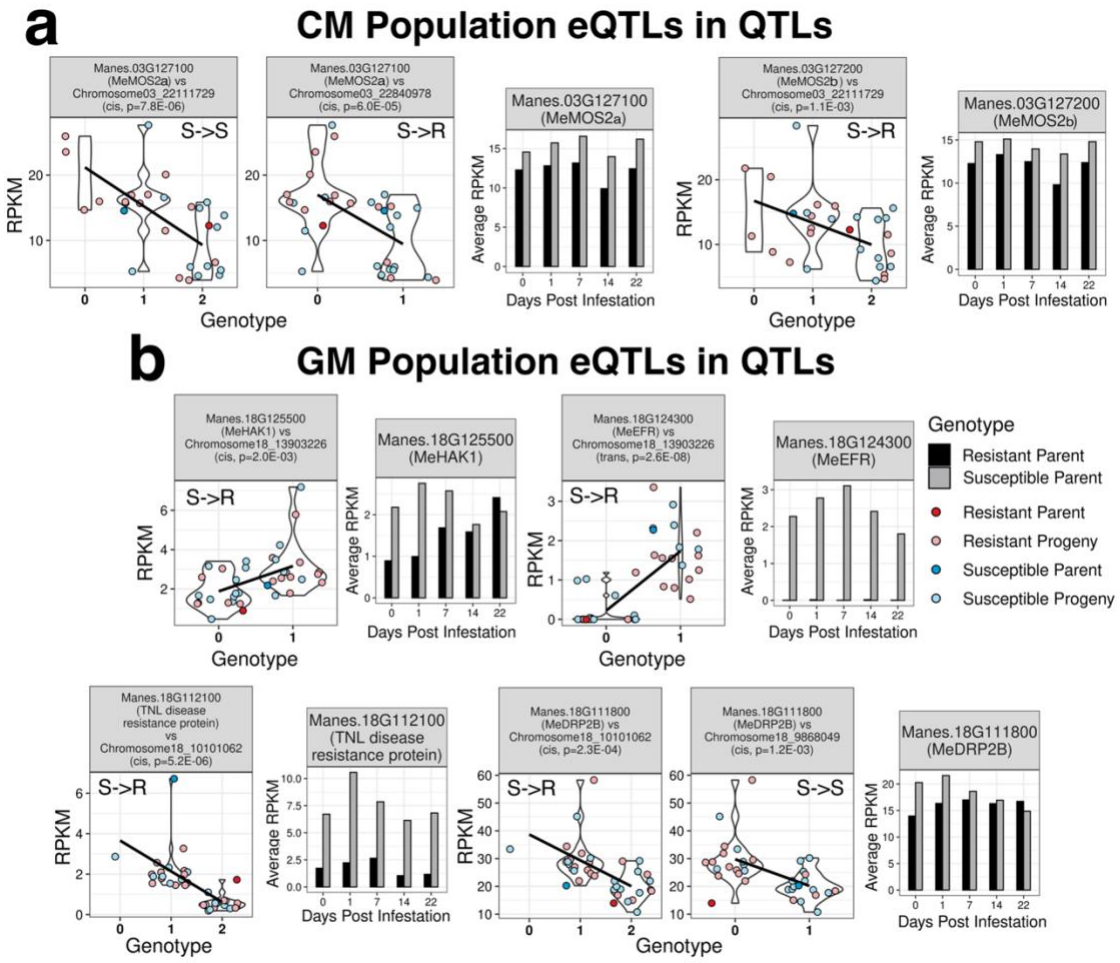

Figure 3.6. eQTLs within QTL regions.

Violin plots displaying the effect of eQTL SNPs within QTL regions on the expression of their target genes in the F_1 progeny and parents are shown. Bar graphs displaying the expression of target genes during whitefly infestation in parental genotypes for the CM (**a**) and GM (**b**) populations are displayed. Genotype classes are denoted as follows: 0 - homozygous for the reference genome; 1 - heterozygous; 2 - homozygous for the alternative allele. Whitefly-resistant genotypes are colored red (ECU72) and light red (resistant progeny) versus blue (COL2246 or 60444) and light blue (susceptible progeny) in violin plots. Expression is displayed as mean RPKM values at 0-22 dpi are colored in black for ECU72 and grey for COL2246 or 60444 in bar graphs. ID of SNP affecting ("versus") target gene, eQTL type (*cis* or *trans*), inheritance class, and *p*-value are provided in each violin plot. Violin plots in each panel are ordered from lowest to highest *p*-value. Trends in expression are shown here. The statistical relevance of the expression data for the eQTL-identified genes can be found in Chapter 2 (Table S2.1).

eQTL clusters

eQTLs and target genes for CM and GM populations were mapped to cassava's 18 chromosomes. This identified eQTLs that were co-located within the genome; importantly, these eQTL clusters were often associated with specific inheritance classes (Figures 3.4-3.5; Table S3.4). From this observation, we sought to investigate the function of eQTLs located in clusters (Table 3.1). To this end, eQTL clusters were defined as regions with at least eight eQTL SNPs or target genes within a 10-kb. While 23 clusters were found in the CM population, only 10 were identified in the GM population (Table S3.4). Among eQTL-identified genes within such clusters, we focus only on those with known roles in defense for further analysis.

CM population clusters spanned chromosomes 1, 4, 6, 7, 10, 11, and 14 and contained 43 eQTL gene targets, of which four have known roles in defense (Figures 3.4 and 3.7a; Tables 3.1 and S3.4). The *cis*- and *trans*-eQTL target *MeTCP9*, a positive regulator of SA biosynthetic gene *ICS1* (Wang et al., 2015c), and the *cis*-eQTL target *MeSUT1*, a sucrose transporter that facilitates root-knot nematode development (Zhao et al., 2018), were both more highly expressed in ECU72 versus COL2246 constitutively and during infestation. In contrast, a *cis*-eQTL identified the ETI-involved immune receptor *MeCAR1*, which recognizes bacterial effectors (Laflamme et al., 2020); *MeCAR1* showed higher expression in COL2246 versus ECU72 at 0-22 dpi. For these three genes however, the 0 dpi expression levels and genotypes of the F_1 progeny and parents were not always well correlated. For example, *MeSUT1* expression at 0 dpi is higher in ECU72 versus COL2246, but is lower in resistant progeny as compared to susceptible progeny. Such expression trends make the role of these genes in whitefly resistance/susceptibility unclear (Figure 3.7a).

cis-eQTL target *MeSOG1* expression levels were well correlated between parents and progeny as ECU72 and resistant progeny had higher expression, while COL2246 and susceptible progeny had lower expression at 0 dpi. In Arabidopsis, *SOG1* is a regulator of between DNA damage and immune response and positive regulator of chitin-responsive genes (Yoshiyama et al., 2020). ECU72 had elevated transcript levels compared to COL2246 at 0-7 dpi and higher transcript levels were detected in the F_1 resistant progeny at 0 dpi. However, by 14-22 dpi, *MeSOG1* expression was higher in COL2246 versus ECU72 suggesting a constitutive/early activation of *MeSOG1* may be important for whitefly resistance (Figure 3.7a).

In the GM population, 10 eQTL clusters were identified residing on chromosomes 2, 3, 11, and 13 with a total of 65 genes associated with these clusters (Figures 3.5 and 3.7b; Tables 3.1 and S3.4). Within these clusters, two defense genes were identified are highlighted here. *MeLIK1*, a LRR receptor-like kinase regulated by CERK1 in the perception of chitin (Le et al., 2014), was identified by a *cis*-eQTL and resides in the eQTL cluster on chromosome 3. *MeLIK1* was more highly expressed in ECU72 and resistant F_1 progeny than in COL2246 and susceptible progeny. *MeLIK1* was inherited from ECU72 to the resistant progeny, and had very elevated transcript levels in ECU72 compared to COL2246 throughout infestation. These data suggest that *MeLIK1* may be important for recognition of whiteflies and subsequent activation of whitefly-resistance responses. In contrast, the *cis*-eQTL target LRR kinase Manes.13G094300 in Cluster GM7 on chromosome 13 was more highly expressed in COL2246 at all times in the infestation time-course (0-22 d) and in susceptible F_1 progeny, suggesting that it may act as a whitefly susceptibility factor (Figure 3.7b). To date, the function of this LRR kinase remains unknown.

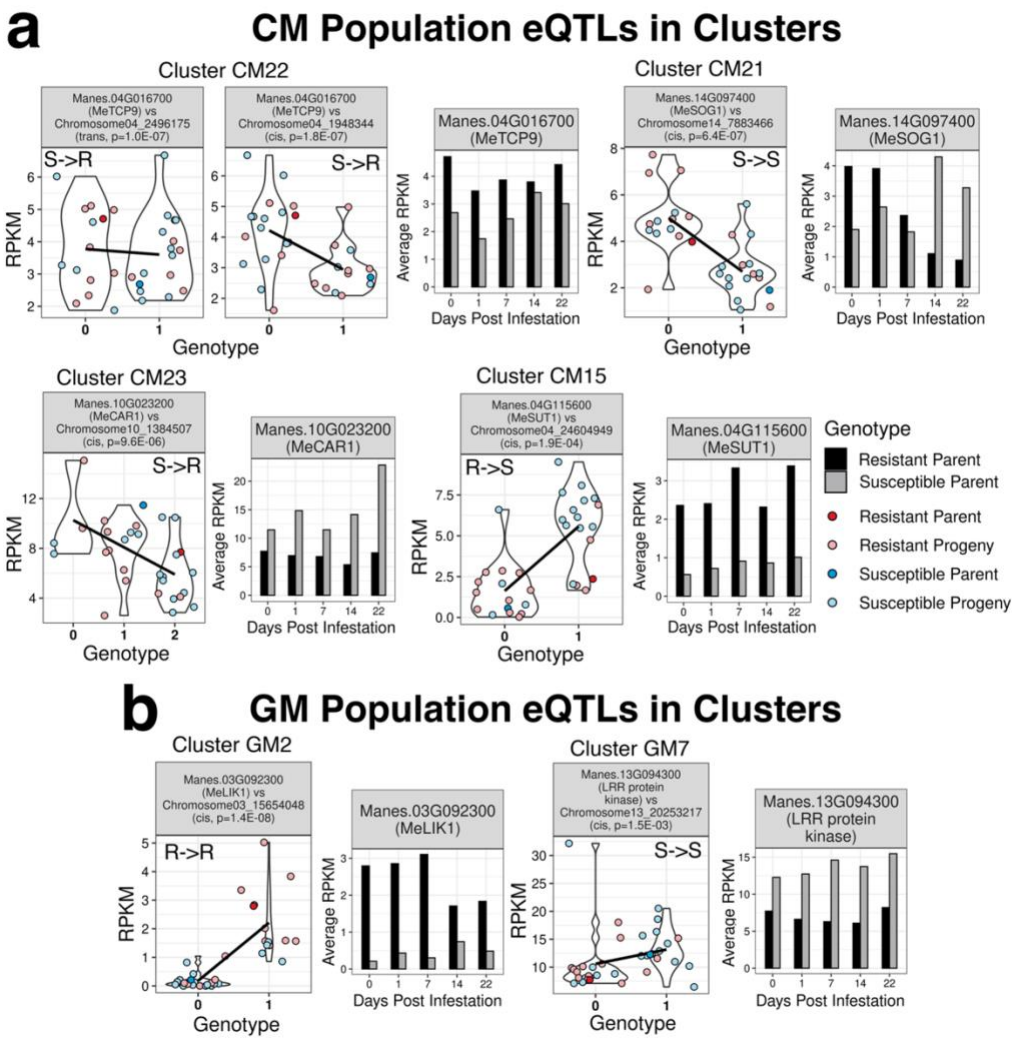

Figure 3.7. eQTLs within eQTL clusters.

Violin plots displaying the effect of eQTL SNPs within eQTL clusters on the expression of their target genes in the F_1 progeny and parents are shown. Bar graphs displaying the expression of target genes during whitefly infestation in parental genotypes for **(a)** CM and **(b)** GM populations. Genotypes are denoted as in Figure 3.6. Whitefly-resistant genotypes are colored red (ECU72) and light red (resistant progeny bulk) versus blue (COL2246 or 60444) and light blue (susceptible progeny bulk) in violin plots. Expression is displayed as average RPKM values and colored in black for ECU72 and grey for COL2246 or 60444 in bar graphs. ID of SNP affecting ("versus") target gene, eQTL type (*cis* or *trans*), inheritance class, and *p*-value are labeled in each violin plot. Violin plots in each panel are ordered from lowest to highest *p*-value. The statistical relevance of the expression data for the eQTL-identified genes can be found in Chapter 2 (Table S2.1).

eQTL hotspots

Another important criterion often used to identify possible functional significance of eQTLs is identifying eQTL hotspots among a set of eQTLs. An eQTL hotspot SNP affecting the expression of numerous genes can indicate the presence of a master regulator (Breitling et al., 2008). Here, we define eQTL hotspots as *cis*- or *trans*-eQTLs affecting the expression of ten or more target genes. All target genes are of the same inheritance class as the eQTL-associated hotspot SNP that targets them. No hotspots were identified within the CM population. In contrast, 109 hotspots were identified in the GM population including 45 R→R, 43 R→S, two S→S, and 19 S→R eQTL hotspots (Table S3.5). To visualize the physical locations of eQTL hotspots, SNPs and target genes for each hotspot were mapped onto a two-genome plot displaying SNP versus target gene locations (Figure 3.8).

While most hotspots targeted 23 genes or less, two R→S inheritance class SNPs in *MeSOAR1* together affected the expression of 119 target genes (Figure 3.8; Table S3.5). *MeSOAR1* is an RNA-binding protein and known master negative regulator of ABA responses in Arabidopsis (Ma et al., 2020). The expression of *MeSOAR1* itself was affected by both *MeSOAR1* *cis*-QTL SNPs (Chromosome03_7177401 and Chromosome03_7177022). SNP Chromosome03_7177022 caused a nonconservative amino acid change from the positively charged lysine to the negatively charged glutamic acid in 60444 (Lys303Glu), which was homozygous for the alternate allele, and one allele of ECU72 (heterozygous). In contrast, the Chromosome03_7177401 SNP was a silent mutation in *MeSOAR1*. These SNPs fell within the second exon of *MeSOAR1*, but are not within any known functional domain of the protein, so their effect on *MeSOAR1*'s function remains unclear.

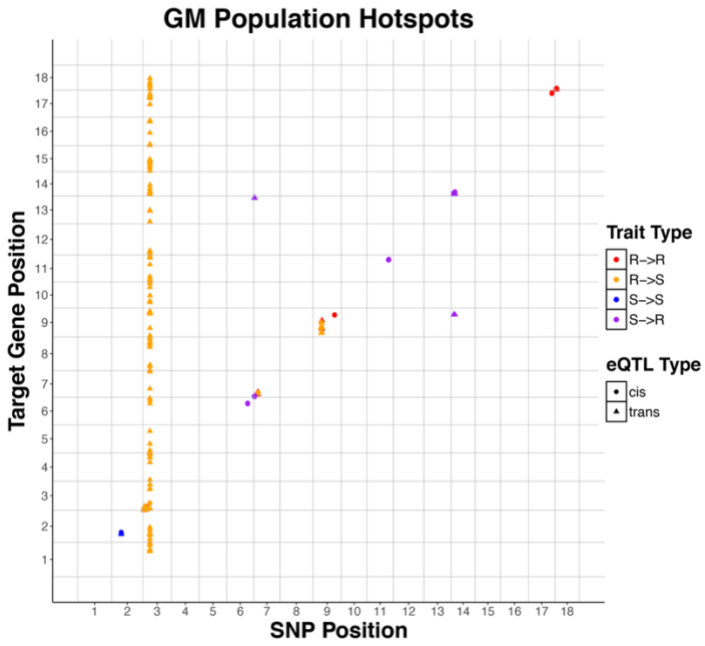

Figure 3.8. eQTL hotspots within the GM population.

Chromosome positions of eQTL hotspots, displaying the SNP position along the x-axis and the positions of target genes along the y-axis. Hotspots are colored according to inheritance class (R→R: red; R→S: orange; S→S: blue, S→R: purple) with shape denoting *cis*- (circle) versus *trans*- (triangle) eQTLs. The locations of five target genes that have not been assigned to cassava chromosomes are not shown. eQTL hotspot SNP and target gene identities and functional bins are provided in Table S3.5.

Among *MeSOAR1*'s 119 targets, 20 genes with known roles in defense were identified (Figures 3.9 and 3.10; Table 3.1) and the effect of the two SNPs within *MeSOAR1* on the expression of such target genes was further examined. Target genes were divided into those with known roles in ABA-, redox-, cell-death-, or other defense-related processes. At 0 dpi, *MeSOAR1* was a genotype DEG, with significantly lower expression in ECU72 versus 60444 (a 0-dpi DEG) (Table 3.1), while all *MeSOAR1 trans*-eQTL targets (excluding *MeERD15*, *MeBBD1*, *MeFITNESS*, and *MePR-5b*) had higher expression in ECU72 versus 60444 at 0 dpi (Figures 3.9 and 3.10). Additionally, such

targets (excluding *MeCcdA* and *MeHXK1*) had higher transcript levels in ECU72 at one or more infestation time points (Figures 3.9 and 3.10). Collectively, these results support that *MeSOAR1* may similarly act as a negative regulator of not only ABA but redox, cell death, and other processes in cassava.

This premise is further supported by the facts that six *MeSOAR1* targets (*MeTRE1*, *MeTHI*, *MeRR2, MeGI, MeROC3, and MeERD15*) are known to be involved in ABA-mediated or -independent stomatal regulation or closure (Aalto et al., 2012, Li et al., 2016a, Van Houtte et al., 2013, Wang et al., 2020). These genes are more highly expressed by 0 or 1 dpi in ECU72 versus 60444, suggesting that ABA-mediated (via *MeROC3, MeGI*, *MeTRE1*, and/or *MeTHI*) or ABA-independent (via *MeRR2*) mechanisms of stomatal closure during early infestation regulated by *MeSOAR1* may be important for resistance to whitefly (Figures 3.9-3.10). Two other *MeSOAR1* targets, *MeADF4* and *MeBBD1,* which promote callose deposition (Mondal et al., 2018, You et al., 2010), were more highly expressed at 7 dpi in ECU72, suggesting that this process may be stimulated by contact with eggs or egg secretions for the purpose of priming defenses for future feeding of early-stage nymphs. As all *MeSOAR1* eQTLs were of the R$\rightarrow$S inheritance class, phenotypes of the resistant and susceptible parents were opposite to those of the resistant and susceptible progeny, making a correlation of *MeSOAR1* with resistance or susceptibility less clear (Figures 3.9-3.10). However, as previous work has implicated ABA-mediated processes are important for whitefly resistance in ECU72 (Chapter 2), we believe these findings still suggest dampened expression of ABA response negative regulator *MeSOAR1* is important for whitefly resistance in ECU72.

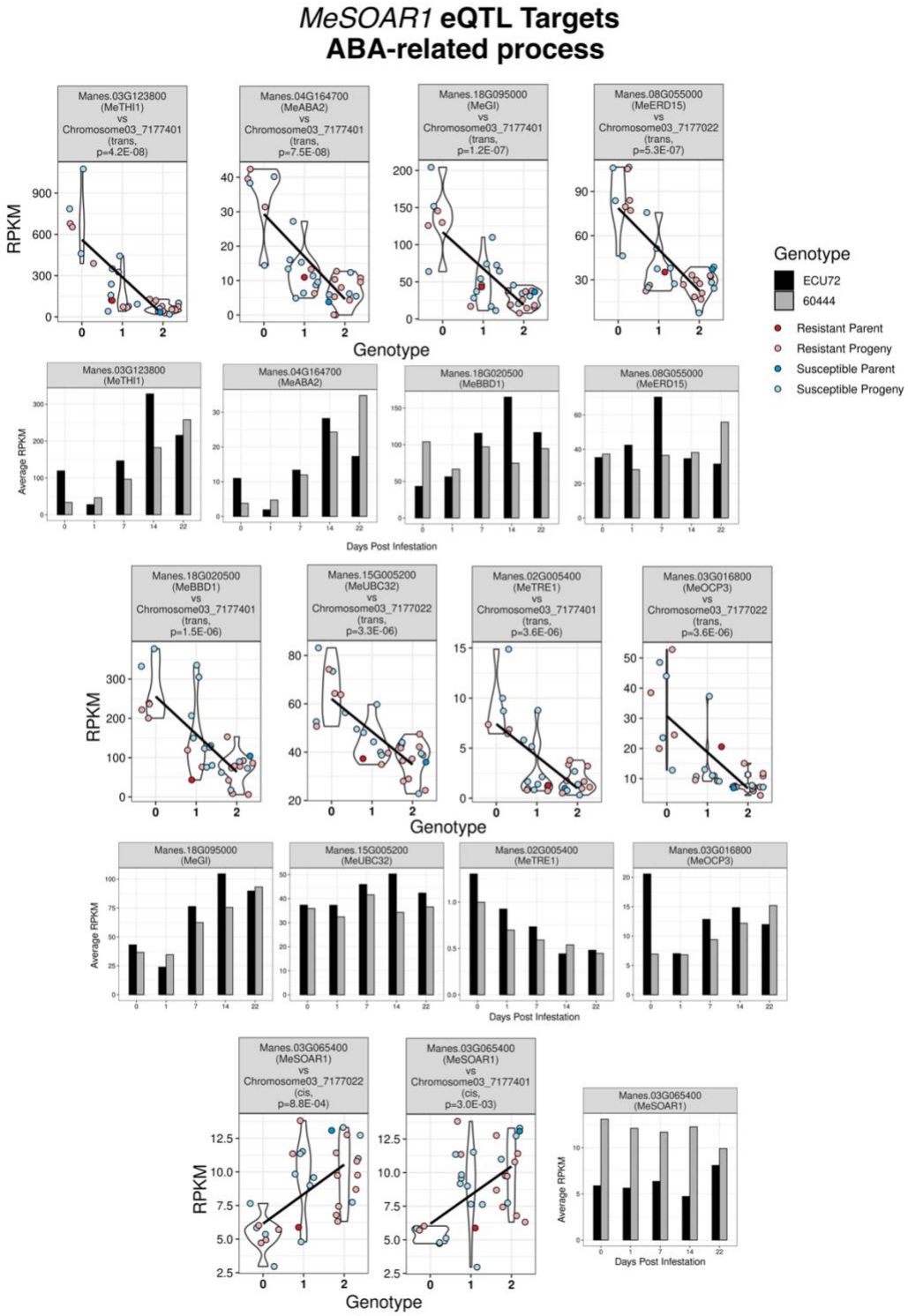
MeSOAR1 eQTL Targets
ABA-related process
Manes.03G123800 (MeTHI1) vs Chromosome03_7177401 (trans, p=4.2E-08)
Manes.04G164700 (MeABA2) vs Chromosome03_7177401 (trans, p=7.5E-08)
Manes.18G095000 (MeGI) vs Chromosome03_7177401 (trans, p=1.2E-07)
Manes.08G055000 (MeERD15) vs Chromosome03_7177022 (trans, p=5.3E-07)
RPKM
Genotype
Genotype
ECU72
60444
Resistant Parent
Resistant Progeny
Susceptible Parent
Susceptible Progeny
Manes.03G123800 (MeTHI1)
Manes.04G164700 (MeABA2)
Manes.18G020500 (MeBBD1)
Manes.08G055000 (MeERD15)
Average RPKM
Days Post Infestation
Manes.18G020500 (MeBBD1) vs Chromosome03_7177401 (trans, p=1.5E-06)
Manes.15G005200 (MeUBC32) vs Chromosome03_7177022 (trans, p=3.3E-06)
Manes.02G005400 (MeTRE1) vs Chromosome03_7177401 (trans, p=3.6E-06)
Manes.03G016800 (MeOCP3) vs Chromosome03_7177022 (trans, p=3.6E-06)
Manes.18G095000 (MeGI)
Manes.15G005200 (MeUBC32)
Manes.02G005400 (MeTRE1)
Manes.03G016800 (MeOCP3)
Manes.03G065400 (MeSOAR1) vs Chromosome03_7177022 (cis, p=8.8E-04)
Manes.03G065400 (MeSOAR1) vs Chromosome03_7177401 (cis, p=3.0E-03)
Manes.03G065400 (MeSOAR1)

Figure 3.9. eQTL hotspot target genes of the SNP-containing gene *MeSOAR1*.

Two separate SNPs within *MeSOAR1* (Chromosome03_7177401 and Chromosome03_7177022) affect the expression of 119 target genes in the GM population, of which nine genes involved in ABA processes are visualized as violin and bar plots. Genotypes are denoted as described in Figure 3.6. Whitefly-resistant genotypes are colored red (ECU72) and light red (resistant progeny) versus blue (60444) and light blue (susceptible progeny). ID of SNP affecting ("versus") target gene, eQTL type (*cis* or *trans*), inheritance class, and *p*-value are labeled in each violin plot. Violin plots in each panel are ordered from lowest to highest *p*-value. All eQTL-associated SNPs and target genes presented here are of the R→S inheritance class. The statistical relevance of the expression data for the eQTL-identified genes can be found in Chapter 2 (Table S2.1).

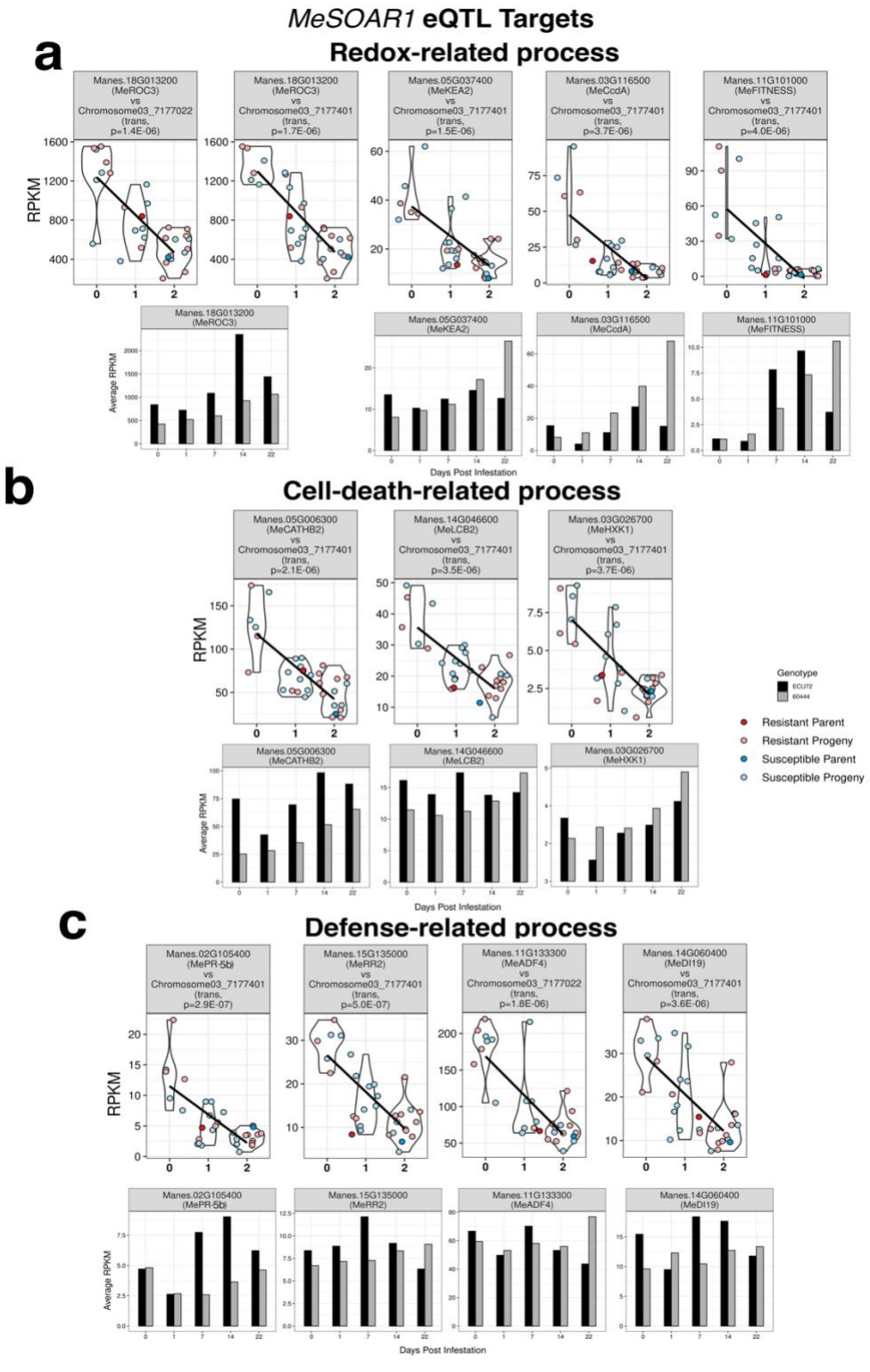
MeSOAR1 eQTL Targets
a
Redox-related process
Manes.18G013200 (MeROC3) vs Chromosome03_7177022 (trans, p=1.4E-06)
Manes.18G013200 (MeROC3) vs Chromosome03_7177401 (trans, p=1.7E-06)
Manes.05G037400 (MeKEA2) vs Chromosome03_7177401 (trans, p=1.5E-06)
Manes.03G116500 (MeCcdA) vs Chromosome03_7177401 (trans, p=3.7E-06)
Manes.11G101000 (MeFITNESS) vs Chromosome03_7177401 (trans, p=4.0E-06)
RPKM
Average RPKM
Days Post Infestation
b
Cell-death-related process
Manes.05G006300 (MeCATHB2) vs Chromosome03_7177401 (trans, p=2.1E-06)
Manes.14G046600 (MeLCB2) vs Chromosome03_7177401 (trans, p=3.5E-06)
Manes.03G026700 (MeHXK1) vs Chromosome03_7177401 (trans, p=3.7E-06)
Genotype
ECU72
60444
Resistant Parent
Resistant Progeny
Susceptible Parent
Susceptible Progeny
c
Defense-related process
Manes.02G105400 (MePR-5bi) vs Chromosome03_7177401 (trans, p=2.9E-07)
Manes.15G135000 (MeRR2) vs Chromosome03_7177401 (trans, p=5.0E-07)
Manes.11G133300 (MeADF4) vs Chromosome03_7177022 (trans, p=1.8E-06)
Manes.14G060400 (MeDI19) vs Chromosome03_7177401 (trans, p=3.6E-06)

Figure 3.10. eQTL hotspot target genes of the SNP-containing gene *MeSOAR1*.

Two separate SNPs within *MeSOAR1* (Chromosome03_7177401 and Chromosome03_7177022) affect the expression of 119 target genes in the GM population, of which those involved in **(a)** redox (four genes), **(b)** cell death (three genes) and **(c)** defense (four genes) processes are visualized as violin and bar plots here. Genotypes are denoted as described in Figure 3.6. Whitefly-resistant genotypes are colored red (ECU72) and light red (resistant progeny) versus blue (60444) and light blue (susceptible progeny). ID of SNP affecting ("versus") target gene, eQTL type (*cis* or *trans*), inheritance class, and *p*-value are labeled in each violin plot. Violin plots in each panel are ordered from lowest to highest *p*-value. All eQTL-associated SNPs and target genes presented here are of the R→S inheritance class. The statistical relevance of the expression data for the eQTL-identified genes can be found in Chapter 2 (Table S2.1).

Other eQTLs with known roles in defense were also identified within the R→R, R→S, S→S, and S→R type eQTL hotspots, fitting into ABA-, ET-, SA-, cell-wall-, redox-, cell-death-, or other defense-related processes (Figures 3.11-3.16; Tables 3.1 and S3.5). In several cases, eQTL-hotspot-SNPs affected the expression of multiple target genes and conversely, eQTL-hotspot-identified target genes were affected by multiple SNPs belonging to one or more inheritance classes (Figures 3.11-3.16).

R→R type eQTLs had SNPs within genes or gene targets involved in ABA-, ET- or defense-related processes (Figures 3.11 and 3.12). Two *cis*-eQTL target genes, which regulate ABA sensitivity, *MeCOL4* and *MeHD2C* (Luo et al., 2012, Min et al., 2015), were associated with SNPs in *MeKNAT3* (Qin et al., 2020), a positive regulator of monolignol biosynthesis, and *MeEIN2*, a positive regulator of ET signaling (Ju et al., 2012), respectively (Figure 3.11). *MeCOL4* transcript levels were higher in 60444 and susceptible progeny at 0 dpi, while *MeHD2C* was more highly expressed in ECU72 and resistant progeny 0-7 dpi, suggesting that control of ABA sensitivity may be important in conferring resistance or susceptibility. Identities of the SNPs affecting these genes also suggest that lignin and ET pathways may have some function in regulating ABA sensitivity (Figure 3.11).

Several genes involved in immunity were also targeted by R→R hotspot SNPs. The *cis*-eQTL target genes *MePDX1* and *MeSSL4*, involved in bacterial resistance (Aoun et al., 2017, Zhang et al., 2015b), were expressed at lower levels in ECU72 and many resistant progeny versus 60444 and many susceptible progeny; these data suggest that the bacterial resistance responses activated in 60444 may be involved in whitefly susceptibility. Three NLRs (Manes.07G044700, Manes.07G044300 and

Manes.07G045400) were also targeted; one (Manes.07G045400) had higher expression in 60444 vs ECU72, although similar trends were not seen in the phenotypes of the progeny (Figure 3.12; Table 3.1).

In other cases, R→R type eQTL hotspots did not show well-correlated target gene expression between parental lines and progeny of the same phenotype, making the role of such eQTLs in resistance or susceptibility unclear (Figures 3.11 and 3.12). Examples include an R→R type *trans*-eQTL SNP within *MeAIRP3*, a negative regulator of ABA signaling (Pan et al., 2020). *MeAIRP3* targeted two *MePERK14* genes resulting in higher *MePERK14a* expression in ECU72 at 0-22 dpi and higher *MePERK14b* expression in ECU72 constitutively and during early infestation (Figures 3.11a). A second example is the *cis*-eQTL SNP in *MeKNAT3* that was associated with higher expression of *MeSHN3* at 0-14 dpi in ECU72; *SHN3* is involved in ET responses (Djemal and Khoudi, 2015) (Figures 3.11c). The third *cis*-eQTL target gene in this category is *MeRD19*. *RD19* is important for resistance mediated by the *R* gene *RRI* and was more highly expressed in ECU72 at 0 dpi (Bernoux et al., 2008). Lastly, *cis*-eQTL target gene *MeEAP3*, involved in glucosinolate trafficking, was more highly expressed in ECU72 at 0-1 dpi (Mao et al., 2017) (Figure 3.12). Although a role in resistance or susceptibly of the genes above cannot be inferred, such eQTL hotspots still provide additional evidence for the possible involvement of ABA, ET, immune, and glucosinolate pathways in cassava's response to whitefly.

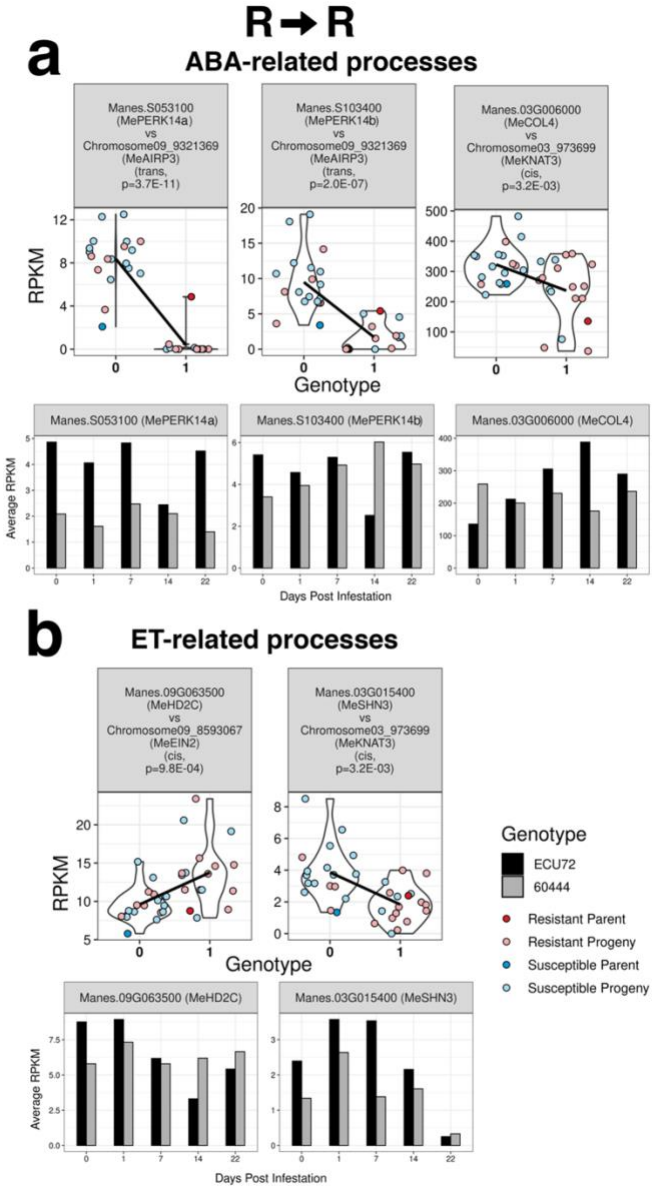
R → R
a
ABA-related processes
Manes.S053100 (MePERK14a) vs Chromosome09_9321369 (MeAIRP3) (trans, p=3.7E-11)
Manes.S103400 (MePERK14b) vs Chromosome09_9321369 (MeAIRP3) (trans, p=2.0E-07)
Manes.03G006000 (MeCOL4) vs Chromosome03_973699 (MeKNAT3) (cis, p=3.2E-03)
RPKM
Genotype
Manes.S053100 (MePERK14a)
Manes.S103400 (MePERK14b)
Manes.03G006000 (MeCOL4)
Average RPKM
Days Post Infestation
b
ET-related processes
Manes.09G063500 (MeHD2C) vs Chromosome09_8593067 (MeEIN2) (cis, p=9.8E-04)
Manes.03G015400 (MeSHN3) vs Chromosome03_973699 (MeKNAT3) (cis, p=3.2E-03)
Genotype
ECU72
60444
Resistant Parent
Resistant Progeny
Susceptible Parent
Susceptible Progeny
Manes.09G063500 (MeHD2C)
Manes.03G015400 (MeSHN3)
Average RPKM
Days Post Infestation

Figure 3.11. eQTL hotspot target genes for R→R inheritance class SNPs for ABA- and ET-related processes.

Violin plots displaying the effect of R→R inheritance class SNPs on the expression of their target genes accompanied by bar graphs displaying the expression of target genes during whitefly infestation in ECU72 and 60444. Target genes shown are categorized as **(a)** ABA-related and **(b)** ET-related processes. Genotypes are denoted as in Figure 3.6. Whitefly-resistant genotypes are colored red (ECU72) and light red (resistant progeny) versus blue (60444) and light blue (susceptible progeny) in violin plots. Expression is displayed as average RPKM values and colored in black for ECU72 and grey for 60444 in bar graphs. ID of SNP affecting ("versus") target gene, eQTL type (*cis* or *trans*), inheritance class, and *p*-value are labeled in each violin plot. Violin plots in each panel are ordered from lowest to highest *p*-value. All eQTL-associated SNPs and target genes presented here are of the R→R inheritance class. The statistical relevance of the expression data for the eQTL-identified genes can be found in Chapter 2 (Table S2.1).

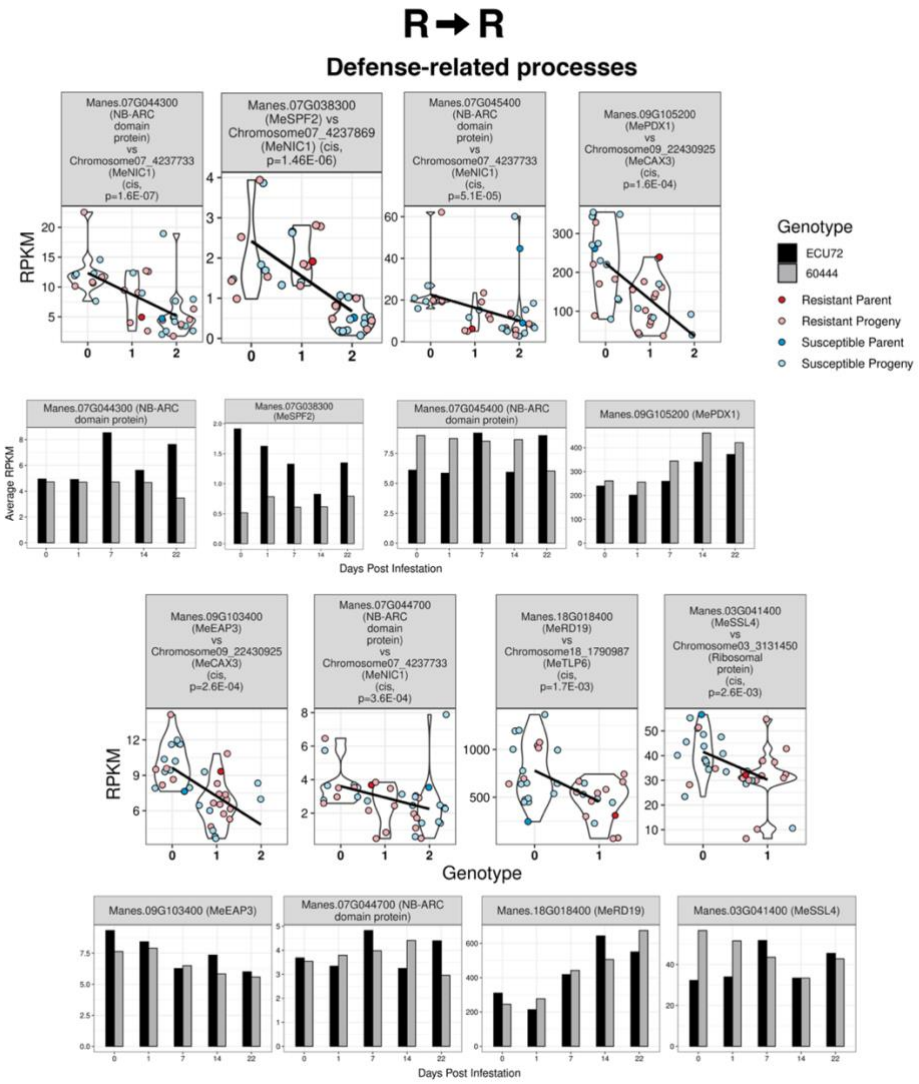

Figure 3.12. eQTL hotspot target genes for R→R inheritance class SNPs for defense-related processes.

Violin plots displaying the effect of R→R inheritance class SNPs on the expression of their target genes accompanied by bar graphs displaying the expression of target genes during whitefly infestation in ECU72 and 60444. Target genes shown are categorized in defense-related processes. Genotypes are denoted as in Figure 3.6. Whitefly-resistant genotypes are colored red (ECU72) and light red (resistant progeny) versus blue (60444) and light blue (susceptible progeny) in violin plots. Expression is displayed as average RPKM values and colored in black for ECU72 and grey for 60444 in bar graphs. ID of SNP affecting ("versus") target gene, eQTL type (*cis* or *trans*), inheritance class, and *p*-value are labeled in each violin plot. Violin plots in each panel are ordered from lowest to highest *p*-value. All eQTL-associated SNPs and target genes presented here are of the R→R inheritance class. The statistical relevance of the expression data for the eQTL-identified genes can be found in Chapter 2 (Table S2.1).

R→S type eQTL hotspots also had SNPs within genes or gene targets involved in ET- or defense-related processes (Figure 3.13). A *cis*-eQTL SNP within *MeEBF1*, a negative regulator of ET signaling (An et al., 2010), resulted in higher 0-14 dpi expression in ECU72 of ET-response gene *MeSHN3* (an ET-responsive transcription factor) and *MeOCP3* (a regulator of callose deposition in response to fungal pathogens) (Garcia-Andrade et al., 2011). *MeHD2C*, a regulator of ABA sensitivity (Luo et al., 2012), was also more highly expressed in ECU72 0-7 dpi due to a *cis*-eQTL SNP in *MeEIN2* (Figure 3.13a). *MeSHN3* and *MeHD2C* are both examples of target genes affected by multiple eQTL-associated SNPs. For the *MeSHN3* eQTL, a high percentage of progeny shared the parental alleles with distinct expression differences seen between genotypes, however as an R→S eQTL it is unclear if this trait is important for resistance or susceptibility. Two NLRs were also *cis*-eQTL targets of an R→S type SNP in heat shock protein *MeHSC70-5*, one with higher (Manes.07G044300) and one with lower (Manes.07G045400) expression in ECU72 at 0 dpi (Figure 3.13b). As R→S type eQTLs do not show correlated target gene expression between parental lines and progeny of the same phenotype, the roles of these target genes (*MeSHN3*, *MeHD2C* and NLRs Manes.07G044300 and Manes.07G045400) in ABA sensitivity, ET response, immunity, and callose deposition in relation to whitefly resistance or susceptibility remains unclear.

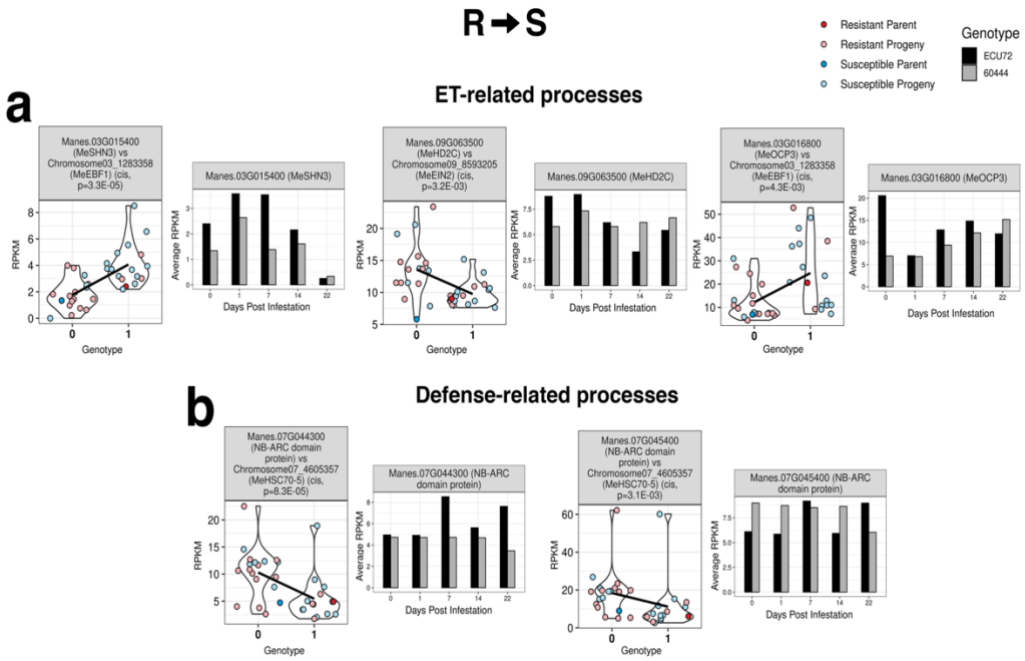

Figure 3.13. eQTL hotspot target genes for R→S inheritance class SNPs involved with ET- and defense-related processes.

Violin plots displaying the effect of R→S inheritance class SNPs on the expression of their target genes accompanied by bar graphs displaying the expression of target genes during whitefly infestation in ECU72 and 60444. Target genes shown are categorized in **(a)** ET-related and **(b)** defense-related processes. Genotypes are denoted as in Figure 3.6. Whitefly-resistant genotypes are colored red (ECU72) and light red (resistant progeny) versus blue (60444) and light blue (susceptible progeny) in violin plots. Expression is displayed as average RPKM values and colored in black for ECU72 and grey for 60444 in bar graphs. ID of SNP affecting ("versus") target gene, eQTL type (*cis* or *trans*), inheritance class, and *p*-value are labeled in each violin plot. Violin plots in each panel are ordered from lowest to highest *p*-value. All eQTL-associated SNPs and target genes presented here are of the R→S inheritance class. The statistical relevance of the expression data for the eQTL-identified genes can be found in Chapter 2 (Table S2.1). Note that hotspot SNPs or target genes with no known roles in defense are not listed in Table 3.1.

Among S→S type eQTLs hotspots, *trans*-eQTL target *MeASK2*, involved in ABA sensitivity (Li et al., 2012a), and an NLR (Manes.07G008700) *cis*-eQTL target were identified (Figure 3.14). Higher expression of *MeASK2* was clearly correlated with susceptibility in 60444 and susceptible progeny, with higher expression in 60444 versus ECU72 maintained throughout infestation. Thus, *MeASK2* regulation of ABA sensitivity may be important in whitefly susceptibility. The role of NLR Manes.07G008700 in resistance or susceptibility was however unclear; ECU72 and 60444 had similar 0 dpi expression levels higher than all F_1 progeny, but resistant progeny had lower expression levels than susceptible progeny at 0 dpi (Figure 3.14).

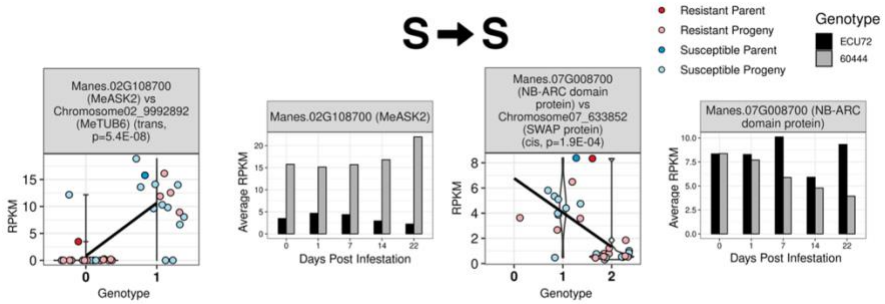

Figure 3.14. eQTL hotspot target genes for S→S inheritance class SNPs associated with defense.

Violin plots displaying the effect of S→S inheritance class SNPs on the expression of their target genes accompanied by bar graphs displaying the expression of target genes during whitefly infestation in ECU72 and 60444. Genotypes are denoted as in Figure 3.6. Whitefly-resistant genotypes are colored red (ECU72) and light red (resistant progeny) versus blue (60444) and light blue (susceptible progeny) in violin plots. Expression is displayed as average RPKM values and colored in black for ECU72 and grey for 60444 in bar graphs. ID of SNP affecting ("versus") target gene, eQTL type (*cis* or *trans*), inheritance class, and *p*-value are labeled in each violin plot. Violin plots in each panel are ordered from lowest to highest *p*-value. All eQTL-associated SNPs and target genes presented here are of the S→S inheritance class. The statistical relevance of the expression data for the eQTL-identified genes can be found in Chapter 2 (Table S2.1). Note that hotspot SNPs or target genes with no known roles in defense are not listed in Table 3.1.

Target genes or SNP-containing genes involved in ABA-, cell-wall-, redox-, and defense-related processes were identified among S→R type eQTL hotspots (Figure 3.15 and 3.16). Negative regulators of ABA signaling, *cis*-eQTL targets *MeROP10* and *MeATI-2* (Hou et al., 2016, Li et al., 2012b), showed similar expression trends, with *MeROP10* more highly expressed at 0-22 dpi and *MeATI-2* more highly expressed 0-14 dpi in ECU72 versus 60444 (Figures 3.15a). A SNP in *MeEX2*, a positive regulator of singlet-oxygen signaling, also regulates *MeROP10*, as well as two other *cis*-eQTL targets in the redox class: a glutaredoxin *MeGRXC1* (Riondet et al., 2012) and wound-responsive gene *MeWR3* (Leon et al., 1998). *MeGRXC1* was more highly expressed, while *MeWR3* was expressed at lower levels in ECU72 at 0-22 dpi (Figure 3.16a). ECU72 expression of *MeGRXC1* was notable higher than all F_1 progeny and 60444. A SNP in the redox sensor glutathione peroxidase *MeGPX2* (Passaia and Margis-Pinheiro, 2015) regulated the expression of two *cis*-eQTL *MeVEP1* target genes, involved in the wounding response (Yang et al., 1997). The *MeVEP1a* and *MeVEP1b* genes were expressed at approximately 9- to 20-fold higher levels in 60444 versus ECU72 throughout the entire infestation time course (Figures 3.16a). All F_1 progeny matching ECU72's genotype displayed very low to undetectable transcript levels. Four *MeMIK2* genes (*MeMIK2a,b* and *d*), all *cis*-eQTL targets, involved in cell wall damage sensing and fungal elicitor perception (Coleman et al., 2021) were also affected by SNPs in *MeGPX2* and *MeVEP1*. All four genes had higher transcript levels in 60444 versus ECU72 at 0-14 dpi. Two other cell-wall-related *cis*-eQTL target genes, *MePMEI* and *MeMSBP2* (Gou et al., 2018, Jolie et al., 2010), instead were more highly expressed in ECU72 versus 60444 at 0-22 dpi (Figure 3.15b). Three *cis*-eQTL target genes were involved in immunity, an NLR with similar expression in parental lines (Manes.07G008700), as well as a pathogen (*MeLRRAC1*) and insect resistance gene

(*MeATS3B*) more highly expressed in 60444 versus ECU72 at 0-22 dpi (Bianchet et al., 2019, Savadogo et al., 2021) (Figure 3.16b). For eQTL-identified genes *MeVEP1a*, *MeVEP1b* and *MeROP10*, resistant progeny had similar 0 dpi transcript levels as 60444, similar to susceptible progeny and ECU72 (Figures 3.15a and 3.16a). However, in S→R type eQTLs, expression of target genes cannot be strictly correlated with resistance or susceptibility. Still, these results suggest redox regulation of ABA, wounding, and cell-wall damage sensing responses may be important during cassava whitefly responses.

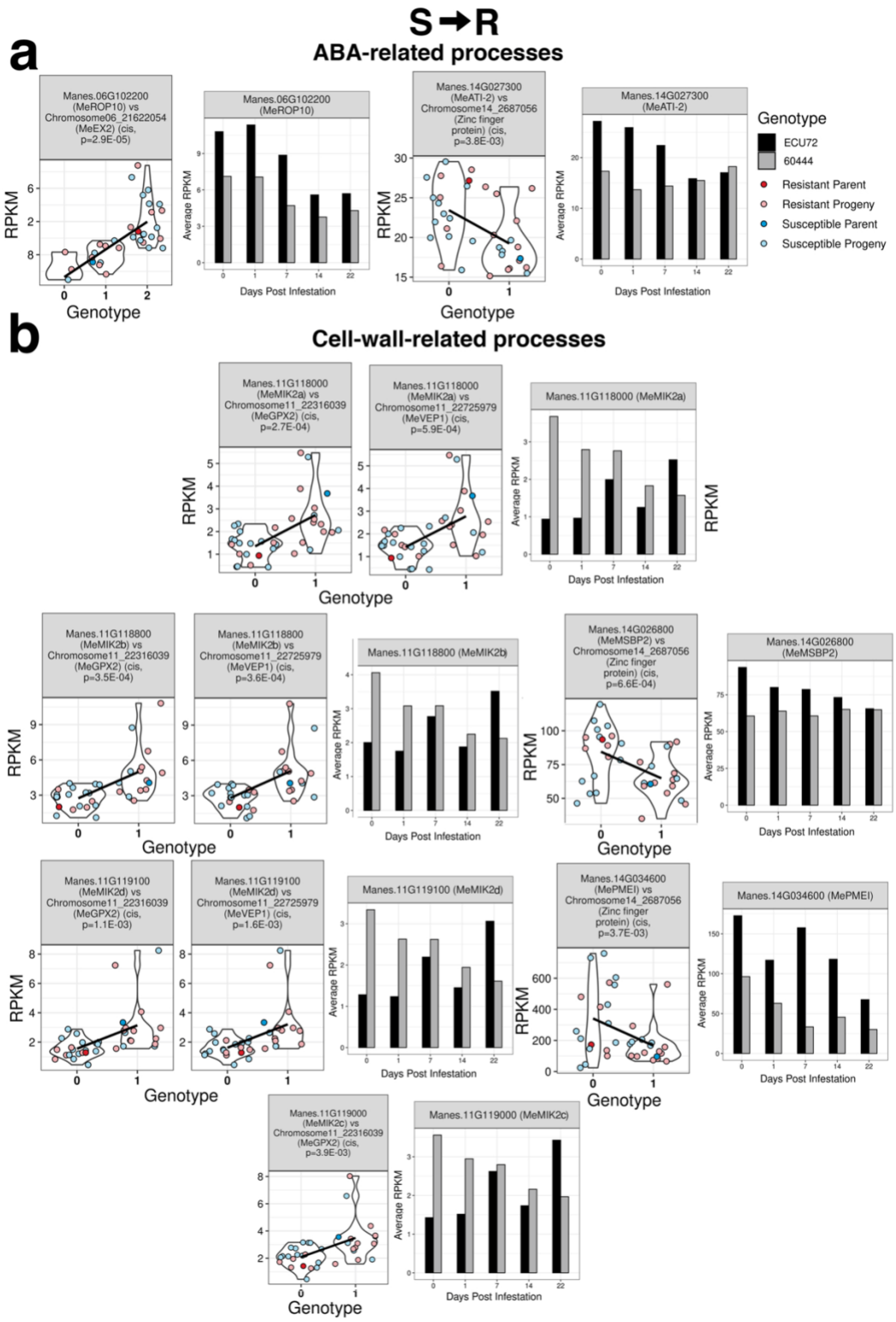
S→R
a
ABA-related processes
Manes.06G102200 (MeROP10) vs Chromosome06_21622054 (MeEX2) (cis, p=2.9E-05)
Manes.06G102200 (MeROP10)
Manes.14G027300 (MeATI-2) vs Chromosome14_2687056 (Zinc finger protein) (cis, p=3.8E-03)
Manes.14G027300 (MeATI-2)
RPKM
Average RPKM
Genotype
Days Post Infestation
Genotype
ECU72
60444
Resistant Parent
Resistant Progeny
Susceptible Parent
Susceptible Progeny
b
Cell-wall-related processes
Manes.11G118000 (MeMIK2a) vs Chromosome11_22316039 (MeGPX2) (cis, p=2.7E-04)
Manes.11G118000 (MeMIK2a) vs Chromosome11_22725979 (MeVEP1) (cis, p=5.9E-04)
Manes.11G118000 (MeMIK2a)
Manes.11G118800 (MeMIK2b) vs Chromosome11_22316039 (MeGPX2) (cis, p=3.5E-04)
Manes.11G118800 (MeMIK2b) vs Chromosome11_22725979 (MeVEP1) (cis, p=3.6E-04)
Manes.11G118800 (MeMIK2b)
Manes.14G026800 (MeMSBP2) vs Chromosome14_2687056 (Zinc finger protein) (cis, p=6.6E-04)
Manes.14G026800 (MeMSBP2)
Manes.11G119100 (MeMIK2d) vs Chromosome11_22316039 (MeGPX2) (cis, p=1.1E-03)
Manes.11G119100 (MeMIK2d) vs Chromosome11_22725979 (MeVEP1) (cis, p=1.6E-03)
Manes.11G119100 (MeMIK2d)
Manes.14G034600 (MePMEI) vs Chromosome14_2687056 (Zinc finger protein) (cis, p=3.7E-03)
Manes.14G034600 (MePMEI)
Manes.11G119000 (MeMIK2c) vs Chromosome11_22316039 (MeGPX2) (cis, p=3.9E-03)
Manes.11G119000 (MeMIK2c)

Figure 3.15. eQTL hotspot target genes for S→R inheritance class SNPs associated with ABA- and cell wall-related processes.

Violin plots displaying the effect of S→R inheritance class SNPs on the expression of their target genes accompanied by bar graphs displaying the expression of target genes during whitefly infestation in ECU72 and 60444. Target genes are categorized in **(a)** ABA- and **(b)** cell wall-related processes. Genotypes are denoted as in Figure 3.6. Whitefly-resistant genotypes are colored red (ECU72) and light red (resistant progeny) versus blue (60444) and light blue (susceptible progeny) in violin plots. Expression is displayed as average RPKM values and colored in black for ECU72 and grey for 60444 in bar graphs. ID of SNP affecting ("versus") target gene, eQTL type (*cis* or *trans*), inheritance class, and *p*-value are labeled in each violin plot. Violin plots in each panel are ordered from lowest to highest *p*-value. All eQTL-associated SNPs and target genes presented here are of the S→R inheritance class. The statistical relevance of the expression data for the eQTL-identified genes can be found in Chapter 2 (Table S2.1). Note that hotspot SNPs or target genes with no known roles in defense are not listed in Table 3.1.

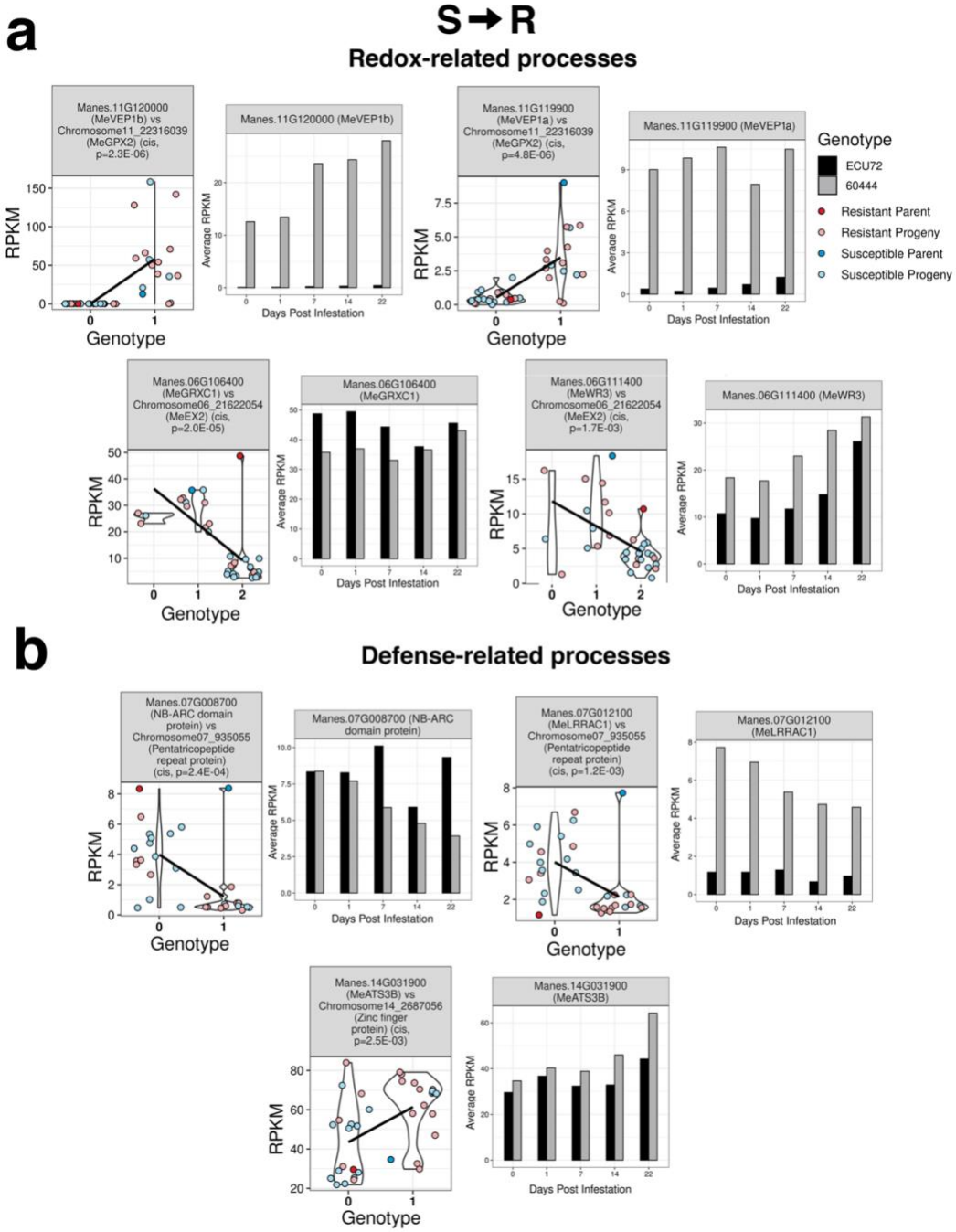
a
S➔R
Redox-related processes
Manes.11G120000 (MeVEP1b) vs Chromosome11_22316039 (MeGPX2) (cis, p=2.3E-06)
Manes.11G120000 (MeVEP1b)
Manes.11G119900 (MeVEP1a) vs Chromosome11_22316039 (MeGPX2) (cis, p=4.8E-06)
Manes.11G119900 (MeVEP1a)
Genotype
ECU72
60444
Resistant Parent
Resistant Progeny
Susceptible Parent
Susceptible Progeny
RPKM
Average RPKM
Genotype
Days Post Infestation
Manes.06G106400 (MeGRXC1) vs Chromosome06_21622054 (MeEX2) (cis, p=2.0E-05)
Manes.06G106400 (MeGRXC1)
Manes.06G111400 (MeWR3) vs Chromosome06_21622054 (MeEX2) (cis, p=1.7E-03)
Manes.06G111400 (MeWR3)
b
Defense-related processes
Manes.07G008700 (NB-ARC domain protein) vs Chromosome07_935055 (Pentatricopeptide repeat protein) (cis, p=2.4E-04)
Manes.07G008700 (NB-ARC domain protein)
Manes.07G012100 (MeLRRAC1) vs Chromosome07_935055 (Pentatricopeptide repeat protein) (cis, p=1.2E-03)
Manes.07G012100 (MeLRRAC1)
Manes.14G031900 (MeATS3B) vs Chromosome14_2687056 (Zinc finger protein) (cis, p=2.5E-03)
Manes.14G031900 (MeATS3B)

Figure 3.16. eQTL hotspot target genes for S→R inheritance class SNPs associated with redox- and defense-related processes.

Violin plots displaying the effect of S→R inheritance class SNPs on the expression of their target genes accompanied by bar graphs displaying the expression of target genes during whitefly infestation in ECU72 and 60444. Target genes are categorized in **(a)** redox- and **(b)** defense-related processes. Genotypes are denoted as in Figure 3.6. Whitefly-resistant genotypes are colored red (ECU72) and light red (resistant progeny) versus blue (60444) and light blue (susceptible progeny) in violin plots. Expression is displayed as average RPKM values and colored in black for ECU72 and grey for 60444 in bar graphs. ID of SNP affecting ("versus") target gene, eQTL type (*cis* or *trans*), inheritance class, and *p*-value are labeled in each violin plot. Violin plots in each panel are ordered from lowest to highest *p*-value. All eQTL-associated SNPs and target genes presented here are of the S→R inheritance class. The statistical relevance of the expression data for the eQTL-identified genes can be found in Chapter 2 (Table S2.1). Note that hotspot SNPs or target genes with no known roles in defense are not listed in Table 3.1.

eQTLs that are 0-h DEGs

Another criteria used to identify eQTLs possibly involved in defense against whiteflies was status as a differentially expressed gene (DEG) at 0 dpi between parental lines (genotype DEGs) (Chapter 2). We identified 47 and 51 eQTL target genes that were genotype DEGs between ECU72 and COL2246 or 60444 at 0 dpi, respectively (Table S3.6). Among these genes, two and five *cis*-eQTL target genes in the CM and GM population eQTLs, respectively, had a known role in defense and were further examined (Figure 3.17; Table 3.1). Target genes *MeABCG40*, an ABA importer (Finkelstein, 2013), and Manes.17G080400, involved in cell-wall-based oomycete susceptibility, were identified in the CM population (Figure 3.17). *MeABCG40* had elevated transcript levels in ECU72 versus COL2246 0-22 dpi and in resistant versus susceptible progeny at 0 dpi; therefore as a R→R eQTL, these data suggest that ABA transport may be important for whitefly resistance. In contrast, the expression of Manes.17G080400, which is an S→R eQTL, did not correlate strictly with resistance or susceptibility in the F_1 progeny, although its transcripts were at higher levels in COL2246 than ECU72 0-22 dpi.

In the GM population, eQTL target genes differentially expressed between parents at 0 dpi involved in cell-wall-, redox-, and defense-related processes were identified (Figure 3.17b-d). Target genes *MeCSLB5*, a cellulose synthase (Richmond and Somerville, 2000), and the glutathione peroxidase *MeGPX1* gene (Passaia and Margis-Pinheiro, 2015) showed clear correlation with susceptibility, with higher expression in COL2246 versus ECU72 at all infestation timepoints and in susceptible versus resistant F_1 progeny at 0 dpi. *MeWAK2*, a receptor that senses pathogen/wounding created pectin fragments (Kohorn et al., 2014), and *MeMOS11*, an mRNA exporter involved in NLR-mediated immunity (Dong et al., 2016), instead did not show clear expression correlation

with resistance/susceptibility, but were more highly expressed in COL2246 versus ECU72 0-22 dpi (Figure 3.17b-c). Together, these results suggest that higher expression of *MeCSLB5* and *MeGPX1* may be indicative of whitefly susceptibility.

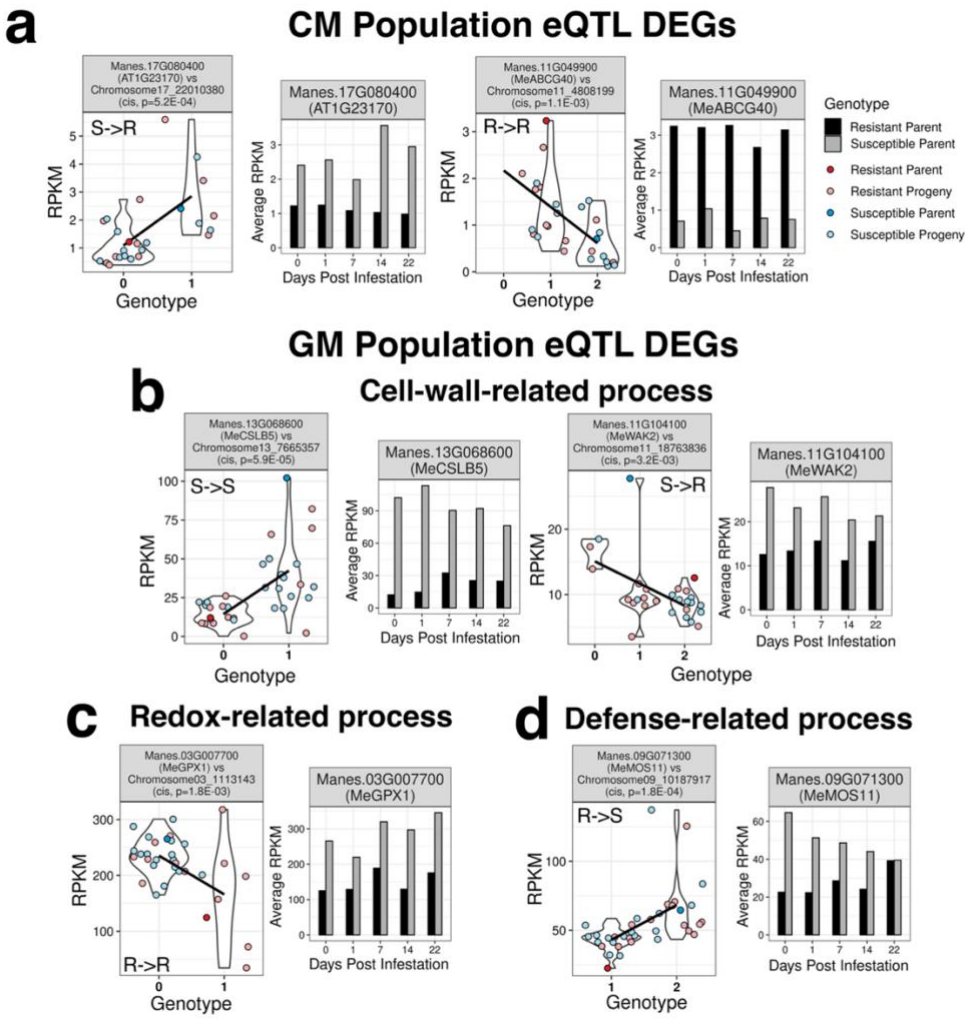

Figure 3.17. eQTLs with target genes classified as 0-h DEGs in the CM and GM populations.

Violin plots displaying the effect of eQTL SNPs on the expression of target genes that are differentially expressed between parents at 0 dpi accompanied by bar graphs displaying the expression of target genes during whitefly infestation in parental genotypes for **(a)** CM and **(b-d)** GM populations. GM population eQTLs are categorized in **(b)** cell-wall-, **(c)** redox-, and **(d)** defense-related processes. Genotypes are denoted as in Figure 3.6. Whitefly-resistant genotypes are colored red (ECU72) and light red (resistant progeny bulk) versus blue (COL2246 or 60444) and light blue (susceptible progeny bulk) in violin plots. Expression is displayed as average RPKM values and colored in black for ECU72 and grey for COL2246 or 60444 in bar graphs. ID of SNP affecting ("versus") target gene, eQTL type (*cis* or *trans*), inheritance class, and *p*-value are labeled in each violin plot. Violin plots in each panel are ordered from lowest to highest *p*-value. The statistical relevance of the expression data for the eQTL-identified genes can be found in Chapter 2 (Table S2.1).

CM and GM population shared eQTLs

To determine if any eQTLs may have an important role in ECU72's whitely resistance in comparison to both whitefly-susceptible genotypes (COL2246 and 60444), eQTLs identified in both CM and GM populations were identified (Figure 3.18; Table 3.1). Of the 309 and 768 eQTL target genes identified in CM and GM population eQTLs (Table S3.2), respectively, only 43 were shared target genes of both populations. While the majority of these genes were unable to be classified into functional bins, bin categories of protein biosynthesis, modification and translocation as well as chromatin organization were most prevalent (Figure 3.18).

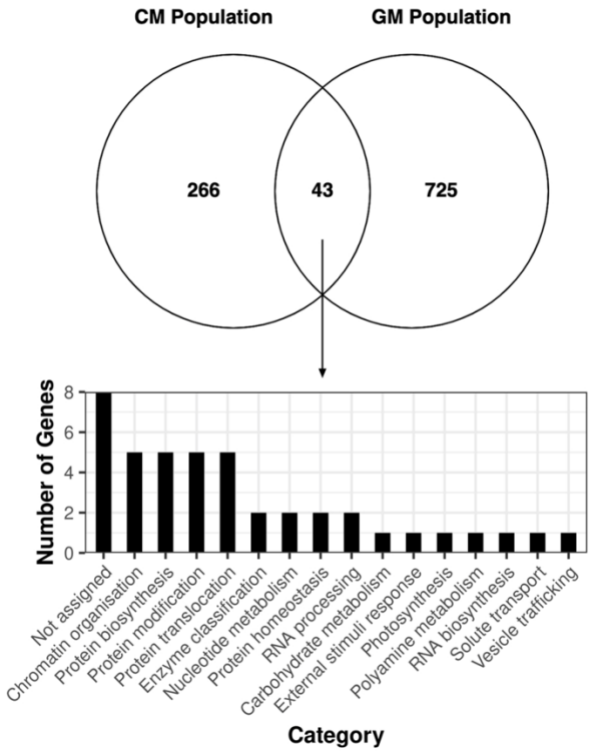

Figure 3.18. Overlap in CM and GM population eQTLs.

Venn diagram displaying numbers of eQTLs shared by or unique to the CM and GM populations. A bar graph depicts the number of eQTL target genes shared between the two populations within each Mercator functional bin. Mercator bins are ordered by the number of genes in each category.

The 43 shared target gene functionalities were additionally examined, identifying four genes involved in either ABA-, ET-, or other defense-related processes (Figure 3.19; Table 3.1). Most striking, *MeASK2*, a regulator of ABA sensitivity, was more highly expressed in COL2246 and 60444 versus ECU72 at 0-22 dpi (Figure 3.19a); in addition, *MeASK2* was a 0-dpi genotype DEG in the comparison of ECU72 to COL2246 (Table 3.1). *MeASK2* was a *cis*-eQTL target in the CM population but was affected by both *cis*- and *trans*-eQTLs in the GM population. For two SNPs affecting *MeASK2* expression in the GM population (Chromosome02_7964174 and Chromosome02_9052239), 60444 0 dpi

transcript levels were much higher than those of any F_1 progeny of the same genotype (Figure 3.19a). Epigenetic regulator of *MeEIN2*, *cis*-eQTL target *MeEEN* (Zander et al., 2019), also had slightly higher transcript levels at 0-1 dpi in both susceptible genotypes (Figure 3.19b). In some cases resistant whereas in other cases susceptible progeny showed higher expression among the different eQTLs identifying *MeEIN2* in the two populations.

cis-eQTL target *MeLRK10L1.2*, a leaf rust resistance gene (Shin et al., 2015), was identified by three SNPs and was more highly expressed in ECU72 compared to COL2246 but not 60444 at 0 dpi. In 60444, *MeLRK10L1.2* shows little temporal response to infestation unlike ECU72 and COL2246. At 1, 14 and 22 dpi, this gene was expressed at lower levels in ECU72 compared to COL2246 (Figure 3.19c). *MeSPF2* was a *cis*-eQTL target in the CM population but affected by both *cis*- and *trans*-eQTLs in the GM population. Strict correlation of *MeSPF2*, which activates whitefly defense protein WRKY33 (Verma et al., 2021, Wang et al., 2019), with resistance was observed in the GM population. Higher expression of *MeSPF2* in ECU72 and resistant progeny versus 60444 and susceptible progeny at 0-22 dpi was observed. Such correlation was not observed for the CM population eQTL targeting *MeSPF2*. Among other eQTL targets shared between populations, strict correlation of target gene expression between parents and progeny of the same phenotype was not observed (Figure 3.19).

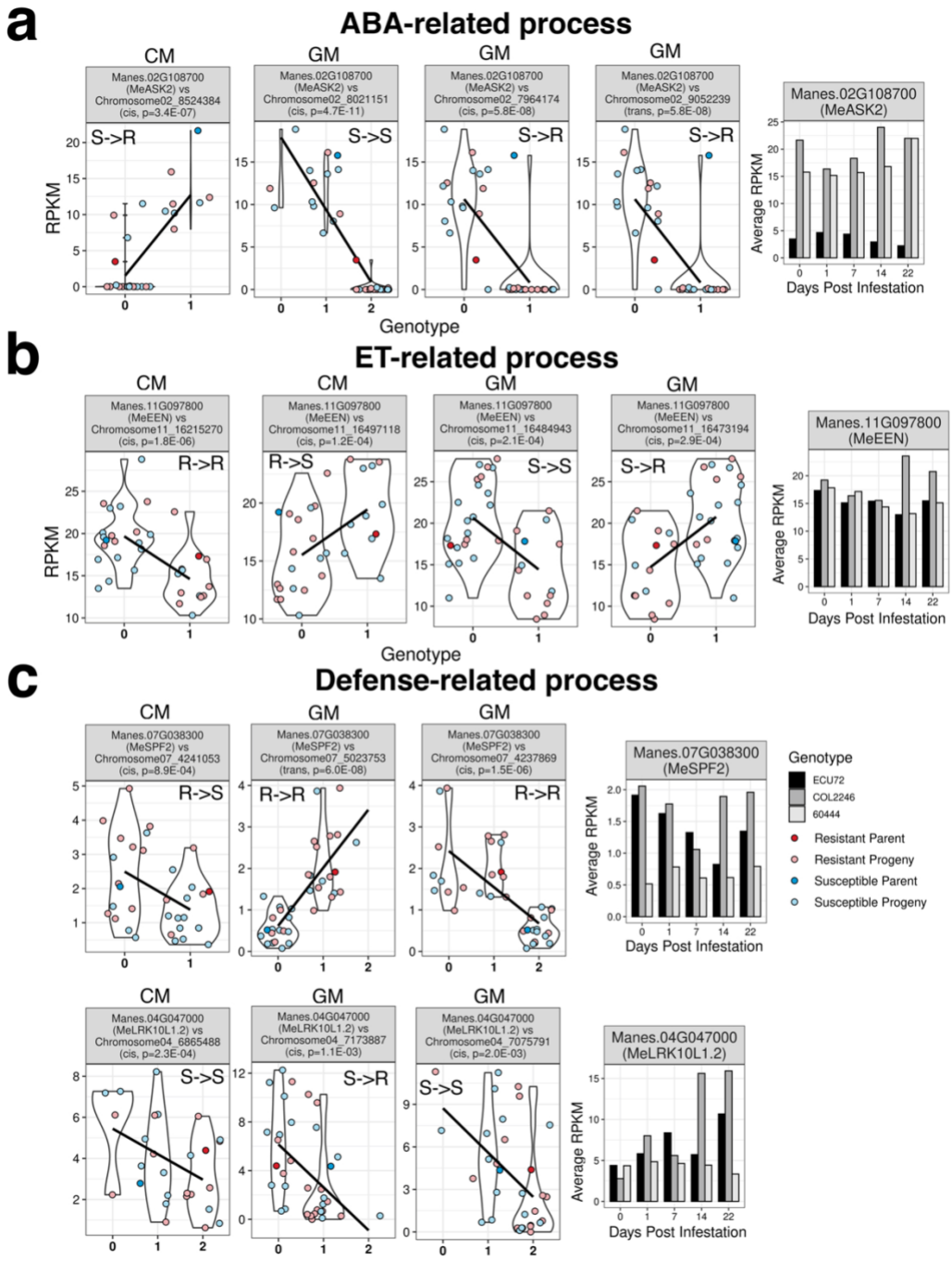
a
ABA-related process
CM
GM
GM
GM
Manes.02G108700 (MeASK2) vs Chromosome02_8524384 (cis, p=3.4E-07)
Manes.02G108700 (MeASK2) vs Chromosome02_8021151 (cis, p=4.7E-11)
Manes.02G108700 (MeASK2) vs Chromosome02_7964174 (cis, p=5.8E-08)
Manes.02G108700 (MeASK2) vs Chromosome02_9052239 (trans, p=5.8E-08)
S->R
S->S
S->R
S->R
RPKM
Genotype
Manes.02G108700 (MeASK2)
Average RPKM
Days Post Infestation
b
ET-related process
CM
CM
GM
GM
Manes.11G097800 (MeEEN) vs Chromosome11_16215270 (cis, p=1.8E-06)
Manes.11G097800 (MeEEN) vs Chromosome11_16497118 (cis, p=1.2E-04)
Manes.11G097800 (MeEEN) vs Chromosome11_16484943 (cis, p=2.1E-04)
Manes.11G097800 (MeEEN) vs Chromosome11_16473194 (cis, p=2.9E-04)
R->R
R->S
S->S
S->R
RPKM
Genotype
Manes.11G097800 (MeEEN)
Average RPKM
Days Post Infestation
c
Defense-related process
CM
GM
GM
Manes.07G038300 (MeSPF2) vs Chromosome07_4241053 (cis, p=8.9E-04)
Manes.07G038300 (MeSPF2) vs Chromosome07_5023753 (trans, p=6.0E-08)
Manes.07G038300 (MeSPF2) vs Chromosome07_4237869 (cis, p=1.5E-06)
R->S
R->R
R->R
Manes.07G038300 (MeSPF2)
Average RPKM
Days Post Infestation
Genotype
ECU72
COL2246
60444
Resistant Parent
Resistant Progeny
Susceptible Parent
Susceptible Progeny
CM
GM
GM
Manes.04G047000 (MeLRK10L1.2) vs Chromosome04_6865488 (cis, p=2.3E-04)
Manes.04G047000 (MeLRK10L1.2) vs Chromosome04_7173887 (cis, p=1.1E-03)
Manes.04G047000 (MeLRK10L1.2) vs Chromosome04_7075791 (cis, p=2.0E-03)
S->S
S->R
S->S
Manes.04G047000 (MeLRK10L1.2)
Average RPKM
Days Post Infestation

Figure 3.19. eQTLs shared in CM and GM populations.

Violin plots displaying eQTLs identified in both CM and GM populations accompanied by bar graphs displaying the expression of target genes during whitefly infestation in parental genotypes. eQTLs are categorized in **(a)** ABA-related, **(b)** ET-related and **(c)** defense-related processes. Genotypes are denoted as in Figure 3.6. Whitefly-resistant genotypes are colored red (ECU72) and light red (resistant progeny) versus blue (COL2246 or 60444) and light blue (susceptible progeny) in violin plots. Expression is displayed as average RPKM values and colored in black for ECU72 and grey for COL2246 or 60444 in bar graphs. Population, ID of SNP affecting ("versus") target gene, eQTL type (*cis* or *trans*), inheritance class, and *p*-value are labeled in each violin plot. Violin plots in each panel are ordered from lowest to highest *p*-value. The statistical relevance of the expression data for the eQTL-identified genes can be found in Chapter 2 (Table S2.1).

Other eQTLs of interest

Lastly, among all eQTL targets in both populations, an additional four *cis*-eQTL target genes not fitting aforementioned criteria but with known roles in defense were identified within the CM or GM populations (Figure 3.20; Table 3.1). In the CM population, higher expression of *MeENO2* was strictly correlated with susceptibility in COL2246 at 0-22 dpi and in susceptible progeny at 0 dpi (Figure 3.20a). An alternative translation product of *AtENO2* positively regulates ABA responses (Kang et al., 2013); for this reason, additional information is required to understand the role of this gene in whitefly susceptibility. In the GM population, eQTL target genes involved in ABA-, ET-, and SA-related processes were identified (Figure 3.20b-d). Expression of *MeOST1*, positive regulator of ABA-mediated stomatal closure (Yoshida et al., 2006), was higher in ECU72 versus COL2246 at 0,1 and 22 dpi. In contrast, expression of *MePR-3d*, ET-induced *in* Arabidopsis (van Loon et al., 2006), and the SA receptor *MeNPR4* (Liu et al., 2020) was lower in ECU72 versus COL2246 at 0-1 dpi, with *MePR-3d* more highly induced in ECU72 7-22 dpi and *MeNPR4* maintaining higher COL2246 expression throughout infestation. There was not strict correlation of these three gene's expression in resistant or susceptible F_1 progeny, so their role in whitefly response remains unclear (Figure 3.20b-d).

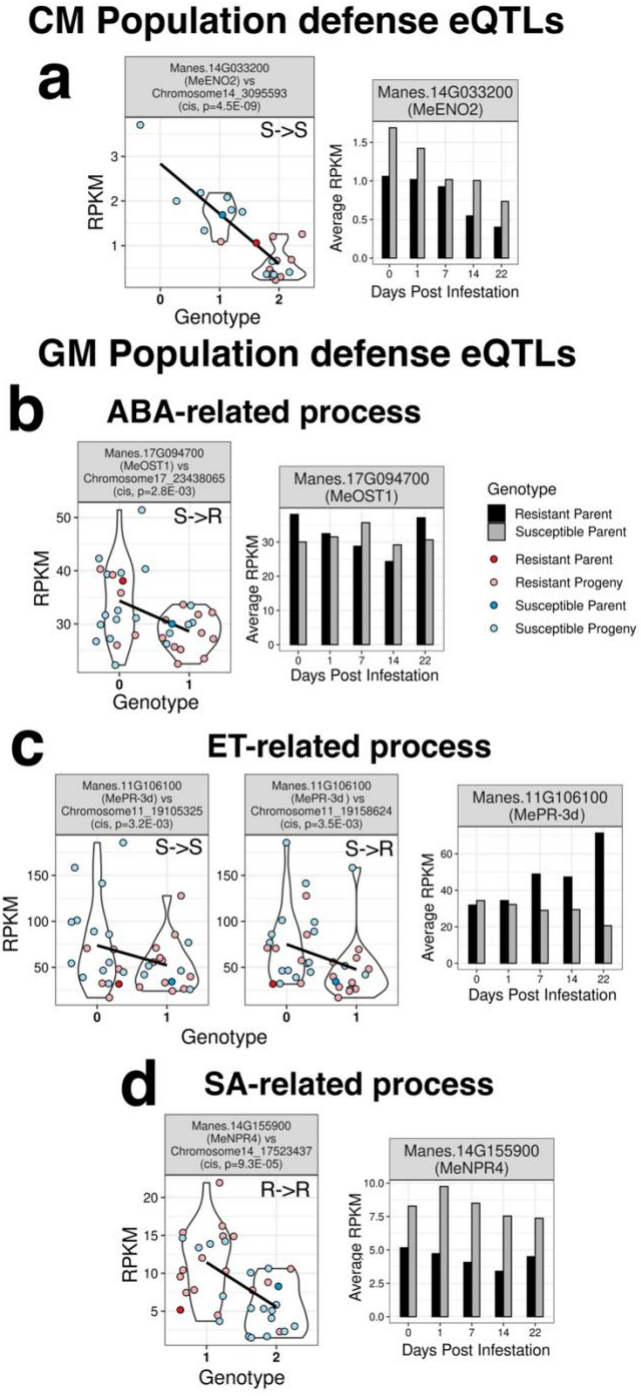
CM Population defense eQTLs
a
Manes.14G033200
(MeENO2) vs
Chromosome14_3095593
(cis, p=4.5E-09)
S->S
RPKM
Genotype
Manes.14G033200
(MeENO2)
Average RPKM
Days Post Infestation
GM Population defense eQTLs
b
ABA-related process
Manes.17G094700
(MeOST1) vs
Chromosome17_23438065
(cis, p=2.8E-03)
S->R
Manes.17G094700
(MeOST1)
Genotype
Resistant Parent
Susceptible Parent
Resistant Parent
Resistant Progeny
Susceptible Parent
Susceptible Progeny
c
ET-related process
Manes.11G106100
(MePR-3d) vs
Chromosome11_19105325
(cis, p=3.2E-03)
S->S
Manes.11G106100
(MePR-3d) vs
Chromosome11_19158624
(cis, p=3.5E-03)
S->R
Manes.11G106100
(MePR-3d)
d
SA-related process
Manes.14G155900
(MeNPR4) vs
Chromosome14_17523437
(cis, p=9.3E-05)
R->R
Manes.14G155900
(MeNPR4)

Figure 3.20. eQTLs with target genes in defense processes.

Violin plots displaying the effect of eQTL SNPs on the expression of target genes involved in defense that were not captured by other eQTL categories are accompanied by bar graphs displaying the expression of target genes during whitefly infestation in parental genotypes for **(a)** CM and **(b-d)** GM populations. GM population eQTLs are categorized in **(a)** ABA-, **(b)** ET- and **(c)** SA-related processes. Genotypes are denoted as in Figure 3.6. Whitefly-resistant genotypes are colored red (ECU72) and light red (resistant progeny) versus blue (COL2246 or 60444) and light blue (susceptible progeny) in violin plots. Expression is displayed as average RPKM values and colored in black for ECU72 and grey for COL2246 or 60444 in bar graphs. ID of SNP affecting ("versus") target gene, eQTL type (*cis* or *trans*), inheritance class, and *p*-value are labeled in each violin plot. Violin plots in each panel are ordered from lowest to highest *p*-value. The statistical relevance of the expression data for the eQTL-identified genes can be found in Chapter 2 (Table S2.1).

eQTL hormone responses

In Chapter 2, we established the temporal response of genes in ECU72 and COL2246 to SA, JA, ET, and/or ABA treatments. Here, we leverage these defense hormone data to determine if the eQTL SNP-containing genes or eQTL target genes with roles in defense (Table 3.1) were hormone responsive (Figure 3.21; Table S3.7). As we only have hormone treatment data for the South American cassava (ECU72 and COL2246) and African cassava genotype 60444 is genetically distinct (Bredeson et al., 2016), this analysis cannot performed with confidence on GM population eQTL targets.

The 13 CM population eQTL target genes with roles in defense (Table 3.1) were classified by their ability to respond to SA, JA, ET, and/or ABA 0.5 to 24 h after hormone treatment (Figure 3.21a; Table S3.7). In ECU72 and COL2246, most of the defense genes were hormone non-responsive. Notably, nine of these target genes were not responsive to any hormone in either genotype (Figure 3.21a), suggesting that if these genes have roles in defense against whiteflies, they are likely be regulated by other defense signals not examined here. Three of the four remaining target genes with roles in defense were hormone-responsive DEGs in both genotypes, with cell wall modification gene Manes.17G080400 only responsive in COL2246 (Figure 3.21a; Table S3.7). Three of the four hormone-responsive DEGs in COL2246 responded only to SA (*MeSOG1*, *MeLRK10L1.2* and Manes.17G080400), all of which are involved in responses to fungi, oomycetes, or chitin (Table 3.1). In contrast, the three hormone-responsive DEGs in ECU72 responded to either ET, SA and JA, or JA and ET.

The complete set of CM and GM population eQTL-identified defense genes (Table 3.1) was also examined for hormone responses; however, as such data is lacking for line

60444, hormone responses are based solely on the responses identified in ECU72's hormone treatment (SA, JA, ET and/or ABA) time-courses (Figure 3.21b). Out of a total of 78 eQTL-associated genes examined, 40 were hormone insensitive in ECU72, including 19 immunity genes: *MeCAR1*, *MeDRP2B*, *MeEAP3*, *MeMOS2a, MeMOS2b*, *MeMOS11*, *MePERK14a*, *MePERK14b*, *MeSPF2*, *MeSUT1*, *MeEFR*, *MeLRRAC1*, *MeVEP1a, MeVEP1b*, four NB-ARC domain-containing proteins, LRR Kinase Manes.13G094300, and the cell wall modification gene Manes.17G080400. Five immunity genes specifically involved in PTI triggered by cell-wall derived DAMPs/HAMPs (*MeMIK2a*, *MeMIK2c*, *MeMIK2d*, *MeWAK2*, and *MeHAK1*) were also hormone non-responsive.

Of the remaining 38 genes showing a hormone response, 18 were responsive to all four hormones (Figure 21b; Table S7). Of these 18 genes, several were involved in immunity (e.g., *MeADF4*, *MeBBD1*, *MeLIK1*, *MeOCP3*, *MeWR3*, and *MeRD19*). Furthermore, many genes involved in ABA response (*MeABA2*, *MeSOAR1*, *MeABCG40*, *MeUBC32*, *MeAIRP3*, *MeATI-2*, *MeENO2*, *MeHD2C*, and *MeROP10*), ABA-mediated stomatal regulation (*MeERD15*, *MeTHI*, *MeGI*, and *MeOST1*) or ABA-mediated callose deposition (*MeBBD1* and *MeOCP3*) were regulated by all or none of the hormones examined (Figure 3.21b; Table S3.7). These data strongly suggest that an unknown signal(s) may regulate the expression of immunity, ABA response, stomatal regulation, and callose deposition genes important for whitefly response in ECU72.

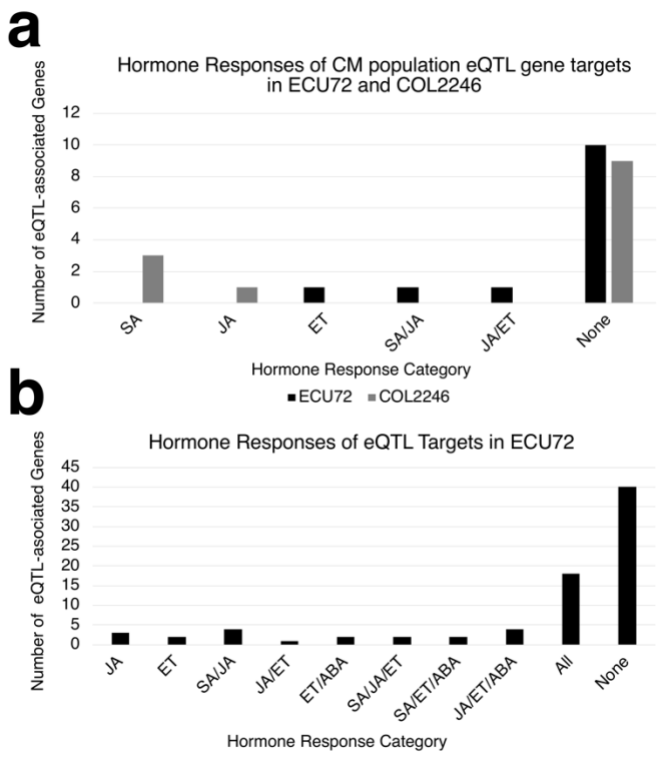

Figure 3.21. Hormone responses of eQTL target genes.

Bar graph displaying the number of defense-related eQTL-associated genes in the CM population differentially expressed following hormone treatment (SA, JA, ET or ABA) of ECU72 or COL2246 **(a).** Bar graph of all eQTLs in the CM and/or GM populations differentially expressed following hormone treatment in ECU72 **(b)**. Hormone response categories are labeled to reflect responses to single or multiple hormones. Some eQTL target genes responded to all four hormones (All) or were hormone-nonresponsive (None).

Discussion

eQTL analysis for whitefly defense gene identification

In this study, the F_1 populations generated by crosses of whitefly-resistant cassava genotype ECU72 with whitefly-susceptible cassava genotypes COL2246 (resulting in the CM population) and 60444 (resulting in the GM population) proved to be valuable resources beyond QTL mapping in the facilitation of RNA-sequencing-based eQTL analyses. In addition to whitefly resistance loci mapped in ECU72, eQTL analysis results were utilized to identify genes with possible roles in cassava's defense against whitefly infestation. In the selection of resistant and susceptible progeny members to use in these analyses, two insights into the nature of whitefly resistance in ECU72 were revealed. Visualization of nymph counts for all progeny in both populations revealed a broad spectrum of phenotypes, demonstrating that whitefly resistance in ECU72 is a quantitative trait (Young, 1996). Additionally, within this spectrum, some progeny displayed higher or lower nymph counts than their respective resistant or susceptible parent. This observation suggests that transgressive segregation for resistance or susceptibility traits has occurred, resulting in resistance factors being inherited by susceptible progeny and susceptibility factors being inherited by resistant progeny. In such cases, the genetic background of the susceptible parent caused resistance factors to be insufficient for resistance, and vice versa (de Los Reyes, 2019). From this observation, it was thus important to examine eQTL-associated SNPs inherited from the resistant parent by susceptible progeny, and from the susceptible parent by the resistant progeny. However, in such cases the association of eQTL-identified genes with resistance or susceptibility to whitefly could not be firmly established.

The numbers of eQTLs identified in the CM and GM populations additionally revealed differences in the relationship of ECU72 to the susceptible parents, COL2246 or 60444. In the GM population (ECU72 x 60444), the total number of eQTLs exceeded that identified in the CM population (ECU72 x COL2246) by over 1,000 eQTLs, with more than twice as many eQTL target genes identified in the GM population. Additionally, while over 100 eQTL hotspots were identified in the GM population, none were found in the CM population. Furthermore, very little overlap (four genes) occurred between eQTL-identified defense genes located in whitefly-resistance QTL regions in the two populations. Two factors may explain these differences. The resistance level of ECU72 and COL2246 is more comparable, as COL2246 is considered more tolerant of whitefly infestation (Dr. Luis Augusto Becerra Lopez-Lavalle and Dr. Adriana Bohorquez, personal communication), whereas 60444 is extremely susceptible to whitefly. Additionally, ECU72 and COL2246, both South American genotypes, are more closely related to each other than ECU72 and the African genotype 60444. Together, the different genetic backgrounds and whitefly susceptibility levels of COL2246 and 60444 may affect gene expression and the detection of SNPs, causing a difference in identified eQTLs or QTL regions. A greater difference in whitefly resistance levels between ECU72 and 60444 may also have enabled the identification of more eQTLs, particularly those with known roles in defense.

Beginning with several hundred eQTL-identified genes in the CM and/or GM populations, several criteria were applied to better identify those genes that may be important for defense against whiteflies (Table 3.1). eQTL-associated genes that may contribute to resistance were identified within QTL regions, as well as eQTL-associated genes appearing in physical clusters throughout the genome. Other criteria included status as a 0-dpi DEG in parent genotypes, as a shared eQTL in both populations or as a known

plant defense gene. As potential transcriptional regulators, eQTL hotspots affecting the expression of many target genes were additionally discovered within the GM but not CM population. The general lower number of eQTLs identified in the CM population may account for this.

Overall, the eQTL-associated defense genes identified using these criteria suggest that immune, defense-signaling and cell-wall-related processes may be important for the response of cassava to whitefly infestation. As relatively few defense-related eQTL-identified genes were identified in the CM population, it is difficult to assess whether certain processes are enriched in either population. Therefore, eQTL-identified genes identified in either population are largely discussed together.

Possible whitefly resistance and susceptibility mechanisms: immunity genes

Criteria used to better identify whitefly defense genes identified many immunity genes including those involved in PTI/ETI responses (*MeMOS2a* and *b*, *MeRD19*, *MeDRP2B*, *MeEFR*, *MeHAK1*, *MeCAR1*, *MeLIK1*, *MeADF4*, *MeMIK1a-d*, *MeRD19*, *MeMOS11*, *MeWAK2*, four NLRs, and one TNL), wounding response (*MeVEP1*, *MeWR3* and *MeWAK2*), chitin response (*MeSOG1* and *MeLIK1*), cell death (*MeHXK1* (Bruggeman et al., 2015), *MeCATHB2*, *MeLCB2*, and *MeEX2*), pathogen/pest resistance (*MeSSL4*, *MeSUT1*, *MeATS3B*, *MeBBD1*, *MeEAP3*, *MeLRRAC1*, *MeOCP3*, *MePDX1*, *MeLRK10L1.2*, *MeSPF2*) and cell wall-modification gene (Manes.17G080400), or uncharacterized defenses (*MePERK14a*, *MePERK14b* and one LRR kinase) (Table 3.1). Additionally, of those eQTL-associated defense genes identified within QTL regions (*MeMOS2a*, *MeMOS2b*, *MeDRP2B*, *MeEFR*, and a TNL), all had known roles in immunity, suggesting that the QTL regions in which they reside (CM population: Chromosome 3, GM

population: Chromosome 18) may be important for immune responses active during infestation.

Among immunity-related eQTL-identified genes, many were PTI receptors involved in the recognition of bacterial PAMPs (*MeEFR*), fungal chitin elicitors (*MeLIK1*), polysaccharide HAMPs (*MeHAK1*), and cell wall DAMPs (*MeWAK2* and likely *MeMIK1a-d*). No studies to date have definitively identified whitefly-derived elicitors, however, alteration of defense signaling in Arabidopsis has been shown to result from whitefly feeding (Zarate et al., 2007), in one case dependent on the presence of the whitefly endosymbiont *Hamiltonella defensa* (Su et al., 2015). Alteration of plant defenses by Hemipterans is well-documented, with some instances of elicitors identified within Hemipteran saliva (Kaloshian and Walling, 2016). It has been speculated that Hemipteran elicitors may be derived from chitinous stylets, cell wall damage resulting from probing or small RNAs or endosymbiont derived molecules within saliva (van Bel and Will, 2016, van Kleeff et al., 2016, Wang et al., 2017). Thus, the identification of these PTI receptors suggests that early perception of unknown whitefly-derived elicitors may be important for defense. It is also interesting to note that four *MIK1* genes were identified in cassava as opposed to the single copy gene in Arabidopsis, which may suggest that an expansion of this gene in cassava could possibly allow for the detection of multiple types of cell wall DAMPs or perhaps detection in specific cell types.

Several ETI receptors (*MeCAR1*, four NLRs and one TNL) or genes involved in ETI (*MeMOS2a* and *b*, *MeADF4*, *MeRD19*, and *MeMOS11*) were additionally found among eQTL-identified genes. While *MOS2* and *MOS11* have broad roles in ETI (Dong et al., 2016, Wu et al., 2013) and the identified NLRs have unknown roles, *CAR1*, *ADF4*

and *RD19* are known to be involved in resistance to various bacterial pathogens (Bernoux et al., 2008, Laflamme et al., 2020, Tian et al., 2009). Many ETI receptors are known to be important for resistance to pests, including many that confer resistance to Hemipterans and one known cloned whitefly *R* gene *Mi1.2* (Walling and Thompson, 2012, Rodriguez-Alvarez et al., 2015). With the identification of three genes involved in ETI against bacteria, it may be possible that effectors derived from whitefly or its endosymbionts trigger an ETI response. Additionally, one or more of the five NLRs identified (*MeCAR1* and NLRs Manes.18G112100, Manes.07G008700, Manes.07G044300, Manes.07G044700, and Manes.07G045400) may potentially be candidate whitefly *R* genes or *R* gene co-regulators (Jacob et al., 2013). Further experiments in transgenic cassava are required to determine this functionality.

While all eQTL-identified immunity genes support the idea that immune responses are important for whitefly responses, only those eQTL-identified genes, which strictly correlate with resistance (resistance factors *MeSOG1*, *MeLIK1* and *MeSPF2*) or susceptibility (susceptibility factors *MeDRP2B*, *MePDX1*, *MeSSL4* and LRR kinase Manes.13G094300) to whiteflies can provide insights into possible resistance or susceptibility mechanisms. In Arabidopsis, LIK1, an interaction partner of the chitin receptor CERK1, acts as a negative regulator of innate immune responses prompted by chitin elicitors (Le et al., 2014). Conversely, the Arabidopsis gene *SOG1* positively regulates chitin-responsive genes in response to DNA damage that occurs during pathogen attack (Yoshiyama et al., 2020). Both *MeLIK1* and *MeSOG1* expression is strictly correlated with resistance, showing higher transcript levels in resistant versus susceptible progeny and in ECU72 versus COL2246 0-22 and 0-7 dpi, respectively. While both genes suggest response to chitin during early infestation is important for resistance,

the needed mechanism of chitin perception, via PTI in the case of *MeLIK1* or via DNA damage response in the case of *MeSOG1*, remains unclear.

The eQTL-identified immunity gene *MeSPF2* was strictly correlated with resistance in the GM but not CM population, with higher expression in ECU72 versus 60444 0-22 dpi. In Arabidopsis, SPF2 activates WRKY33 via sumolyation (Verma et al., 2021). *WRKY33* is a known positive regulator of whitefly resistance that is targeted by whitefly *B. tabaci* effector Bsp9, which is produced by whiteflies harboring *Tomato Yellow Leaf Curl Virus* for the purpose of further viral spread (Wang et al., 2019). Our results suggest that *MeSPF2* may also be important for activating *WRKY33* responses prior to and throughout infestation needed for ECU72's resistance to whitefly.

MeDRP2B, *MePDX1* and *MeSSL4* were identified as whitefly susceptibility factors, with higher expression in susceptible versus resistant progeny and in 60444 versus ECU72 0-14, 0-22 and 0-1 dpi, respectively. In Arabidopsis, while *PDX1* is important for vitamin B6-mediated bacteria and fungi resistance (Zhang et al., 2015b), *SSL4* is a susceptibility factor against bacteria *Ralstonia solanacearum* (Aoun et al., 2017). *MeDRP2B* is instead involved in response to bacterial PAMPs, and was located within a whitefly resistance QTL region found in both CM and GM populations (Ekanayake et al., 2021). It may be possible that in cassava, one or more of these three genes activate ineffective defense mechanisms, contributing to whitefly susceptibility. The LRR kinase Manes.13G094300 was also identified as a susceptibility factor, however its function is unknown.

Possible whitefly resistance and susceptibility mechanisms: defense-signaling genes

Defense signaling genes involved in SA (*MeTCP9, MePR-5b* and *MeNPR4*), ET (*MeSHN3*, *MeEBF1*, *MeEIN2*, *MePR-3d*, and *MeEEN*), ABA (*MeABA2*, *MeASK2*, *MeATI-2*, *MeBBD1*, *MeCOL4*, *MeERD15*, *MeGI*, *MeHD2C*, *MeOCP3*, *MeROP10*, *MeTHI*, *MeTRE1*, *MeSOAR1*, *MeAIRP3*, *MeABCG40*, *MeUBC32*, *MeENO2*, *MeROC3*, and *MeOST1*), and ROS (*MeCcdA* (Motohashi and Hisabori, 2010), *MeGRXC1*, *MePDX1*, *MeRR2*, *MeROC3*, *MeFITNESS*, *MeKEA2*, *MeEX2*, *MeGPX1*, and *MeGPX2*) responses were also found among eQTL-identified genes (Table 3.1). While the roles of *TCP9* or *NPR4* in plant whitefly responses have not been specifically investigated, SA responses have been shown to be active in several plant-whitefly interactions. Plants susceptible to whiteflies such as Arabidopsis, tobacco, and lima bean deploy SA responses during whitefly infestation (Zarate et al., 2007, Zhang et al., 2009, Zhang et al., 2017). In response to whitefly infestation, JA/ET responses have also been documented in the responses of whitefly-resistant cotton (Li et al., 2016b). eQTL-identified genes involved in SA, JA and ET responses were not strictly correlated with resistance or susceptibility to whitefly, but nevertheless suggest an importance of these pathways in cassava's response to whitefly infestation.

Of the 38 eQTL-identified defense-signaling genes, 23 were involved in ABA responses. Twenty of these genes were identified by GM population eQTL hotspots, 17 of which were specifically target genes of SNPs residing within *MeSOAR1*. In Arabidopsis, *SOAR1*, a pentatricopeptide repeat (PPR) RNA-binding protein, is a known master negative regulator of ABA signaling. SOAR1 acts downstream of putative ABA receptor ABAR, interacting with exoribonuclease USB1 to regulate spliceosome assembly and

alternative splicing of ABA signaling genes (Ma et al., 2020). SOAR1 is also known to negatively regulate ABA signaling gene *ABI5* at the translational level, via its role in repressing assembly of the cap-binding complex (Bi et al., 2019). Our results support this role for *MeSOAR1* as a negative regulator of ABA signaling in cassava, as its expression is inversely related to the expression of its target genes involved in ABA responses. *MeSOAR1* expression is significantly lower at 0 dpi in ECU72 versus 60444, and expression levels of *MeSOAR1* targets, involved in ABA, redox, cell death, and other defense processes, are mainly higher in ECU72 versus 60444. Thus, it appears that dampened expression of the negative regulator *MeSOAR1* results in activation of ABA responses in ECU72, which may contribute to whitefly resistance. While expression of *MeSOAR1* or its targets is not strictly correlated with resistance, these results together with previous evidence of induced ABA-pathway genes and higher ABA levels in whitefly-infested ECU72 compared to COL2246 (Chapter 2) imply that whitefly resistance in ECU72 is (at least in part) ABA-mediated. Additional targets in redox, cell death, and other defense processes also suggest these *MeSOAR1*-regulated processes may be important for resistance. Supporting this theory, involvement of ABA in whitefly resistance has also been documented in tomato, cabbage and eggplant (Broekgaarden et al., 2018, Esmaeily et al., 2020, Esmaeily et al., 2021).

Among *MeSOAR1* targets, several were found to be involved in stomatal regulation (*MeTRE1*, *MeTHI*, *MeRR2, MeGI, MeROC3, and MeERD15*) or callose defenses (*MeADF4* and *MeBBD1*), in most cases mediated by ABA (excluding *MeRR2*, *MeGI* and *MeADF4*). Positive regulators of stomatal closure *MeTRE1*, *MeGI, MeROC3, MeTHI,* and *MeRR2* (Li et al., 2016a, Van Houtte et al., 2013, Wang et al., 2020) were more highly expressed in ECU72 versus 60444 at 0 dpi, with higher expression in ECU72

continued at multiple time points during infestation. In plant-pathogen interactions, early-onset closing of stomata, triggered by PAMP perception can confers stomatal immunity - blocking pathogen entrance via stomata (Sawinski et al., 2013). Similar mechanisms of stomatal immunity have yet to be identified in plant-insect interactions, however alteration of stomatal aperture has been documented in such interactions. In tomato and soybean, caterpillar feeding has been shown to cause stomatal closure within 48 hours, and is speculated to impede the release of certain herbivore-induced plant volatiles involved in plant defenses (Lin et al., 2021); this may be attributed to the wounding incurred after larval feeding and the transient water deficit that occurs prior to wound healing. As female whitefly ovipositors cut into the epidermal surface to insert eggs via their pedicels (Byrne and Bellows, 1991), a transient and very mild water-deficit response could be occurring. An alternative theory is that stomatal closure alters turgor pressure, which may affect survival of whitefly eggs that are heavily dependent on water from the leaf (Voigt et al., 2019, Buckner et al., 2002, Byrne and Bellows, 1991), or perhaps interfere with transport of phloem photosynthates to be ingested through the stylet (Walker, 2000). Together our results suggest that stomatal regulation may be important for defense against whiteflies; however, the mechanism by which stomatal regulation may affect whitefly survival/feeding is unknown.

Our results also suggest callose defenses may be important for defense against whiteflies. ETI regulator *ADF4* and nuclease *BBD1* are both positive regulators of callose-mediated bacterial or fungal resistance (Tian et al., 2009, You et al., 2010). In addition to pathogens, callose defenses can be effective against insects. Plants can obstruct the feeding of phloem-feeding insects by occluding phloem sieve elements via callose deposition (Will et al., 2013). In Arabidopsis, *ADF4* has been shown to confer callose-

based resistance to green peach aphid (Mondal et al., 2018), and has even been shown to respond to a microbial elicitor and regulate a *R* gene, suggesting a role for *ADF4* in PTI/ETI signaling (Porter et al., 2012). Additionally, callose defenses have been previously suggested as important for plant defense against whiteflies, as *B. tabaci* effector BtFer1 dampens callose-based defenses in tomato, resulting in higher whitefly survival rates (Su et al., 2019). Thus, *MeADF4* or *MeBBD1* may be important for callose-based defenses against whiteflies.

Aside from *MeSOAR1* targets, other ABA-response genes included whitefly resistance factors *MeHD2C* and *MeABCG40* and whitefly susceptibility factors *MeCOL4*, *MeASK2* and *MeENO2,* which showed clear correlation with resistance and susceptibility, respectively. *ABCG40* is an ABA importer (Finkelstein, 2013), whereas Arabidopsis T-DNA insertion lines for histone deacetylase *HD2C* showed increased sensitivity to ABA (Luo et al., 2012). Similarly, whitefly susceptibility factors *MeCOL4* and *MeASK2* have been shown to lower and raise ABA sensitivity in Arabidopsis, respectively (Li et al., 2012a, Min et al., 2015). In Arabidopsis, the alternative translation product of *ENO2* is a positive regulator of ABA responses, making while its role in ABA responses at the transcript level is unclear. The eQTL identification of these genes suggests that level of sensitivity to ABA perception and transport of ABA may be important for whitefly resistance in ECU72.

In addition to defense hormones, several eQTL-identified genes were involved in ROS-associated processes (*MeCcdA*, *MeGRXC1*, *MePDX1*, *MeRR2*, *MeEX2*, *MeGPX1*, and *MeGPX2*). Expression of the redox sensor glutathione peroxidase *MeGPX1* (Passaia and Margis-Pinheiro, 2015) was strictly correlated with susceptibility, with higher

expression in COL2246 than ECU72 0-22 dpi. Additionally, a SNP within *MeEX2*, known in Arabidopsis to regulate singlet-oxygen retrograde signaling (Chen et al., 2015), was identified as an eQTL hotspot. Though a particular role is unclear, the identification of this gene suggests that perception of cell redox state and perhaps ROS signaling may be important in the response of COL2246 to whitefly infestation. Redox genes, antioxidant enzymes, and ROS responses during plant-whitefly interactions has also been previously suggested in cassava, Arabidopsis, cotton, pepper, and eggplant (Esmaeily et al., 2020, Kempema et al., 2007, Li et al., 2016b, Mwila et al., 2017, Wu et al., 2019).

Lastly, two *PR* genes (*PR-3d* and *PR-5b*) (Irigoyen et al. (2020), that are common markers of plant defense to various pests/pathogens, were identified by eQTLs (van Loon et al., 2006). *PR-5* is SA-responsive in Arabidopsis (van Loon et al., 2006), however, *PR-5* orthologs in other plants are regulated differently. For example, in tobacco, *PR-5* is regulated by SA in addition to ET and JA (Niki et al., 1998). Supporting their role in cassava whitefly responses as suggested here, many *MePR-3* (including *Me-PR3d*) and *MePR-5* genes are induced or repressed in response to *A. socialis* infestation of whitefly-susceptible genotypes COL2246, COL1468, TME3, and 60444 (Irigoyen et al., 2020).

Possible whitefly resistance and susceptibility mechanisms: cell-wall-related genes

Genes involved in cell wall-related processes (*MeHAK1*, *MeMSBP2*, *MePMEI*, *MeKNAT3*, *MeWAK2*, *MeCSLB5*, and cell wall modification gene Manes.17G080400) were additionally found among eQTL-identified genes (Table 3.1). Three of these six genes (*MeWAK2*, *MeCSLB5*, and Manes.17G080400) were DEGs between parental genotypes at 0 dpi, suggesting that their constitutive expression levels may be important

for whitefly defense. Cellulose biosynthetic gene *MeCSLB5* showed strict correlation with susceptibility, with higher expression in 60444 versus ECU72 0-22 dpi. In Arabidopsis, reduction in cellulose synthase activity has been found to be important for lignification or secondary cell wall biosynthesis, as well as initiating defense signaling as a resistance mechanism against pathogens (Caño-Delgado et al., 2003, Hernández-Blanco et al., 2007). Thus, lower expression of *MeCSLB5* in ECU72 versus 60444 may be important for resistance in ECU72, perhaps as a means of being primed for responding to changes in cell wall integrity. It is also important to note, that while not strictly correlated with resistance, two genes involved in regulation of lignin biosynthesis were identified. MSBP2 regulates lignin biosynthesis by acting as a scaffold to cytochrome P450 enzymes involved in monolignol biosynthesis (Gou et al., 2018), while the transcription factor *KNAT3* is a positive regulator of monolignol biosynthesis (Qin et al., 2020). Additionally, *MeMSPB2* was more highly expressed in ECU72 versus 60444 at 0-22 dpi, suggesting it may be important for resistance. In a metabolomics study comparing whitefly-infested leaves of ECU72 and COL2246, higher lignin levels prior to and during infestation in ECU72 were associated with resistance (Perez-Fons et al., 2019). Cell wall fortification has also been previously suggested as important for plant defense against whiteflies in cassava (Chapter 2, (Irigoyen et al., 2020)) and cotton (Ibrahim, 2016, Li et al., 2019a, Li et al., 2016b). Together, these results suggest that cell-wall-based defenses, including fortification of cell walls with lignin, may be important for whitefly resistance in ECU72.

Hormone responses of eQTL-identified defense genes

Among all eQTL-identified genes in ECU72 and genes identified by eQTLs in the CM population in ECU72 and COL2246 (Table 3.1), most genes were nonresponsive to SA, JA, ET and/or ABA or responded to all four hormones. The observation that most

eQTLs responded to all or none of these hormones suggests that another defense signal or signals may be active. For example, ROS are involved the regulation of many defense-hormone responses and are a more general initial response to stress than activation of particular defense hormone pathways (Li and Loake, 2016). Another possibility is that unknown PAMPs/DAMPs/HAMPs generated in the plant-whitefly interaction may also act as a defense signal. Further studies examining cassava responses to possible whitefly- or damage-derived elicitors, ROS, calcium (Ca^{2+}), small peptides, and lipids are required to better understand which unknown signals are required for the regulation of eQTL-identified genes. Additionally, eQTL-identified genes responsive to all or no hormones were predominantly involved in immunity or ABA responses, including ABA-mediated stomatal regulation and callose deposition, suggesting that these processes may be regulated by an unknown signal.

Together, our eQTL analysis of cassava mapping populations identified genes involved in immunity, defense-signaling (particularly ABA responses), and cell-wall-related processes as possibly involved in cassava's defense against whiteflies. In particular, strict correlation of gene expression in parental lines and progeny identified potential whitefly resistance factors involved in chitin perception/response (*MeSOG1* and *MeLIK1*) (Le et al., 2014, Yoshiyama et al., 2020) and ABA responses (*MeHD2C* and *MeABCG40*) (Finkelstein, 2013, Luo et al., 2012). Although not strictly correlated with resistance, eQTL-identified genes involved in regulation of monolignol biosynthesis (*MeKNAT3* and *MeMSBP2*), regulation of ABA signaling (*MeSOAR1*) and whitefly/aphid resistance (*MeSPF2* and *MeADF4*) are also likely involved in whitefly resistance due to previous evidence of such a role presented by Perez-Fons et al. (2019), in Chapter 2 and from the Arabidopsis literature, respectively (Mondal et al., 2018, Verma et al., 2021, Wang et al.,

2019). These nine genes are promising candidates for testing in transgenic cassava to confirm their possible roles in resistance to whiteflies.

Materials and Methods

Plant growth and crosses

Crosses between cassava (*Manihot esculenta*) genotypes ECU72 (Ecuadorian, whitefly-resistant) and COL2246 (Colombian, whitefly-susceptible), as well as ECU72 and 60444 (African, whitefly-susceptible), were performed at CIAT (Cali, Colombia) for the purpose of mapping whitefly resistance QTLs. Parent and F_1 progeny plants were grown at CIAT under greenhouse conditions with leaves collected from non-infested three-month-old plants according to Irigoyen et al. (2020). For F_1 plants, leaves were collected from single plants. For the parental genotypes, leaves were collected from three independent infestation time-course experiments with the whitefly *Aleurotrachelus socialis* Bondar (0, 1, 7, 14, and 22 days). Leaves from three plants were pooled for each timepoint.

RNA extraction

Sample identities for the top eleven to seventeen most resistant and susceptible F_1 progeny from the ECU72 x COL2246 and ECU72 x 60444 crosses selected for eQTL analysis are available in Table S3.8. RNA extraction and quality assessment RNAs from the F_1 progeny samples and the parental samples from the infestation time courses was performed at CIAT as described by Behnam et al. (2019) and Irigoyen et al. (2020).

Gene expression analysis

RNAs were shipped to UCR for the construction of cDNA libraries for RNA sequencing. Methods for library construction, sequencing, read pre-processing, and alignment are described in Irigoyen et al. (2020). The fifteen RNA samples for each parental genotype, initially assessed for gene expression only, were sequenced using single-end 50- to 75-bp reads; in contrast, F_1 progeny samples were sequenced using paired-end 150-bp reads for improved SNP detection during genotyping. A single, non-infested sample was sequenced for each progeny member. Sequenced libraries for parental genotypes and progeny resulted in 15-56 million and 5-17 million aligned reads, respectively. Gene expression values were calculated as RPKM using the R package systemPipeR (Backman and Girke, 2016). The three non-infested biological replicates for each parent genotype were used to calculate an average RPKM value; one RPKM value for each parental genotype and each progeny member was thus used for eQTL analysis. Trends in expression are displayed for these analyses, however, status of eQTL-identified genes as temporal or genotype DEGs is not shown but is available in Chapter 2 (Table S2.1).

SNP calling

The open source software STAR (Dobin et al., 2013), optimized for variant detection, was used to align RNAseq reads. The R package GATK (Poplin et al., 2018) was then used to perform SNP calling of parental genotype samples (15 per genotype) and progeny samples using RNAseq reads. SNPs were filtered by mapping quality (MQ = 40) and having at least three reads supporting genotype calls (DP = 3), resulting in the identification of 760,143 SNPs. Further filtering retained only those SNPs with variant

missing call rates of < 0.2 and minor allele frequency (MAF) of > 0.05, resulting in 121,778 good quality SNPs.

eQTL calling

For the CM population and its parents (ECU72 and COL2246) and the GM population and its parents (ECU72 and 60444), eQTLs were identified using the R package MatrixeQTL (Shabalin, 2012). Prior to the analysis, genes expressed at low levels and with insufficient support for genotype calls were removed. Genes with RPKM <1 in one or both parental genotypes and RPKM <1 in all progeny members were removed. In addition, SNPs with differing genotype calls across a parent's 15 samples were removed, as well as SNPs with no genotype calls among the progeny. Additionally, to better identify eQTLs underlying parental differences, SNPs with identical genotypes in both parents were removed. To identify eQTLs with sufficient phenotypic support in the progeny, SNPs with less than 2/3 of progeny genotypes matching either the resistant or susceptible parent were removed. Resulting eQTLs were deemed significant if they met *p*-value ≤ 0.05 and FDR ≤ 0.05 criteria.

eQTL gene nomenclature and visualizations

For all cassava genes shown, gene names were obtained from available Phytozome orthologs in Arabidopsis (Goodstein et al., 2012). For cassava genes without identified gene names (in TAIR version 10 (Berardini et al., 2015)) or orthologs in Arabidopsis, manual annotation was performed using Protein BLAST (Altschul et al., 1990) or PFAM domain (El-Gebali et al., 2019) searches as indicated (Table S3.2). Cassava gene functional bins were annotated using the online program Mercator4 version 2.0 (Schwacke et al., 2019). Two-genome plots, which display the chromosomal positions

of each eQTL SNP (x-axis) and their target gene(s) (y-axis), were created using the R/Shiny application shinyChromosome (Yu et al., 2019). Chromosome physical maps of eQTL positions were created using the online program Phenogram (Wolfe et al., 2013). Bar graphs visualizing eQTL gene expression levels and GO terms, as well as violin plots visualizing eQTLs, were created with the R package ggplot2 (Wickman, 2016). A Venn diagram displaying eQTL overlap between the two populations was constructed using the online program VENNY (Oliveros, 2007). Whitefly-resistance QTL regions for the CM and GM populations were provided by CIAT (Dr. Luis Augusto Becerra Lopez-Lavalle, Dr. Adriana Bohorquez and Dr. Vianey Paola Barrera, unpublished data).

Supplemental Tables

Table S3.1. CM and GM population significant eQTLs.

Table S3.2. CM and GM population eQTL associated gene annotation.

Table S3.3. CM and GM population eQTL genes within QTL regions.

Table S3.4. eQTL cluster SNP and gene annotations and Mapman bins for the CM and GM populations.

Table S3.5. GM population eQTL hotspot SNPs and their target genes.

Table S3.6. eQTL genes differentially expressed between parental genotypes at 0 dpi.

Table S3.7. Hormone-responsive eQTL-associated genes.

Table S3.8. Lists of CM and GM population resistant and susceptible progeny.

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Conclusions

Whiteflies are a food security and economic threat to global agriculture, as whitefly infestation and vectoring of plant viral diseases reduces the yields of many of its 1,000+ crop and ornamental plant hosts (EFSA, 2013). Yield losses of the subsistence crop cassava due to superabundant whitefly populations in sub-Saharan Africa in recent decades have been particularly devastating for small-shareholder farmers (FAO, 2018). Previous strategies to mitigate whitefly-inflicted cassava yield losses using insecticides or virus-resistant cassava varieties have been ineffective, as whiteflies are generally able to develop insecticide resistance and virus-resistant cassava varieties remained susceptible to whiteflies. The African Cassava Whitefly Project (ACWP) was formed to identify strategies for whitefly control for African cassava. The ACWP focuses on identifying sources of whitefly resistance in cassava to be introduced in African cassava lines through breeding. A promising source of resistance to whitefly has been found in Ecuadorian cassava genotype ECU72. Together, teams at CIAT, UCR and RHUL have been researching whitefly resistance in ECU72 at the genetic, transcriptomic and metabolic levels, respectively.

As a part of the ACWP, the main goal of my Dissertation has been to identify processes/genes important for whitefly resistance in ECU72 as compared to whitefly-susceptible Colombian genotype COL2246. To this end, I have defined, analyzed and integrated with other data sets the transcriptome response of ECU72 and COL2246 to whitefly infestation and defense hormone treatment. In the three chapters of my Dissertation, my goals were to (1) present an initial characterization of the transcriptomic response of *Pathogenesis-related* (*PR*) genes to whitefly infestation, SA, JA, and biotic

stressors in whitefly-susceptible cassava; (2) define the global transcriptomic responses of ECU72 and COL2246 to whitefly infestation, SA, JA, ET, and ABA; and (3) identify possible whitefly defense genes in ECU72 through eQTL analyses.

In Chapter 1, we provided the first genome-wide identification of *PR* gene families in cassava and characterized cassava's *PR* gene transcript-level response to whiteflies, SA, JA, and other biotic stressors. Utilizing four susceptible cassava genotypes (COL2246, COL1468, TM3, and 60444) with diverse genetic backgrounds, we identified core transcriptome responses to whitefly infestation. *PR* genes were originally identified as being induced by pathogen and pest attack (van Loon et al., 2006). In contrast, and quite surprisingly, cassava breaks with this dogma. In cassava, many *PR* genes were downregulated in response to whitefly and other biotic stressors, suggesting novel biological functions of such *PR* genes or evolution of unique *PR* gene regulation programs specific to cassava. Gene expression programs identified for *PR* families *2*, *5*, *7*, and *9* predominated the response to whiteflies, SA, JA, and most other biotic stresses. This suggests that their functionalities (pathogen cell wall degradation and host cell wall reinforcement) (Abad et al., 1996, Lagrimini et al., 1987, Slusarenko et al., 2000, Van Loon and Van Strien, 1999) may play an important role in cassava defense responses.

Correlation and phylogenetic analyses uncovered additional similarities in whitefly/microbe responses with positive correlations in differentially expressed *PR* genes when whitefly responses were compared to bacteria and *Cassava Brown Streak Virus* responses. Comparison of *PR* family composition among plant species revealed cassava-specific expansions within selected *PR* clades. Notably, clades *PR-2e* and *PR-3g* contained genes with cassava-specific expansions that were associated with

whitefly/microbe responses. Our results suggested that *PR* gene responses may be comparable among whiteflies and certain microbes due to similar perception of the whitefly stylet (chitin, apoplast movement) or of whitefly saliva components (elicitors, effectors or endosymbionts) (Kaloshian and Walling, 2016, Schwessinger and Zipfel, 2008).

Definition of the SA- and JA-dependent transcriptomes of cassava revealed that whitefly, bacteria and fungi *PR* gene responses are largely coordinately regulated by both SA and JA. These results show that *PR* genes typically used to report activation of the SA and JA pathways in Arabidopsis cannot be used in cassava. Our genome-wide analysis of cassava's *PR* gene families also emphasizes the need for evaluating the *PR* gene regulatory programs in other crops to develop an understanding of the utility of *PR* genes as defense sentinels.

In Chapter 2, we conducted a more global and comparative analysis, defining the transcriptomes of ECU72 and COL2246 to whitefly infestation. This was combined with the analysis of the ECU72 and COL2246 transcriptome responses to treatments with four defense hormones (SA, JA, ET, and ABA) to characterize their mechanisms of resistance or susceptibility to whiteflies. Comparison of defense-hormone-responsive transcriptomes revealed that while responses to JA, ET and ABA were similar in both genotypes, SA treatment elicited a faster, more prolonged response in ECU72 than in COL2246. These genotypes additionally displayed a striking reciprocal regulation of most SA-responsive genes. SA responses were additionally largely antagonistic with responses to JA, ET and ABA in ECU72 but not COL2246. While the mechanisms that mediate the major reprogramming of SA responses in ECU72 and COL2246 are currently unknown, such results drove an inquiry into possible regulators of hormone crosstalk in cassava. Based

on their pivotal regulatory roles in the model plant Arabidopsis (Caarls et al., 2015, Li et al., 2013a, Li et al., 2019b) and their dynamic expression programs in ECU72 versus COL2246 in response to whitefly infestation and/or SA treatment, *MeWRKY70*, *MeNPR1* and *MeGRX480* were identified as possible hubs to mediate the crosstalk between SA and other defense-hormone pathways. The marked differences in SA responses discovered in the cassava genotypes ECU72 and COL2246 has not been reported frequently, but was seen in a pairwise comparisons SA responses in seven Arabidopsis ecotypes (van Leeuwen et al., 2007). Similarly in cacao, partial reciprocity of gene expression was observed in SA-treated oomycete-resistant versus oomycete-susceptible genotypes (Fister et al., 2015). Therefore, the variation in hormone networks may also exist in other plant species as well. Such hormonal fine tuning among varieties may reflect adaptations to specific suites of pests, pathogens or environmental conditions.

To place cassava's hormone responses in a broader context, we sought to compare cassava responses to the SA- and JA-responsive transcriptomes of Arabidopsis. A survey of the Arabidopsis literature indicated that existing transcriptomes of SA- or JA-treated Arabidopsis were highly variable, likely due to differences in treatment concentrations or plant age (Hickman et al., 2017, Pauwels et al., 2008, Sawant et al., 2009, Singh et al., 2015, Thibaud-Nissen et al., 2006, Yang et al., 2017). As existing studies were also not aligned with time points used in our cassava time courses and for better comparisons, we generated transcriptomes to follow the response of Arabidopsis to SA and JA treatment over a 24 h time course. Comparison of the two species revealed that many SA- and JA-responsive genes in cassava were unresponsive to these hormones in Arabidopsis. Furthermore, of SA- or JA-responsive DEGs detected in both cassava and Arabidopsis, many genes showed divergent temporal and directional

responses in the two species. Together, our observations provide evidence of intra- and inter-species variation in hormone responses, an understudied phenomenon also observed in Arabidopsis and cacao (Fister et al., 2015, Proietti et al., 2018, van Leeuwen et al., 2007). Visualization of SA, JA, ET, and ABA pathway gene expression in the two species additionally found, as in Chapter 1, that several sentinel genes defined in Arabidopsis used to indicate hormone pathway activation are not indicators of similar responses in cassava. We hope our findings will dissuade the inference of hormone responses using Arabidopsis sentinels in studies of other plant species.

Whitefly-responsive transcriptomes revealed a clear shift in responses from early (0-7 dpi, adult feeding, oviposition, and egg residency) to late (14-22 dpi, nymph feeding) phases of infestation. Integrative analyses of whitefly and hormone responses also suggested whitefly resistance to be ABA-mediated in ECU72 and whitefly susceptibility to be SA-mediated in COL2246. Higher ABA levels (peaking at 0.5 dpi) and late-infestation induction of ABA signaling genes in ECU72 versus higher SA levels and late-infestation induction of SA-signaling genes in COL2246 support this hypothesis. The importance of SA responses during infestation additionally suggests that the previously mentioned hormone-crosstalk regulatory genes may be important for whitefly resistance. A large proportion of whitefly-regulated genes were also found to be hormone insensitive or responsive to all hormones, suggesting that unknown signals, such as whitefly elicitors, effectors or other defense signals, may also regulate cassava's response to whitefly infestation (Kaloshian and Walling, 2016, Wang et al., 2019, Xu et al., 2019).

Enrichment and clustering analyses of whitefly- and hormone-responsive genotype DEGs was utilized to further characterize cassava's response to whiteflies. At

0-7 dpi, several defense processes were enriched among genes more highly expressed in ECU72 versus COL2246, revealing cell wall processes like lignin biosynthesis, cell wall remodeling and cell wall-elicitor response to be important for ECU72's early defenses against whitefly adults and/or eggs. A role for lignin-based defenses in ECU72 is supported by the metabolomics data reported by Perez-Fons et al. (2019).

In contrast, few processes were enriched among genes more highly expressed in COL2246 versus ECU72 until 14-22 dpi. At these times, we observed a surge in enriched processes including SA signaling, immune signaling, carbohydrate metabolism, and sesquiterpenoid biosynthesis as COL2246's response to feeding by 1^{st}-, 2^{nd}-and 3^{rd}-instar nymphs. We hypothesize that while ECU72 is able to deter the development of eggs and early-stage nymphs during early phases of whitefly infestation using ABA- and cell-wall-based defenses, COL2246's defenses are activated more slowly. This results in a much higher nymph load by late infestation timepoints (14-22 dpi) and a surge of ineffective SA and immune responses to these feeding nymphs.

In Chapter 3, RNA-sequencing-based eQTL analyses were performed to identify genes important for cassava's defense against whiteflies utilizing non-infested F_1 quantitative trait loci (QTL) mapping populations resulting from crosses of ECU72 with COL2246 (the CM population) or with the African genotype 60444 (the GM population). A spectrum of resistance/susceptibility phenotypes observed in these populations demonstrated that whitefly resistance is quantitative. The identification of F_1 progeny that were more resistant or susceptible than the resistant and susceptible parents demonstrated the occurrence of transgressive segregation. This prompted the analysis of progeny resistance traits dominantly inherited from the susceptible parent and vice versa.

eQTL analysis revealed the presence of more eQTLs, eQTL-identified genes, and eQTL hotspots in the GM population than the CM population. While the reason for these differences is not known, they may result from differences in the genetic backgrounds or susceptibility levels of COL2246 and 60444. To better identify genes important for defense against whiteflies, eQTL-identified genes meeting various criteria that imply a role in defense were selected for further analysis. Such criteria included eQTLs shared in both populations, location within an eQTL cluster or whitefly-resistance QTL, or status as a known defense gene, hotspot, or genotype DEG at 0 dpi.

eQTL-identified genes identified several processes as being potentially involved in cassava's defense against whiteflies, including immunity, defense-signaling and cell-wall-related processes. Standing out was a GM population eQTL hotspot at *MeSOAR1*, a master negative regulator of ABA responses (Ma et al., 2020). *MeSOAR1* targeted 119 genes, including many genes involved in ABA, redox, cell death, and other defense-related processes. Lower expression of *MeSOAR1* in ECU72 versus 60444 allowed for higher expression of ABA-response genes in the resistant genotype, including many genes involved in ABA-mediated stomatal closure or callose deposition. Other eQTL-identified genes included regulators of monolignol biosynthesis and genes known to be involved in resistance to whiteflies or aphids. Several genes showed strict correlation of parent and progeny 0-dpi expression with resistance, suggesting they may act as whitefly resistance factors. Such genes include chitin perception/response genes and ABA response genes. Together these genes are suggested as possible candidates for evaluation in transgenic cassava to confirm their possible roles as whitefly resistance factors.

Collectively, this Dissertation contributes to our understanding of the genetic basis of cassava's response to whiteflies. These data will better inform the introduction of resistance traits from ECU72 to locally-adapted, high-yielding African cassava lines in whitefly-affected regions of sub-Saharan Africa. Whitefly- and defense-hormone-responsive transcriptomes first defined here and by Irigoyen et al. (2020) will additionally serve as a useful tool for the cassava-defense community to better characterize cassava's response to various yield-threatening pests/pathogens. Themes emerging among the three Chapters of this Dissertation as factors that may be important for whitefly resistance in ECU72 include perception of unknown whitefly elicitors, cell-wall-based defenses and ABA-based defenses. Evaluation of genes proposed by this Dissertation to be involved in such processes is necessary to confirm that these processes are important for the whitefly resistance mechanism of ECU72. Such evaluations may be performed in transgenic cassava or through utilizing T-DNA lines corresponding to orthologous genes in Arabidopsis.

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