An Investigation of Morphological, Genetic, and Metabolomic Factors Impacting Insect Herbivory Resistance in *Vitis labrusca* Grapevine

Dissertation

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By

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Abstract

Grapevine (*Vitis*) is the world's most valuable fruit crop, therefore, reducing yield losses to stressors is paramount. *Vitis labrusca*, a wild North American grapevine, is well adapted to its local environment, exhibiting stout pathogen resistance. Meanwhile, *Vitis vinifera* grapevine, grown worldwide for winemaking, is native to Europe and is highly susceptible to biotic stressors, particularly fungal and insect pests. *V. labrusca* has been long utilized in *Vitis* breeding programs to imbue resistance. Therefore, in this dissertation, we determined if *V. labrusca* acc. 'GREM4' was more insect herbivory resistant than *V. vinifera* cv. 'PN40024' and investigated the morphological, genetic, and metabolomic factors which may contribute to resistance. In an herbivory choice assay, Japanese beetles (*Popillia japonica*), a major pest of grapevine, preferred to feed upon 'PN40024' compared to 'GREM4'. Further, increased leaf area was consumed on 'PN40024' compared to 'GREM4' in a time course (30min, 1h, and 4h) feeding assay. These results reported 'GREM4' is resistant to Japanese beetle herbivory compared to 'PN40024'. To determine morphological adaptations that may impact defense, trichomes were next investigated. Trichome densities were greater on 'GREM4' compared to 'PN40024' leaves. In trichome-focused herbivory studies, beetles exhibited a preference for lower trichome density sides of leaves and, when provided tissues with equal

trichome densities for both ‘GREM4’ and ‘PN40024’, more leaf tissue was still lost from ‘PN40024’ compared to ‘GREM4’. These results report that trichomes play a role in resistance but are not the sole factor. Therefore, we conducted a comparative transcriptomic analysis to identify differences in gene expression upon insect herbivory between the two species. When comparing constitutive expression differences prior to insect herbivory, genes with greater expression in ‘GREM4’ were enriched in secondary metabolite biosynthesis while enrichment in genes related to plant-pathogen interactions were identified in both species. Upon insect herbivory, the number of significantly differentially expressed genes (DEGs) was lowest in ‘GREM4’ at 30min and highest at 4h while the opposite was observed in ‘PN40024’. By 4h, many defense-related DEGs were identified in ‘GREM4’ compared to relatively few in ‘PN40024’. Systemic responses revealed a greater number of DEGs related to defense and signaling in ‘GREM4’ compared to ‘PN40024’. In both herbivory and systemic responses, flavonoid, phenylpropanoid, acyltransferase, and signaling-pathway genes were identified in greater numbers, or exclusively, in ‘GREM4’ compared to ‘PN40024’. To determine the impact of these transcriptomic alterations on metabolite levels, a comparative untargeted metabolomic study was conducted. Constitutively higher levels of metabolites, such as flavonoids, phenylpropanoids, and terpenes, were identified in ‘GREM4’ compared to ‘PN40024’ leaves while, after 1h of herbivory, a greater number of significantly differentially accumulating metabolites (DAMs) were identified in ‘PN40024’ compared to ‘GREM4’. Constitutively and inducibly increased levels of metabolites with insect

repellent and insecticidal properties were observed in ‘GREM4’ compared to ‘PN40024’. Candidate genes and metabolites reported may be employed in future functional studies to determine their impact on resistance. Findings presented herein will inform research endeavors to increase insect herbivory resistance of *Vitis*, and likely other crops.

Dedication

To Mom, Pop-pop, Grammy, Granddad, and Nana who cannot be here with me in person but are smiling down on me from heaven and are proud of the achievements I've accomplished in my life through Christ.

To Dad and Nick, for always being there to support me each in their own ways and being my unwaveringly most ardent supporters through thick and thin.

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"A ship is safe in harbour, but that is not what ships are for."

- John A. Shedd

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Publications

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Field of Study

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Chapter 1 - Introduction

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Status: In Preparation

Abstract

Insect herbivory causes roughly 25% of crop loss each year. Plants have evolved an extensive array of adaptations to defend against insect herbivory and thrive in their local environments. Understanding the diversity of insect herbivory defenses that have evolved across plants provides the opportunity to integrate them into crops to decrease insect herbivory damage. In this review, we detail a variety of insect herbivory defensive adaptations in cultivated plants, highlighting recent findings in plant physical and chemical defenses, as well as genetic and phytohormonal responses to insect herbivory. We also discuss implications for applying this knowledge to crop breeding, biotechnology, and management practices to mitigate insect herbivory-related yield losses, contributing to global food security.

Introduction

Biotic stressors curtail production of agricultural goods that are critical components of diets globally. Bacterial, fungal, and viral pathogens, in conjunction with insects and animals, are 'biotic stressors' and collectively invoke between 17 to 30% of merchantable crop losses globally each year (Gimenez et al., 2018; Moustafa-Farag et al., 2019; Savary et al., 2019). Biotic stressors decrease yields by damaging salable portions of plants and reducing photosynthetic capacity which, in turn, diminishes food security and profits (Hunter and Hinds, 1904; Dalal et al., 2006; Oerke, 2006; Ni et al., 2007; Pfeiffer, 2012; Chen et al., 2019; International Journal of Molecular Sciences, 2020; Naegele et al., 2020; United State Department of Agriculture - National Agricultural

Statistics Service, 2020; Li et al., 2021). For example, insect pests, such as locusts, aphids, grubs, beetles, and thrips, survive by ingesting sugars and other critical plant metabolites, either by directly eating herbaceous plant tissues (chewing mouthpart insects) or by puncturing the vasculature of the plant to extract photosynthates (piercing-sucking mouthpart insects). Adding in abiotic factors, such as untimely cold-snaps or flooding events, plants must endure many abuses to survive a growing season and reproduce.

Insects, which are found across every continent and environment, are highly diverse, both genetically and morphologically, and are the most abundant group of animals on the planet (Sharma et al., 2017). The Food and Agriculture Organization of the United Nations (FAO) estimates that insect pests of crops are responsible for 25% of total crop yield losses worldwide (Singh and Kaur, 2018; FAO and Sarkozi, 2019). Despite the intensive and long-standing cultivation of domesticated crops, which has allowed for advancement in insect herbivory protection practices, insect pests still pose a substantial threat to cultivated crop quality and yields. The study and identification of insect herbivory defensive traits across plant life can help drive advances in commercially important crops. This review outlines a handful of key defenses observed in commercially and scientifically relevant plants against insect herbivores.

Plant-Insect Interactions – A Co-Evolutionary Arms Race

Insects and plants have co-evolved over 350 million years in an arms race to feed upon, or defend against, one another in a delicate balance for survival (War et al., 2012).

Plants are generally resilient to abiotic and biotic stressors due to heterogeneous gene pools and natural selection. However, just as plants have evolved defenses against insect pests, insects have evolved adaptations to overcome plant defenses in a relationship of predative and reciprocal defensive co-evolution known as an 'evolutionary arms race' (Peiffer et al., 2009; Medel et al., 2010; War et al., 2012; Bortesi and Fischer, 2015; Lev-Yadun, 2016, 2021; Endara et al., 2017). An evolutionary arms race occurs when an evolutionary advantage is gained by a predator or host and the other party must adapt itself to overcome, or circumvent, the adaptation or face extinction. This process occurs at the population level over many generations due to random genome alterations of individuals via mutation, homologous recombination, and chromosome segregation which, by chance, confer increased fitness.

For example, if a plant were to produce a new toxin lethal to an herbivorous beetle pest, then, over many generations, the beetle species as a population adapts. Supposing the beetles must eat the plant, only beetles with a genetic composition which provides the ability to detoxify the compound will survive through natural selection. The genetic composition which conferred the ability to survive the selection event will be passed on to the next generation, thus perpetuating the advantageous genetic makeup. Over generations, the number of herbivorous beetles in the population that can overcome the plant toxin will become great enough to harm the plant population. The plant population will, therefore, undergo the same natural selection process to adapt and overcome this herbivory pressure. Perhaps the surviving individuals will possess stiffer

leaves or greater cuticle thickness to reduce feeding. Thus, the cycle begins anew. This foundational principle can be observed across all forms of life. In plants, natural selection has resulted in a vast array of unique and advantageous adaptations to overcome insect herbivores.

Since plants are sessile, a wide array of morphological, physiological, and chemical defenses has evolved. These defensive measures may either be produced constitutively or inducibly when specific criteria are met. Constitutive defenses are always produced or present affording constant protection at the expense of energy costs. Inducible defenses are only produced or present upon induction by a stimulus, typically stress, thus, is more energetically efficient but at the cost of delayed defense in the face of a stressor (Rasmann et al., 2015). Further, plants may invoke direct or indirect means of defense. Direct means of defense typically physically interact with the stressor directly in an obvious manner. Direct defensive measures include trichomes or toxic compounds that are ingested (Chen, 2008). Indirect defenses typically impart defense against a stressor through interaction with other individuals, their ecosystem, or other biological pathways (Kessler and Heil, 2011). For example, the release of volatile organic compounds (VOCs), which often attract beneficial insects or inform neighboring plants of the stress a nearby plant is facing, is an indirect defense (Kessler and Heil, 2011). All plant defense responses can be categorized as direct or indirect and constitutive or inducible. Table 1.1 displays this relationship in a matrix and provides an example of each type of defense.

Perception of Insect Feeding

While mechanical damage is a vital signal for insect defense perception within plants (damage-associated molecular patterns (DAMPs)), another integral cue is found in insect oral secretions – herbivore-associated molecular patterns (HAMPs). HAMPs are molecules produced by herbivorous pests which plants recognize and identify as indicators of active herbivory (Grissett et al., 2020). Otherwise known as 'elicitor compounds', the most well studied subset of HAMPs are fatty acid-amino acid conjugates (FACs). FACs include volicitin, free peptides, specific enzymes, caeliferins, and other associated compounds (Wu and Baldwin, 2009; Allmann and Baldwin, 2010; Yoshinaga et al., 2010; War et al., 2012; Allmann et al., 2013; Engelberth and Engelberth, 2019; Grissett et al., 2020). FACs are found in oral secretions (OS) of feeders, and differ between species, thereby allowing the plant to invoke species-specific responses (Yoshinaga et al., 2010). After recognition of elicitors by the plant, signaling cascades initiate biological processes which have been evolutionarily tailored to defend the plant from the specific threat, such as, for example, the induction of genes which produce an insecticidal compound *in planta* (Ahmad et al., 2011; Zhang et al., 2022a).

While FACs play an important role in insect feeding perception by plants, not all plants can detect these molecules. An exploration in the *Solanaceae* discovered mixed abilities of species to perceive FACs (Grissett et al., 2020). Eggplant (*Solanum melongena*), bell pepper (*Capsicum annuum*), model tobacco (*Nicotiana benthamiana*), cultivated tobacco (*N. tabacum*), jasmine tobacco (*N. alata*), woodland (nightshade)

tobacco (*N. sylvestris*), petunia (*Petunia hybrida*), and two wild tomato species (*Solanum chilense* and *S. corneliomulleri*) were all responsive to FACs produced by tobacco hornworm (*Manduca sexta*). However, potato (*Solanum tuberosum*), *Nicotiana knightiana* (unnamed semi-wild tobacco accession), cultivated tomato (*Solanum lycopersicum*), and multiple wild tomato species (*Solanum cheesmaniae, S. pimpinellifolium, S. chmielewskii, S. neorickii*) were found unresponsive. Plant recognition of FACs likely varies depending on both the insect and plant species in question.

Plants invoke 'tailored' defense responses to the insects they encounter. Appel et al. (2014) reported differential gene expression in *Arabidopsis* which altered uniquely upon attack by different insect herbivore species and by feeding type (Appel et al., 2014). While mechanical wounding and insect damage treatments both elicited differential gene expression, caterpillar feeding elicited greater than 3 times more significantly differentially expressed genes (DEGs) than mechanical wounding suggesting unique responses tailored to combating insect herbivory beyond a baseline tissue damage response. When reviewing the transcriptomic response in *Arabidopsis* between feeding by piercing-sucking mouthpart insects (aphids) and chewing mouthpart insects (caterpillars), only 18% of DEGs were shared between the responses to the two feeding types. Even when comparing the response between two different caterpillar pests (beet armyworm (*Spodoptera exigua*) versus caterpillar-stage cabbage butterfly (*Pieris rapae*)) or two different aphid pests (green peach aphid (*Myzus persicae*) versus cabbage aphid

(*Brevicoryne brassicae*)), only 33% of DEGs were shared between the responses to the two caterpillar species while only 11% were shared between the two aphid species. Overall, this study reports distinct, insect-specific defense responses within *Arabidopsis*. Tailored defense responses unique to specific insect pests have also been observed in rice (Deng et al., 2022), *Eruca sativa* (Ogran et al., 2019), soybean (Romero et al., 2020), and other plants (Musaqaf et al., 2023), lending support to the notion that insect-specific responses are observed in plants upon herbivory from pest to pest. These responses have evolved to exact effective defense while balancing use of energetic resources.

While this review is not exhaustive, it explores key plant defensive adaptations that have evolved to defend against insect herbivory, including phytohormonal responses, physical defenses, and chemical defenses.

Insect Herbivory Defensive Mechanisms

The Role of Plant Phytohormones in Insect Herbivory Defense

Plant hormones (phytohormones) are secondary metabolites and critical signaling molecules which serve as essential components in the processes of plant development, stress response, and stress mitigation, including insect herbivory response and defense. Alterations in phytohormone regimes often coincide with defense responses and trigger specific genes and pathways to combat or mitigate the stress (Aerts et al., 2021; Zhao et al., 2021). Seven plant hormones discussed in this chapter include auxin, cytokinin (CK), gibberellins (GA), abscisic acid (ABA), ethylene (ETH), jasmonic acid (JA), and salicylic acid (SA) (Aerts et al., 2021; Zhao et al., 2021). JA and SA are two important

plant hormones implicated in biotic stress response, including insect herbivory, but ABA and ETH also play a role (Meents et al., 2019; Costarelli et al., 2020). Phytohormone levels in tissues fluctuate dynamically to induce tailored, evolutionarily-adapted plant responses through synergistic interactions known as 'cross-talk' in which differing combinations, ratios, or concentrations of phytohormones accumulate *in planta* to invoke unique responses to stressors (Aerts et al., 2021). The best understood example of cross-talk exists between JA and SA, both of which are master regulators of plant biotic defense and are usually antagonists to one another (Aerts et al., 2021; Weeraddana and Evenden, 2022). Upon recognition or damage by necrotrophic pathogens and chewing insects, JA levels increase and SA levels decrease to engage defense responses uniquely adapted to combating such stressors (Costarelli et al., 2020; Aerts et al., 2021; Weeraddana and Evenden, 2022). In response to biotrophic pathogens, and in some cases piercing-sucking insects, SA levels increase while JA decreases (Geuss et al., 2018; Costarelli et al., 2020; Aerts et al., 2021; Weeraddana and Evenden, 2022).

In rice (*Oryzae sativa*) damaged by brown plant hopper (*Niaparvata lugens*), phytohormone genes were differentially expressed, and an increase in phytohormones was detected, in a resistant line compared to a non-resistant line in which connection networks reported strong correlations between phytohormone induction and insect herbivory defense (Zhang et al., 2022a). Interestingly, SA levels increased in both the resistant and susceptible line upon hopper herbivory. However, the resistant lines accumulated greater concentrations of SA compared to susceptible lines. Auxin

concentrations were also different between resistant and susceptible rice upon herbivory wherein indole-3-acetic acid (IAA) significantly decreased in resistant rice but decreased negligibly in susceptible rice, compared to controls. Follow up experiments which applied exogenous IAA, SA, or water to brown plant hopper-susceptible plants after six days of hopper feeding reported IAA applications expedited plant death while SA improved plant health and vigor, when compared to the water-only control. Essential regulator of SA biosynthesis *NPR1* (*non-expressor of pathogenesis-related genes 1*) was strictly up-regulated in resistant rice while multiple IAA biosynthesis regulating genes were down-regulated strictly in susceptible rice. These results report SA is a major phytohormonal contributor to insect defense signaling in rice and that decreased IAA concentrations were critical for rice to survive herbivory events. As for other phytohormones, genes *ABF* (*ABRE binding factor*) and *PYL* (*pyrabactin resistance 1-like protein*) of the ABA biosynthetic pathway, *ARR-A* (*type-A response regulator*) of the CK biosynthetic pathway, and *PIF4* (*phytochrome interacting factor 4*) of the GA biosynthetic pathway were all significantly down-regulated in both resistant and susceptible rice, suggesting a minimized role of ABA, CK, and GA in insect herbivory defense in rice regardless of susceptibility. In another study, transgenic rice which overexpressed a flavonoid biosynthesis gene, *OsF3'H* (*Flavonoid 3-monoxygenase*), produced a greater quantity of flavonoids, as well as SA, compared to WT susceptible rice (Jan et al., 2022). Genes implicated in SA and ETH biosynthesis were up-regulated in the overexpressor plant which was ultimately found to be resistant to white-backed

plant hopper (*Sogatella furcifera*), a serious piercing-sucking mouthpart pest of rice in Asia (Insecticide Resistance Action Committee, 2022). These results provide further evidence of SA biosynthesis gene up-regulation and JA biosynthesis gene down-regulation which have resulted in resistance to piercing-sucking mouthpart insect feeding and support JA/SA cross-talk (Aerts et al., 2021; Jan et al., 2022; Zhang et al., 2022a).

In bittersweet nightshade (*Solanum dulcamara*), upon beet armyworm feeding, jasmonic acid-isoleucine (JA-Ile), JA, and ABA levels increased, along with increased trypsin protease inhibitor activity (Geuss et al., 2018). Trypsin proteases function as critical enzymes in insect digestion. In plants, trypsin protease inhibitors play roles in multiple homeostatic biological functions and are constitutively present at low levels in plant tissues, but, upon insect herbivory, they function to impede insect digestion by blocking hydrolytic enzymes (Pandey et al., 2022). Exogenous applications of methyl-jasmonate (MeJA) to plants which had not been fed upon increased trypsin protease inhibitor activity in nightshade, a response recapitulated when afflicted by armyworms (Geuss et al., 2018). Additionally, when fed upon by armyworms, increased JA, JA-Ile, and ABA levels were observed compared to unafflicted controls, in addition to up-regulation of multiple JA biosynthesis-related genes, with a smaller proportion up-regulated associated with IAA.

When SYSTEMIN, a bioactive peptide which greatly amplifies a JA production signal in the JA pathway, is silenced in tomato, tobacco hornworm larvae that fed on *systemin*-silenced plants exhibited three times greater growth than larvae which fed on

WT plants (Orozco-Cardenas et al., 1993; Montero-Vargas et al., 2018). Quantifiable levels of multiple protease inhibitors were identified in non-silenced WT plants by the second day of hornworm feeding (Orozco-Cardenas et al., 1993). However, quantifiable levels of protease inhibitors could not be identified in SYSTEMIN-silenced tomatoes until after six days. At all times, WT plants had two to five times more protease inhibitors compared to silenced plants up to and through completion of the study on the fourteenth day. Considering the integral role of JA in insect herbivory defense signaling, especially that of JA as an essential distal wounding response signal (Schilmiller and Howe, 2005), a lack of JA production signal amplification by SYSTEMIN is likely the root cause for the increased larval feeding in the *systemin*-silenced line, illustrating the importance of JA and SYSTEMIN in robust insect herbivory defense in tomato. In addition to the critical role of SYSTEMIN to induction of JA production in tomato, such a role is also observed in other *Solanaceae*, and perhaps in other plants as well (Pearce et al., 2001, 2007, 2009; Ryan and Pearce, 2003; Schilmiller and Howe, 2005; Chen et al., 2008; Montero-Vargas et al., 2018).

Phytohormones are critically important secondary metabolites and signaling molecules in plants leveraged to regulate growth and development and mediate responses to stress such as insect herbivory. Exploring the various responses of phytohormones *in planta* to insect herbivory is important to fully understanding and leveraging robust insect herbivory defense and, as such, are implicated in all insect herbivory defense responses discussed in the following sections.

Physical Defenses

Plants possess a suite of physical defenses against insects. Physical defenses are morphological or anatomical adaptations which increase fitness by deterring insect pests (Hanley et al., 2007). The following section will cover trichomes and rigidity of tissues (sclerophylly) - two major types of physical insect herbivory defenses in plants.

Trichomes

Trichomes are physical structures that often serve as the first line of defense against many abiotic and biotic stressors (Guo et al., 2022). Trichomes have many advantageous functions including providing defense against infections or herbivory, preventing cellular damage due to UV exposure, and regulating leaf temperature and transpiration (Hunter and Hinds, 1904; Peiffer et al., 2009; Tooker et al., 2010; War et al., 2012; Liu et al., 2016; Zhou et al., 2017; Fambrini and Pugliesi, 2019; Kono and Shimizu, 2020; Wang et al., 2021; Nassour and Ayash, 2021; Singh et al., 2021; Guo et al., 2022; Jia et al., 2022).

One of the earliest scientific reports connecting trichomes with insect herbivory resistance was in 1904 by Hunter & Hinds who carried out Mexican boll weevil (*Anthonomus grandis*) resistance trials and noted that Egyptian cottons, such as *Gossypium barbadense*, with trichome-less stems, were severely damaged by weevils, while American cottons, such as *Gossypium hirsutum*, which exhibited many trichomes, underwent little feeding damage (Hunter and Hinds, 1904).

The genetic pathway which underlies trichome formation is complex and regulated by multiple genes. Trichome development genes such as *GLABRA1* (*Gl1*), *GLABRA2* (*Gl2*), *GLABRA3* (*Gl3*), and *Transparent TESTA GLABRA* (*TTG1*) generally positively regulate formation of trichomes, whereas expression of *CAPRICIOUS* (*CPC*), *Trichomeless1* (*TCL1*), *Trichomeless2* (*TCL2*), and others inhibit trichome formation (Yan et al., 2012; Liu et al., 2020c). *Arabidopsis gl1*, *gl3*, or *ttg1* mutants exhibited significantly decreased quantities of trichomes compared to WT plants and beet armyworm or *Spodoptera frugiperda* (fall armyworm) caterpillars which fed on any of the three mutants gained significantly more weight than those which fed upon WT (Song et al., 2022). This study supports previous mutant work which also reported *gl1* mutant plants exhibited significantly increased damage by chewing mouthpart insects compared to WT (Sato et al., 2019). These studies together support the role of Gl1 in trichome formation and insect herbivory defense. Additionally, feeding by beet armyworm and fall armyworm was found to have significantly increased transcript accumulation of *Gl1* and *Gl3* in WT *Arabidopsis* (*TTG1* expression also increased, but not significantly), suggesting trichome production could be a defense response to herbivory (Song et al., 2022). In soybean, *Gl1, Gl2, Gl3, and TTG1* all play a critical role trichome formation (Liu et al., 2020c). *Glyma.01G240100* (*Gl2*) was identified as being responsible for the high trichome density phenotype *Pd1* (dense pubescence) in soybean and encodes the final transcription factor in the trichome development process. As such, Gl2 is known to directly trigger trichome formation and Gl1, Gl3, TTG1, MYB23, and EGL3 are known

to bind to the promotor of *Gl2* to induce expression (Liu et al., 2020c; Wang et al., 2021; Song et al., 2022). To that end, overexpression of *Gl2* in soybean has resulted in more than three times the number of trichomes on stems and petioles (Liu et al., 2020c). Together, these studies, along with others, report strong evidence that *Gl1, Gl2*, *Gl3,* and *TTG1* are important genes implicated in insect-herbivory defense.

Functionally, trichomes inhibit insect pests in multiple ways. One way trichomes aid in defense is acting as a physical barrier between the plant surface and the insect (Massee, 1924; Peiffer et al., 2009; War et al., 2012; Fambrini and Pugliesi, 2019; Singh et al., 2021). It was found that bird cherry-oat aphid (*Rhopalosiphum padi*) preferentially consumed older leaves, with lower trichome densities, of undomesticated emmer wheat (*Triticum turgidum*) compared to younger leaves with high densities (Singh et al., 2021). Observations from the study noted that trichomes physically obstructed aphids from feeding on the younger leaves. Considering many trichome-abundant plants display increased trichome densities on immature leaves compared to mature, this could serve as a mechanism to protect vulnerable, immature leaves from herbivory.

Trichomes can also aid in defending the plant through conversion of physical interactions into biological signals. Tooker et al. 2010 and Peiffer et al. 2009, in concurrent studies, discovered trichomes function in transducing defensive signaling in tomato (*Solanum lycopersicum*) suggesting this phenomenon may be found in species across plant life and is not constrained solely to a few unique plants such as the Venus flytrap (*Dionaea muscipula*) or sundew (*Drosera*) as was previously postulated (Peiffer

et al., 2009; Tooker et al., 2010). In their studies, it was found that defense genes, including JA biosynthesis genes, were significantly up-regulated after tobacco hornworm, *Heliothis virescens* (tobacco budworm) and *Helicoverpa zea* (corn earworm) caterpillars and moths were permitted to crawl on tomato leaves for 10 minutes. Mechanical disruption by hand induced similar effects and induced large accumulations of reactive oxygen species such as H_2O_2 (Peiffer et al., 2009). They further reported H_2O_2 is a key mediator in induction of *PIN2* (*Proteinase inhibitor 2*), a wounding response defense gene in tomatoes (Graham et al., 1985; Peiffer et al., 2009). In this interaction, the physical act of an insect walking on trichomes cause trichomes to "buckle" (i.e. - tip over or break off) which initiates transduction of a biological signal resulting in a defense response (Liu et al., 2016; Zhou et al., 2017). In two complimentary studies by Liu et al. 2016 and Zhou et al. 2017 with *Arabidopsis* trichomes, they collectively reported trichome buckling altered defense signaling and elicitor accumulations (Liu et al., 2016; Zhou et al., 2017). Physiologically, Liu et al. discovered trichome buckling alters cytosolic Ca^{2+} and apoplastic pH in cells surrounding buckled trichomes, thusly identifying the cellular-level alterations implicated in transducing the mechanical disturbance into a chemical signal (Liu et al., 2016). Genetically speaking, Zhou et al. hypothesized these putative signaling reporters induce up-regulation of defensive genes and pathways (Zhou et al., 2017).

Trichomes are also recognized as sinks for heavy metals which could confer additional insecticidal defensive capabilities (Sarret et al., 2006; Bothe and Słomka,

2017). Glandular trichomes, which are found in roughly 30% of plants, can store and secrete heavy metals, which aid in insect defense in plants such as tomato, tobacco, sunflower, and other wild and domesticated plants (Kang et al., 2010; Glas et al., 2012; Gao et al., 2018; Morimoto, 2019; Li et al., 2020b; Feng et al., 2021; Wang et al., 2021; Guo et al., 2022). Interestingly, recent studies report non-glandular trichomes may also exhibit heavy-metal sequestration capabilities. When two plant species with strictly non-glandular trichomes, *Arabidopsis* and Chinese cabbage (*Brassica rapa*), were exposed to stimulation via beet armyworm larvae or a paint brush, significantly and rapidly increased uptake and storage of cadmium was observed in leaves and leaf trichomes under both types of stimulation compared to unstimulated controls (Guo et al., 2022). Further, *Arabidopsis gl1-1* mutants (which lacked substantial quantities of trichomes compared to WT *Gl1-1*) exposed to armyworm or mechanical stimulation did not exhibit increased cadmium uptake nor storage. Heavy metal tolerance/detoxification genes were significantly up-regulated in WT *Gl1-1* including *AtHMA2*, *AtPCS1, AtNRAMP3*, and *AtCOPT2* upon stimulation compared to unstimulated controls, and thus, may play a role in increasing heavy metal concentrations in trichomes. These results not only suggest trichomes play a role in heavy metal tolerance *in planta*, but storage and secretion of heavy metals by trichomes may be a selective advantage against insect herbivory as noted by others (Sarret et al., 2006; Kang et al., 2010; Glas et al., 2012; Bothe and Słomka, 2017; Gao et al., 2018; Morimoto, 2019; Li et al., 2020b; Feng et al., 2021; Wang et al., 2021; Guo et al., 2022).

Trichome density does not always correlate with resistance to insect pests. Studies in chrysanthemum (*Chrysanthemum* × *morifolium* Ramat.) infested by western flower thrips (*Frankliniella occidentalis*) and in cotton (*Gossypium hirsutum*) infested with sweet potato whitefly (*Bemisia tabaci*) reported increased trichome densities positively correlated with nymph and egg quantities on leaves (Butler et al., 1991; Chu et al., 2003; Chen et al., 2020). Chu et al. postulated the microclimate within the boundary layer created by the trichomes is more humid, relative to trichome-less tissues, which is more amiable to eggs and nymphs (Chu et al., 2003). Others hypothesize increased trichome densities provide safety for oviposited eggs and newly hatched nymphs from predators. In a survey of 19 wild and cultivated tomato accessions of varying morphology, insect resistance and trichome density were sometimes positively correlated with insect defensive capacities of accessions over *B*. *tabaci* and thrips, but other times were negatively correlated (Kortbeek et al., 2021). Exudates secreted by glandular trichomes were found to play a larger role in defense than density of trichomes alone (Kortbeek et al., 2021), which is supported by previous research (Dimock and Kennedy, 1983; Weston et al., 1989). As a final counterexample, a study by Sato et al. found a significant negative correlation between trichome densities on leaves of field-grown *Arabidopsis* and feeding by chewing mouthpart insects, but no correlation was found for piercing-sucking mouthpart feeders (Sato et al., 2019). These studies demonstrate trichomes may be more effective at deterring some insect pests over others. However,

overall, trichomes are viewed as important and effective insect-herbivory defensive structures *in natura.*

Sclerophylly

Plants have evolved the capacity to 'harden' their tissues in response to abiotic and biotic stresses. Sclerophylly translates to 'hard-leaved' and sclerophylly index is calculated by dividing the dry mass of a tissue by its area (Read and Sanson, 2003; Batjuka and Škute, 2021). Increased rigidity of tissue is typically concurrent with leaf thickening and is primarily brought about via increased sclerification (lignification of plant cell walls, followed by cell death) of vascular bundle sheaths and tissue margins paired with increased cuticle thickness (Turner, 1994; Peeters, 2002; Lobregat et al., 2018; Ribeiro et al., 2021). Lignin stiffens plant secondary cell walls, providing structural support and conferring heightened stress mitigation (Zhang et al., 2021b). As sclerification increases, so does tissue strength and rigidity (Choong et al., 1992). Alterations in sclerification can be influenced by abiotic factors, such as elevation, drought, extreme temperatures, and UV light (Jordan et al., 2005; Lobregat et al., 2018; Ogran et al., 2019; Soriano et al., 2019; Mickky et al., 2020; Batjuka and Škute, 2021; Llerena-Zambrano et al., 2021; Bordbar et al., 2022).

Sclerophylly aids in insect herbivory defense via hardening of tissues in both constitutive (pre-emptive sclerification of tissues) and inducible fashions (Ogran et al., 2019; dos Santos et al., 2020; Ribeiro et al., 2021; Silva et al., 2021; Zhang et al., 2021b). Catingueira (*Cenostigma pyramidale*), an animal feedstock, displayed an inverse

relationship between sclerophylly index and insect herbivory in a study of variably sclerified leaves (Ribeiro et al., 2021). Similarly, a study in maize revealed fall armyworm (*Spodoptera frugiperda*) leaf damage was inversely correlated with sclerophylly at growth stage V12 (dos Santos et al., 2020). Among 38 different plant species found in Brazilian forests, a negative correlation was identified between sclerified mass and insect herbivory damage of leaves (Silva et al., 2021). While these three studies indicate the role of sclerophylly as a constitutive defense against insect herbivory, sclerification is also known to act inducibly. Zhang et al. 2022 found lignin content, sinapyl alcohol (lignin biosynthetic pathway intermediate) levels, and expression of genes associated with lignin biosynthesis increased upon brown plant hopper feeding in resistant rice compared to susceptible (Zhang et al., 2022a). They hypothesized increased lignification prevented successful phloem penetration by plant hopper stylets, and thus, conferred heightened insect herbivory resistance. 'Sclerophyllization pathway genes' in arugula (*Eruca sativa*), including suberin, lignin, and putrescine synthesis genes, were up-regulated when mechanically wounded and treated with oral secretions from Egyptian cotton leafworm (*Spodoptera littoralis*) or cabbage butterfly (Ogran et al., 2019). Overall, sclerification of tissues has been shown repeatedly to play an important role in plant rigidity and insect herbivory defense in a wide array of plants.

Sclerophylly not only decreases the ability of insects to physically consume tissues and access photosynthates, but also reduces digestibility of plant materials to insects (Morrow, 1983; Martin, 1991; Choong et al., 1992; Ribeiro et al., 2021).

Considering cellulose, a major component of sclerification, is not digestible by most insects, it is hypothesized that herbivorous insects not only avoid highly sclerophyllous tissues because they are challenging to penetrate, but are also inferior in nutritional value per quantity ingested compared to low-sclerophylly feeding sources, further disincentivizing feeding (Morrow, 1983; Martin, 1991; Choong et al., 1992; Hochuli, 1996; Coetzee et al., 1997; Peeters, 2002; Read and Sanson, 2003; Hanley et al., 2007).

The genes which underly sclerification fall mostly within the phenylpropanoid biosynthetic pathway – a critical upstream pathway of both the flavonoid and lignin pathways – and the lignin biosynthetic pathway (Xie et al., 2018; Chen et al., 2022; Wang et al., 2022; Zhang et al., 2022a). The phenylpropanoid pathway begins by phenylalanine ammonia-lyase (PAL) catalyzing the conversion of phenylalanine to cinnamic acid, which is then converted to p-coumaric acid by Chalcone-4-hydrolase (C4H) (Xie et al., 2018; Zhang et al., 2022a). 4-coumarate-CoA ligase (4CL) primarily converts p-coumaric acid to p-coumaroyl-CoA in the phenylpropanoid pathway in advance of p-coumaroyl-CoA usage in the flavonoid biosynthesis pathway (via catalyzation by CHS) or the lignin biosynthesis pathway (via catalyzation by HCT) (Xie et al., 2018; Zhang et al., 2022a). Notably, 4CL also catalyzes at least 4 other known reactions nested within the lignin pathway (Xie et al., 2018; Zhang et al., 2022a). Hydroxycinnamoyl-CoA Shikimate (HCT) catalyzes the crucial conversion of the end-product of the phenylpropanoid pathway, p-coumaroyl-CoA, to p-coumaroyl shikimic acid, one of two intermediaries in the first step of the lignin biosynthetic pathway (Xie et

al., 2018; Kriegshauser et al., 2021). HCT also catalyzes the reaction converting caffeoyl shikimic acid to caffeoyl-CoA just one step later (Kriegshauser et al., 2021; Zhang et al., 2022a). Other genes, such as *CCoAMT*, *CCR*, *CAD*, *F5H*, and *COMT* play important roles downstream in producing G and S lignin.

Since lignin is also a critical component of plant cell walls, these aforementioned genes also significantly impact normal plant growth, development, and morphology. For example, a quadruple *PAL* mutant in *Arabidopsis* led to reduced lignin content and decreased SA accumulation (Huang et al., 2010). Reduction of *4CL* expression in rice and Monterey pine (*Pinus radiata*) resulted in reduced lignin content and shorter plants (Wagner et al., 2009; Gui et al., 2011). Non-functional *HCT* in *Physcomitrium patens* (Physcomitrella) resulted in total loss of the plant cuticle, degrading its overall defensive capacity, in addition to alterations in phenolic compounds compared to WT (Kriegshauser et al., 2021). Similarly, *HCT* knock-out mutants in *Arabidopsis* resulted in reduced growth, a thinner cuticle, and decreased accumulation of phenylpropanoid-related molecules (Kriegshauser et al., 2021). These results illustrate that phenylpropanoid/lignin pathway genes are critical to plant development, but, as can be seen from these examples, contribute to defensive structures as well, such as the cuticle and sclerophylly, as well as defensive compounds, like SA and phenolic compounds, highlighting their extensive importance in herbivory defense.

A number of studies in rice have illustrated the importance of phenylpropanoid/lignin pathway genes in insect herbivory defense. Seven of the nine

total *PAL* genes were up-regulated in resistant rice infested with brown plant hopper compared to both susceptible infested and resistant un-infested rice (He et al., 2019). Further, knock down of *OsPAL* genes in resistant rice resulted in significantly increased hopper damage. When *OsPAL8* was overexpressed in a susceptible line, lignin and resistance to hopper feeding increased significantly. *OsMYB30*, which encodes a transcription factor, was found to positively regulate *OsPAL* gene family members' responses to insect herbivory in resistant rice compared to non-resistant. In another study, *4CL* and *HCT* were two of four total DEGs that were exclusively significantly up-regulated in resistant compared to non-resistant rice afflicted by brown plant hopper (Zhang et al., 2022a). Lignin accumulation was significantly higher in resistant rice compared to non-resistant at all timepoints queried and thickness of sclerenchyma and vascular bundles was positively correlated with increased lignin accumulation *in planta* suggesting increased lignin deposition in plant vasculature, decreasing the risk of penetration by insects with piercing-sucking mouthparts. These studies report that phenylpropanoid/lignin genes are differentially expressed in response to insect herbivory and sclerification/lignin accumulation contribute to insect herbivory defense in rice.

The role of phenylpropanoid pathway genes in insect herbivory defense has been studied in other crops as well. Resistant wheat (*Triticum aestivum*) exhibited significant up-regulation of all four *PAL* genes upon orange wheat blossom midge (*Sitodiplosis mosellana*) feeding versus resistant plants not exposed to feeding (Wang et al., 2022). Meanwhile, two of four *PAL* genes were up-regulated when susceptible plants were

exposed to feeding compared to no feeding controls. When exposed to feeding, the resistant wheat also exhibited significantly increased accumulations of cinnamic acid, and other downstream products of the phenylpropanoid pathway, compared to resistant plants not exposed to feeding. RNA-seq and qRT-PCR results showed *4CL* expression significantly increased upon midge feeding in resistant wheat plants as well. In cassava infested with two-spotted spider mites (*Tetranychus urticae*) *4CL* and *PAL* were consistently, and constitutively, up-regulated and increased levels of p-coumaroyl-CoA were found in resistant compared to susceptible plants at zero days (just prior to feeding initiation), one day, and eight days after feeding (Chen et al., 2022). In grapevine, resistance to *Acrida chinensis* (oriental longheaded grasshopper) in hybrid grapevine 'Kyoho' was in part attributed to up-regulation of *PAL* (Jia et al., 2022). Interestingly, *PAL* transcripts were only significantly up-regulated in mature insect-damaged leaves and not in young insect-damaged leaves. These studies support the role of phenylpropanoid/lignin pathway genes in insect herbivory defense across a variety of commercially important plants. Overall, sclerophylly is an important adaptation which aids plants in defending against insect herbivory by hardening tissues preventing insect damage.

Chemical Defensive Compounds

Compounds have evolved in plants which, through their presence in tissues, have come to deter insect herbivory through their ingestion, contact, or perception by insect pests. While a vast array of compounds have evolved in plants for defense, this section

will outline recent findings in chemical defensive compounds related to flavonoids and terpenes.

Flavonoids

Flavonoids are responsible for many of the red, purple, and blue pigments in plants, and also exhibit antioxidant and insecticidal properties that are important in biotic and abiotic stress mitigation and insect defense respectively (Bate-Smith, 1969; Nicholson et al., 1987; Lo et al., 1999; Pourcel et al., 2007; Men et al., 2022). Most flavonoid pathway genes were originally identified via cloning studies in maize and other plants in the 1980's (Dooner et al., 1985; Beld et al., 1989; Chandler et al., 1989). More recent studies have also reported their importance in pigmentation and insect defense. Perilla mint is an exceptional example to illustrate the relationship between flavonoid pathway genes, pigmentation, and flavonoid accumulation. In perilla leaves which display red or purple coloration, flavonoid biosynthetic pathway genes *CHS, FSH, CHI, F3H, F3'H, DFR, ANS, 3-GT, ACT, 5-GT*, and *MAT* and phenylpropanoid biosynthetic pathway genes *PAL, C4H,* and *4CL* were all up-regulated and significantly increased accumulations of flavonoids were identified in such leaves compared to green perilla leaves which displayed significantly reduced flavonoid and phenylpropanoid gene expression (Xie et al., 2022).

Flavonoid compounds are widely recognized as insecticidal. For example, *Cascabela peruviana* (aka *Thevetia peruviana* (Luckynut)) extracts exhibit antibacterial, antifungal, and insecticidal properties and flavonoids are a major component of the

extract (Men et al., 2022). Artificial diets containing extracts from luckynut significantly increased mortality of fruit flies and induced morality in 80% of larvae after seven days of feeding, in addition to many developmental defects and decreased body weight of surviving flies. Therefore, it is unsurprising that flavonoids accumulate upon insect herbivory, as seen in the examples below.

Under insect herbivory, flavonoids have been shown to accumulate to protect the plant from insect pests. Zhang et al. 2022 found flavonoid content significantly increased in an insect-herbivory resistant rice cultivar, but significantly decreased in a susceptible cultivar, when attacked by brown plant hopper (*Niaparvata lugens*) (Zhang et al., 2022a). Many stimulus recognition genes were significantly up-regulated in the resistant variety but were significantly down-regulated in the susceptible cultivar, with the most enriched pathways being phenylpropane biosynthesis, flavonoid biosynthesis, and plant hormone signaling transduction genes. In addition, flavonoid-biosynthesis-related metabolites broadly accumulated in the resistant cultivar, but broadly decreased, or did not change, in the susceptible cultivar. *Chalcone synthase* (*CHS*) and *Anthocyanidin reductase* (*ANR*) were two of four genes which were significantly, and exclusively, up-regulated in the resistant cultivar compared to the susceptible. CHS is the first enzyme in the flavonoid biosynthetic pathway converting p-Coumaroyl-CoA, one of the final products of the phenylpropanoid pathway, to naringenin chalcone, the first intermediary in the flavonoid pathway (Chen et al., 2022; Zhang et al., 2022a). When *CHS* was overexpressed in rice protoplasts, flavonoids significantly increased resulting in greater mortality of hoppers

which fed on *CHS* overexpressed protoplasts compared to WT (Zhang et al., 2022a). Anthocyanidin reductase converts cyanidin to epigallocatechin - one of many end products of the flavonoid pathway (Chen et al., 2022; Zhang et al., 2022a). Artificial diets spiked with epigallocatechin resulted in increased mortality of feeding hoppers (Zhang et al., 2022a).

Pyramiding *ANR* and another flavonoid pathway gene - *dihydroflavonol 4-reductase* (*DFR*) - from *Camellia sinensis* (tea) in transgenic tobacco resulted in increased accumulations of flavan-3-ols, earlier flowering, improved yields, and decreased herbivory over WT plants (Kumar and Yadav, 2017). Dihydroflavonol 4-reductase catalyzes dihydroflavonol to leucoanthocyanidins which are subsequently converted to anthocyanidins by ANS (Anthocyanidin synthase) (Kumar and Yadav, 2017; Chen et al., 2022; Zhang et al., 2022a). Of 10 genes implicated directly in the flavonoid pathway and upstream steps (*PAL, CHS, CHI, F3'H, DFR, FLS, ANR1, ANR2, LAR,* and *ANS*), all 10 were found to be significantly up-regulated in the pyramided tobacco plants (Kumar and Yadav, 2017). Further, pyramided lines exhibited decreased tobacco cutworm (*Spodoptera* litura) herbivory damage and larval growth was additionally retarded compared to controls.

Another study, which examined flavonoids in *Manihot esculenta* (cassava) after herbivory by two-spotted spider mite, found insect-herbivory resistant cassava exhibited an increase, compared to susceptible cassava, in all 16 flavonoids assayed in their study during at least one time point, with the majority of flavonoids consistently higher at all

timepoints (Chen et al., 2022). Nearly all known flavonoid biosynthesis genes, including core phenylpropanoid and flavonoid biosynthesis genes *PAL*, *4CL, CHS, F3'H, FLS, ANS, ANR,* and *LAR* (*leucoanthocyanidin reductase*, which catalyzes the reaction of leucoanthocyanidin to catechin), were significantly up-regulated in the resistant compared to the susceptible (Chen et al., 2022; Zhang et al., 2022a). Transgenic cassava which overexpressed *ANR* or *LAR* exhibited heightened spider mite resistance compared to WT cassava (Chen et al., 2022).

UDP-7-O-glucosyltransferase (UGT) catalyzes the final step in the production of multiple flavonoid biosynthetic products (Dong et al., 2020; Zhang et al., 2022b). *UGT* in soybean (*Glycine max*) was found to reside within a QTL, which was the major determinant of insect herbivory defense (Zhang et al., 2022b). When *GmUGT* was knocked-out (KO) from soybean, significantly decreased *Helicoverpa armigera* (cotton boll worm) and tobacco cutworm herbivory was observed compared to WT plants. When fed upon, KO mutants displayed significantly increased accumulations of 25 flavonoids and significant decreases in 15 others compared to WT controls which were also afflicted. Flavonoid pathway genes *CHS, CHR,* and *CYP81E11* were significantly up-regulated upon insect herbivory in the KO mutant compared to WT. These results suggest silencing of *GmUGT* may be necessary to shift flavonoid production within the pathway to produce more robust insect-defensive flavonoids in soybean.

In sorghum, some varieties display red/purple pigmentation upon stress, which others lack, due to a flavonoid-accumulation-affecting MYB transcription factor encoded

by *y1* (*yellow seed 1*) (Ibraheem et al., 2015). Y1 activates *CHS, DFR,* and other genes, in the flavonoid biosynthesis pathway in sorghum and maize. In sorghum, the *y1-rr* (functional) allele of *y1* results in accumulation of 3-DFs and 3-DAs (flavonoid compounds) in leaves whereas plants containing the *y1-ww* (non-functional) allele did not (Ibraheem et al., 2015; Kariyat et al., 2019). Corn leaf aphids (*Rhopalosiphum maidis*) preferentially colonized *y1-ww* plants over *y1-rr* plants in choice feeding assays and artificial diets spiked with flavonoids from *y1-rr* plants resulted in increased aphid mortality compared to diets spiked with flavonoid extracts from *y1-ww* plants (Kariyat et al., 2019). Further work by Chatterjee et al. 2022 reported maize and sorghum flavonoids, most notably 3-DFs and 3-DAs, are insecticidal to fall armyworm (*Spodoptera frugiperda*), as seen when both artificial diets spiked with 3-DAs and exogenous applications of 3-DAs to leaves before feeding significantly increased morality and significantly decreased body weight of armyworms in both experiments (Chatterjee et al., 2022). Further, maize with an overexpression allele (*U-E*) for *ZmUfo1-1,* which results in hyper-activation of *P1* (*y1* homolog in maize), accumulated large quantities of flavonoids and exhibited heightened insect herbivory resistance compared to maize with the silencing allele (*U-S*) which accumulated normal levels of flavonoids and was not herbivory resistant. Transgenic maize containing *y1-rr* from sorghum also exhibited significantly increased resistance to armyworm compared to WT non-*y1-rr* controls. In these studies, 3-DAs, 3-DFs, and total phenolic compounds increased upon armyworm herbivory in all lines. However, *U-E* and *y1-rr* lines had a greater

concentration of these compounds compared to *U-S* and WT respectively. In addition to these examples, increased flavonoid accumulation during insect herbivory has also been noted in insect-herbivory resistant varieties of wheat, tea, and other plants (Li et al., 2020b; Wang et al., 2022).

Yet, flavonoid biosynthesis gene expression is not always positively correlated with insect-herbivory resistance. For example, multiple flavonoid biosynthesis genes, such as *F3'H* and *DFR*, were down-regulated in resistant sorghum when fed on by sugarcane aphids (*Melanaphis sacchari*) compared to uninfested controls after 5 days of *ad libitum* aphid herbivory (Tetreault et al., 2019). Similar results were observed at 15 days but with different flavonoid pathway genes down-regulated. Another example can be seen in bittersweet nightshade (*Solanum dulcamara*) in which oviposition of beet armyworm eggs into the plant increased expression of *ANS* (as did oviposition with subsequent feeding) (Geuss et al., 2018). However, feeding alone elicited down-regulation of *ANS*. It is not surprising that not all genes in all species function identically to insect herbivory, especially when considering the highly complex nature of metabolite production *in planta* which is often a highly dynamic and interconnected process. In order for certain metabolites to be favored products, others, as resources are finite, must logically diminish in quantity as fewer resources are allocated towards their production. Nevertheless, further investigation is necessary to understand these complex processes more fully. The results outlined in this section across multiple economically important crops demonstrate flavonoids play a significant role in insect herbivory defense.

Terpenes

Terpenes are biological compounds comprised of typically one to six isoprene (C_5H_8) subunits forming monoterpenes ($C_{10}H_{16}$), sesquiterpenes ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$), and triterpenes ($C_{30}H_{48}$) (Zhang et al., 2018; Hosseini and Pereira, 2023). These compounds are further diversified based on additions of functional groups (Hosseini and Pereira, 2023) and are involved in flavor (Pieroni et al., 2023) as well as biological processes including primary metabolism (Saadat et al., 2023), allelopathy (De Martino et al., 2010), and plant signaling (Loughrin et al., 1997; Girón-Calva et al., 2014). Most notably, many terpenes exhibit insect repellent and insecticidal properties (Liu et al., 2020a; de Albuquerque Lima et al., 2021; Zavala-Gómez et al., 2021; Sun et al., 2022; Wang et al., 2023a).

Monoterpenes

Monoterpenes are differentially released during insect herbivory or wounding events compared to control conditions and have been shown to act as insect herbivory defensive compounds (Lewinsohn et al., 1991; Giunta et al., 2016; Chiu et al., 2017; Phschiutta et al., 2017; Quan et al., 2018; Giunti et al., 2020; de Albuquerque Lima et al., 2021; Zavala-Gómez et al., 2021; Diass et al., 2021; Paczkowski et al., 2021; Pavela et al., 2021). Upon infestation of *Olea europaea* cv. 'Ottobractica', 'Sinopolese', and 'Roggianella' (olive) by *Bactrocera oleae* (olive fruit fly), over 70 different VOCs were formed (Giunti et al., 2020). Highly infested olives produced chiefly three monoterpenes, (*E*)-β-ocimene, β-myrcene, and limonene, all of which deterred *B. oleae* in bioassays.

Highly infested plants were found to emit the greatest quantities of monoterpenes with increased maturity level also positively correlated to their emissions. *Terpene synthase* (*TPS*) genes are integral enzymes in the formation of terpenes, including monoterpenes, in plants (Qiao et al., 2022). Qiao et al (2022) identified *TPS* genes which were up-regulated in *Camellia sinensis* cv. 'Shuchazao' (tea) upon *Ectropis obliqua* (Chinese tea black arch bug; aka - tea looper, tea geometrid moth) herbivory (Qiao et al., 2022). Three *TPS* genes were selected for in-depth study of *CsTPS08, CsTPS10,* and *CsTPS58*. *CsTPS08* and *CsTPS10* were found to significantly increase expression continuously – even up to 12 hours after feeding ceased – compared to a control not exposed to feeding. Expression of *CsTPS58* significantly increased, up to six hours after feeding ceased, to levels greater than *CsTPS08* and *CsTPS10* but drastically decreased in expression afterwards, compared to a control not exposed to feeding. In another study, when essential oil extracts from *Minthostachys verticillata* and *Eucalyptus globus* were assayed for their chemical composition, monoterpenes limonene, 1,8-cineole, (-)-menthone, and pulegone were the preeminent constituents (Phschiutta et al., 2017). In feeding bioassays, pulegone, a monoterpenoid, induced 100% morality in grapevine mealybug, but limonene (15.33% mortality), (-)-menthone (35.24% mortality), and 1,8-cineole a (26.67% mortality) also contributed to mortality in grapevine mealybug, confirming insecticidal and deterrent qualities of 1,8-cineole previously reported (Phschiutta et al., 2017; Quan et al., 2018; de Albuquerque Lima et al., 2021; Diass et al., 2021; Zavala-Gómez et al., 2021). These results suggest that monoterpenes are implicated in insect

herbivory responses in olive, tea, *M. verticillata*, and *E. globus,* with strong implications in other plants, as well.

Diterpenes

Similar to monoterpenes, diterpenes also play important roles in insect herbivory defense (Ralph et al., 2006; Schmelz et al., 2011; Heiling et al., 2012; Zhang et al., 2017; Oh et al., 2017; Macel et al., 2019; Maharijaya et al., 2019; Morimoto, 2019; Li et al., 2020a; Zheng et al., 2022; Sun et al., 2022; Antoine et al., 2023). Diterpenes are also known to exhibit insect repellent and insecticidal properties. Trichome exudates from yellow bartsia (*Parentucellia viscosa*) and Mediterranean lineseed (*Bellardia trixago*) were previously known to exhibit insect herbivory repellent and insecticidal activity (Morimoto, 2019). Analysis of these extracts found mono-, sesqui-, and diterpenes were major constituents of the exudates and diterpene kolavenic acid, when isolated, exhibited antifeedant activity to tobacco cutworm. Meanwhile, in maize, six kauralexins (diterpenes) increased in accumulation upon European stem borer (*Ostrinia nubilalis*) attack. Two of the kauralexins selected for additional testing were found to exhibit antifeedant activity (Schmelz et al., 2011). Of 20 diterpenes isolated from monk's hood (*Aconitum apetalum*) and *Aconitum franchetii*, 11 exhibited medium to high antifeedant activity against beet armyworm, the greatest being chasmanthinine (Zhang et al., 2017). In another study which isolated a separate set of 20 diterpenes from peacock flower (*Caesalpinia pulcherrima*), 15 exhibited antifeedant activity while eight exhibited insecticidal activity (Li et al., 2020a). In pepper, several diterpene glycosides have been

shown to be positively correlated with resistance to thrips in a screen of diverse *Capsicum* accessions (Macel et al., 2019). Further exploration identified two diterpene glycosides, which were members of an mQTL (metabolomic-QTL) that co-localized with a major QTL for resistance in pepper identified previously, that were negatively correlated with thrips survival (Maharijaya et al., 2019). In cotton, feeding by cotton boll worm for 6h or 12h prior to cotton aphid (*Aphis gossypii*) attack resulted in reduced aphid damage, suggesting defensive priming (Zheng et al., 2022). Transcriptomic analysis revealed significant functional enrichment of phytohormone signal transduction, phenylpropanoid biosynthesis, and terpene biosynthesis genes wherein many of the terpene biosynthesis genes were associated with diterpene production.

While the terpene biosynthetic pathway is complex (Ralph et al., 2006; Huang et al., 2015), some genes have been shown to display a connection to diterpene synthesis. Unsurprisingly, considering the high number of *TPS* genes implicated throughout the terpene biosynthetic pathway in various enzymatic roles (Ralph et al., 2006; Huang et al., 2015), *TPS* gene family members are known to play important roles in diterpene synthesis. In Sitka spruce (*Picea sitchensis*) genes related to terpene biosynthesis were induced upon mechanical wounding, weevil herbivory for 3h, budworm herbivory for 3h, and budworm herbivory for 52h (Ralph et al., 2006). Although genes varied between responses, of 22 total *TPS* gene family members, 20 were up-regulated in at least one insect treatment, many being up-regulated consistently across treatments. In rice, 25 *TPS* genes were differentially expressed upon Asiatic rice borer (*Chilo suppressalis*) feeding

(Sun et al., 2022). The majority of these genes were up-regulated and implicated in diterpene synthesis. Some such genes included *TPS8, TPS13, TPS38,* and *CYP99A3.* Additionally, up-regulation was observed of genes implicated in the ent-kaurane branch of the diterpene biosynthetic pathway. While the ent-kaurane branch of the pathway primarily functions to produce ent-kaurane, a precursor to gibberellin (Salazar-Cerezo et al., 2018), some studies have reported insecticidal activity of ent-kaurane (Antoine et al., 2023). For example, in coffee (*Coffea arabica*), cafestol, an ent-kaurane diterpene, was the most highly accumulating terpene in the endosperm of seed which are known to experience little insect damage in the field (Antoine et al., 2023). When artificial diets of oriental fruit fly (*Bactrocera dorsalis*) were spiked with cafestol, reduced pupation rate and decreased mass of pupae and adults was observed. Meanwhile, in the aforementioned Stika spruce study, *Geranylgeranyl diphosphate synthase* (*GGPPS*), which is responsible for catalyzing the reaction to produce Geranylgeranyl diphosphate, which is the penultimate intermediate to diterpene formation, exhibited significantly increased expression upon mechanical wounding, weevil herbivory, and budworm herbivory for 52h (Ralph et al., 2006). Illustrating the importance of *GGPPS* to insect resistance, when *GGPPS* was silenced in coyote tobacco (*Nicotiana attenuata*), insect herbivory increased, 17-hydroxygeranyllinalool diterpene glycoside concentrations decreased, and tobacco hornworm larvae grew up to 10 times larger (Heiling et al., 2012).

One possible biological mechanism by which diterpenes may harm insects is through inhibition of growth regulating hormones *in insectum.* In an insect growth

inhibition screen focusing on insect juvenile hormone, diterpene 7-oxodehydroabietic from Japanese pinus (pine) (*Pinus densiflora*) was found to inhibit juvenile hormone activity (Oh et al., 2017). In addition, 7-oxodehydroabietic was found to impair larval growth of Indian meal moth (*Plodia interpuncetlla*).

Collectively, these studies report the deterrent and lethal effects of mono- and diterpenes on insect pests and their capacity to enhance plant defense. Terpenes are an important insect herbivory defensive chemical in many plants and warrant continued exploration to further elucidate their varied roles in plant-insect interactions.

Genetic Responses in Insect Herbivory Defense

The subset of genes outlined in Table 1.2 play critical roles within specific biological pathways highlighted in this review. However, it does not encompass the entirety of responses across all plant species to insect herbivory. Typically, biotic stress, including insect herbivory, elicits differential expression in hundreds to thousands of genes. The defense responses against insect herbivory exhibit variability depending on factors such as plant species, population or accession, insect pest, tissue type, stage of maturity (both in the plant and insect), as well as environmental conditions. For these reasons, continued investigation of plant responses to insect herbivory is imperative to capture unique responses and enhance our understanding of these intricate interactions.

Discussion

Insect pests are major threats to global food security, therefore identifying means to improve insect herbivory resistance in crop plants is imperative. All *in planta* insect resistance capabilities are derived from naturally occurring mutations, of which, some are beneficial and give rise to novel and effective defenses. Generation of novel insect defensive capabilities can occur in any plant species, whether wild or domesticated. Therefore, it is logical to review the great variety of insect herbivory defenses across plant life to identify adaptations to incorporate into cultivated crops. Farmers have identified insect herbivory resilient plants in their fields dating back to ancient times and preferentially planted seed from such individuals in the following seasons to attain higher yields with decreased pest damage. Today, this selection still occurs on vast scales to identify unique individuals with an array of advantageous traits, including insect herbivory resistance. Molecular breeding and genetic engineering have improved this process by allowing efficient introduction of advantageous genes into established cultivars' genomes. This review provided an overview of major plant insect herbivory defenses, including genetics, pathways, and phytohormonal drivers.

Phytohormones, such as JA, SA, auxin, and others, are critical signaling molecules in plants that regulate plant development and are implicated in practically all plant responses, including insect herbivory resistance. Phytohormonal responses to a stressor vary by species, tissue, maturity, stress, and more. Depending on the insect feeding type, typically, JA levels increase while SA levels decrease, or conversely, SA

levels increase while JA levels decrease. Phytohormones are master regulators of downstream defensive processes and, on the whole, phytohormones vary uniquely to respond to differing insect herbivores and evoke different responses in different plant species. Strategic applications of plant hormones by growers to prime plant defenses in advance of insect herbivory could aid in defending against insect damage.

Physical defenses are some of the most well studied insect herbivory defensive mechanisms. Trichomes aid in combating many abiotic and biotic stresses, including insect herbivory, through physically protecting the leaf surface, releasing insect herbivory defensive compounds, and acting as signaling structures which relay insect presence. Increased trichome quantities *in planta* generally indicate increased insect-herbivory resistance. Sclerophylly, or the hardening of tissues, has been shown to increase upon insect herbivory and contributes to insect herbivory defense in many crops, typically constitutively. While reducing edibility of plants to insects through heightened sclerophyllization or trichome densities may reduce damage due to insect feeding, it is imperative to consider possible decreased palatability to human consumers as well – depending on the tissue and species in question. Such ramifications may include considerations of safe levels of heavy metal ingestion which are sequestered in trichomes for defense, potential consumer aversion to a stiffer leafy-green in a salad, or the off-putting mouthfeel of a more trichome-dense vegetable. Implications on crop fruit/grain quality and yield should also be considered as heightened energetic resource allocation to physical defenses could affect grain/fruit fill or quantity.

Chemical defensive compounds are present in plant tissues and are ubiquitous across plant life but vary greatly in composition, quantity, and efficacy across plant species. Chemical defensive compounds include flavonoids, terpenes, as well as many others not covered in this review. While their activities are highly diverse, and some play roles in various functions *in planta*, all the compounds outlined in this review exhibit, or impact accumulation of, compounds with insect repellent or insecticidal properties. Chemical defensive compounds could be incorporated into plants to produce the compound innately (plant-incorporated-protectants) or as an active ingredient in foliar-applied chemicals (insecticides). While chemical defensive compounds have proven highly effective at preventing insect damage to plants, resistance against such compounds inevitably will arise in insect populations over time. Trait stacking (pyramiding), the technique of incorporating multiple genes or traits into one plant to provide multiple forms of defense against a stress, is one strategy which provides heightened defense in conjunction with increased durability and longevity of the resistance genes/traits, resulting in successful implementation over a longer period with greater effectiveness.

The genes which underlie the defensive mechanisms outlined in this review should be considered prime targets for genetic enhancement to confer insect herbivory defenses to susceptible crops. A list of potential targets can be found in Table 1.2. Vastly different transcriptomic responses were observed in *Arabidopsis* when fed upon by piercing sucking versus chewing mouthpart insects and differences were even observed between species within mouthpart groups (Appel et al., 2014). These results

indicated that *Arabidopsis*, and likely other plants, mount unique defensive responses against individualized threats implying defense responses are fine-tuned to combat different pests. Given these considerations, it is plausible that integrating a defense-related transgene into a plant may effectively target only a limited subset of closely related insect pests, leaving other insects largely unaffected. While this approach could offer advantages in safeguarding pollinators and other beneficial organisms, by the same merit the potentially narrow scope could present a significant challenge in achieving comprehensive protection against insect herbivory. Introduction of genes by genetic engineering, or novel genetic material via breeding efforts, are crucial undertakings in crop plant improvement. Technologies such as RNAi and CRISPR/Cas9 increase the ease with which transgenes can be introduced, or their transcription altered, paving the way for advancements in both foundational and applied research ventures. However, it is vitally important to consider that not all genes and/or transformation events will produce desired outcomes when introduced to another species, including genes associated with insect herbivory resistance. Various factors contribute to this phenomenon including: insufficient up or downstream genes/proteins in a pathway to facilitate progression of the reaction and production of the desired output product, inadequate or suppressed transcription/translation of transcripts/proteins from such genes, lack of induction during insect herbivory, differences in codon usage between species impacting protein primary structure, inadequate cellular reaction conditions for the non-native reaction to occur, protein misfolding, transgene insertion into a repressed region of the genome or into a

critical gene, or various other genetic and biochemical factors which complicate transgenic crop improvement.

Selection is the ultimate driver of evolution and is responsible for the range of defensive adaptations we observe in plant life today. Through evolution, plants that best deterred or killed herbivorous insect pests, or otherwise survived insect herbivory events, passed on their advantageous traits and effective genetic compositions to the next generation. Insect pests which plants encounter are extremely diverse and are determined by the environments they inhabit. Therefore, plants experienced distinct evolutionary pressures giving rise to the wide variety of insect herbivory defensive adaptations seen today. The diversity of insect herbivory defenses found across plants provide great opportunities to identify, understand, and exploit various means of defense. Considering the vital importance to decrease crop losses due to insect herbivory, future research efforts to identify insect herbivory resistant species, cultivars, and accessions, investigate the factors which underly resistance observed in these plants, and incorporate such traits into crops will continue to be a key area of research for the foreseeable future.

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Tables

Table 1.1 – Matrix of Plant Protective Strategies

	Constitutive	**Inducible**
Direct	**Constitutive-Direct** Trichomes prevent insect feeding on plant leaves	**Inducible-Direct** Upon herbivory, monoterpenes are produced in the tissues being fed upon by the insect
Indirect	**Constitutive-Indirect** A compound is released throughout all developmental stages that deters an insect pest	**Inducible-Indirect** When sensing insect herbivory, a compound is released into the air attracting insects that are natural predators of the attackers

Table 1.2 – Putative and Known Genes Implicated in Insect-Herbivory Defense Response Across Diverse Plants

Gene Functional Name	Implication or Function	Species Cited in Literature
<u>Trichome-related</u>		
Gl1 **(*GLABRA1*)**	Critical trichome development gene Induces expression of *Gl2,* the final gene in the biosynthetic pathway prior to induction of trichome development Implicated in heavy metal uptake and deposition in trichomes upon mechanical stimulation and insect presence, and in heavy metal stress mitigation Implicated in chewing-mouthpart insect herbivory defense Up-regulated upon insect herbivory	*Arabidopsis* (Sato et al., 2019; Guo et al., 2022; Song et al., 2022) Soybean (Liu et al., 2020c)
Gl2 **(*GLABRA2*)**	The final gene in the biosynthetic pathway prior to induction of trichome development	Soybean (Liu et al., 2020c)
Gl3 **(*GLABRA3*)**	Key regulator of environmental-stress-mediated trichome alterations throughout development Induces expression of *Gl2* Implicated in chewing-mouthpart insect herbivory defense Up-regulated upon insect herbivory	Soybean (Liu et al., 2020c) *Arabidopsis* (Yan et al., 2012; Song et al., 2022)

Continued…

(Table 1.2 continued)

***TTG* (*Transparent testa GLABRA*)**	Induces expression of *Gl2* Implicated in chewing-mouthpart insect herbivory defense Implicated in conferring high trichome density Overexpression in transgenic plants resulted in increased trichome densities	*Arabidopsis* (Song et al., 2022) Soybean (Liu et al., 2020c)
<u>Trichome-Deposition and Secretion of Chemicals-related</u>		
***AtHMA2* (*Arabidopsis heavy metal ATPase 2*); *PCS1* (*Phytochelatin synthesis 1*); *NRAMP3* (*Natural resistance-associated macrophage protein 3*); *COPT2* (*Copper transporter 2*)**	Four independent genes sharing the same response in *Arabidopsis* Hypothetically implicated in heavy metal stress mitigation Indirectly implicated in heavy metal ion deposition in trichomes contributing to hypothesized insect herbivory defense Up-regulated when stimulated with paint brush to simulate insect movement	*Arabidopsis* (Guo et al., 2022)
<u>Lignin Biosynthesis-related</u>		
***HCT* (*Hydroxycinnamoyl-CoA shikimate*)**	Catalyzes the first step converting the end-product of the phenylpropanoid pathway, p-Coumaroyl-CoA, to p-coumaroyl shikimic acid; also catalyzes the conversion of caffeoyl shikimic acid to caffeoyl-CoA Necessary for cuticle development Up-regulated upon insect herbivory in some insect-herbivory resistant plants, while in other resistant plants, it was constitutively moderately expressed	Sorghum (Tetreault et al., 2019) Physcomitrella (Kriegshauser et al., 2021) Tea (Li et al., 2020b) Rice (Zhang et al., 2022a)

Continued…

(Table 1.2 continued)

Phenylpropanoid Biosynthesis-related		
***PAL* (*Phenylalanine ammonia-lyase*)**	Catalyzes the reaction to form cinnamic acid – an intermediary compound in the phenylpropanoid pathway crucial to insect-herbivory inducible lignin biosynthesis Up-regulated by insect herbivory in insect-herbivory resistant plants Mutants exhibited decreased lignin content as well as salicylic acid accumulation	Rice (He et al., 2019; Dong et al., 2020) Wheat (Wang et al., 2022) Cassava (Chen et al., 2022) Grapevine (Jia et al., 2022) Tobacco (Kumar and Yadav, 2017) *Arabidopsis* (Huang et al., 2010)
***4CL* (*4-coumarate-CoA ligase*)**	Primarily converts p-coumaric to p-coumaroyl-CoA in the last step of the phenylpropanoid pathway; also catalyzes at least four separate reactions within the lignin pathway Up-regulated in some insect-herbivory resistant plants upon insect herbivory, in other resistant plants, it was constitutively highly expressed Decreased expression results in reduced lignin content and shorter plants	Wheat (Wang et al., 2022) Cassava (Chen et al., 2022) Tea (Li et al., 2020b) Rice (Gui et al., 2011; Zhang et al., 2022a) Sorghum (Tetreault et al., 2019) Monterey Pine (Wagner et al., 2009)
Flavonoid Biosynthesis-related		

Continued…

(Table 1.2 continued)

***y1*; Allele -rr (*Yellow Seed 1*; Allele: red pericarp, red glumes)**	MYB transcription factor which activates *CHS, DFR,* and other genes in the flavonoid synthesis pathway Lines with the functional '*-rr'* allele accumulate 3-DAs (insecticidal agent), 3-DFs (insecticidal agent), and greater accumulations of total phenolic compounds compared to non-functional '-*ww*' allele The '-*rr*' allele confers enhanced insect herbivory resistance	Sorghum (Kariyat et al., 2019) Maize (Chatterjee et al., 2022)
***ZmUfo1-1*; Allele: U-E (*Unstable factor for orange 1-1*; Allele: Ufo-Expressor)**	Lines with the '*U-E*' allele hyper-activate the sorghum *y1* ortholog in maize, *P1* Lines with the '*U-E'* allele accumulate flavonoids, including 3-DAs (insecticidal agent), 3-DFs (insecticidal agent), and other phenolic compounds at heightened levels compared to WT '*U-S'* allele The '*U-E*' allele confers enhanced insect herbivory resistance	Maize (Chatterjee et al., 2022)
***CHS* (*Chalcone synthase*)**	First gene in the flavonoid synthesis pathway, thus, a critical gatekeeping gene Catalyzes p-Coumarocyl-CoA into Naringenin Chalcone within flavonoid biosynthesis pathway Up-regulated by insect herbivory (in insect-herbivory resistant plants) and UV-B Up-regulation in protoplasts of rice resulted in increased flavonoid accumulation and increased brown plant hopper mortality Up-regulated in perilla displaying strong red/purple leaf pigmentation paired with increased flavonoid accumulation	Rice (Dong et al., 2020; Zhang et al., 2022a) Tobacco (Kumar and Yadav, 2017) Cassava (Chen et al., 2022) Soybean (Zhang et al., 2022b) Liverwort (Soriano et al., 2019) Tartary Buckwheat (Huang et al., 2019) Tea (Li et al., 2020b) Perilla (Xie et al., 2022)

Continued…

(Table 1.2 continued)

***F3'H* (*Flavonoid 3-monoxygenase*)**	Catalyzes the formation of kaempferol from naringenin in the flavonoid synthesis pathway Implicated the re-routing of flavonoid biosynthetic pathway intermediates upon specific stresses resulting in different end-product flavonoids Often upregulated upon insect herbivory in insect herbivory resistant plants Up-regulated in perilla displaying strong red/purple leaf pigmentation paired with increased flavonoid accumulation	Grapevine (Jia et al., 2022) Cassava (Chen et al., 2022) Tobacco (Kumar and Yadav, 2017) Tartary Buckwheat (Huang et al., 2019) Rice (Dong et al., 2020; Jan et al., 2022) Sorghum (Tetreault et al., 2019) Perilla (Xie et al., 2022)
***FLS1* (*Flavonol synthase/Flavone 3-hydroxylase 1*)**	Catalyzes the reaction converting dihydrokaempferol to kaempferol in the flavonoid synthesis pathway as well as the reaction converting dihydromyricetin to myricetin; also known to catalyze the reaction converting dihydroquercetin to quercetin Known to broadly mediate flavonoid biosynthesis in plant life Up-regulated upon insect herbivory in insect herbivory resistant plants	Grapevine (Jia et al., 2022) Tobacco (Kumar and Yadav, 2017) Cassava (Chen et al., 2022) Tartary Buckwheat (Huang et al., 2019)
***LAR* (*Leucoanthocyanidin reductase*)**	Converts leucocyanidin to catechin in the flavonoid synthesis pathway Up-regulated upon insect herbivory in resistant plants Overexpression leads to enhanced insect herbivory resistance	Cassava (Chen et al., 2022) Tobacco (Kumar and Yadav, 2017)

Continued…

(Table 1.2 continued)

***ANS* (*Anthocyanidin synthase*)**	Converts leucocyanidin to cyanidin in the flavonoid synthesis pathway Critical to flavonoid formation Often upregulated upon insect herbivory of insect-herbivory resistant plants Expression down-regulated in the presence of *FtMYB8* Up-regulated in perilla displaying strong red/purple leaf pigmentation paired with increased flavonoid accumulation	Cassava (Chen et al., 2022) Tartary Buckwheat (Huang et al., 2019) Bittersweet Nightshade (Geuss et al., 2018) Eggplant (Chen et al., 2018) Maize (ul Malook et al., 2019) Tobacco (Kumar and Yadav, 2017) Rice (Dong et al., 2020) Perilla (Xie et al., 2022)
***ANR* (*Anthocyanidin reductase*)**	Catalyzes the reaction converting cyanidin to epigallocatechin (insecticidal agent) in the flavonoid synthesis pathway When pyramided with *DFR* in transgenic tobacco multiple physiological traits were positively affected, including yield Up-regulated by insect herbivory in insect herbivory resistant plants Overexpression results in heightened insect herbivory defense	Cassava (Chen et al., 2022) Tobacco (Kumar and Yadav, 2017) Rice (Zhang et al., 2022a)

Continued…

(Table 1.2 continued)

***DFR* (*Dihydroflavonol 4-reductase*)**	Catalyzes the reaction converting dihydroquercetin to leucocyanidin in the flavonoid synthesis pathway When pyramided with *ANR* in transgenic tobacco multiple physiological traits were positively affected, including yield Often upregulated by insect herbivory in insect herbivory resistant plants Overexpression results in heightened insect herbivory defense Up-regulated in perilla displaying strong red/purple leaf pigmentation paired with increased flavonoid accumulation	Tartary Buckwheat (Huang et al., 2019) Tobacco (Kumar and Yadav, 2017) Sorghum (Tetreault et al., 2019) Perilla (Xie et al., 2022)
***UGT* (*UDP-7-O-glucosyltransferase*)**	Catalyzes one of multiple possible final steps in flavonoid synthesis to convert anthocyanidins (insecticidal agent) to anthocyanins (insecticidal agent) Implicated in insect herbivory resistance Knock out results in insect herbivory resistance in soybean via altered flavonoid-intermediate usage in the flavonoid biosynthetic pathway ultimately resulting in different end products which confer heightened resistance Pathway rerouting is also observed in rice due to the presence of different UGT alleles in different parents	Soybean (Zhang et al., 2022b) Rice (Dong et al., 2020)
Terpene Biosynthesis-related		

Continued…

(Table 1.2 continued)

TPS **(*Terpene synthase*)**	Gene family members are implicated in the majority of steps in the terpene biosynthetic process including the formation of mono-, sesqui-, di-, and triterpenes Up-regulated upon insect herbivory	Tea (Qiao et al., 2022; Sun et al., 2022) Sitka spruce (Ralph et al., 2006)
GGPPS ***(Geranylgeranyl diphosphate synthase)***	Penultimate intermediate in the formation of diterpenes Implicated in insect herbivory resistance Knock out results in insect herbivory susceptibility in coyote tobacco via decreased diterpene accumulation Up-regulated upon mechanical damage, weevil, and budworm herbivory in Sitka spruce	Sitka spruce (Ralph et al., 2006) Coyote tobacco (Heiling et al., 2012)
<u>Genes Implicated in a Response to Insect Herbivory – Genes Not Yet Mentioned</u>		
PIN2 **(*Proteinase inhibitor 2*)**	Implicated in wounding defense and insect locomotion response Up-regulated upon insect-herbivory and insect locomotion	Tomato (Graham et al., 1985; Peiffer et al., 2009; Tooker et al., 2010)
SYSTEMIN	A bioactive molecule critical to amplifying JA signals throughout the plant Overexpression leads to significantly increased levels of JA When silenced via RNAi, insect herbivory significantly increased in silenced plants compared to controls; JA and protease inhibitor levels also significantly decreased	Tomato (Orozco-Cardenas et al., 1993; Montero-Vargas et al., 2018)

Chapter 2 - Trichomes and Unique Gene Expression Confer Insect Herbivory Resistance in *Vitis labrusca* Grapevines

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Abstract

Grapevine (*Vitis*) is one of the world's most valuable fruit crops, but insect herbivory can decrease yields. Identifying insect herbivory resistance genes and pathways is critical to mitigating these losses. *Vitis labrusca* is a wild North American grapevine species which has been used in grapevine breeding programs to increase abiotic and biotic stress resistance of hybrid grapevines, making it a valuable genetic resource for sustainable viticulture. In this study, we evaluated the resistance of *V. labrusca* acc. 'GREM4' and *Vitis vinifera* cv. 'PN40024' grapevines to *Popillia japonica* (Japanese beetle) herbivory. In our study, herbivory assays indicated 'GREM4' was more resistant to beetle herbivory than 'PN40024'. When investigating the role of physical defenses, 'GREM4' exhibited higher leaf trichome density, which contributed to insect herbivory resistance, but did not entirely explain the phenotype. A comparative transcriptomic study between 'GREM4' and 'PN40024' revealed 'GREM4' exhibited greater constitutive basal (0h) expression of genes related to defense response and secondary metabolite biosynthesis compared to 'PN40024'. Under herbivory, 'GREM4' had a greater number of total differentially expressed genes compared to 'PN40024'. Genes up-regulated in 'GREM4' were enriched in terpene biosynthesis, flavonoid biosynthesis, phytohormone signaling, and disease defense-related functions. The majority of genes implicated in insect herbivory defense were orthologous with specific expression patterns in 'GREM4' and 'PN40024', but some paralogous and genome-specific genes also contributed to conferring resistance. Our findings suggest that a

combination of trichomes and unique expression of secondary metabolite and pathogen defense genes are crucial for insect herbivory resistance in 'GREM4'.

Introduction

Grapes are the most valuable fruit crop globally (Food and Agriculture Organization of the United Nations (FAO), 2023). In the United States the wine industry alone had a $275B impact on economy in 2022 (John Dunham & Associates of New York City, 2023). Insect pests invoke up to 30% of crop loss each year globally, decreasing yields (Singh and Kaur, 2018; FAO and Sarkozi, 2019). *Popillia japonica* (Japanese beetle) is a major polyphagous invasive pest in North America and Europe, damaging plants of both commercial and non-commercial uses, including grapevine (Fleming, 1976; Smith et al., 1996; Potter and Held, 2002; Mercader and Isaacs, 2003; Gu and Pomper, 2008; The United States Department of Agriculture and USDA-APHIS, 2015; European and Mediterranean Plant Protection Organization, 2016; MacGregor et al., 2016). Improved resistance of cultivated grapevines to Japanese beetles, and other insect pests, would decrease inputs, costs, and crop damage while increasing yields in this multi-billion-dollar industry.

Vitis labrusca is a grapevine native to North America and is highly fit in its local environment. *Vitis labrusca* is cold-hardy and resistant to pathogens (Kortekamp and Zyprian, 1999; Gabler et al., 2003; Dami, 2007; Cadle-Davidson, 2008; Gee et al., 2008; Nascimento-Gavioli et al., 2019; Todaro and Longstroth, 2019). Conversely, *Vitis vinifera*, a species cultivated across the globe and well adapted to European biomes, is

highly susceptible to abiotic and biotic stresses endemic to North America (Moio and Etievant, 1995; Dami et al., 2005; Smith, 2005; Dami, 2007; Cadle-Davidson et al., 2011; Qiu et al., 2015). *Vitis labrusca* has been widely employed in grapevine breeding programs to introduce these adaptive traits into hybrids (Smith, 2005; Qiu et al., 2015). Intriguingly, grapevine varieties bred from North American species experienced decreased insect herbivory in the field. Hybrid grapevines with majority *V. labrusca* genetic background exhibited greater resistance to Japanese beetle whereas *V. vinifera* cultivars and hybrids with little *V. labrusca* genetic background exhibited greater damage (Mercader and Isaacs, 2003). Further, hybrids bred from other North American grapevine species also exhibit decreased Japanese beetle herbivory and decreased mealybug (*Planococcus ficus*) infestation compared to European grapevines (Gu and Pomper, 2008; Naegele et al., 2020). These results suggest the genetic composition of *V. labrusca* provides an advantage for insect herbivory resistance.

Insect herbivory defense has been well studied in many plant species, though plant responses and their efficacy can differ depending on the pest and the plant. The classes and quantities of secondary metabolites produced in defense of insect herbivory can vary between plants, but terpenes have been reported to have insecticidal properties. For example, essential oils containing terpenes derived of Cassumunar ginger (aka – Plai) (*Zingiber cassumunar*) displayed insect repellent and larvicidal properties against Asiatic tiger mosquito (*Aedes albopictus*) (Li et al., 2021). In rice, 25 *Terpene synthase* (*TPS*) genes, which are critical in catalyzing terpene synthesis, were differentially expressed

upon Asiatic rice borer (*Chilo suppressalis*) herbivory and overexpressing a *TPS* gene (*Beta-ocimene synthase* (*OCS*)) in both tobacco and soybean resulted in enhanced resistance to tobacco cutworm (*Spodoptera litura*) (Sun et al., 2022; Han et al., 2023). Additionally, other secondary metabolites, such as flavonoids, play important roles in insect herbivory defense and resistance such as observed in wheat, rice, tea, sorghum, and maize (Kumar and Yadav, 2017; Kariyat et al., 2019; Chatterjee et al., 2022; Zhang et al., 2022a; Lv et al., 2023). For example, in resistant cassava (*Manihot esculenta*), increased accumulations of phenylpropanoid and flavonoid pathway compounds were identified upon two-spotted spider mite (*Tetranychus urticae*) herbivory and led to greater resistance when overexpressed (Chen et al., 2022). While terpenes, flavonoids, and other secondary metabolites are critical to insect herbivory defense, physical adaptations, such as trichomes, hair-like structures on the surface of plant tissues, also provide increased defense against pathogens and insect pests (Smith, 2005; Xu et al., 2011; de Queiroz et al., 2020; Yin et al., 2022). High trichome densities have led to decreased insect herbivory in wheat, *Datura stramonium,* and soybean, among other plants (Valverde et al., 2001; de Queiroz et al., 2020; Singh et al., 2021). These observations suggest specialized morphological and chemical defenses have evolved in resistant compared to susceptible plants which contribute to differences in the success of the defense.

Limited studies have been conducted in grapevine to identify the unique adaptive defenses involved in deterring insect herbivory in North American wild grapevine species. A comparative genomic study between *V. labrusca, V. riparia,* and *V. vinifera*

varieties identified genome-specific genetic variation linked to adaptive traits, laying the foundation for discovering the genetics that underlie adaptive differences (Li and Gschwend, 2023). In an herbivory study, oriental longheaded grasshopper feeding on *V. vinifera* x *V. labrusca* hybrid 'Kyoho' induced transcriptomic, phytohormonal, and metabolomic alterations after 72h of feeding, with increased expression of genes implicated in reactive oxidative species (ROS) production, flavonoid biosynthesis, insect and physical damage response, and lignin biosynthesis, among others (Jia et al., 2022). In a *V. riparia* hybrid, a QTL associated with phylloxera resistance was found to contain disease resistance genes, such as *Resistance to Phytophthora sojae 5* (*Rps5*), which suggests genes canonically associated with pathogen resistance may also impact insect herbivory defense (Yin et al., 2022). A comparative study of the genetic responses of *V. labrusca* and *V. vinifera* to insect herbivory is still needed to identify specific defenses exhibited by *V. labrusca* contributing to its resistance to insect herbivory.

In this manuscript, we conducted a comprehensive, comparative study to determine the phenotypic differences and transcriptomic responses of *V. labrusca* acc. 'GREM4' ('GREM4') and *V. vinifera* cv. 'PN40024' ('PN40024') to insect herbivory. We tested 'GREM4' and 'PN40024' for insect herbivory resistance via Japanese beetle feeding assays and determined the role of trichome density in deterring insect herbivory (Muza et al., 2002; MacGregor et al., 2016). Additionally, we conducted a quantitative comparative transcriptomic study to determine transcriptomic responses, and functional implications, for each species ('GREM4' vs. 'PN40024') in response to Japanese beetle

herbivory and identified specific responses in ‘GREM4’ that likely contribute to insect herbivory resistance.

Materials and Methods

Plant Materials

Vitis labrusca acc. ‘GREM4’ (PI-588583) and *Vitis vinifera* cv. ‘PN40024’ (DVIT-908) grapevine cuttings were acquired from the United States Department of Agriculture at Geneva, NY and Davis, CA, respectively, in 2021 and 2022 (Prins and Agricultural Research Service - United States Department of Agriculture, 2018; Grape Genetics Research Unit, 2020). ‘PN40024’ was selected due to its role as the *V. vinifera* reference cultivar/reference genome since 2007 while ‘GREM4’ was selected due to the availability of a reference genome sequence and its resistance to pathogens, suggesting broad fitness in its local environment (Jaillon et al., 2007; Cadle-Davidson, 2008; Li and Gschwend, 2023). Both species were propagated from cuttings and grown in greenhouses at The Ohio State University, Columbus OH, USA under 16hr light:8hr dark. Experiments took place between the months of July and October.

Insect Collections

Popillia japonica (Japanese beetles) were collected from The Ohio State Waterman Agricultural and Natural Resources Laboratory, Columbus OH, USA between the months of July and October of 2021 and 2022. Beetles were collected using “Spectracide Bag-A-Bug Japanese Beetle Trap2” pheromone traps (Spectrum Brands, 2023) in a soybean field which had not been sprayed with insecticides. Beetles were kept

in a 16.5 x 16.5 x 30in 'bug dorm' (Educational Science, 2019) within a growth chamber overnight and semi-starved (one small *V. vinifera* leaf provided to prevent death due to starvation or dehydration) and were used for experiments the following day. The growth chamber was set to a 16hr light:8hr dark cycle at 25ºC and 21ºC, respectively.

Herbivory Preference Study

Fifteen semi-starved Japanese beetles were placed in a bug dorm inside a growth chamber as previously described. Three mature, similar-sized, attached 'GREM4' and 'PN40024' leaves were concurrently introduced into the bug dorm. The experiment permitted 19hrs of *ad libitum* feeding (6PM-1PM the following day) and was replicated four times between August and September of 2021. Pictures of the leaves were taken before and after feeding and total leaf areas were measured using ImageJ, with the difference in mm^2 representing the area of feeding (AOF), i.e. - the area in mm^2 eaten by Japanese beetles (Schneider et al., 2012). Holes made completely through the leaf and noticeable tissue loss along the leaf margin were included in the AOF calculation. Significance was determined using MiniTab21 via a one-sided two-sample t-test (variances unequal) (Minitab 21 Statistical Software, 2010).

Herbivory Time Course Study

One semi-starved Japanese beetle was placed in a transparent, mesh 11cm x 10cm bag, which was then placed over one mature attached leaf of either 'GREM4' or 'PN40024', and beetles were permitted to feed for 30min, 1h, or 4h (Figure 2.1A). 30min was chosen since transcriptomic differences *in planta* have been observed within

20min after encountering a stress (Pandey et al., 2017). 4h was chosen since defensive compounds were found to increase consistently up to 4h in a previous insect herbivory study (Köllner et al., 2010). Feeding timing began once visible damage to the leaf was observed. All experimental 'runs' (an attempt at collecting feeding data by placing a beetle in a bag on a leaf) were conducted in a greenhouse August through September of 2021 and 2022, between 9:00AM and 3:00PM daily. Plants were not used again for at least four days between runs to ensure *in planta* responses captured were not a vestige of prior feeding. If a beetle did not feed within a 4h timeframe the run was considered 'unsuccessful'. Additional runs were needed for some time points to attain the desired experimental replicates, thus 'GREM4' had more experimental attempts, since many 'GREM4' runs were unsuccessful (scored as an AOF of zero). Replicates for each condition are as follows: 'GREM4' 30min = 19; 'GREM4' 1h = 20; 'GREM4' 4h = 20; 'PN40024' 30min = 8; 'PN40024' 1h = 9; 'PN40024' 4h = 9.

After each run, leaves were photographed, placed inside 50mL conical tubes, then plunged into liquid nitrogen. Leaves were stored at -80ºC until RNA isolation for RNA-sequencing (see "RNA Isolation and Sequencing"). '0h' control leaves were also collected, but from a different plant than the herbivory samples to avoid confounding transcriptomic responses due to the removal of a leaf.

AOF measurements were ascertained as previously described (see "Herbivory Preference Study"). The differences between 'GREM4' and 'PN40024' AOF for each herbivory time point was determined via a two-sample t-test (unequal variances) using

MiniTab21. The feeding success rate for 'GREM4' and 'PN40024' was also reported as the percentage of successful feeding runs out of the total number of runs.

Leaf Trichome Density Observations

Trichome densities were recorded for the adaxial and abaxial sides of three immature and three mature 'GREM4' and 'PN40024' leaves, all plants being grown in the greenhouse, three measurements each, 72 in total. Monochrome images were obtained using a digital Nikon Eclipse 80*i* microscope at 10x magnification with a Nikon DS_QiMc Digital Sight camera at the Molecular and Cellular Imaging Center - South, The Ohio State University. Images were scored by three independent scorers based on the Organisation Internacionale Vitis de la Vigne et du Vin (OIV) 'Mature leaf: density of prostrate hairs between main veins on lower side of blade' scale, where a score of '1' indicated no trichomes were present, while '9' was extremely high trichome density (Alercia et al., 2001). All trichomes, both prostrate and erect, were included in scoring. Significance was determined via a one-way ANOVA (Games-Howell with grouping, equal variances, confidence level 95%, error rate 0.05%) using MiniTab21.

Herbivory Under Equal Trichome Densities Study

The adaxial sides of mature 'GREM4' and 'PN40024' leaves were found to not significantly differ in trichome densities (see "Results"). As such, one beetle was restricted to the adaxial side of a mature leaf in both species via a transparent plastic container with small holes for air movement and allowed to feed for 1h (Figure 2.1B and Figure 2.1C). Experiments were performed in the greenhouse between August and

September of 2022. Photos of the leaves were taken before and after feeding and used to calculate the AOF as previously described (see "Herbivory Preference Study"). Runs where beetles forced their way onto the abaxial side and fed were excluded. Ten replicates were collected per species. Significance was determined via a one-sided two-sample t-test (variances equal), using MiniTab21.

'GREM4' Herbivory Under Differing Trichome Densities Study

Experimental conditions were identical to the "Herbivory Under Equal Trichome Densities Study", but Japanese beetles were presented with the adaxial or abaxial sides of mature 'GREM4' leaves, which significantly differed in trichome density (see "Results"). A total of 10 abaxial and 21 adaxial replicates were performed in July 2023. Differing numbers of replicates were due to beetles occasionally forcing their way to the non-presented side of the leaf, resulting in greater adaxial feeding datapoints. Significance was determined as previously described (see "Herbivory Under Equal Trichome Densities Study").

RNA Isolation and Sequencing

RNA was isolated from the 30min, 1h, and 4h 'Herbivory' and 0h 'Control' leaf samples collected during the 2021 "Herbivory Time Course Study". RNA from leaf samples was isolated using a Sigma-Aldrich Spectrum Plant Total RNA Kit (Millipore-Sigma, 2023) and RNA quality and quantity were determined via Nanodrop (Desjardins and Conklin, 2010), Qubit (Invitrogen/ThermoFisher Scientific/Life Technologies Holdings Pte Ltd, 2021), and a formaldehyde gel. A total of 32 samples (four herbivory

replicates for each of the three time points, plus four 0h samples, for both species) were submitted to Novogene (Novogene Co. Ltd, n.d.) for individual library preparation and Illumina NovaSeq 6000 paired-end RNA sequencing (150bp , 20M reads per sample). RNA-seq reads were subjected to quality control assessments via FastQC (Andrews, 2023) and removal of adapters and poor quality reads via Trimmomatic (Bolger et al., 2014).

Vitis labrusca acc. 'GREM4' Gene Annotation Generation

To ensure a high quality gene annotation for downstream transcriptomic analysis, the *Vitis labrusca* acc. 'GREM4' genome annotation (Li and Gschwend, 2023) was updated using 'GREM4' RNA-seq data to improve annotation accuracy and can be found on GitHub at https://github.com/cdixo/Vitis-labrusca-Version-2-Genome-Annotation.git as Version 2. Gene annotation was completed using the repeat masked 'GREM4' primary genome sequence assembly and employing Funannotate assisted by a publicly available container (Korf, 2004; Majoros et al., 2004; Lomsadze et al., 2005; Stanke et al., 2008; Manni et al., 2021; Konkel, 2022; Stajich and Palmer, 2022; Li and Gschwend, 2023). BUSCO was run on the 37,443 annotated genes and 96.7% of the 1,375 BUSCO genes were detected, suggesting a high-quality annotation. Additional information can be found in Figure 2.2 and on GitHub at https://github.com/cdixo/Inter-species-and-Herbivory-Publication.git .

Orthologous, Paralogous, and Genome-specific Gene Identification

Orthologous genes were identified between 'GREM4' and 'PN40024' using OrthoFinder V2.2.5, DIAMOND, and custom scripts (Emms and Kelly, 2019; Buchfink et al., 2021). Additional information can be found in Figure 2.3 and on GitHub at https://github.com/cdixo/Inter-species-and-Herbivory-Publication.git . A subset of orthologous genes were manually checked and verified for accuracy using NCBI BLAST (Agarwala et al., 2016).

Genes which did not have an orthologous gene identified between the two genomes were characterized as either paralogous or genome-specific. Paralogous genes did not have a direct corresponding ortholog in the other species but did share sequence similarity to other gene(s) within the same species (i.e. - were grouped into the same orthogroup by OrthoFinder). Genome-specific genes did not have a corresponding ortholog in the other species nor a paralog within the same species.

When investigating gene families which differed in size in which the additional gene family members were significantly differentially expressed upon beetle herbivory, gene families were defined as per orthogroups reported via OrthoFinder (Emms and Kelly, 2019). If multiple genes with different names were clustered into one orthogroup, the gene name present most frequently was used to name the group.

RNA-seq Read Alignment, Transcriptomic Analysis, and Enrichment Analysis

RNA-seq reads were aligned to their respective genomes using STAR (Dobin et al., 2013). CoCo via 'coco correct_counts' was used to create the count matrix (to better

account for multi-mapping reads) (Deschamps-Francoeur et al., 2019). DESeq2 was used to identify differentially expressed genes (DEGs) (Love et al., 2014). DEGs were identified at each time point (30min, 1h, and 4h) independently and then combined for downstream analysis. Throughout analyses, significant p- and p-adj values were defined as ≤ 0.05 whereas |log2foldchange| was ≥ 2. RNA-seq read quality statistics are found in Table 2.1. Additional information on the pipeline and programs used to analyze the RNA-seq data can be found in Figure 2.4 and on GitHub at https://github.com/cdixo/Inter-species-and-Herbivory-Publication.git . BioVenn, BioInfoRx, and molbiotools were used to identify DEGs conserved between transcriptomic comparisons and to create Venn diagrams (Hulsen et al., 2008; BioInfoRx, 2023; Molbiotools, 2023).

Inter-species transcriptomic comparisons were conducted by three different methods (Figure 2.5). The first method simply determined if DEGs identified between insect herbivory samples compared to 0h in one species were also independently determined to be DEGs in the other species. This analysis was conducted by reviewing the names of the DEGs identified, for any herbivory time point, between the two species, to determine if the DEG (gene name) was present in both lists by running an intersection command. This method was called 'Overlap Analysis'. The second method was an 'Interaction Analysis' which identified genes that, upon evaluating the interaction between the genotype ('GREM4' or 'PN40024') and the treatment (herbivory or 0h), were determined to be significantly differentially expressed. Functionally, this method

explored the change in log2FoldChange (Δlog2FoldChange) between 'GREM4' and 'PN40024' for a gene, i.e. - identified genes with significantly different responsiveness to insect herbivory between the two species. Technically, first, log2FoldChange values were generated for the 30min, 1h, or 4h herbivory samples, compared to 0h, for all orthologous genes in their respective species. Then, these log2FoldChange values were compared between species to identify log2FoldChange values that were significantly different ($|\Delta log2foldchange| \geq 2$; p-adj ≤ 0.05). A third method to compare inter-species expression was required, since a significantly different Δlog2FoldChange could be reported for a gene between the two species without the gene ultimately being differentially expressed between the two species (see Figure 2.5). For this reason, 'Cross-Reference Analysis' was also conducted. Functionally, this method identified which genes had significantly different expression at a time point between species. Technically, this analysis first identified DEGs from herbivory for 30min, 1h, or 4h time points compared to 0h for all orthologous genes (via DESeq2), in their respective species, and then compared expression (read count values) at the noted time point between species to identify expression values that were significantly different via DESeq2 (p-adj $\leq$ 0.05; $|log2foldchange| \geq 2$). Notably, in both interaction analysis and cross-reference analysis, genes must be DEGs in both species between herbivory and 0h to then be scrutinized in the second step of the comparison.

Enrichment analyses identified Gene Ontology (GO) terms enriched in various gene datasets. Over-Representation Analysis (ORA) identified GO term enrichment of

DEGs. ORA was conducted via 'enricher' (clusterProfiler) with a post-hoc 'gsfilter' (DOSE) (Yu et al., 2015; Wu et al., 2021). Enrichment was also conducted using Kyoto Encyclopedia of Genes and Genomes (KEGG) via the KEGG Orthology-Based Annotation System-intelligent (KOBAS-i) (Kanehisa and Goto, 2000; Bu et al., 2021). Additional information can be found in Figure 2.4 and on GitHub at https://github.com/cdixo/Inter-species-and-Herbivory-Publication.git .

Intra-species transcriptomic comparisons broken down by time point, between 30min, 1h, and 4h time points, are discussed in Chapter 3.

Results

Herbivory Preference Study

To determine if 'GREM4' was resistant to Japanese beetle herbivory, we performed a feeding preference study between 'GREM4' and 'PN40024'. For all studies herein, resistance is defined as one species exhibiting significantly decreased Japanese beetle herbivory damage compared to the other species. When 15 Japanese beetles were provided the choice to feed on either 'GREM4' or 'PN40024' leaves, significantly greater herbivory damage, measured by AOF, was observed for 'PN40024', with 17.79% (±1.19% S.E.) of the leaf area fed upon, compared to 2.40% for 'GREM4' (±0.34% S.E.; $p = 0.037$) (Figure 2.6A, Figure 2.7A, and Figure 2.7B). These results demonstrate Japanese beetles preferred feeding on 'PN40024' over 'GREM4'.

Herbivory Time Course Study

Next, we aimed to determine if, given no choice, Japanese beetles would still feed less on 'GREM4' over time, compared to 'PN40024'. We conducted an insect herbivory time course study which restricted single Japanese beetles to either one 'GREM4' or one 'PN40024' attached leaf and allowed the beetles to feed for 30min, 1h, or 4h. A significantly greater AOF was calculated for 'PN40024' compared to 'GREM4' at all time points (p-value ≤ 0.05) (Figure 2.6B-D). AOF also increased in both species from 30min to 4h, but little difference was observed between 1h and 4h of feeding. These results report that, under 30min, 1h, and 4h of herbivory, 'GREM4' experienced less AOF than 'PN40024', suggesting resistance to Japanese beetle herbivory.

We also recorded the number of successful (feeding) and unsuccessful (no feeding) time course runs. The majority of unsuccessful runs occurred with Japanese beetles restricted to feeding on 'GREM4' leaves (Figure 2.6E) ('GREM4' = 15 successful, 44 unsuccessful runs, 25% success rate; 'PN40024' = 22 successful, 4 unsuccessful runs, 85% success rate). Therefore, not only was the AOF on 'GREM4' leaves lower, but, for the majority of the runs, the starved beetles did not feed at all. Together, these findings provide compelling evidence that 'GREM4' leaves are resistant to Japanese beetle herbivory compared to 'PN40024'.

Leaf Trichome Density and Herbivory Studies

We next investigated the defensive mechanisms which contribute to insect herbivory resistance in 'GREM4'. Trichomes are a well-known insect herbivory

defensive adaptation, and trichome densities visibly differed between ‘GREM4’ and ‘PN40024’ (Smith, 2005; de Queiroz et al., 2020; Yin et al., 2022). Therefore, we performed detailed trichome density observations on the adaxial and abaxial sides of ‘GREM4’ and ‘PN40024’ immature and mature leaves. Leaves were scored using a trichome density scale (see “Methods”), where a score of ‘1’ was devoid of trichomes while ‘9’ meant trichome density was extremely high (Alercia et al., 2001). Significantly greater trichome densities were observed in ‘GREM4’ compared to ‘PN40024’ in all comparisons, except for the adaxial side of ‘GREM4’ mature leaves (Figure 2.8A). In ‘GREM4’, trichome density averages on both sides of the immature leaves and on the abaxial side of mature leaves ranged from 8.26 to 9.00, whereas the mature adaxial side was significantly less (2.19). ‘PN40024’ trichome density scores for both sides of mature and immature leaves were between 1.00 and 3.59. These findings indicate greater trichome densities were found overall on ‘GREM4’ leaves compared to ‘PN40024’. Therefore, increased trichome density may contribute to insect herbivory resistance in ‘GREM4’.

To evaluate the impact of trichome density on ‘GREM4’ herbivory defense, we next permitted Japanese beetles to only feed on the adaxial (low trichome density) or abaxial (high trichome density) side of ‘GREM4’ leaves. There was no significant difference between AOF on the ad- vs. abaxial sides of the leaves ($p = 0.307$) (Figure 2.8B), but a feeding preference was observed during the study. Though beetles were placed on the ad- or abaxial side of the leaf, they were not completely restricted in their

movement (see "Methods"). Therefore, some beetles did not feed on the presented side and instead transitioned to the non-presented side of the leaf to feed. Of beetles placed on the adaxial side of the leaf, 33% transitioned to and fed on the opposite side of the leaf (abaxial side) while 72% of beetles placed on the abaxial side transitioned to and fed on the opposite side (adaxial side) (Figure 2.8C). Though the AOF was not significantly different between the two sides, these findings report that the Japanese beetles preferentially avoided the high trichome density side of the leaves, which supported the hypothesis that trichomes aid in deterring insect herbivory on 'GREM4'.

Since trichome densities were greater on 'GREM4' leaves than 'PN40024' leaves, and considering the results from the above experiment, we additionally assessed if trichome density was the sole factor conferring heightened insect herbivory resistance in 'GREM4'. Trichome densities on the adaxial surfaces of mature 'GREM4' and 'PN40024' leaves were not significantly different (Figure 2.8A). Therefore, Japanese beetles were restricted to feed only on 'GREM4' and 'PN40024' adaxial sides of leaves. Under equal trichome density, beetles still fed about three times more on 'PN40024' leaves ($9.80 \pm 2.68 mm^2$ S.E.) compared to 'GREM4' (3.29 ± 1.25 mm^2 S.E. ($p = 0.029$) (Figure 2.8D). These results report other factors, beyond trichomes, are also implicated in insect herbivory resistance in 'GREM4'.

Inter-species Transcriptomic Responses

Orthologous Genes between 'GREM4' and 'PN40024'

Orthologous genes were identified to compare expression between 'GREM4' and 'PN40024'. 23,337 orthologous genes were identified between 'GREM4' (37,443 total annotated genes) and 'PN40024' (35,133) (Table 2.2). An additional 12,898 'GREM4' and 8,435 'PN40024' paralogous genes, genes with homology with other genes in the same species, but did not have an ortholog in the other species, (i.e. - additional gene family members), were identified. This left 'GREM4' with 1,168 (3.12%) genome-specific genes and 'PN40024' with 3,321 (9.45%). The expression of orthologous genes could be compared directly between species, but genome-specific and paralogous genes could not, as they were only identified in one of the two genomes. Nonetheless, they may play important roles in conferring insect-herbivory resistance. All three categories of genes were investigated and are discussed below.

Basal Expression Differences at 0h

First, expression of 'GREM4' and 'PN40024' orthologous genes at 0h was compared to identify differences in basal expression to determine constitutively differentially expressed genes (Table 2.3, Table 2.4, and Table 2.5). 1,373 of 23,377 (5.87%) orthologous genes had significantly higher expression in 'GREM4' compared to 'PN40024' at 0h, while 1,146 (4.90%) had significantly lower expression in 'GREM4' compared to 'PN40024' (Table 2.9, Figure 2.9A, and Table 2.5). Overall, these findings

indicate differences in basal transcriptomic states exist between ‘GREM4’ and ‘PN40024’.

Of genes with significantly higher expression in ‘GREM4’ compared to ‘PN40024’, two enriched KEGG pathways were identified (Table 2.6) - ‘plant-pathogen interaction’ (34 implicated DEGs) and ‘biosynthesis of secondary metabolites’ (95 implicated DEGs). Of DEGs with lower expression in ‘GREM4’ compared to ‘PN40024’ (higher expression in ‘PN40024’ at 0h), only one pathway was enriched - ‘plant-pathogen interaction’ (25 implicated DEGs) (Table 2.6). DEGs with greater expression in ‘GREM4’ compared to ‘PN40024’ at 0h were enriched in secondary metabolite biosynthesis, which was not identified of DEGs with greater expression in ‘PN40024’ compared to ‘GREM4’ at 0h, and likely contributed to defense against insect herbivory and other biotic stress. Though DEGs with greater expression in ‘GREM4’ compared to ‘PN40024’ at 0h and DEGs with greater expression in ‘PN40024’ compared to ‘GREM4’ at 0h were both enriched in pathway ‘plant-pathogen interaction’, a greater number of DEGs contributed to the enrichment identified in ‘GREM4’. While it was found that a greater number of DEGs had higher expression in ‘GREM4’ at 0h compared to ‘PN40024’ the lack of enrichment in other pathways (besides the three mentioned) suggest these genes are broadly distributed across a large number of biological processes.

Due to their integral role in plant defense signaling, we investigated if JA and SA pathway genes were significantly differentially expressed at 0h in ‘GREM4’ compared to ‘PN40024’ (Geuss et al., 2018; Costarelli et al., 2020; Aerts et al., 2021; Weeraddana and

Evenden, 2022). Seven JA and four SA pathway genes were significantly differentially expressed (Figure 2.10). Overall, JA and SA biosynthesis gene transcript accumulation was skewed towards greater expression in 'GREM4' compared to 'PN40024'. All seven JA DEGs and three of four SA DEGs had higher constitutive expression in 'GREM4' compared to 'PN40024', which could initiate downstream defensive pathways conferring heightened responses to insect and pathogen attacks.

These findings report genes implicated in defense signaling, pathogen response, and secondary metabolite biosynthesis are constitutively expressed at a higher level in 'GREM4' compared to 'PN40024' and may contribute to the increased insect herbivory resistance.

Insect Herbivory

The total number of DEGs at 30min, 1h, and 4h of Japanese beetle herbivory (compared to 0h) were determined for 'GREM4' and 'PN40024' and combined between time points (duplicates removed). A total of 690 (549 up-regulated and 141 down-regulated) DEGs were identified in 'GREM4' under herbivory, while a total of 502 (447 up-regulated and 55 down-regulated) DEGs were identified in 'PN40024' (Table 2.9), thus, more genes were both up and down-regulated in 'GREM4' under herbivory compared to 'PN40024'. These identified DEGs could have been orthologous, paralogous, or genome-specific, which we investigated below.

We first identified genes with significantly different expression between 'GREM4' and 'PN40024' upon Japanese beetle herbivory (Table 2.3, Table 2.4, and

Table 2.5). We conducted these analyses via three methods - 'Overlap Analysis', 'Interaction Analysis', and 'Cross-Reference Analysis' (see "Materials and Methods" and Figure 2.5) - to identify DEGs, and identify candidate genes, likely contributing to increased insect herbivory resistance in 'GREM4'.

Overlap Analysis

Overlap analysis identified orthologous genes which were significantly up or down-regulated under herbivory compared to 0h at any time point in both species. Out of the total 1,192 DEGs identified under insect herbivory in 'GREM4' and 'PN40024', 911 had orthologs in both genomes. Of these 911 orthologs, only 108 DEGs were significantly differentially expressed in both 'GREM4' and 'PN40024' (Figure 2.9B and Table 2.7) and overlapping DEGs were enriched in genes involved in 'sequence-specific DNA binding' (Table 2.8). 495 of the orthologous genes were only differentially expressed in 'GREM4' (Figure 2.9B and Table 2.7) and ORA enrichment analysis revealed 'hydrolase activity, acting on ester bonds' as the only functional enrichment in these DEGs (Table 2.8). Nonetheless, genes implicated in other pathways were identified in this list as well, including lipid formation, terpene biosynthesis, and peroxidase activity. 308 orthologs were only differentially expressed in 'PN40024' (Figure 2.9B and Table 2.7) and nine functional enrichments were identified including xyloglucan-related terms, 'cell wall biogenesis', and 'calcium ion binding' (Table 2.8). These results report that, although the majority of differentially expressed genes under herbivory were orthologous, only about 12% were significantly differentially expressed in both species,

suggesting specialized expression patterns are observed for the majority of these orthologous genes in each species under insect herbivory.

Interaction Analysis

The interaction analysis identified orthologous genes with a significant Δlog2FoldChange between 'GREM4' and 'PN40024'. Out of 23,377 orthologous genes, only 78 had a significant Δlog2FoldChange between 'GREM4' and 'PN40024', and 58% had a greater Δlog2FoldChange in 'GREM4' compared to 'PN40024' (Table 2.9, Table 2.5, and Table 2.7). The top 10 genes that were identified via the interaction analysis had a $|\Delta log2FoldChange| \geq 20$ and a p-adj ≤ 0.01 and were implicated in terpene biosynthesis, disease and pathogen resistance, and wax biosynthesis (Table 2.10 and Table 2.7). Eight of these 10 genes had greater Δlog2FoldChange in 'GREM4' and are candidate genes for insect herbivory resistance and future study.

Cross-reference Analysis

The cross-reference analysis identified orthologous genes which were significantly differentially expressed during herbivory compared to 0h and had significantly different expression between 'GREM4' and 'PN40024' under herbivory (read count value) at the coincidental time point. When combining all up and down-regulated DEGs across all time points, 82 such genes were identified in 'GREM4' compared to 'PN40024' (Table 2.9 and Table 2.7). Comparatively, in 'PN40024', only 48 genes were identified under the same parameters (Table 2.9 and Table 2.7). Of the 82 'GREM4' genes, the top 12 had a $|log2FoldChange| \geq 20$ and a p-adj ≤ 0.01 (Table 2.11).

These 12 DEGs were implicated in phytohormonal response, disease/fungal resistance, terpene biosynthesis, and flavonoid biosynthesis and are candidate genes for insect herbivory resistance and future study.

Overall, all three comparative methods reported genes implicated in processes and pathways with obvious implications in insect herbivory defense and some genes (Table 2.10 and Table 2.11) have been reported as candidates for future insect herbivory resistance functional validation studies. The methods cooperatively identified genes of interest by either capturing genes overlooked, or refining a pool identified, by another method (Figure 2.9C). Sixteen DEGs were captured by all three methods and were thus very strong candidates to confer insect herbivory resistance (Figure 2.9C and Table 2.12). These 16 genes were implicated in disease resistance, insect herbivory resistance and response, biotic stress response, JA and SA, pollen-related functions, and photosynthesis under stress.

Functions of Genome-specific and Paralogous Genes

The inter-species analyses conducted above only compared gene expression differences under herbivory for genes with an ortholog in both species. But, genome-specific and paralogous genes, for which a direct ortholog could not be identified, are also of interest since they are major contributors to genetic novelty.

Genes which were only identified in ‘GREM4’ or ‘PN40024’ were identified as ‘genome-specific genes’. In ‘GREM4’, 1,168 genome-specific genes were identified (Figure 2.9D, Table 2.2, and Table 2.7), and while no functional enrichments via ORA

were identified (Table 2.8), one KEGG pathway was enriched of 'plant-pathogen interactions' (34 genes) (Table 2.6). This result suggests some 'GREM4' genome-specific genes contribute to interactions with pathogens, but the rest are distributed across a myriad of metabolic pathways, with, for example, 9% being found to be involved specifically in secondary metabolite biosynthesis. Of the 690 total DEGs identified in the 'GREM4' herbivory samples compared to 0h, only eight (2%) were genome-specific (Figure 2.9B) representing <1% of all genome-specific genes in 'GREM4' (Figure 2.9D) and are listed in Table 2.13. In 'PN40024', 3,321 genome-specific genes were identified (Figure 2.9E, Table 2.2, and Table 2.7), but while no KEGG pathways were significantly enriched (Table 2.6), one functional enrichment was identified of 'cytochrome complex assembly' (Table 2.8). This result suggests 'PN40024' genome-specific genes, alike 'GREM4', engage in a broad range of functions and pathways. Of the 502 total 'PN40024' herbivory DEGs, only 15 (3%) were genome-specific (Figure 2.9B) representing <1% of all genome-specific genes (Figure 2.9E).

Next, we investigated paralogous genes (e.g. – extra gene copies unique to a species). 12,898 paralogous genes were detected in 'GREM4' (Figure 2.9D, Table 2.2, and Table 2.7) which were enriched in 30 functional enrichments including 'signal transduction', 'lignin catabolic process', terpene-related terms, acyltransferase-related terms, and 'transcription coactivator activity' (Table 2.8). Of the 690 herbivory DEGs in 'GREM4', 79 (11%) were paralogous genes (Figure 2.9B) representing <1% of all paralogous genes in 'GREM4' (Figure 2.9D). Four functional enrichments were

identified in these 79 genes of ‘signal transduction’, ‘biosynthetic process’, and two acyltransferase-related terms (Table 2.8). When identifying the topmost significantly differentially expressed genes via parameters of a |log2FoldChange| ≥ 20 and a p-adj ≤ 0.01, two ‘GREM4’ herbivory DEGs which were paralogs (Table 2.13). In ‘PN40024’, 8,435 paralogous genes were identified (Figure 2.9E, Table 2.2, and Table 2.7) and were enriched in 35 ORA functional terms including ‘DNA integration’, cellulose-related terms, and ‘response to auxin’ (Table 2.8). 71 (14%) of the total 502 ‘PN40024’ herbivory DEGs were paralogous genes (Figure 2.9B) representing <1% of all paralogous genes (Figure 2.9E). ‘Apoplast’ was the only functional enrichment in these 71 genes (Table 2.8). These results report ‘GREM4’ had a greater number of paralogous genes, indicating more gene family expansions and/or fewer gene family contractions compared to ‘PN40024’, and a portion of those genes were differentially expressed under insect herbivory, suggesting a role in defense response.

Gene family expansions can give rise to genes with novel or specialized functions, expression patterns, or activity. To explore how such genes could impact insect herbivory defense, we investigated two gene families which differ in gene family size between ‘GREM4’ and ‘PN40024’ and displayed significantly different expression upon Japanese beetle herbivory. The *TPS1*-orthogroup gene family was identified via OrthoFinder and is implicated in terpene biosynthesis. The *TPS1*-orthogroup gene family differs in gene family members between ‘PN40024’ (four genes) and ‘GREM4’ (eight genes) and two genes unique to ‘GREM4’, *Terpene synthase 1-2* (*TPS1-2*)

(*Vitla_GREM4_19g60.31*) and *Terpene synthase 1-3* (*TPS1-3*) (*Vitla_GREM4_19g59.46*), experienced increased expression upon beetle herbivory (Figure 2.11A). As for constitutive expression, *TPS1-3* displayed the highest expression of any family member in 'GREM4'. The second gene family explored was *Phenylalanine lipase* (*PAL*) – a gene encoding an enzyme which catalyzes the reaction converting phenylalanine to cinnamic acid in the phenylpropanoid pathway which is critical to both flavonoid and lignin biosynthesis (Koukol and Conn, 1961). Four gene family members were identified in the *PAL* gene family in 'PN40024' while 12 were identified in 'GREM4', four of which were differentially expressed upon insect herbivory - *PAL1-4* (*Vitla_GREM4_16g7.31*), *PAL1-5* (*Vitla_GREM4_16g8.34*), *PAL1-6* (*Vitla_GREM4_16g8.37*), and *PAL1-8* (*Vitla_GREM4_16g7.35*) (Figure 2.11B). When reviewing constitutive expression, 'GREM4' novel gene *PAL1-11* (*Vitla_GREM4_8g123.37*) was expressed thousands of times greater than most other genes at 0h. These results suggest that *PAL* and *TPS* paralogous genes unique to 'GREM4' are involved in a response to insect herbivory, and in some cases, are constitutively expressed at high levels. It is likely these genes are important in conferring heightened insect herbivory defense via terpene, flavonoid, lignin, or other phenolic compound production.

Though some paralogous (11% and 14%) and genome-specific (2% and 3%) genes were differentially expressed under herbivory in both 'GREM4' and 'PN40024', it is striking that 87% and 83% of the DEGs during herbivory in 'GREM4' and 'PN40024'

were orthologous genes (Figure 2.9B). Additionally, only 108 of the 603 'GREM4' and 416 'PN40024' total orthologous herbivory DEGs were differentially expressed during herbivory in both species, suggesting differential expression of orthologous genes is key in imparting insect herbivory resistance in 'GREM4' (Figure 2.9B).

Taken together, these findings suggest that the heightened insect herbivory resistance of 'GREM4' compared to 'PN40024' is greatly due to unique expression patterns of orthologous genes in 'GREM4' and, to a lesser degree, expression of paralogous and genome-specific genes involved in plant-pathogen interactions and secondary metabolism. Additional functional studies are necessary to fully elucidate the impact of paralogous and genome-specific insect herbivory response candidate genes in Table 2.13.

Discussion

Plants are sessile organisms, so the evolution of defensive measures to counteract threats, including insect herbivory, is essential for survival and reproduction. Defenses against herbivory are diverse and include trichomes, lignified tissue, thick waxy cuticles, chemical defenses such as insecticidal or repellent secondary metabolites, and volatile organic signaling compounds (Peeters, 2002; Kariyat et al., 2019; Meents et al., 2019; Silva et al., 2021; Singh et al., 2021; Han et al., 2023). *V. labrusca* is commonly used in grapevine breeding programs to instill resistance to biotic and abiotic stresses, but the underlying contributors to this resistance are not well understood. In this study, we evaluated *V. labrusca* acc. 'GREM4' and *V. vinifera* cv. 'PN40024' for herbivory

resistance against Japanese beetle, determined the role of trichomes in herbivory defense, and identified genes involved in responses to insect herbivory.

‘GREM4’ is Resistant to Japanese Beetle Herbivory

‘GREM4’ exhibited increased Japanese beetle herbivory resistance compared to ‘PN40024’ in both our choice and no-choice experiments, across multiple feeding time points, as less total leaf area was damaged in ‘GREM4’ compared to ‘PN40024’. Our results support previous reports that *V. labrusca*-hybrid grapevines exhibited decreased Japanese beetle herbivory compared to *V. vinifera* (Mercader and Isaacs, 2003). Past studies have also reported heightened insect herbivory resistance in other North American wild grapevines; A screen of North American grapevine species and hybrid *Vitis* cultivars for mealybug resistance found *V. vinifera* lines were highly infested with mealybugs, while North American hybrids experienced little infestation (Naegele et al., 2020). Insect herbivory resistance has been widely identified in wild relatives of other crops, such as wild soybean (*Glycine soja*), exotic cotton landraces (*Gossypium hirsutum*), and maize landraces (*Zea mays*) (Du et al., 2022; Abel et al., 2023; Conzemius et al., 2023). Wild plant species/accessions often exhibit heightened resistance to biotic and abiotic stress, and consequently, have long been employed in breeding programs as sources of novel genetic material to imbue advantageous traits to elite lines (Smith, 2005; Qiu et al., 2015). In general, *V. labrusca* is highly fit in its local environment against pathogens and adverse weather conditions, so it was not surprising our herbivory experiments found that *V. labrusca* accession ‘GREM4’ was more resistant to Japanese

beetles herbivory compared to ‘PN40024’ (Gabler et al., 2003; Dami, 2007; Cadle-Davidson, 2008; Gee et al., 2008; Nascimento-Gavioli et al., 2019; Todaro and Longstroth, 2019).

Trichome Density Contributes to Insect Herbivory Resistance

Since trichomes are well-known plant adaptations that aid in defense against insect herbivory, we tested whether increased trichome density was responsible for conferring heightened insect herbivory resistance in ‘GREM4’ compared to ‘PN40024’ (Smith, 2005; de Queiroz et al., 2020; Yin et al., 2022). Our results determined that leaf trichome densities were significantly greater on ‘GREM4’ leaves compared to ‘PN40024’, which is consistent with previous ampelographic studies of trichomes in *Vitis* (Kortekamp and Zyprian, 1999; Gerrath et al., 2015; Ma et al., 2016; MacGregor et al., 2016). When beetles were placed on high trichome density sides of ‘GREM4’ leaves, they moved to the low trichome density side of the leaf to feed 72% of the time suggesting trichomes deter Japanese beetle herbivory.

The impact of trichomes on insect defense in crop plants is well established. For example, high trichome densities have resulted in decreased insect damage in wheat, *Datura stramonium*, and soybean (Valverde et al., 2001; de Queiroz et al., 2020; Singh et al., 2021). Trichomes appear to contribute to insect herbivory defense in grapevines. However, insect size and mouthpart type seem to determine their effectiveness. In interspecific grapevine ‘GE1025’ for example, a weak negative correlation was identified between phylloxera severity traits and trichome density of leaves, phylloxera being a

small piercing-sucking mouthpart insect (Yin et al., 2021). Anecdotally, *V. labrusca* hybrid 'Edelweiss' experienced decreased phylloxera damage due to its high trichome density, as well (Yin et al., 2021). Large, chewing mouthpart insects, such as Japanese beetles, are most deterred by high trichome densities, as supported by Johnson et al. in which *V. vinifera* acc. 'Mars', with high trichome density, had the least amount of feeding damage in a *V. vinifera* panel (Dami et al., 2005; Johnson et al., 2010). Our results also support Japanese beetle herbivory is deterred by the high trichome density in 'GREM4'.

Importantly, in our study when Japanese beetles were strictly allowed to feed on mature adaxial sides of 'GREM4' and 'PN40024' leaves with similarly low trichome densities, there was still significantly less (~3 times less) AOF in 'GREM4' than 'PN40024'. This finding indicates trichomes are not the only factor contributing to herbivory resistance in 'GREM4'.

Defense Response Genes are Constitutively Expressed at Higher Levels in 'GREM4' compared to 'PN40024'

Constitutive defense in plants is a phenomenon where defensive structures, compounds, etc. are always produced or present, even when the stress is not experienced, to provide an immediate level of protection when encountered (Rasmann et al., 2015). In our study, a comparison between basal transcript accumulation levels of 'GREM4' (0h) compared to 'PN40024' (0h) revealed 2,519 DEGs between the two species. DEGs with greater expression in 'GREM4' compared to 'PN40024' were enriched in pathways

involved in ‘plant-pathogen interaction’ and ‘biosynthesis of secondary metabolites’. It is not uncommon for genes implicated in pathogen interaction and resistance to be differentially expressed under insect herbivory, as they often serve multiple roles in biotic stress response, including insect herbivory, in a variety of plants including grapevine (Ralph et al., 2006; Huang et al., 2015; Ederli et al., 2020; Zhou et al., 2020; Jan et al., 2022; Yin et al., 2022). Considering *V. labrusca* is resistant to many pathogens we propose it is likely these genes play a role in conferring heightened constitutive defense against pathogens, as well as insects, in ‘GREM4’ (Gabler et al., 2003; Cadle-Davidson, 2008; Gee et al., 2008; Nascimento-Gavioli et al., 2019). While ‘plant-pathogen interactions’ was also enriched in genes with higher constitutive expression in ‘PN40024’, a greater number of such genes, and different genes, were more highly expressed in ‘GREM4’. Additionally, the pathway ‘biosynthesis of secondary metabolites’ was also enriched in DEGs with constitutively increased expression in ‘GREM4’ compared to ‘PN40024’ and the implicated 149 genes were mainly associated with terpene, carotenoid, phenylalanine-tyrosine-tryptophan, flavone-flavanol, stilbenoid, and flavonoid biosynthesis (Darzi et al., 2018). Considering the role of terpenes, flavonoids, and other secondary metabolites in insect herbivory defense, it seems likely that increased basal expression of these genes translates to increases in such metabolites, conferring heightened constitutive defense against insect herbivory, though metabolomic tests are required to verify this hypothesis (Loughrin et al., 1997; Chatterjee et al., 2022;

Chen et al., 2022; Jia et al., 2022; Sun et al., 2022; Zhang et al., 2022a; Han et al., 2023; Wang et al., 2023a).

Heightened constitutive expression resulting in insect defense has been observed in other species. Sitka spruce (*Picea sitchensis*) genotypes with resistance to spruce weevil (*Pissodes strobi*) constitutively expressed over 2,000 genes at greater levels, and had twice as many constitutively expressed genes associated with defense-related GO terms, than susceptible genotypes prior to insect herbivory (Whitehill et al., 2021). In alfalfa (*Medicago sativa*) resistant to thrips, when compared to a susceptible line under non-insect herbivory conditions, the resistant line had higher levels of flavonoid compounds (Zhang et al., 2022c). In wheat (*Triticum aestivum*), a variety resistant to maize weevil (*Sitophilus zeamais*) constitutively produced multiple compounds, including flavonoids and benzoxazinoids, at levels greater than the susceptible variety (Lv et al., 2023). Overall, the high constitutive expression of defense genes in 'GREM4' relative to 'PN40024' likely provides greater immediate defense against Japanese beetle herbivory.

Unique Genes and Gene Expression are Implicated in 'GREM4' Insect Herbivory Defense

In our study, the majority of genes which were differentially expressed under herbivory were orthologous between 'GREM4' and 'PN40024' suggesting differences in gene regulation is a crucial factor in conferring heightened insect herbivory resistance in 'GREM4' compared to 'PN40024'. Genomic studies have reported extensive structural

differences between ‘GREM4’ and ‘PN40024’, likely impacting its fitness (Li and Gschwend, 2023). Structural variation, including duplications, insertions, and deletions, as well as small indels and SNPs, impact gene content, gene zygosity, and gene regulation between ‘GREM4’ and ‘PN40024’ (Li and Gschwend, 2023). This previously identified genetic variation between ‘GREM4’ and ‘PN40024’ in genic and regulatory regions likely contributed to the observed differential expression of orthologous genes under insect herbivory in our study through the modification or degeneration of cis-regulatory elements, or the genes themselves, leading to differential defense responses.

Genome-specific and paralogous genes are unique to a species, often exhibiting novel or specialized functions or regulation that give rise to distinctive phenotypes and were found to comprise a relatively small percentage of total DEGs upon herbivory in our study (13% in ‘GREM4’ and 17% in ‘PN40024’), but, nonetheless, play a role in insect herbivory defense. Segmental duplications result in paralogous genes and were reported as key drivers of genome evolution and diversification in ‘GREM4’ (Li and Gschwend, 2023). Segmental duplications were previously found to have contributed to rapidly amplified gene families involved in environmental response in ‘GREM4’, including defense response genes (Li and Gschwend, 2023). Our study found paralogous genes in ‘GREM4’ contributed to insect herbivory defense responses. For example, *PAL1,* an enzyme critical to the phenylpropanoid pathway, had 12 gene copies in ‘GREM4’ but only four in ‘PN40024’ and the *TPS1*-orthogroup gene family, implicated in terpene biosynthesis, had eight gene copies in ‘GREM4’ but only four in ‘PN40024’. These

novel paralogs had heightened expression upon insect herbivory in 'GREM4', likely resulting in increased flavonoid and/or lignin and terpene production, in turn increasing defense. Metabolomic analysis is necessary to confirm this connection. These results further support the premise that duplicated genes impact responses to environmental stress and contribute to increased plant fitness.

Examples of gene family expansions playing a role in insect herbivory resistance have been observed in other species. *Threonine deaminase* (*TD1*), a gene which encodes an enzyme critical in the formation of isoleucine, is an example of a gene duplication event resulting in a paralog with novel function. TD2 (the paralog of *TD1* in tomato) had lower isoleucine biosynthetic capacity compared to TD1, but, uniquely, impaired insect digestion while *TD1* significantly increased in expression upon MeJA application and wounding (Chen et al., 2007; Gonzales-Vigil et al., 2011). Another example is the expansion of the *Lipoxygenase* (*LOX*) gene family, which is important in various biological processes including ROS, JA, and defense (Zhu-Salzman et al., 2008). In wheat, 44 *LOX* gene family members were identified compared to only 6-13 in other plants (Wang et al., 2023b). After 48-72h of English grain aphid herbivory in a resistant genotype, *LOX5, LOX7, LOX10, LOX24, LOX29,* and *LOX33* were up-regulated but had lower expression in a susceptible genotype (Wang et al., 2023b). These studies provide support that gene family paralogs can exhibit differential responses to insect herbivory compared to other family members and contribute to resistance. Overall, our study reports paralogous and genome-specific genes in 'GREM4' likely play a role in

conferring insect herbivory resistance in ‘GREM4’. However, altered expression of orthologous genes, which constituted the majority of DEGs under herbivory, appear to be the major contributors.

Key Genes, Processes, and Pathways Implicated in ‘GREM4’ Insect Herbivory Response

The DEGs involved in defense responses to insect herbivory in ‘GREM4’ were especially enriched in functions related to secondary metabolite biosynthesis, phytohormone signal transduction, and pathogen defense. These genes, processes, and pathways play a role in conferring the heightened insect herbivory resistance observed in ‘GREM4’.

Phytohormones are critical signaling molecules essential to plant development, stress response, and insect herbivory defense (Geuss et al., 2018; Costarelli et al., 2020; Aerts et al., 2021; Weeraddana and Evenden, 2022). In our study, we identified multiple DEGs under herbivory involved in ethylene (ETH), SA, and JA biosynthesis and regulation, many of which were highly expressed in ‘GREM4’ compared to ‘PN40024’. Alterations in phytohormone accumulations signal downstream defense responses, such as secondary metabolite biosynthesis.

Secondary metabolites are key defensive compounds produced by plants in response to insect herbivory. The pathway ‘biosynthesis of secondary metabolites’ was enriched in both ‘GREM4’ and ‘PN40024’ herbivory responses, but, in ‘GREM4’, greater numbers of genes (87 compared to 48) were associated with this pathway. ‘Biosynthesis of secondary metabolites’ was also enriched in DEGs with greater

expression in ‘GREM4’ compared to ‘PN40024’ under basal conditions. Genes associated with secondary metabolite biosynthesis were also identified via overlap analysis, interaction analysis, and cross-reference analysis and were identified as candidate genes (see Table 2.10, Table 2.11, Table 2.12, and Table 2.13).

Terpenes are a class of secondary metabolites which contribute to insect herbivory resistance in plants and play roles in flavor, signaling, and development (Shahidi et al., 1999; Liu et al., 2020a; de Albuquerque Lima et al., 2021; Li et al., 2021; Pieroni et al., 2023; Wang et al., 2023a). Insect herbivory of leaves revealed enrichment of DEGs implicated in terpene-related functions and pathways in ‘GREM4’, but not in ‘PN40024’. Interaction analysis and cross-reference analysis revealed terpene biosynthesis genes as some of the topmost significantly differentially expressed genes upon insect herbivory, likely contributing increased insect herbivory resistance in ‘GREM4’. Some of these candidate genes include *Beta-amyrin synthase isoform X2 / Camelliol C synthase 1* (*BAS isoform X2/CAMS1*) (*Vitla_GREM4_10g108.60*)*, TPS1-like* (*Vitla_GREM4_19g14.9*), and *Cytochrome P450 monooxygenase, family 716, subfamily A, polypeptide 1 / Beta-amyrin 28-monooxygenase-like* (*CYP716A1*) (*Vitla_GREM4_18g311.31*). Terpenes have been reported to play roles in insect herbivory resistance in grapevine and other crops. In *V. labrusca* x *V. riparia* hybrid ‘Beta’, volatile terpene production increased in the days following Japanese beetle herbivory of leaves (Loughrin et al., 1997). Genes implicated in terpene biosynthesis also undergo expression alterations in response to insect herbivory. *TPS* genes, which are

implicated in terpene biosynthesis, for example, were up-regulated in rice (*Oryza sativa*) upon Asiatic rice borer (*Chilo suppressalis*) herbivory and in tea (*Camellia sinensis*) upon tea geometrid (*Ectropis obliqua*) feeding (Liu et al., 2020a; Sun et al., 2022). Additionally, *D-limonene synthase* (a terpene biosynthesis gene) maize mutants experienced increased corn borer damage, reinforcing the importance of terpene genes in insect herbivory defense (Wang et al., 2023a). Downstream analyses are necessary to determine if considerations such as the quantity or unique activities of terpenes produced in 'GREM4' impart the heightened insect herbivory resistance.

Flavonoids are widely recognized as insect herbivory defensive compounds in plants and are broadly insecticidal (Kariyat et al., 2019; Chatterjee et al., 2022; Chen et al., 2022; Zhang et al., 2022a). In our study, genes involved in the flavonoid biosynthesis pathway were exclusively enriched in 'GREM4' leaf herbivory DEGs compared to 'PN40024'. Some flavonoid biosynthesis genes were exclusively identified as DEGs in 'GREM4', including *Flavonol synthase 2* (*FLS2*) (*Vitla_GREM4_10g70.44*) and *Flavonol synthase/Flavanone 3-hydroxylase* (*FLS/F3H*) (*Vitla_GREM4_13g47.35*). Flavonoid biosynthesis genes *Flavanone 3-hydroxylase* (*F3H*) (*Vitla_GREM4_4g210.29*) and *UDP-glucosyl transferase 88A1* (*UGT88A1*) (*Vitla_GREM4_16g200.49*) were specifically identified as 'GREM4' candidate genes. Increased expression of genes implicated in flavonoid biosynthesis and accumulation have been observed upon insect herbivory, such as seen in oriental longheaded grasshopper herbivory of *V. vinifera* x *V. labrusca* hybrid 'Kyoho' (Jia et al., 2022). Flavonoids have also been documented as

insect herbivory defense compounds as flavonoids extracted from sorghum were insecticidal to fall armyworm (*Spodoptera frugiperda*) and increased mortality was observed when feeding upon maize overproducing flavonoids compared to wild-type lines (Chatterjee et al., 2022). In insect herbivory resistant rice, flavonoid accumulations significantly increased upon brown planthopper (*Niaparvata lugens*) feeding but significantly decreased in a susceptible cultivar (Zhang et al., 2022a). These results suggest flavonoids are likely key contributors in conferring heightened insect herbivory resistance in 'GREM4'. Future metabolomic analyses are necessary to validate this finding.

Genes implicated in disease resistance, pathogen response, plant-pathogen interactions, and other related processes and pathways, were widely implicated in 'GREM4' insect herbivory responses but were not as prominently observed in 'PN40024' in our study. Pathogen defense-related genes were enriched in 'GREM4' compared to 'PN40024' under basal conditions, overlap analysis, interaction analysis, and cross-reference analysis, and included genes such as *RPP13-like-1*, *Resistance to P. syringae Pv. Maculicola 1-like* (*RPV1-like*) (*Vitla_GREM4_18g256.38*), *GDSL esterase/lipase 2* (*GLIP2*) (*Vitla_GREM4_10g58.5*), and *Beta glucosidase 16* (*BGLU16*) (*Vitla_GREM4_13g317.61*). Pathogen resistance genes were some of the topmost significantly differentially expressed genes upon insect herbivory and thusly identified as candidate genes, including *Putative Resistant to P. syringae 2* (*RPS2*) (*Vitla_GREM4_12g237.26*), *BGLU16, Pectin methylesterase inhibitor 25* (*PMEI25*)

(*Vitla_GREM4_13g203.16*), and *GDSL esterase/lipase 1* (*GLIP1*) (*Vitla_GREM4_19g86.34*). Further, genome-specific genes in 'GREM4' were enriched in functions related to 'plant-pathogen interactions', while 'PN40024' was not. While response of genes implicated in pathogen resistance may appear unexpected, such genes have been reported to play roles in a variety of biotic stress and defense responses. For example, upon insect herbivory, increased expression of genes implicated in the production of disease resistance compounds, such as protease inhibitors, glucanases, chitinases, and peroxidases has been observed in pepper, rice, and tobacco and have been shown to contribute to insect resistance in other crops (Pechan et al., 2002; Kanno et al., 2005; Gomi et al., 2010; Lee et al., 2012; Fescemyer et al., 2013; Javadi Khederi et al., 2018; Liu et al., 2020b; Lokya et al., 2020; Anwer et al., 2023). In a *V. riparia* hybrid grapevine, a quantitative trait loci (QTL) associated with phylloxera resistance was found to contain disease resistance genes, such as *Rps5* and *Ca^{2+}-responsive phospholipid-binding protein* (*Bonzai*), supporting the premise that pathogen defense genes play a role in insect herbivory defense (Yin et al., 2022). Overall, broadly observed up-regulation and enrichment of pathogen defense genes in 'GREM4' upon beetle herbivory, and lack thereof in 'PN40024', suggests genes associated with pathogen resistance contribute to insect herbivory resistance in 'GREM4'. It is unknown if the expression of these genes directly or indirectly contributes to the production of compounds that deter insect herbivory, help protect the plant from opportunistic pathogens that invade through the

newly broken tissue, or a combination of both. Additional studies are needed to parse apart this complex interaction.

Conclusion

In conclusion, our study determined that *V. labrusca* acc. 'GREM4' exhibits greater resistance to insect herbivory compared to *V. vinifera* cv. 'PN40024'. High trichome densities found in 'GREM4' compared to 'PN40024' were shown to explain some, but not all, of the insect herbivory resistance phenotype observed of 'GREM4'. 'GREM4' had higher basal expression of genes involved in defense response and secondary metabolism, likely conferring constitutive defense to insect herbivory. Under insect herbivory, genes involved in secondary metabolism, including terpene and flavonoid biosynthesis, and plant-pathogen interaction genes were enriched in 'GREM4', but not in 'PN40024', indicating the putative importance of these genes in conferring insect herbivory resistance in 'GREM4'. In 'GREM4' and 'PN40024', a comparable, but small, number of paralogous and genome-specific genes were implicated in insect herbivory defense responses underscoring their significance. Investigation into two gene families related to insect defense with additional paralogs in 'GREM4' revealed the paralogous genes were differentially expressed upon insect herbivory. Differential expression of orthologous genes is likely the major contributor to the insect herbivory resistance phenotype observed in 'GREM4'. Overall, these results provide evidence and candidate genes for tapping into genetic variation of wild grapevines to enhance

herbivory resistance in cultivated grapevine varieties and provide metabolic pathways to explore for implications in insect herbivory resistance in other species.

Acknowledgements

We thank the USDA/ARS at Geneva, NY, USA and Davis, CA, USA for providing 'GREM4' and 'PN40024' plant material. We thank Novogene for providing RNA-sequencing services. We thank Ben Phillips, Dr. Rachel Combs-Giroir, Rosemary Ball, Gurkirat Singh, Molly Dougherty, and Katie Fulcher for their help in plant care, data collection, and data analysis. We thank Dr. Bo Li, Dr. Rachel Combs-Giroir, Dr. Zachary Konkel, and Dr. Mike Sovic for bioinformatic assistance. We thank Dr. Laura Kubatko for methodological insights and assistance with statistical analysis of herbivory studies. We thank the Ohio Supercomputer Center for use of computing resources throughout this project. We thank Dr. JC Jang, Dr. Rachel Combs-Giroir, and Gurkirat Singh for their constructive comments on the manuscript. We thank Michael Anderson and the Howlett Greenhouse staff for their support in maintaining our grapevines and Glenn Mills and the Waterman Agricultural and Natural Resources Laboratory for allowing us to collect beetles at the farm.

Availability of Data and Materials

The datasets presented in this article can be found within the main text and supplementary materials. The transcriptomic data underlying the results of this article are available at NCBI (https://www.ncbi.nlm.nih.gov/) via BioProject number

PRJNA1070606 (reviewer link: https://urldefense.com/v3/__https://dataview.ncbi.nlm.nih.gov/object/PRJNA1070606?reviewer=nuagim9ji1gab51imbf42u5768__;!!KGKeukY!2DWAGpy2ayyt_GRKl6ae8bwk1TjvzlYq5Gpl7jOzTUIpUOyBhvHhHjEm3DYrw0gXUMhLLAbCOtgMOg1NY8VRkQ$), or by request. The genome annotation can be found on GitHub at https://github.com/cdixo/Vitis-labrusca-Version-2-Genome-Annotation.git . Custom codes used for analyses and creation of the updated genome annotation are also available on GitHub at https://github.com/cdixo/Inter-species-and-Herbivory-Publication.git .

Funding

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Figures

Figure 2.1 – Herbivory Experiments Experimental Design Images.

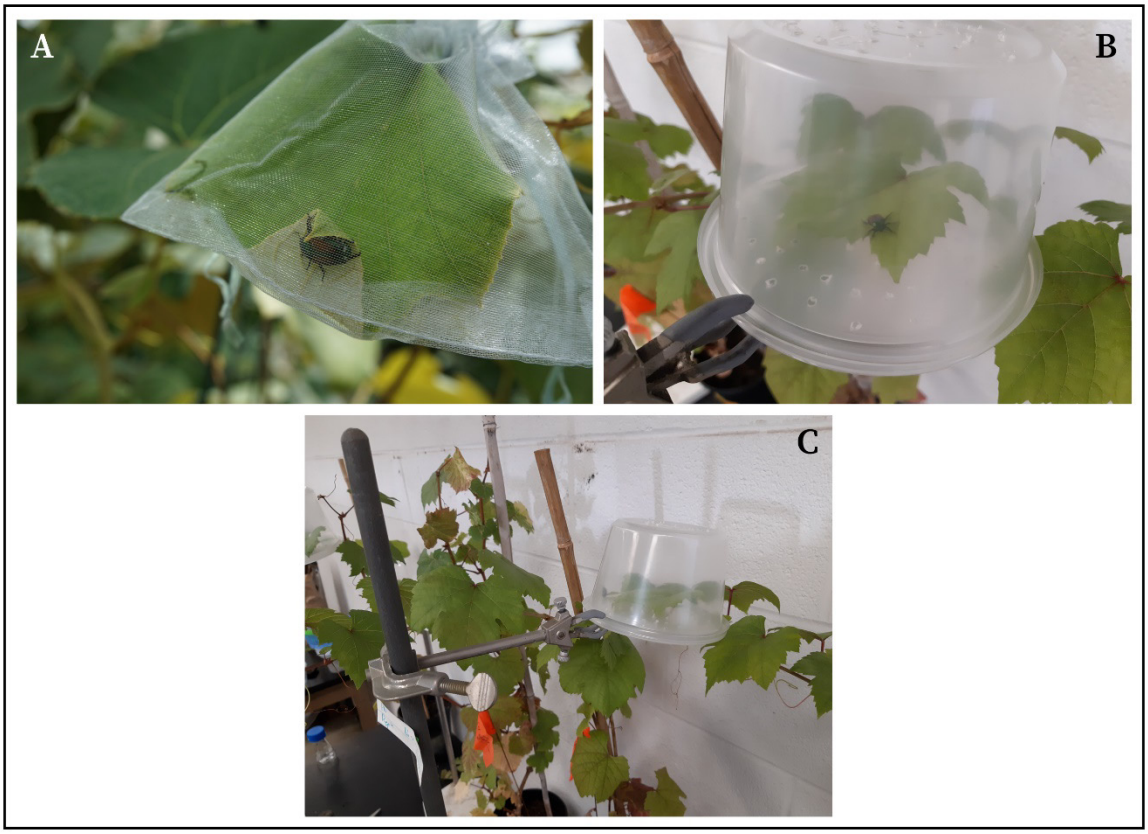
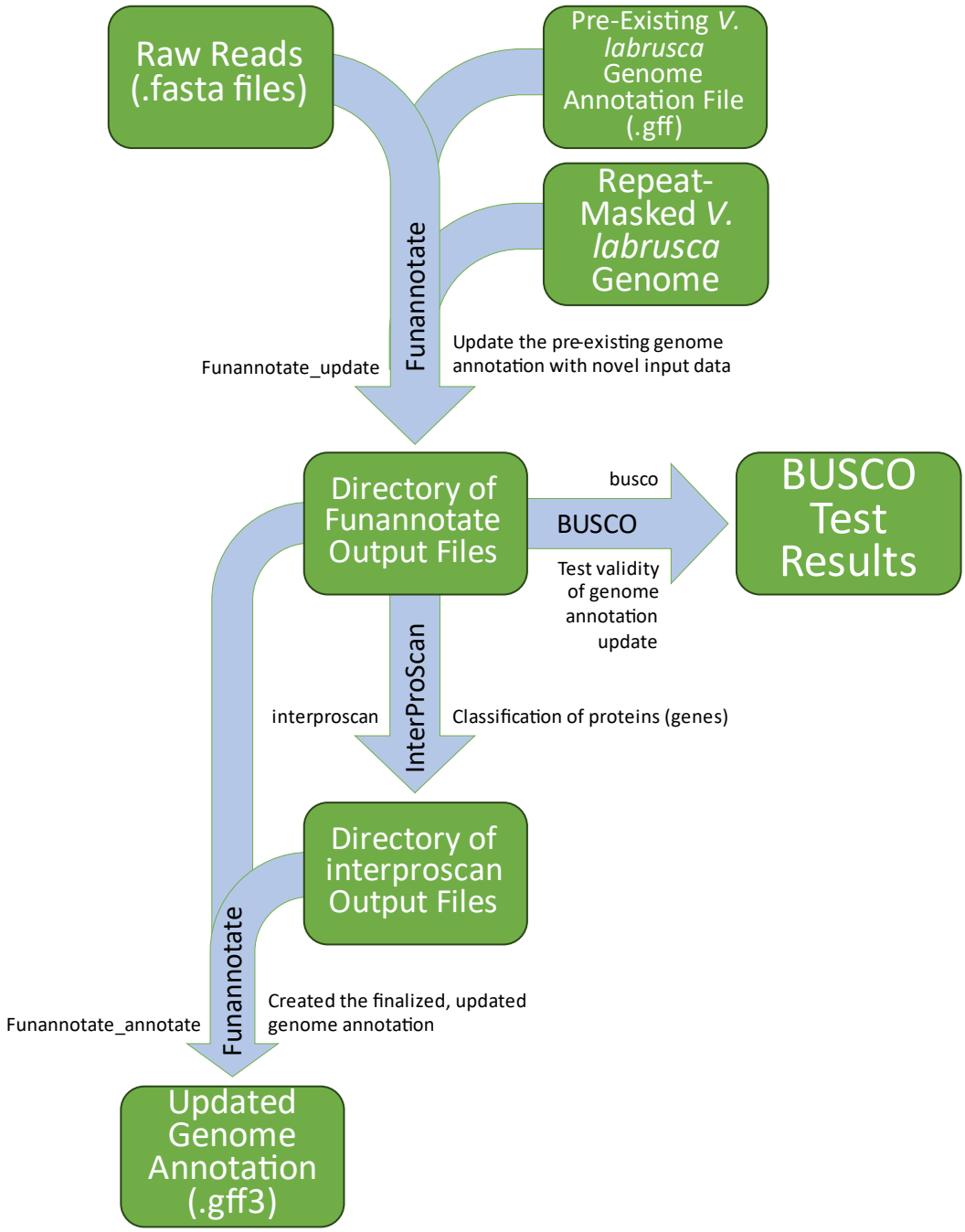


Figure 2.1 Caption – Images shown are from the herbivory time course and trichome experiments. A. Japanese beetle in bag during an herbivory time course study run on a 'GREM4' grapevine leaf. B & C. Japanese beetle inside the container which was used for the herbivory under equal trichome densities and herbivory under differing trichome densities studies. Both images shown are 'PN40024' leaves from the herbivory under equal trichome densities

Figure 2.2 – Genome Annotation Pipeline

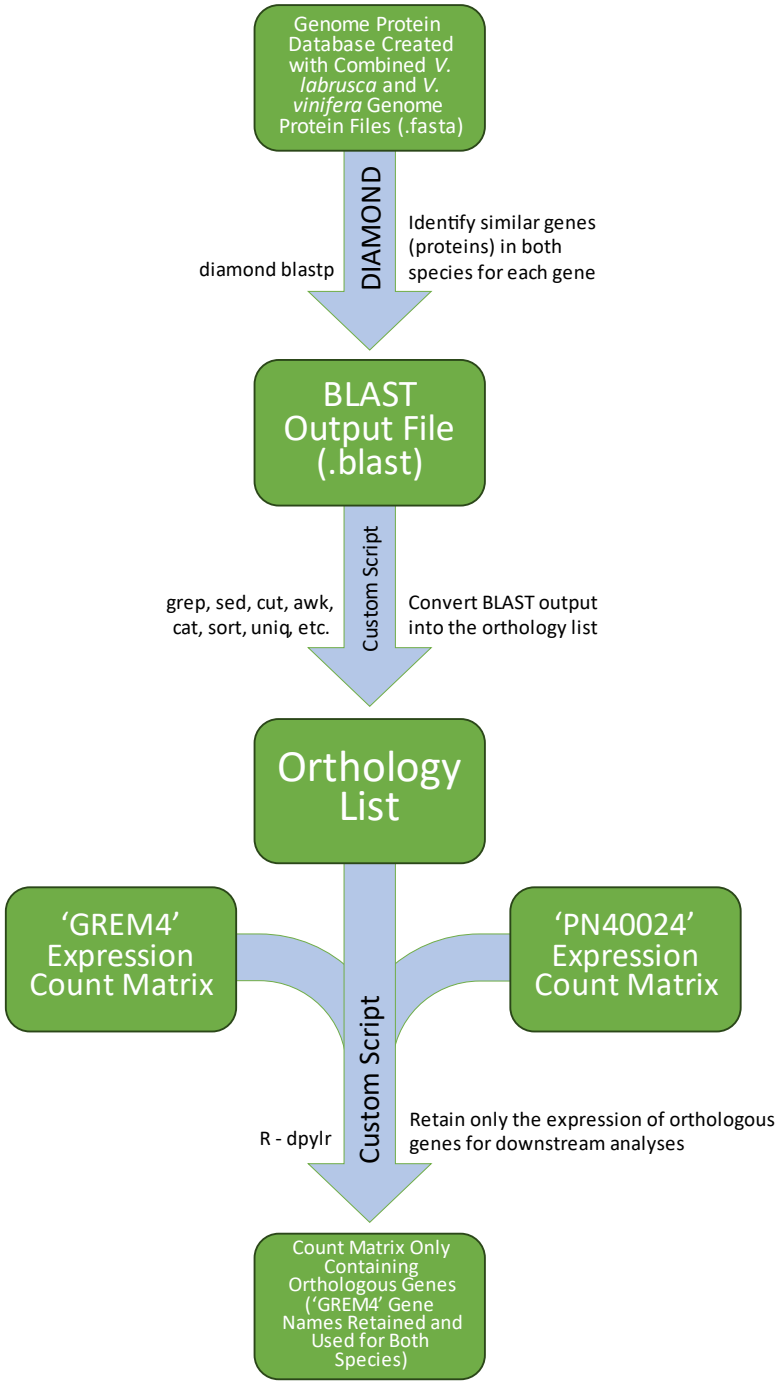


Continued…

(Figure 2.2 continued)

Figure 2.2 Caption – Bioinformatic workflow for updating the pre-existing *Vitis labrusca* acc. 'GREM4' gene annotation. Pipeline encompasses the addition of RNA-seq reads for additional predictive power, as well as the pre-existing gene annotation, to update the annotation of the repeat-masked V. *labrusca* 'GREM4' genome via Funannotate. The resulting updated genome annotation was tested with BUSCO for the presence of conserved single-copy orthologs. Green boxes represent an object, file, or directory while blue arrows represent a script or command being conducted.

Figure 2.3 – Gene Orthology Identification Pipeline

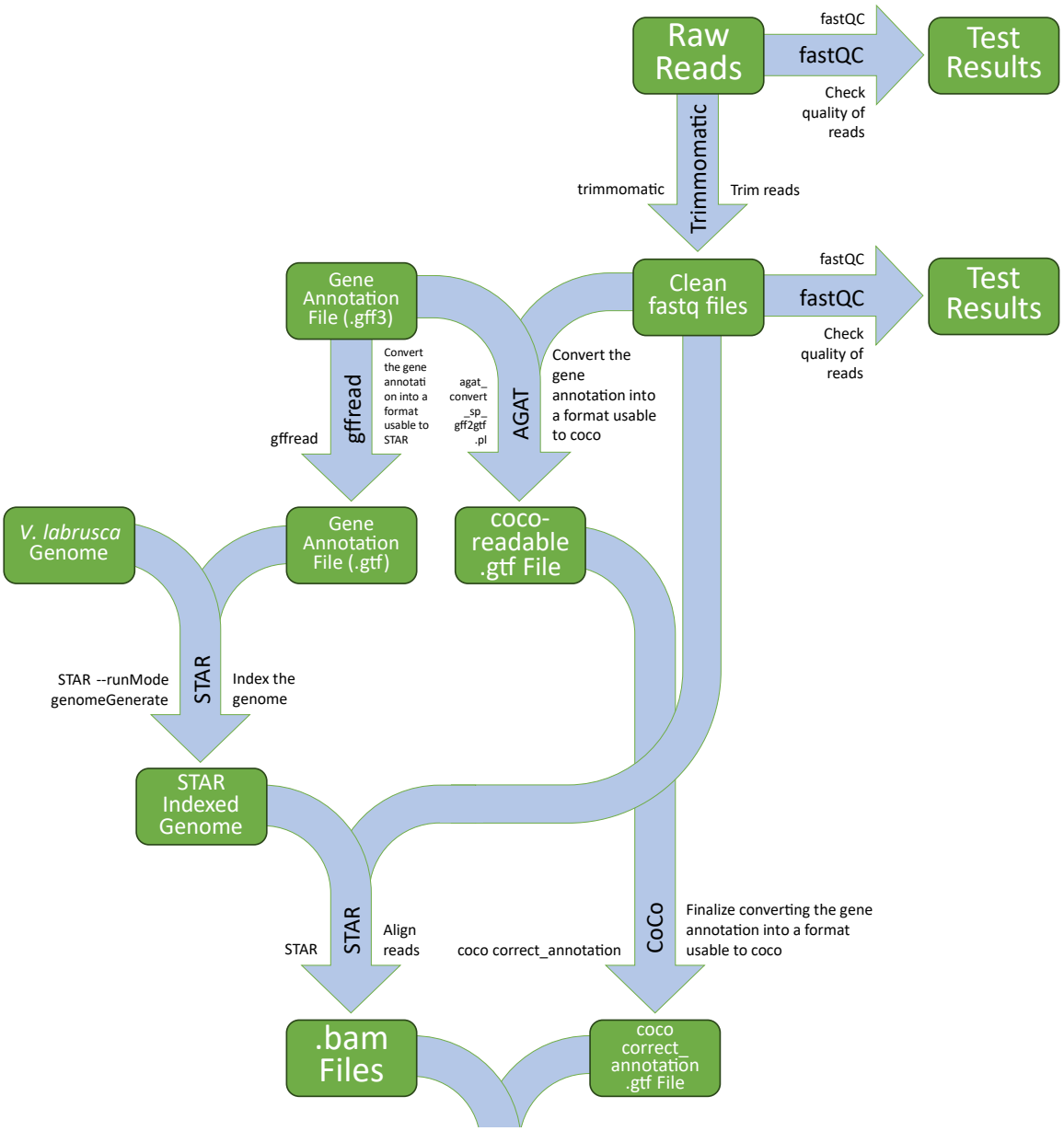


Continued…

(Figure 2.3 continued)

Figure 2.3 Caption – Bioinformatic workflow for identification of orthologous genes. The resulting count matrix, which only contained orthologous genes, was used for inter-species analyses. Green boxes represent an object, file, or directory while blue arrows represent a script or command being conducted.

Figure 2.4 – RNA-seq, DEG Identification, and Enrichment Pipeline



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(Figure 2.4 continued)

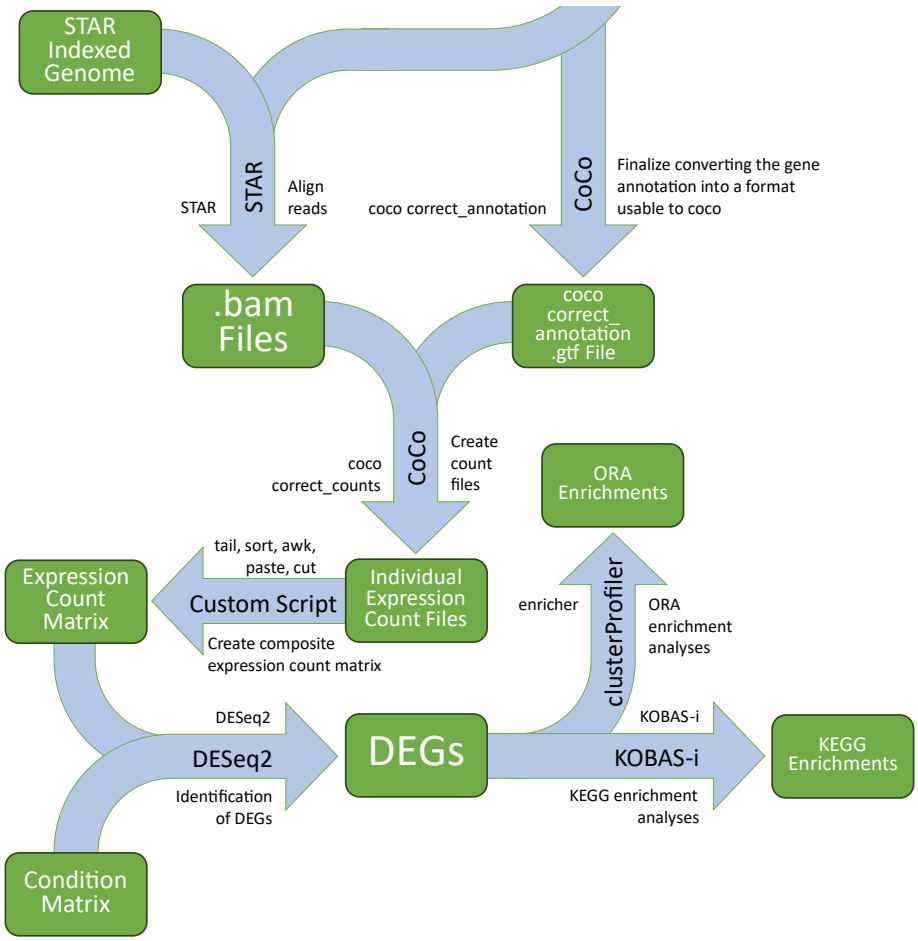
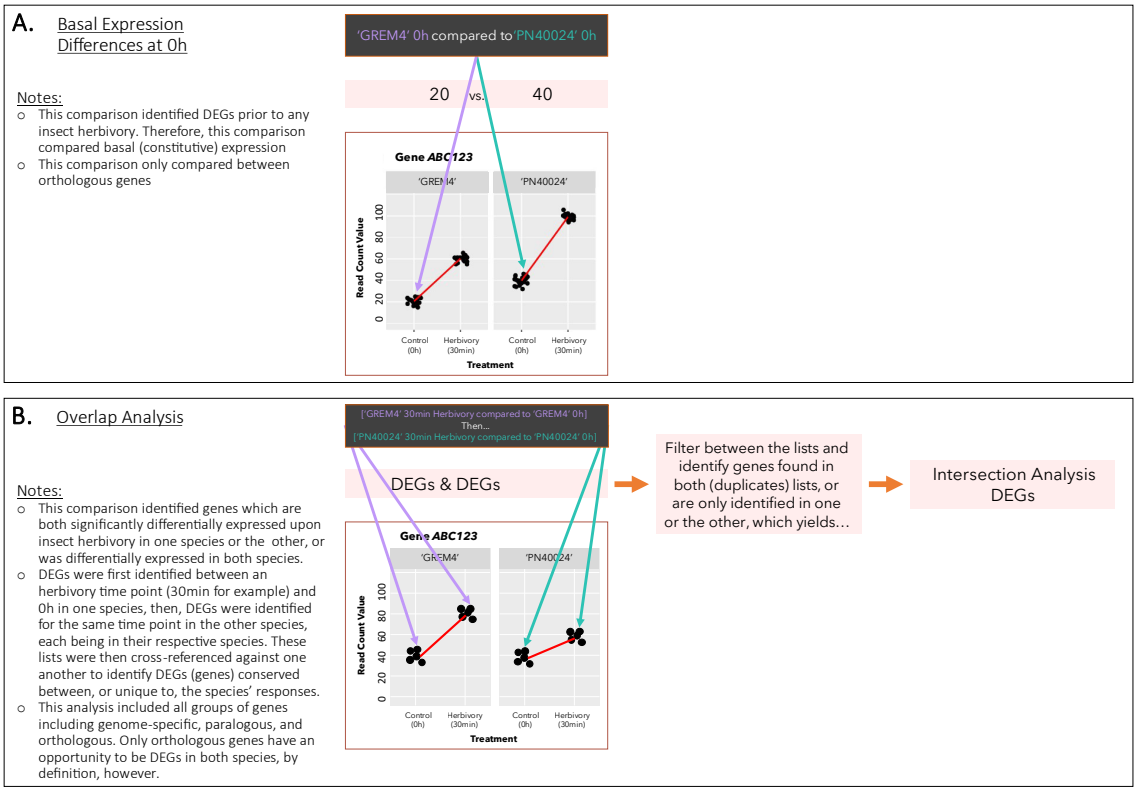


Figure 2.4 Caption – Bioinformatic workflow for RNA-seq data analysis. Pipeline encompasses cleaning of reads, quality control, read alignment, count matrix creation, DEG identification, and enrichment analysis. Green boxes represent an object, file, or directory while blue arrows represent a script or command being conducted.

Figure 2.5 – Graphical Depictions of Inter-species Comparison Methods



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(Figure 2.5 continued)

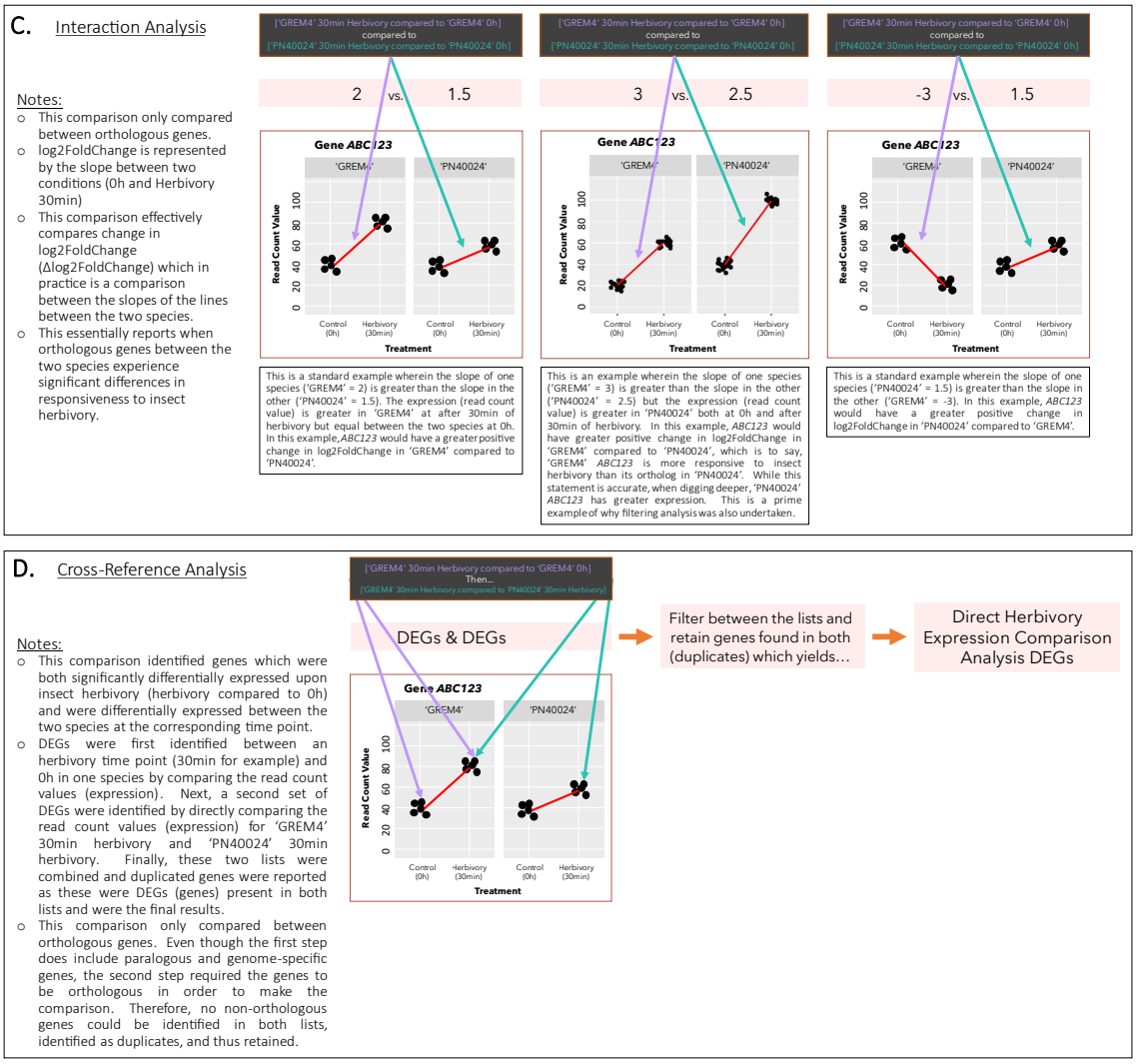
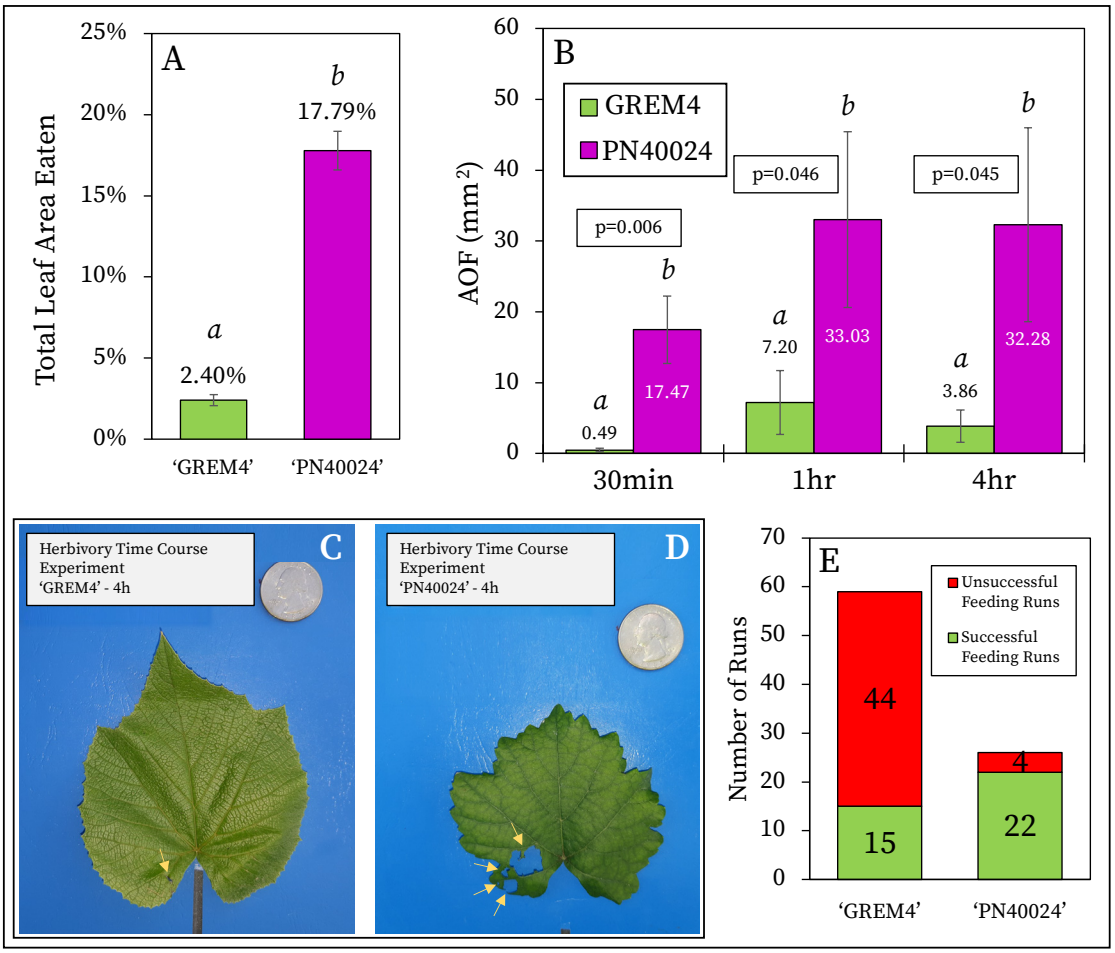


Figure 2.5 Caption – A. Description of Identifying Basal Expression Differences at 0h. B. Description of the Overlap Analysis. C. Description of the Interaction Analysis. D. Description of the Cross-Reference Analysis.

Figure 2.6 – Insect Herbivory Study Results



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(Figure 2.6 continued)

Figure 2.6 Caption – A. The percentage of total leaf area eaten by Japanese beetles for ‘GREM4’ and ‘PN40024’ in the herbivory preference study, where Japanese beetles were permitted to feed upon either species *ad libitum* ($p = 0.037$; $n = 4$). Error bars show the standard error. B. Herbivory time course study average area of feeding (AOF) by Japanese beetles on ‘GREM4’ and ‘PN40024’ at 30min, 1h, and 4h. Significance is represented by differing letters and was calculated independently at each timepoint. Error bars show the standard error. C & D. Representative images of Japanese beetle feeding damage on (C) ‘GREM4’ and (D) ‘PN40024’ mature leaves from the herbivory time course study after 4h of feeding. Arrows indicate locations of feeding damage and a quarter was used to indicate scale. E. Japanese beetle feeding success rate during the herbivory time course study. A run in which a Japanese beetle fed was considered ‘successful’ while a run with no feeding was ‘unsuccessful’.

Figure 2.7 – Herbivory Preference Study Feeding Images

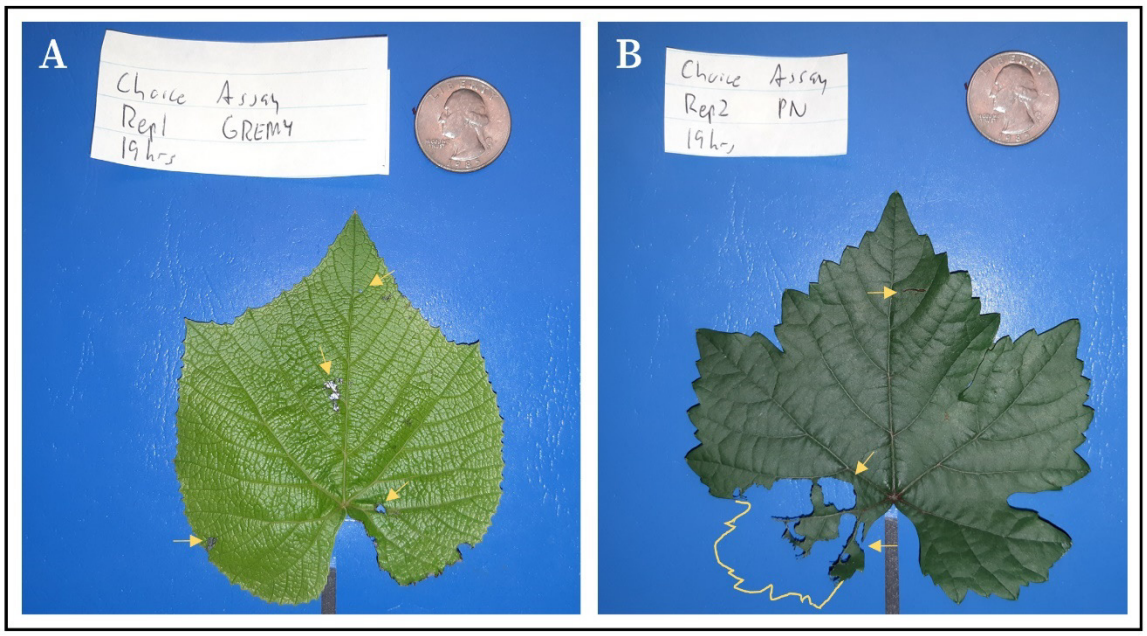
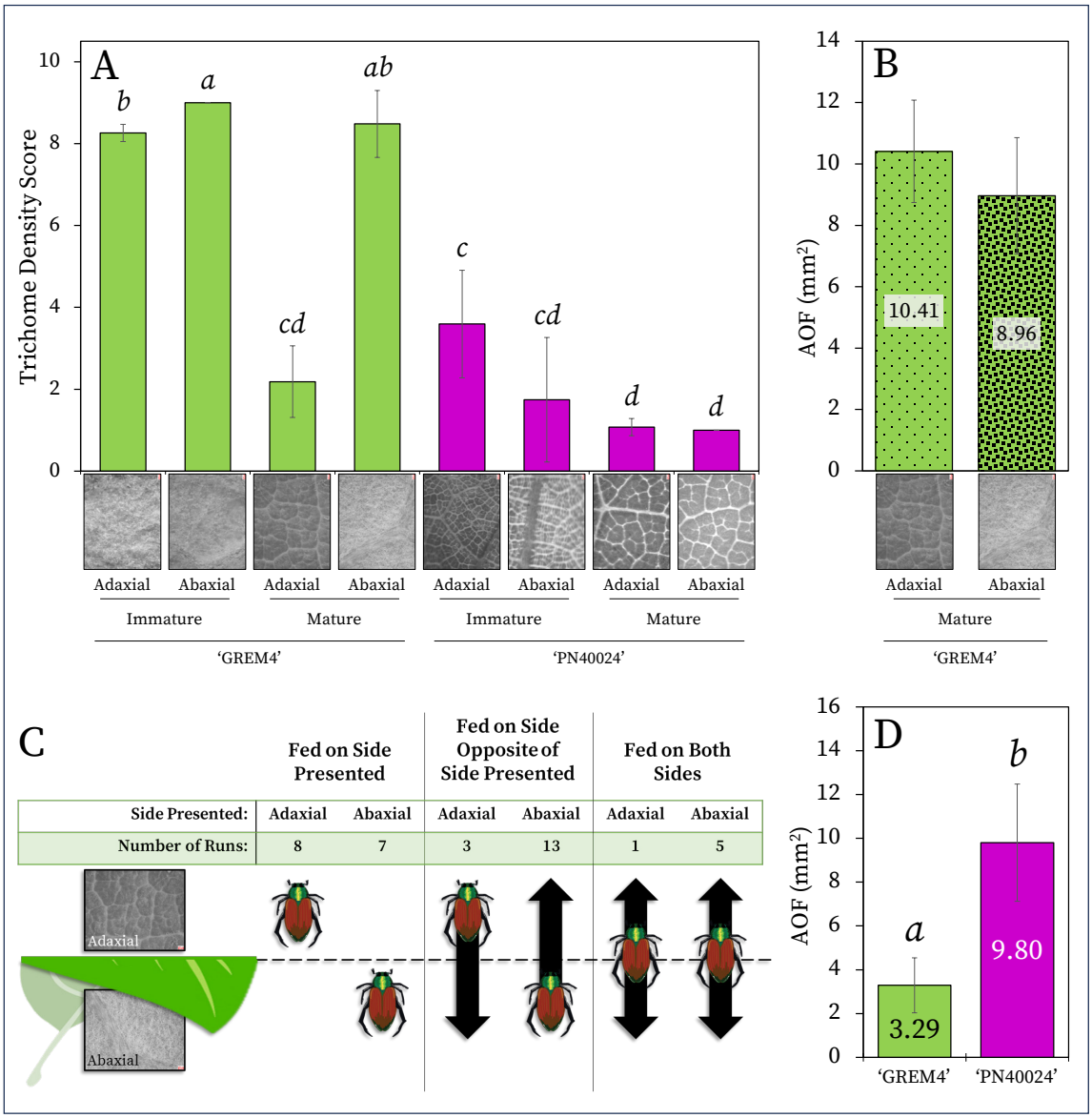


Figure 2.7 Caption – A & B. Representative images depicting damage from Japanese beetles in (A) 'GREM4' and (B) 'PN40024' leaves after 19h of feeding in the herbivory preference study. Arrows indicate locations of feeding damage while the yellow outline indicates the leaf margin before feeding. Feeding area was recorded to determine AOF.

Figure 2.8 – Leaf Trichome Density Study Results



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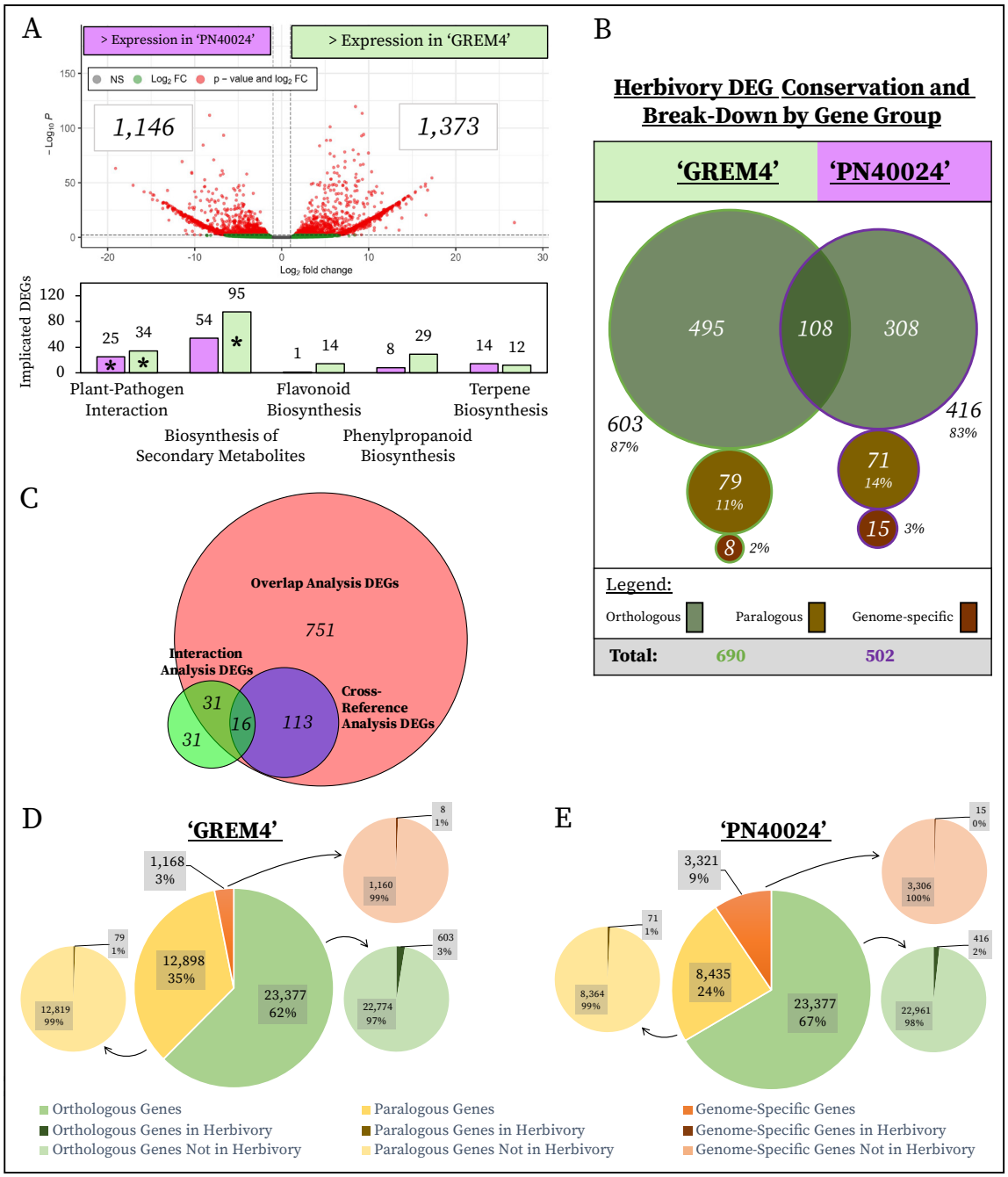
(Figure 2.8 continued)

Figure 2.8 Caption – In all figures, significance is denoted by differing letters above the bar graph. The error bars denote standard errors. A. Leaf trichome density scores. Nine images (data points) were recorded per side, maturity, and species, resulting in 72 total images ($p = <0.001$ where $N = 72$). Both ad- and abaxial sides of leaves were scored for trichome density based on the OIV 'Mature leaf: density of prostrate hairs between main veins on lower side of blade' scale. Representative images taken under 10x magnification are inlayed to illustrate the differences in trichome densities. B. Average AOF per ad- or abaxial side of the leaf when trichome densities were significantly different. No significance was found ($p = 0.307$; $n = 21$ (adaxial), 10 (abaxial)). C. Feeding preference of Japanese beetles when presented differing trichome densities in 'GREM4'. The side of the leaf which the beetle was placed and the number of runs in which each feeding outcome occurred are reported in the table. Arrows point in the direction in which the beetles moved during the experiment. D. Average AOF per grapevine species when trichome densities were not significantly different between the adaxial sides of the mature leaves of 'GREM4' and 'PN40024' ($p = 0.029$, $n = 10$).

Figure 2.9 – Inter-species Comparisons Results



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(Figure 2.9 continued)

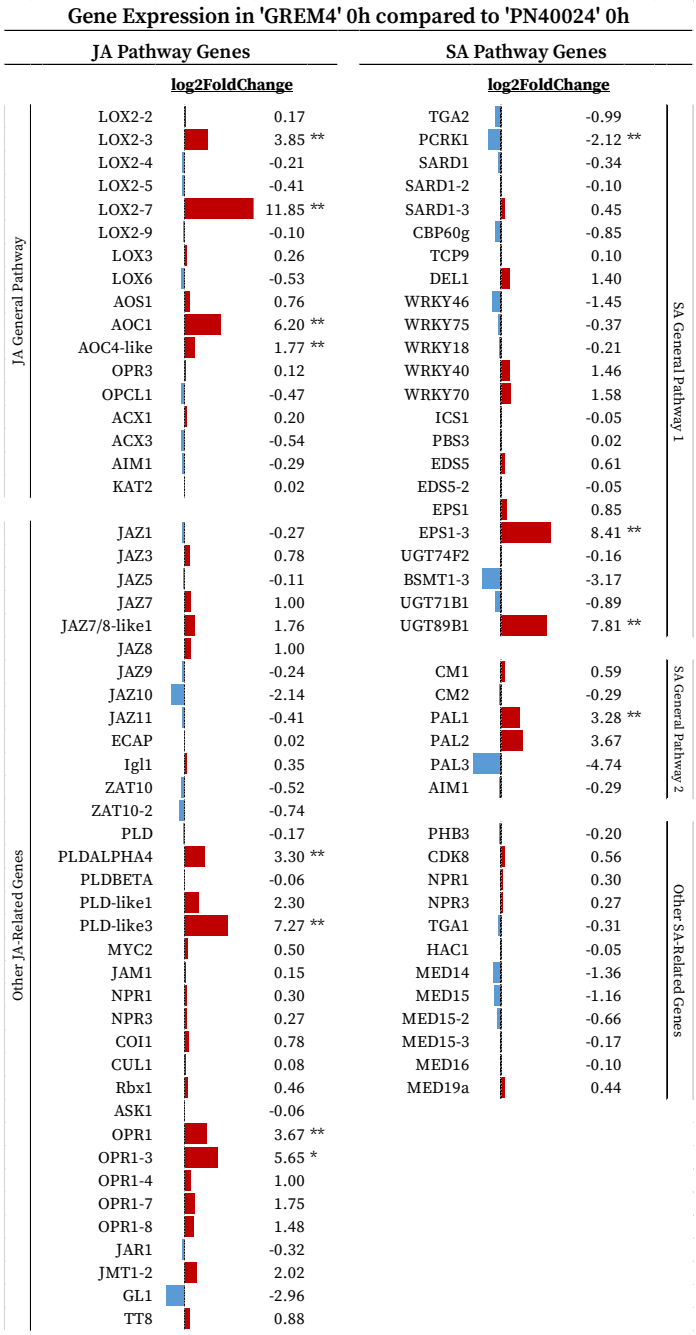
Figure 2.9 Caption – A. Volcano plot of DEGs identified via DESeq2 in ‘GREM4’ compared to ‘PN40024’ under basal (0h) conditions with bar plot below displaying numbers of DEGs implicated in significantly enriched and other noteworthy pathway enrichments. In the volcano plot, the dashed horizontal line represents the p-adj threshold of ≤ 0.05 and the two dashed vertical lines denote the |log2foldchange| threshold of ≥ 2. Dots to the right of the vertical dashed line and above the horizontal dashed line are genes which experienced statistically significantly greater expression in ‘GREM4’ compared to ‘PN40024’. Dots to the left of the vertical line and above the horizontal line are genes which experienced statistically significantly lower expression in ‘GREM4’ compared to ‘PN40024’. In the bar plot, KEGG pathway enrichments are noted along the x-axis and the number of DEGs implicated in each enrichment are noted on the y-axis. Enrichments with asterisks within the bars were significantly enriched while those without were not significantly enriched but were displayed as they are key insect herbivory defensive pathways. Green bars correspond to enrichments in genes with greater expression in ‘GREM4’ compared to ‘PN40024’ while purple bars correspond to enrichments in gene with greater expression in ‘PN40024’ compared to ‘GREM4’. B. Diagram representing the breakdown of DEGs identified by orthologous (green), paralogous (brown), and genome-specific genes (maroon) upon insect herbivory in both ‘GREM4’ (green bordered, leftmost circles) and

Continued…

(Figure 2.9 continued…)

‘PN40024’ (purple border, rightmost circles) as well as conservation between groups. Numbers of herbivory DEGs are reported in addition to the percentages of the total number of herbivory DEGs (‘GREM4’ = 690; ‘PN40024’ = 502) in each respective species. C. Venn diagram representing the conservation of DEGs identified using the three different inter-species orthologous gene analysis methods. D & E. Breakdown of orthologous (green palate), paralogous (yellow palate), and genome-specific genes (orange palate) implicated in herbivory responses (compared to 0h) in ‘GREM4’ and ‘PN40024’. Small break-out pie charts display the number of DEGs identified under insect herbivory (the darker-colored small slice) out of the total genes within the group.

Figure 2.10 – JA and SA Pathway Gene Expression in ‘GREM4’ 0h Compared to ‘PN40024’ 0h.



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(Figure 2.10 continued)

Figure 2.10 Caption – Gene expression presented via log2FoldChange. Significance presented via p-adj where * = ≤ 0.10 and ** = ≤ 0.05. Red bars indicate greater expression in 'GREM4' compared to 'PN40024' whereas blue bars indicate lower expression in 'GREM4' compared to 'PN40024'. Genes are positioned in the order in which they are implicated in each biosynthetic pathway.

Figure 2.11 – Expression Within Expanded Gene Family Examples.

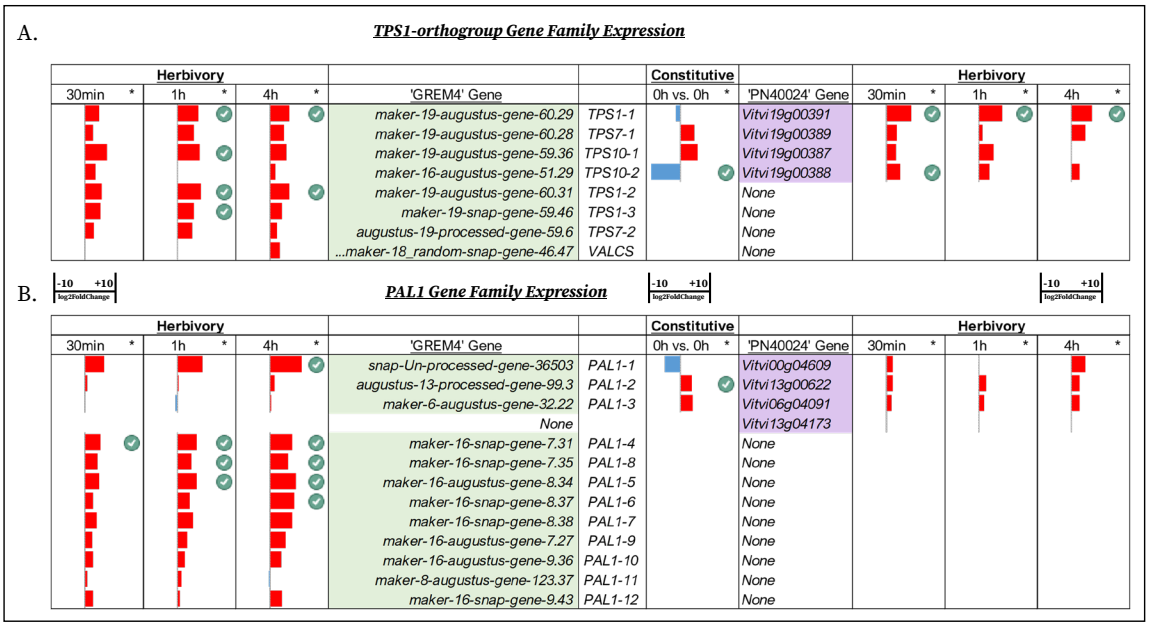

Figure 2.11 Caption – Expanded (paralogous) gene family members' expression under insect herbivory in 'GREM4' and 'PN40024' in the *TPS1*-orthogroup (A) and *PAL1* (B) gene families. All genes in each gene family are listed along with their expression. Expression is relayed via horizontal bar graphs which illustrate expression via log2FoldChange where red is increased expression and blue is decreased. Expression is reported for both insect herbivory (insect herbivory (broken down by time point) compared to 0h) and for constitutive (basal) expression. Green check marks indicate the change in expression was significantly different for the gene family member at the respective time point where significance was determined as p-adj ≤ 0.05.

Tables

Table 2.1 – RNA-seq Read Quality Statistics After Quality Control and Cleanup.

	Raw Reads[a]	Raw Data[b]	Effective(%)[c]	Error(%)[d]	Q20(%)[e]	Q30(%)[e]	GC(%)[f]
Max	100.36MB	15.1	98.88	0.03	98.26	94.71	47.29
Average	53.70MB	8.1	98.06	0.03	98	94.09	46.06
Median	51.98MB	7.8	98.35	0.03	98.01	94.1	46.11
Min	41.49MB	6.2	94.94	0.02	97.55	93.16	44.17

Footnotes: All statistical descriptions provided here are as per defined by Novogene. a. Raw reads: total amount of reads of raw data, each four lines taken as one unit. For paired-end sequencing, it equals the amount of read1 and read2, otherwise it equals the amount of read1 for single-end sequencing. b. Raw data: (Raw reads) * (sequence length), calculating in G. For paired-end sequencing like PE150, sequencing length equals 150, otherwise it equals 50 for sequencing like SE50. c. Effective: (Clean reads/Raw reads)*100%. d. Error: base error rate. e. Q20 & Q30: (Base count of Phred value > 20 or 30) / (Total base count). f. GC: (G & C base count) / (Total base count).

Table 2.2 – Genome and Orthology Data for *Vitis labrusca* acc. 'GREM4' and *Vitis vinifera* cv. 'PN40024'.

Category	Quantities	
	***Vitis labrusca* acc. 'GREM4'**	***Vitis vinifera* cv. 'PN40024'**
Genes	37,443	35,133
Proteins	40,277	41,160
Total Orthologous Pairs Identified	23,377	
Paralogous Genes in Each Species	12,898	8,435
Genome Specific Genes in Each Species	1,168	3,321

Table 2.3 – Expression Results for All Genes

File available at the permanent repository hosted by figshare at

https://figshare.com/s/9e92483448cd44239028

Table 2.4 – Gene Functional Annotation.

File available at the permanent repository hosted by figshare at

https://figshare.com/s/9e92483448cd44239028

Table 2.5 – DEG Expression Results.

File available at the permanent repository hosted by figshare at

https://figshare.com/s/9e92483448cd44239028

Table 2.6 – KEGG Pathway Enrichments.

File available at the permanent repository hosted by figshare at

https://figshare.com/s/9e92483448cd44239028

Table 2.7 – Gene Conservation Lists.

File available at the permanent repository hosted by figshare at

https://figshare.com/s/9e92483448cd44239028

Table 2.8 – ORA GO Term Enrichments.

File available at the permanent repository hosted by figshare at

https://figshare.com/s/9e92483448cd44239028

Table 2.9 – Numbers of Genes Identified in Transcriptomic Comparisons Between *V. labrusca* acc. 'GREM4' and *V. vinifera* cv. 'PN40024'.

Comparison	Details			#
Basal Expression Differences at 0h	Inter-species	'GREM4' 0h compared to 'PN40024' 0h	Increased Expression in 'GREM4' Compared to 'PN40024'	1373
			Decreased Expression in 'GREM4' Compared to 'PN40024'	1146
Insect Herbivory	'GREM4'	Herbivory (All Time Points Combined) compared to 0h	Up-regulated	549
			Down-regulated	141
	'PN40024'	Herbivory (All Time Points Combined) compared to 0h	Up-regulated	447
			Down-regulated	55
Overlap Analysis	Inter-species	Unique to 'GREM4'	Number of DEGs	495
		Unique to 'PN40024'	Number of DEGs	308
		Conserved in Both 'GREM4' and 'PN40024'	Number of DEGs	108
Interaction Analysis	Inter-species	'GREM4' compared to 'PN40024'	Increased Expression in 'GREM4' Compared to 'PN40024'	45
			Decreased Expression in 'GREM4' Compared to 'PN40024'	33
Cross-Reference Analysis	Inter-species	'GREM4' compared to 'PN40024'	Number of DEGs	82
		'PN40024' compared to 'GREM4'	Number of DEGs	48

Table 2.10 – *V. labrusca* acc. 'GREM4' Candidate Insect Herbivory Resistance Genes from the Interaction Analysis.

#	⇅	Biological Implication:	Abbreviated Gene Name:	*V. labrusca* acc. 'GREM4' Gene:	Full Gene Name:
1	↑	**Terpene Biosynthesis**	*BAS isoform X2/CAMS1*	*Vitla_GREM4_10g108.60*	*Beta-amyrin synthase isoform X2 / Camelliol C synthase 1*
2	↑	**Putative Pathogen Resistance**	*RPS2*	*Vitla_GREM4_12g237.26*	*Putative Resistant to P. syringae 2*
3	↓	**Disease Resistance; SAR and ETH Induction**	*GLIP2*	*Vitla_GREM4_10g58.5*	*GDSL esterase/lipase 2*
4	↑	**Phosphate Transport**	*PHO1-like 3*	*Vitla_GREM4_1g132.33*	*Phosphate 1-like 3*
5	↓	**Wax Biosynthesis**	*CER1/22*	*Vitla_GREM4_15g100.37*	*Eceriferum 1/22*
6	↑	**Disease Resistance**	*RPP13-like*	*Vitla_GREM4_13g144.42*	*Putative disease resistance RPP13-like*
7	↑	**Disease Resistance**	*PR1-like 1*	*Vitla_GREM4_3g126.4*	*Pathogenesis-related protein 1-like 1*
8	↑	**Disease Resistance**	*N-like 1*	*Vitla_GREM4_00g37057*	*TMV resistance protein N-like protein 1*
9	↑	**Terpene Biosynthesis**	*TPS1-like*	*Vitla_GREM4_19g14.9*	*Terpene synthase 1-like*
10	↑	-	-	*Vitla_GREM4_00g74.30*	-

Table 2.11 – *V. labrusca* acc. 'GREM4' Candidate Insect Herbivory Resistance Genes from the Cross-Reference Analysis.

#	↑↓	Biological Implication:	Abbreviated Gene Name:	*V. labrusca* acc. 'GREM4' Gene:	Full Gene Name:
1	↑	-	-	*Vitla_GREM4_14g4.6*	-
2	↑	**Response to SA; Cell Wall Formation**	*GRP5-like 1*	*Vitla_GREM4_7g96.2*	*Glycine rich protein 5-like 1*
3	↑	**Response to SA; Cell Wall Formation**	*GRP5-like 2*	*Vitla_GREM4_7g95.10*	*Putative Glycine rich protein 5-like 2*
4	↓	**Disease Resistance; SAR and ETH Induction**	*GLIP2*	*Vitla_GREM4_10g58.5*	*GDSL esterase/lipase 2*
5	↑	**Phytohormone Regulation; Antioxidant and Defense Metabolite Biosynthesis**	*CYP-like*	*Vitla_GREM4_15g170.54*	*Cytochrome P450-like*
6	↑	**JA Biosynthesis**	*AOS3*	*Vitla_GREM4_3g53.38*	*Allene oxide synthase 3*
7	↑	**Fungal Defense; Glucosinolate Processing**	*BGLU16*	*Vitla_GREM4_13g317.61*	*Beta glucosidase 16*
8	↑	-	-	*Vitla_GREM4_5g213.11*	-

Continued…

(Table 2.11 continued)

9	↑	**Disease Resistance; SAR and ETH Induction**	*GLIP1*	*Vitla_GREM4_19g86.34*	*GDSL esterase/lipase 1*
10	↑	**Pectin Cell Wall Remodeling; Pathogen Resistance**	*PMEI25*	*Vitla_GREM4_13g203.16*	*Pectin methylesterase inhibitor 25*
11	↑	**Terpene Biosynthesis**	*CYP716A1*	*Vitla_GREM4_18g311.31*	*Cytochrome P450 monooxygenase, family 716, subfamily A, polypeptide 1 / Beta-amyrin 28-monooxygenase-like*
12	↑	**Flavonoid Biosynthesis**	*F3H*	*Vitla_GREM4_4g210.29*	*Flavanone 3-hydroxylase*

Table 2.12 – *V. labrusca* acc. 'GREM4' Insect Herbivory Genes Identified by All Three Transcriptomic Comparison Methods.

Biological Implication:	**Abbreviated Gene Name:**	***V. labrusca* acc. 'GREM4' Gene:**	**Full Gene Name:**
Disease Resistance; SAR and ETH Induction	*GLIP2*	*Vitla_GREM4_10g58.5*	*GDSL esterase/lipase 2*
-	-	*Vitla_GREM4_14g4.6*	-
Response to SA; Cell Wall Formation	*GRP5-like 1*	*Vitla_GREM4_7g96.2*	*Glycine rich protein 5-like 1*
Pollen Grain Compatibility	*RKFL1*	*Vitla_GREM4_10g56.41*	*Receptor-like kinase in flowers 1*
Biotic Stress Response	*HSP*	*Vitla_GREM4_13g82.26*	*Class I heat shock protein*
Cuticular Wax Formation	*MAH1*	*Vitla_GREM4_14g270.32*	*Mid-chain alkane hydroxylase 1*
Photosynthesis Under Senescence and High-Light	*FTSH6*	*Vitla_GREM4_14g293.28*	*FTSH protease 6*
Pathogen Resistance; Abiotic Stress Tolerance; Plant Development	*BAG6*	*Vitla_GREM4_15g196.45*	*BCL-2-associated athanogene 6*
Possible Implication in Flavonoid Biosynthesis/Insect Resistance	*UGT88A1*	*Vitla_GREM4_16g200.49*	*UDP-glucosyl transferase 88A1*
SA/MeSA Regulation	*SAMT2*	*Vitla_GREM4_00g36975*	*Salicylate carboxymethyl transferase 1*
-	-	*Vitla_GREM4_4g0.9*	-
ER-related; Intra-cellular Transport; Ion Transport	*ER body-like protein*	*Vitla_GREM4_00g188.10*	*ER body-like protein*

Continued…

(Table 2.12 continued)

Biotic Stress Response	*HSP-2*	*Vitla_GREM4_8g87.15*	*Class I heat shock protein – 2*
Protein Binding	*XIAO*	*Vitla_GREM4_9g148.35*	*Putative inactive leucine-rich repeat receptor kinase XIAO*
Insect Herbivory Resistance; JA and JA-Ile Biosynthesis; Pollen Chemi-attractance	*MIK2*	*Vitla_GREM4_13g250.49*	*MDIS1-interacting receptor like kinase 2*
Pathogen Resistance; Insect Herbivory Response	*RLP27-like*	*Vitla_GREM4_8g145.10*	*Receptor like protein 27-like*

Table 2.13 – *V. labrusca* acc. 'GREM4' Paralogous or Genome-specific Candidate Insect Herbivory Resistance Genes.

Biological Implication:	**Abbreviated Gene Name:**	***V. labrusca* acc. 'GREM4' Gene:**	**Full Gene Name:**	**Gene Group:**
Reactive Oxidative Species	*LOX1*	*Vitla_GREM4_6g20.0*	*Lipoxygenase 1*	*Genome-specific*
Stigmasterol Biosynthesis	*CYP710A11*	*Vitla_GREM4_10g73.23*	*Cytochrome P450 monooxygenase, family 710, subfamily A, polypeptide 11*	*Genome-specific*
Reactive Oxidative Species	*Predicted protein HHK36*	*Vitla_GREM4_14g248.28*	*Predicted protein HHK36 (Peroxidase)*	*Genome-specific*
MeJA Conversion to JA	*MJE1*	*Vitla_GREM4_00g37214*	*Methyl jasmonate esterase 1*	*Genome-specific*
-	-	*Vitla_GREM4_11g38.27*	-	*Genome-specific*
-	-	*Vitla_GREM4_18g79.1*	-	*Genome-specific*
-	-	*Vitla_GREM4_6g24.28*	-	*Genome-specific*
-	-	*Vitla_GREM4_13g88.14*	-	*Genome-specific*

Continued…

(Table 2.13 continued)

Wax Biosynthesis; Development	*WSD1*	*Vitla_GREM4_12g40.2*	*O-methyltransferase (Wax synthase/acyl-CoA:diacylglycerol acyltransferase)*	*Paralogous*
Disease Resistance; SAR and ETH Induction	*GDSL-like*	*Vitla_GREM4_18g317.26*	*GDSL-like lipase/Acylhydrolase*	*Paralogous*

Chapter 3 - Insect Herbivory Time Course Reveals Unique Temporal Transcriptomic Responses in Herbivory and Systemic Leaves of Resistant *Vitis labrusca* and Susceptible *Vitis vinifera*

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Status: In Preparation

Abstract

Grapevine (*Vitis*) is a crop of global importance grown on over 19M acres worldwide. Biotic stressors, including insect pest *Popillia japonica* (Japanese beetle), threaten yields of this high value crop. Japanese beetles are pests of grapevine that primarily consume leaf tissues, decreasing photosynthetic capacity of the plant, and thus, yield. *Vitis labrusca,* which is native to northern North America and highly fit in its local environment, has previously been shown to be resistant to Japanese beetles (accession 'GREM4'). Conversely, *Vitis vinifera,* which is widely used for winemaking but generally lacks fitness in North America, was found to be susceptible to Japanese beetles (cultivar 'PN40024'). For this reason, we carried out an insect herbivory time course transcriptomic study to identify the genes, processes, and pathways which differ over time that underly this agronomically valuable phenotype. Major transcriptomic differences were observed between 'GREM4' and 'PN40024' in both direct herbivory and systemic leaves after 30min, 1h, and 4h of beetle herbivory. The number of significantly differentially expressed genes (DEGs) increased over time in 'GREM4' under herbivory but decreased in 'PN40024'. In systemic leaves, hundreds of DEGs were identified in 'GREM4' across all time points, but DEGs decreased over time to only 11 by 4h in 'PN40024'. More DEGs involved in insect herbivory defense were identified in 'GREM4' compared to 'PN40024'. Comparisons between early (30min) and late (4h) response genes revealed that, while the early transcriptomic response in 'PN40024' and 'GREM4' exhibited some DEGs involved in insect herbivory defense, by the late

response, ‘GREM4’ exhibited a large number of DEGs associated with insect herbivory defense, while fewer were observed in ‘PN40024’. Across herbivory and systemic responses, genes associated with flavonoids, phenylpropanoids, acyltransferases, and signaling-pathway genes were implicated in the response in greater numbers, or exclusively, in ‘GREM4’ compared to ‘PN40024’. Therefore, we hypothesize such genes and pathways are important in insect herbivory response in ‘GREM4’ and, likely, play a role in conferring insect herbivory resistance. These results provide insight into the genetic variation, processes, and pathways which underly insect herbivory resistance in *Vitis* and provide candidate genes, and biological pathways, to target for future functional studies and breeding efforts to advance cultivar development and yields.

Introduction

Grapes have been a part of human diets globally for centuries (Myles et al., 2011; Qiu et al., 2015) and are an important agricultural economic driver across the world, especially in the U.S. (United State Department of Agriculture - National Agricultural Statistics Service, 2020). The extraordinarily high value of grapevine necessitates vigilant and intensive management, as even minimal quantitative losses can translate into large losses monetarily. Therefore, protecting grapevines from yield-reducing stresses is paramount. Japanese beetles (*Popillia japonica*) are a major pest of grapevine in the U.S. (Hornberger et al., 2021), are polyphagous, and are invasive to North America and Europe (Fleming, 1976; Smith et al., 1996; Potter and Held, 2002; Mercader and Isaacs, 2003; Gu and Pomper, 2008; The United States Department of Agriculture and USDA-

APHIS, 2015; European and Mediterranean Plant Protection Organization, 2016; MacGregor et al., 2016). *Vitis labrusca* grapevine, which is native to the northeastern U.S. and southeastern Canada, is highly fit in its local environment and is resistant to both abiotic (e.g. - cold hardiness (Dami, 2007; Todaro and Longstroth, 2019) and biotic (pathogen resistance (Kortekamp and Zyprian, 1999; Gabler et al., 2003; Cadle-Davidson, 2008; Gee et al., 2008; Nascimento-Gavioli et al., 2019)) stressors while *Vitis vinifera*, which is widely cultivated for berries and wines (Food and Agriculture Organization of the United Nations (FAO), 2023), is susceptible to many pests and pathogens (Dami et al., 2005; Smith, 2005; Dami, 2007; Cadle-Davidson et al., 2011). Recent reports note that wild *Vitis* species (Cochetel et al., 2023), including *Vitis labrusca* (Li and Gschwend, 2023), harbor unique genomic features compared to *V. vinifera* that likely confer heightened resistance to abiotic and biotic stresses. These genomic features include structural variations, decisive single nucleotide polymorphisms, and increased numbers of transposable elements (Cochetel et al., 2023; Li and Gschwend, 2023). Additionally, previous findings from Chapter 2 noted resistance to Japanese beetle herbivory in *Vitis labrusca* acc. 'GREM4', in both choice and no-choice herbivory assays, which persisted over time periods of 30min, 1h, and 4h. Meanwhile, *Vitis vinifera* cv. 'PN40024' was found to be susceptible (Chapter 2). Significant up-regulation of genes associated with terpene biosynthesis, flavonoids biosynthesis, and plant-pathogen response and resistance were identified in 'GREM4' compared to 'PN40024'. Differences in constitutive (basal, 0h) expression were also identified in

which ‘GREM4’ had greater expression of genes related to secondary metabolite biosynthesis and plant-pathogen interaction and defense compared to ‘PN40024’, even before feeding began.

The reaction of plants to biotic stress is characterized by dynamic processes. Upon perceiving stress, inducible responses initiate in the plant to activate defense, including against insect herbivory. These responses often involve modifications to the transcriptome which ultimately lead to changes in the proteome and subsequent metabolome and the accumulation of specific metabolites. Among these metabolites are compounds known for their insect repellent or insecticidal properties, such as flavonoids (Ibraheem et al., 2015; Kumar and Yadav, 2017; Kariyat et al., 2019; Chatterjee et al., 2022; Chen et al., 2022; Men et al., 2022; Zhang et al., 2022a, 2022b), phenylpropanoids (He et al., 2019; Dixit et al., 2020; Chen et al., 2022; Wang et al., 2022; Zhang et al., 2022a, 2022c), and terpenes (Lewinsohn et al., 1991; Giunta et al., 2016; Chiu et al., 2017; Phschiutta et al., 2017; Quan et al., 2018; Giunti et al., 2020; Liu et al., 2020a; de Albuquerque Lima et al., 2021; Zavala-Gómez et al., 2021; Diass et al., 2021; Paczkowski et al., 2021; Pavela et al., 2021; Sun et al., 2022; Wang et al., 2023a). A limited number of studies have explored transcriptomic responses over the time frame of 0-4h following insect herbivory in plants. In susceptible maize (*Zea mays*) cultivar ‘B73’, under fall armyworm (*Spodoptera exigua*) herbivory, 41% of differentially expressed genes (DEGs) were conserved between 1h and 4h (Tzin et al., 2017). DEGs which were consistently up-regulated across all time points (1h, 4h, 6h, and 24h) in the

study were enriched in the biological processes of suberin biosynthesis, phenylpropanoid biosynthesis, and jasmonic acid (JA) signaling (Tzin et al., 2017). Another study solely exploring volatile accumulations of indole (a terpene precursor (Zhang et al., 2018; Hosseini and Pereira, 2023)), ethylene (ETH), JA, and sesquiterpenes (a terpene sub-family (Zhang et al., 2018; Hosseini and Pereira, 2023)) in maize cultivar 'Delprim' upon fall armyworm herbivory found that indole, sesquiterpenes, and JA accumulations all increased significantly from 0h to 4h (Schmelz et al., 2003). However, no intermediate time points nor transcriptomic data were collected (Schmelz et al., 2003). Similar responses are observed in other plants, such as seen in rice (*Oryzae sativa*) afflicted by rice leaf roller (*Cnaphalocrocis medinalis*), of which ~38% of DEGs were conserved from 30min to 3h of herbivory (Zhuang et al., 2022). Genes which were up-regulated from 30min to 3h included those related to JA biosynthesis (*JAZ8* (*Jasmonate ZIM-domain protein 8*)*, JAZ9, JAZ11,* and *OPR1* (*12-oxophytodienoate reductase 1*)), reactive oxidative species (multiple *LOX* (*Lipoxygenase*) gene family members), and phenylpropanoid biosynthesis (*4CL6* (*4-coumarate-CoA ligase-like 6*)*, SHT1* (*Spermidine hydroxycinnamoyl transferase 1*)*, PAL6* (*Phenylalanine ammonia lyase 1-4*)*,* and *PAL7*) (Zhuang et al., 2022). These studies demonstrate alterations in plant transcriptomes from ~30min to 4h in response to insect herbivory. However, these investigations did not compare responses of a resistant and a susceptible plant. Most studies to date which have conducted RNA-seq to explore transcriptomic response to insect herbivory over time have employed sampling timescales of many hours or even days (Broekgaarden et al.,

2007; Li et al., 2016; Tetreault et al., 2019; Dixit et al., 2020), while no studies could be identified conducting a transcriptomic study exploring insect herbivory response in a time course-dependent manner in *Vitis*. As such, a knowledge gap exists in the understanding of transcriptomic responses to insect herbivory across a timescale of 4h, how feeding effects transcriptomic responses in tissues elsewhere on the plant, and how these responses differ between resistant and susceptible species.

Another important aspect of plant defense are systemic responses. Systemic response is an inducible response which prepares unafflicted tissues for a stress not yet directly experienced to prevent further damage (Zhou et al., 2020). For example, white cabbage butterfly (*Pieris rapae*) larvae feeding in *Arabidopsis* revealed calcium ion signaling fluctuations in leaves adjacent to feeding in only one to two minutes after the first bites (Toyota et al., 2018). It was found that *Glutamate receptor-like 3* (*GLR3*) gene family members were critical in conveying this signal to distal leaves (Toyota et al., 2018). Systemic responses, such as the aforementioned example, are widely recognized for their role in enhancing insect herbivory resistance such as seen in cotton (*Gossypium hirsutum*). Mechanical damage of the most mature cotton leaves were found to result in increased accumulations of defensive compounds gossypol, heliocides, and terpenes in immature leaves, with terpene β-ocimene exhibiting the greatest increase (Mamin et al., 2023). When fall armyworms (*Spodoptera frugiperda*) were provided immature leaves from plants which were primed by mechanical damage or leaves which were not primed in a choice-assay, armyworms preferentially fed upon the unprimed leaves (Mamin et al.,

2023). In tea (*Camellia sinensis*) attacked by tea geometrid (*Ectropis obliqua*), multiple KEGG pathway enrichments were identified in up-regulated DEGs in systemic leaves including phenylpropanoid biosynthesis, flavonoid biosynthesis, amino-acid metabolism, and aromatic compounds (Zhou et al., 2020). DEGs associated with calcium ion signaling, ROS, and phytohormonal regulation were also differentially expressed (Zhou et al., 2020). Similar responses have been identified in many other plants as well (ul Malook et al., 2019, 2021; Malhotra et al., 2022; Meza-Canales et al., 2022; Xue et al., 2022; Tong et al., 2023) but seemingly not within *Vitis* to date. As such, investigating the temporal variation of systemic response between a resistant and susceptible grapevine upon insect herbivory is an intriguing question.

Herein, we conduct a comparative, time course transcriptomic study to investigate the response of resistant ('GREM4') and susceptible ('PN40024') grapevine leaves to insect herbivory to identify temporal alterations in gene expression after 30min, 1h, and 4h of feeding. Further, we explore the systemic transcriptomic response in 'GREM4' and 'PN40024' after 30min, 1h, and 4h of herbivory. Together, we aim to identify genes, functions, and pathways that may play a role in insect herbivory defense.

Materials and Methods

Plant Materials

Vitis labrusca acc. 'GREM4' (PI-588583) and *Vitis vinifera* cv. 'PN40024' (DVIT-908) grapevine cuttings were acquired from the United States Department of Agriculture at Geneva, NY and Davis, CA, respectively, in 2019 (Prins and Agricultural

Research Service - United States Department of Agriculture, 2018; Grape Genetics Research Unit, 2020). 'PN40024' was selected due to its role as the *V. vinifera* reference cultivar/reference genome (Jaillon et al., 2007) while 'GREM4' was selected due to the availability of a reference genome sequence (Li and Gschwend, 2023) and its resistance to pathogens, suggesting broad fitness in its local environment (Cadle-Davidson, 2008). Both species were propagated from cuttings and grown in greenhouses at The Ohio State University, Columbus, OH USA under 16hr light:8hr dark and a temperature of ~>10°F than ambient for Ohio, USA. At time of experimentation, the plants were two- to three-year-old rooted vegetative plants. Experiments took place from the end of August through mid-September 2021.

Insect Collections

Popillia japonica (Japanese beetles) were collected from The Ohio State Waterman Agricultural and Natural Resources Laboratory, Columbus OH, USA from the end of August through mid-September 2021. Beetles were collected using "Spectracide Bag-A-Bug Japanese Beetle Trap2" pheromone traps (Spectrum Brands, 2023) in a soybean field which had not been sprayed with insecticides. Beetles were kept in a 16.5 x 16.5 x 30in 'bug dorm' (Educational Science, 2019) within a growth chamber overnight and semi-starved (one small *V. vinifera* leaf provided to prevent death due to starvation or dehydration) and were used for experiments the following day. The growth chamber was set to a 16hr light:8hr dark cycle at 25ºC and 21ºC, respectively.

Herbivory Time Course Study

One semi-starved Japanese beetle was placed in a transparent, mesh 11cm x 10cm bag, which was then placed over one mature attached leaf of either 'GREM4' or 'PN40024', and beetles were permitted to directly feed upon the leaf for 30min, 1h, or 4h (Figure 3.1 & Figure 3.2A). Feeding timing began once visible damage to the leaf was observed. This was the 'Herbivory' treatment. 'Systemic' leaves were also collected after 30min, 1h, and 4h of feeding (at the same time as the herbivory leaf) from a leaf which only had a bag placed over it with no beetle. The systemic leaf was of the same maturity, size, and cane position as the herbivory leaf. However, while the systemic and herbivory samples were from the same plant, they were collected from separate canes. Control leaves ('0h') were also collected just prior to herbivory but were obtained from a separate plant than those used for herbivory and systemic samples to prevent potential confounding effects on transcriptomic responses stemming from leaf removal. For the same reason, while herbivory and systemic samples were collected in pairs from the same plant (e.g. – 'GREM4' Herbivory 1h Rep 2 was collected at the same time from the same plant as 'GREM4' Systemic 1h Rep 2), time points (30min, 1h, and 4h) and biological replicates (1 through 4), were collected from separate plants (i.e. – the 30min, 1h, and 4h time points were not occurring simultaneously on a single plant). A total of 56 samples (four herbivory and four systemic replicates for each of three time points, plus four 0h samples, for both species) were collected. All experimental 'runs' (an attempt at collecting feeding data by placing a beetle in a bag on a leaf) were conducted in the

greenhouse August through September of 2021, between 9:00AM and 3:00PM daily. Plants were not used again for at least four days between runs to ensure *in planta* responses captured were not a vestige of prior feeding. After each run, leaves were photographed (Figure 3.2B), placed inside 50mL conical tubes, then plunged into liquid nitrogen. Leaves were stored at -80ºC until RNA isolation for RNA-sequencing.

The 30min time point was chosen since transcriptomic differences *in planta* have been observed within 20min after encountering a stress (Pandey et al., 2017) while 4h was chosen since defensive compounds were found to increase consistently up to 4h in a previous insect herbivory study (Köllner et al., 2010). These time points also aligned with the designation of 'early' and 'late' response time points in a previous study (Zhuang et al., 2022).

RNA Isolation and Sequencing

RNA was isolated from the 30min, 1h, and 4h herbivory, systemic, and 0h (control) leaf samples collected during the herbivory time course study described above. RNA was isolated from leaf samples using a Sigma-Aldrich Spectrum Plant Total RNA Kit (Millipore-Sigma, 2023) and RNA quality and quantity were determined via Nanodrop (Desjardins and Conklin, 2010), Qubit (Invitrogen/ThermoFisher Scientific/Life Technologies Holdings Pte Ltd, 2021), and a formaldehyde gel. All 56 samples were submitted to Novogene (Novogene Co. Ltd, n.d.) for library construction for each sample and Illumina NovaSeq 6000 paired-end RNA sequencing (150bp reads, 20M read per library). RNA-seq read quality statistics were identical to those reported in

Chapter 2. RNA-seq reads were subjected to quality control assessments via FastQC (Andrews, 2023) and removal of adapters and poor quality reads via Trimmomatic (Bolger et al., 2014).

RNA-seq Analysis

A graphical representation of the pipeline can be seen as Figure 2.4. In short, RNA-seq reads were aligned to their respective genomes using STAR (Dobin et al., 2013). The *Vitis vinifera* cv. 'PN40024' 12x.2 genome sequence (Canaguier et al., 2017) and v4.56 annotation (an improved version of the v4 annotation (Velt et al., 2023) updated by IGGP (The Institut National de la Recherche Agronomique, 2022) and Ensembl (Ensembl Plants release 58 and EMBL-EBI, 2024)) was used for 'PN40024' while the *Vitis labrusca* acc. 'GREM4' gene annotation (Chapter 2) was used for 'GREM4'. 37,443 genes models were present in 'GREM4' hypothetically translating into 40,277 proteins, meanwhile, 35,133 genes models were present in 'PN40024' hypothetically translating to 41,160 proteins. CoCo (Deschamps-Francoeur et al., 2019) via 'coco correct_counts' was used to create the count matrix (to better account for multi-mapping reads). DESeq2 (Love et al., 2014) was used to identify significantly differentially expressed genes (DEGs). DEGs were identified at each time point (30min, 1h, and 4h) compared to 0h controls. Throughout analyses, significant p- and p-adj values were defined as ≤ 0.05 whereas |log2foldchange| was ≥ 2. Leaves under insect herbivory directly (herbivory) and indirectly (systemic) were collected and transcriptomic responses analyzed in both species. A third comparison investigated differences between

herbivory and systemic samples via two different methods – 1) comparison of expression (read count values) directly between the two conditions (e.g. – 'GREM4' 1h Herbivory compared to 'GREM4' 1h Systemic) and 2) overlap analysis to identify DEGs conserved or unique between herbivory compared to 0h and systemic compared to 0h comparisons – both methods being conducted via DESeq2. Additional information on the pipeline and programs used to analyze the RNA-seq data, can be found in Chapter 2 and on GitHub at https://github.com/cdixo/Inter-species-and-Herbivory-Publication.git . BioInfoRx (BioInfoRx, 2023), BioVenn (Hulsen et al., 2008), Upset (Lex et al., 2014), and molbiotools (Molbiotools, 2023) were used to identify DEGs conserved or unique between transcriptomic comparisons and to create Venn diagrams.

Over-Representation Analysis (ORA) was used to identified Gene Ontology (GO) term enrichment in sets of DEGs via 'enricher' (clusterProfiler (Wu et al., 2021)) with a post-hoc 'gsfilter' (DOSE (Yu et al., 2015)) wherein enrichments with < 5 implicated DEGs were removed. Pathway enrichment of DEGs was conducted using Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000) via the KEGG Orthology-Based Annotation System-intelligent (KOBAS-i) (Bu et al., 2021). Significance was determined by p.adj ≤ 0.05. Additional information can be found in Chapter 2 and on GitHub at https://github.com/cdixo/Inter-species-and-Herbivory-Publication.git .

Results

To determine what defenses contribute to 'GREM4' insect herbivory resistance, we conducted a comparative, time course transcriptomic study to investigate the gene expression responses of 'GREM4' and 'PN40024' leaves to insect herbivory. Expression results for all genes may be seen as Table 3.1 while expression data for DEGs only may be seen as Table 3.2. Functional annotations may be found in Chapter 2 as Table 2.4. Three types of samples were collected: 1) Leaves from plants that did not undergo insect herbivory (0h – the experimental control), 2) Leaves that were fed on by Japanese beetles (herbivory), and 3) Leaves attached to the same plant as the herbivory sample but a different cane which did not directly undergo herbivory (systemic) (Figure 3.1). Herbivory and systemic samples were collected at 30min, 1h, and 4h after feeding initiation. Four replicates per accession, sample, and time point were sequenced. Downstream analyses were performed to determine the biomolecular processes, pathways, and genes responsible for the differential insect herbivory resistance observed between 'GREM4' and 'PN40024'.

Insect Herbivory Response

First, we sought to identify genes which were significantly differentially expressed under Japanese beetle herbivory in 'GREM4' and 'PN40024' after 30min, 1h, and 4h of feeding by comparing gene expression at each time point to 0h (control) within each species. In both species, under insect herbivory, considerably more DEGs were up- than down-regulated across all time points (Figure 3.3A). Additionally, the number of

total DEGs increased from 204 at 30min to 384 at 4h in ‘GREM4’, but the number of total DEGs decreased in ‘PN40024’ from 278 at 30min to 131 by 4h. These results suggest more genes were implicated in an immediate response to feeding in ‘PN40024’, but, ‘GREM4’ had a sustained, and even enhanced, transcriptomic response to feeding as time progressed.

Candidate genes associated with insect herbivory response in 'GREM4' were identified by screening DEGs, retaining only those with a $|\log2FoldChange| \geq 20$ and a $p.adj \leq 0.01$. This process resulted in 26 DEGs which were generally implicated in pathogen defense, phytohormones, flavonoids, terpenes, and cell wall formation (Table 3.3).

Additionally, considering the importance of immediate and latent response in plant defense, comparisons between early (30min) and late (4h) transcriptomic response were undertaken. In ‘GREM4’, 79 (16%) of 509 total unique DEGs between the two timepoints were conserved (Figure 3.3B, Figure 3.4A, and Figure 3.5A). Meanwhile, in ‘PN40024’, only 36 (10%) of 373 total unique DEGs were conserved. The top 10 DEGs with the greatest change in expression, independently at 30min or 4h, were identified and can be seen as Table 3.4. Interestingly, all such genes were up-regulated. The early response in ‘PN40024’ included genes associated with pathogen resistance (*Ethylene-responsive transcription factor 17-1* (*ERF017-1*) (*Vitvi04g00190*) and *ERF017-2* (*Vitvi11g00046*)), phytohormones (*EXORDIUM* (*EXO*) (*Vitvi18g00424*), *ABA repressor 1-like* (*ABR1-like*) (*Vitvi18g01617*), and *12-oxophytodienoate reductase 11* (*OPR11*)

(*Vitvi18g04622*)) and cell wall remodeling (*Xyloglucan endotransglucosylase/Hydrolase 2* (*XTH2*) (*Vitvi05g02108*)). The early response in 'GREM4' was comprised of genes associated with ETH (*ABR1-like* (*Vitla_GREM4_18g199.44*) and *Wax inducer 1* (*WIN1*) (*Vitla_GREM4_9g74.35*)), terpenes (*Terpene synthase 14-1* (*TPS14-1*) (*Vitla_GREM4_00g152.53*)), and defense signaling (*Cysteine-rich receptor-like kinase 26* (*CRK26*) (*Vitla_GREM4_00g37471*)). The top 10 DEGs in the late response in 'PN40024' were predominantly associated with growth and reproduction (*Phospholipase A2 family protein* (*PLA2-ALPHA*) (*Vitvi11g01098*) and *Early nodulin-75-1, -2,* and *-3* (*ENOD2-1*, *-2,* and *-3*)) and pathogen defense (*Transcription factor MYB78* (*MYB78*) (*Vitvi05g00166*) and *L-type lectin-domain containing receptor kinase (L-type LecRK) VII.1* (*LecRK VII.1*) (*Vitvi12g00300*)). In 'GREM4', the top 10 late response DEGs were associated with SA (*Salicylate carboxymethyl transferase 1* (*SAMT2*) (*Vitla_GREM4_4g197.9*), JA (*(E)-2-methylbutanal oxime monooxygenase* (*CYP71E7/AOS*) (*Vitla_GREM4_6g74.45*)), terpenes (*TPS14-1*), stilbenes (*Stilbene synthase 1-4* (*STS1-4*) (*Vitla_GREM4_16g175.45*) and phenylpropanoids (*PAL1-1*) (*Vitla_GREM4_16g7.31*). Notably, *ABR1-like* was conserved between all top 10 lists in both 'GREM4' and 'PN40024' in both early and late responses. Additionally, *TPS14-1* was also found in the top 10 in both the early and late response in 'GREM4'. Together, these results report that, some of the topmost differentially expressed genes in both 'GREM4' and 'PN40024' were associated with defense-related functions. However, by

the late response, four of the 10 top DEGs were related to growth and development in 'PN40024', which may hamper defense.

Next, we determined which DEGs identified after 30min, 1h, and 4h of herbivory were conserved or unique between time points. In 'GREM4', a total of 690 non-duplicated DEGs (549 up-regulated, 141 down-regulated) were identified, and, of those, 63 (9%) were conserved across all time points while 191 (28%) were conserved between at least two (Figure 3.3B, Figure 3.4A, and Figure 3.5A). All lists of DEGs which were conserved or unique between comparisons, or otherwise screened throughout this manuscript, can be found in Table 3.5. Some DEGs conserved between herbivory time points in 'GREM4' included genes related to phenylpropanoids (*PAL1-4*), flavonoids (*Flavonol synthase* (*FLS2*) (*Vitla_GREM4_10g70.44*) and *Flavonol synthase/Flavanone 3-hydroxylase* (*FLS/F3H*) (*Vitla_GREM4_13g47.35*)), terpenes (*TPS14-1* and *TPS14-2* (*Vitla_GREM4_00g152.55*)), phytohormones (*Allene oxide synthase* (*AOS1*) (*Vitla_GREM4_18g96.22*), *WRKY-domain containing protein 48* (*WRKY48*) (*Vitla_GREM4_5g75.27*), *ABR1-like, Ethylene-responsive transcription factor 110* (*ERF110*), and *Aconitase 3* (*ACO3*) (*Vitla_GREM4_12g68.45*)), and senescence (*Senescence-related gene 1* (*SRG1*) (*Vitla_GREM4_2g70.38*)). In 'GREM4', 72% of all DEGs were only significantly differentially expressed at one time point. When investigating time points individually at 30min, 1h, and 4h in 'GREM4', 35%, 51%, and 64% of DEGs were unique to the time point, respectively (Figure 3.3B). Comparatively, in 'PN40024', 502 total non-duplicated DEGs (447 up-regulated, 55 down-regulated)

were identified, but only 17 (3%) were conserved across all time points while 87 (17%) were conserved between at least two time points (Figure 3.3B, Figure 3.4B, and Figure 3.5B). The majority (83%) of DEGs in 'PN40024' were only identified at one time point wherein at 30min, 1h, and 4h, 73%, 65%, and 64% of DEGs were unique to the time point (Figure 3.3B). Additionally, the majority (65%) of 'GREM4' DEGs detected at 30min were also differentially expressed in at least one other time point, yet only 27% of DEGs identified at 30min in 'PN40024' displayed this attribute. Together, these results indicate a greater conservation of genes differentially expressed across 30min, 1h, and 4h in 'GREM4' compared to 'PN40024' in response to Japanese beetle herbivory, but in both species, the majority of DEGs were only detected at one time point suggesting specific temporal responses.

Enrichment analysis revealed specific biological processes and pathways which were significantly over-represented in lists of DEGs in 'GREM4' and 'PN40024' under insect herbivory over time. When all up- and down-regulated herbivory DEGs from all time points were combined and queried for Gene Ontology (GO) term enrichment via Over-Representation Analysis (ORA), 13 total enrichments were identified including 'acyltransferase activity', 'lyase activity', 'L-phenylalanine catabolic process', 'ethylene-activated signaling pathway', and terpene synthesis-related terms (All ORA enrichment results throughout the manuscript may be seen in full in Table 3.6). In 'PN40024', 12 terms were enriched including xyloglucan-related terms, cell wall remodeling-related terms, and 'ethylene-activated signaling pathway'. Pathways enriched amongst DEGs

were also assessed via Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment via KEGG Orthology-Based Annotation System-intelligent (KOBAS-i). In 'GREM4', when combining all time points and up- and down-regulated DEGs, 14 enriched pathways were identified including 'biosynthesis of secondary metabolites', 'flavonoid biosynthesis', 'plant-pathogen interaction', 'phenylpropanoid biosynthesis', 'plant hormone signal transduction', 'MAPK signaling pathway', and terpene biosynthesis-related pathways (All KEGG enrichment results throughout the manuscript may be seen in full in Table 3.7). In 'PN40024', seven of eight enriched pathways identified were shared with 'GREM4'; The unique enrichment was 'isoquinoline alkaloid biosynthesis' while shared enrichments included 'biosynthesis of secondary metabolites', 'plant-pathogen interaction', 'plant hormone signal transduction', and 'monoterpenoid biosynthesis'. Interestingly, in these shared pathways, in most cases, more DEGs were implicated in 'GREM4' compared to 'PN40024'. For example, a maximum of 87 genes were implicated in the 'biosynthesis of secondary metabolites' pathway in 'GREM4' compared to a maximum of only 48 in 'PN40024'. It is likely that many of these pathways enriched upon Japanese beetle herbivory in 'GREM4' contributed to the increased herbivory resistance phenotype.

Next, we identified enrichments under insect herbivory on individual time points, as well as conservation between them. Enrichments were only identified for up-regulated herbivory DEGs in both species, while no significant enrichments were identified in down-regulated DEGs when separating out lists of DEGs by time point. In 'PN40024',

the number of enrichments identified in up-regulated DEGs decreased over time from 12 (30min) to three (1h) to two (4h) (Figure 3.6). In 'GREM4', the number of functional enrichments increased from zero (30min) to 11 (1hr and 4hr). The only two functional enrichments identified in 'PN40024' at 4h were 'extracellular region' and 'methylation'. Meanwhile, by 4h in 'GREM4', 11 functional enrichments were identified in 'GREM4' including terms related to secondary metabolite biosynthesis such as terpenes and 'L-phenylalanine catabolic process'. In 'GREM4', seven of 15 total enrichments were conserved between 1h and 4h. Meanwhile, in 'PN40024', only two of 14 total enrichments were conserved between more than one time point. When comparing enrichments at time points between the two species, only two of 26 total functional enrichments were conserved - 'sequence-specific DNA binding' and 'ethylene-activated signaling pathway'. Some of the 12 enrichments unique to 'GREM4' included 'terpene-synthase activity', 'L-phenylalanine catabolic process', and acyltransferase-related terms while some of the 12 enrichments unique to 'PN40024' were 'xyloglucan metabolic process', 'cell wall organization', and 'methylation'. Together, enrichments reveal differing processes and pathways implicated in defense responses in both species which, additionally, suggest an increasing response in 'GREM4' compared to a decreasing response in 'PN40024'.

Systemic Response to Insect Herbivory

A systemic response is an inducible defense initiated in afflicted tissue(s) by a stress, including insect herbivory, which provides stauncher protection in unafflicted

tissues (Zhou et al., 2020). Considering the importance of systemic responses in plant defense, we next explored transcriptomic differences between systemic and control (0h) leaves in each species.

Compared to 0h, 1,120 total DEGs were identified in 'GREM4' systemic leaves compared to only 116 in 'PN40024' (Figure 3.7A). The greatest number of total systemic DEGs in 'GREM4' was observed after 1h, with 460, while the lowest was 269 at 4h. Meanwhile, the greatest number of total systemic DEGs for 'PN40024' at any time point was only 66 (1h), while the lowest number was only 11 at 4h. The majority of DEGs in both species were up-regulated at each time point, aside from 'GREM4' at 4h in which up- and down-regulated genes were roughly equivalent, with 131 and 138, respectively. These findings report that systemic leaves in 'GREM4' exhibited a far greater transcriptomic response at every time point compared to 'PN40024'.

Candidate genes associated with systemic response in 'GREM4' were identified by screening DEGs, retaining only those with a $|\log2FoldChange| \geq 10$ and a $p.adj \leq 0.01$. This process resulted in nine DEGs which were generally implicated in plant development, insect defense, pathogen defense, and phytohormones (Table 3.8).

It was previously identified that the *PAL1* gene family was larger in 'GREM4' compared to 'PN40024' and that some additional family members exhibited significantly increased expression upon Japanese beetle herbivory (Chapter 2). Due to this fact, we explored the transcriptomic response of *PAL1* gene family members in systemic leaves. Four of the 12 *PAL1* gene family members in 'GREM4' were significantly up-regulated

in systemic leaves, of which, three were unique to 'GREM4' being *PAL1-4* (*maker-16-snap-gene-7.31*), *PAL1-7* (*maker-16-snap-gene-8.38*), and *PAL1-8* (*maker-16-snap-gene-7.35*) (Figure 3.8). Meanwhile, in 'PN40024', none of the four total *PAL1* genes were significantly differentially expressed in systemic leaves. These results report that *PAL1* gene family members unique to 'GREM4' play a role in systemic insect herbivory defense, in addition to their role in defense under direct herbivory (Chapter 2).

We next determined DEGs conserved or unique between time points in systemic comparisons. In 'GREM4' systemic leaves, 20 (2%) of 888 total non-duplicated DEGs were conserved between all three time points while 212 (24%) were identified between at least two (Figure 3.7B, Figure 3.4C, and Figure 3.5A). Some DEGs conserved between systemic time points in 'GREM4' were implicated in phenylpropanoids, flavonoids, terpenes, JA, ETH, and other pathways. Comparatively, in 'PN40024', only one of 106 total non-duplicated DEGs was conserved between all time points, *Cinnamoyl-CoA reductase 1-like* (*CCR1-like*), while only nine DEGs were conserved between at least two (Figure 3.7B, Figure 3.4D, and Figure 3.5B). These results indicate DEGs involved in a systemic response are more conserved between time points in 'GREM4' than 'PN40024', but, overall, most systemic response genes are time point-specific in both species.

Enrichment analysis of DEGs revealed processes and pathways which were significantly enriched in systemic responses. When combining all up- and down-regulated DEGs across all time points in 'GREM4', GO term enrichment via ORA revealed 16 functional enrichments including 'defense response to fungus', 'ethylene-

activated signaling pathway', calcium ion transport-related terms, 'lipid transport', 'peroxidase activity', 'biosynthetic process', and acyltransferase-related terms. In 'PN40024', only eight total functional enrichments were identified, of which, four were related to protein remodeling, while two others included 'response to hydrogen peroxide' and 'response to heat'. Similar results were observed from KEGG pathway enrichment. In 'GREM4', when combining all up- and down-regulated DEGs across all time points, 12 enriched pathways were identified including 'plant-pathogen interaction', flavonoid biosynthesis-related terms, 'phenylpropanoid biosynthesis', 'MAPK signaling pathway', and 'plant hormone signal transduction'. Interestingly, no pathway enrichments in 'GREM4' were shared with 'PN40024', in which only three pathways enriched: 'protein processing in endoplasmic reticulum', 'sesquiterpenoid and triterpenoid biosynthesis', and 'alpha-linolenic acid metabolism'. Notably, when conducting pathway enrichment analysis on down-regulated DEGs only from all time points in 'PN40024', 'biosynthesis of secondary metabolites' was found to be enriched suggesting genes associated with secondary metabolite biosynthesis are generally down-regulated in 'PN40024' systemic tissues. Overall, fewer processes and pathways were enriched in 'PN40024' compared to 'GREM4' suggesting a less robust response in systemic leaves to insect herbivory. These results suggest that genes implicated in pathogen defense, oxidative stress signaling, ethylene processes, flavonoid biosynthesis, the phenylpropanoid pathway, and pathways implicated in plant-pathogen interactions may play a role in systemic defense in insect herbivory resistant 'GREM4'.

Next, we identified enrichments in DEGs from systemic leaves from individual time points, as well as conservation between them. In 'PN40024', the number of enrichments identified transitioned from one at 30min, to seven at 1h, to zero at 4h (Figure 3.9). Meanwhile, in 'GREM4', 13 enrichments were identified at both 30min and 1h, which declined to six at 4h. Five enrichments were conserved across at least two time points in 'GREM4' including acyltransferase-related terms. Meanwhile, in 'PN40024', no enrichments were conserved across time points. When comparing enrichments at time points between the two species, interestingly, no enrichments were conserved between them. Some of the notable enrichments, of 26 total, unique to 'GREM4' were 'signal transduction', 'defense response to fungus', 'cell surface receptor signaling pathway', acyltransferase-related terms, and even 'biosynthetic process'. Some of the eight total enrichments unique to 'PN40024' were 'response to hydrogen peroxide', 'response to heat', and 'protein folding'. Together, these results report that the response to insect herbivory in 'GREM4' results in the differential expression of many different genes associated with an array of biological processes and pathways with connections to insect herbivory defense and signaling, whereas, in 'PN40024' fewer genes associated with biological functions or pathways were identified and were generally associated with abiotic stress response or protein folding. Also, interestingly, enrichments identified in down-regulated DEGs accounted for almost a quarter of enrichments in 'GREM4' whereas no enrichments were identified of down-regulated genes in 'PN40024'.

Herbivory Compared to Systemic Response

We next compared the transcriptomic responses between herbivory and systemic leaves to determine if responses differ between herbivory and systemic response in 'GREM4' and 'PN40024'.

Direct Comparison Analysis

First, we compared the transcriptomes of herbivory leaves at each time point to their corresponding systemic leaf at the same time point (e.g. – 'GREM4' 30min herbivory compared to 'GREM4' 30min systemic). In 'GREM4' at 30min, 212 DEGs were identified suggesting the transcriptomes of herbivory and systemic leaves differed in response to feeding (Figure 3.10A). However, by 4h, only five DEGs were identified indicating transcriptomic responses were similar between direct herbivory and systemic tissue after 4h of feeding. Surprisingly, in 'PN40024', only five total DEGs were identified across all time points, which indicated a similar transcriptomic response between herbivory and systemic leaves across time. These results report that, while initially the transcriptomes of herbivory and systemic leaves in 'GREM4' were different, by 4h, they were extremely similar. Meanwhile, in 'PN40024', the response in herbivory and systemic leaves was practically not different at all, regardless of time point.

Enrichment via ORA could only be conducted on 'GREM4' 30min and 1h herbivory compared to systemic comparisons, due to too low of numbers of DEGs being present to conduct the analysis in the other four comparisons ('GREM4' 4h and all 'PN40024' comparisons). The 'GREM4' 1h herbivory compared to 1h systemic

comparison yielded no functional enrichments, while 12 enriched terms were identified in DEGs at 30min with greater expression in herbivory compared to systemic leaves in 'GREM4', which included 'enzyme inhibitor activity', 'lignin catabolic process', 'lipid metabolic process', and metal-ion binding-related terms.

Overlap Analysis

As a second approach to identify genes that were either conserved or unique between herbivory and systemic leaves, we compared lists of DEGs identified in herbivory and systemic samples at 30min, 1h, and 4h (compared to 0h). This method allowed us to identify DEGs that were shared or unique between the two conditions. In 'GREM4', when all time points and up- and down-regulated DEGs were combined, 315 of 1,263 total (25%) non-duplicated DEGs were identified in both herbivory and systemic responses (Figure 3.10B & Figure 3.5A). Some of the DEGs in both herbivory and systemic tissues in 'GREM4' included *TPS14-2*, *AOS1*, *Pleiotropic drug resistance 12* (*ABCG40*) (*Vitla_GREM4_9g56.37*), *PAL1-4*, and *Wall-associated receptor kinase-like 8* (*WAK-like 8*) (*Vitla_GREM4_13g33.24*) suggesting terpenes, phenylpropanoids, and pathogen-response genes are common across herbivory and systemic responses in 'GREM4'. Five functional enrichments were identified for these DEGs conserved between herbivory and systemic responses: acyltransferase-related terms, 'biosynthetic process', 'ethylene-activated signaling pathway', and 'sequence-specific DNA binding'. In 'PN40024', 89 of 519 (17%) non-duplicated DEGs were conserved between herbivory and systemic samples (Figure 3.10B & Figure 3.5B). Some of the DEGs expressed in

both herbivory and systemic tissues in 'PN40024' included *SAMT1* (AKA – *BMST1*) (*Vitvi04g02117*), *Receptor-like protein kinase HAIKU2* (*IKU2*) (*Vitvi03g00783*), and *ERF2* (*Vitvi02g01780*) suggesting genes implicated in phytohormones are common between herbivory and systemic responses in 'PN40024'. Only one functional enrichment was identified for this set of genes of 'extracellular space'. Numbers of conserved and unique DEGs in 'GREM4' and 'PN40024', when all herbivory and systemic DEGs are separated out by time point and treatment, can be seen as Figure 3.5. These results report that genes associated with known insect herbivory defensive processes were identified in both herbivory and systemic leaves in 'GREM4'. However, in 'PN40024', while a similar percentage of DEGs were conserved between herbivory and systemic leaves, the total number of DEGs itself was ~225 DEGs less, and these DEGs were mostly associated with phytohormones.

Discussion

Discussion Introduction

Plants must withstand and defend against damage from insect herbivory to survive and reproduce. Defensive measures have evolved in plants to counteract biotic stressors, including insect herbivory. *Vitis labrusca* hybrids have displayed insect resistance in the field (Mercader and Isaacs, 2003) and true-breeding *V. labrusca* acc. 'GREM4' was previously found to be insect herbivory resistant, in part due to increased densities of trichomes, but in part due to other biomolecular factors (Chapter 2). To further elucidate these factors, we investigated the temporal response to insect herbivory

in the transcriptomes of resistant 'GREM4' and susceptible *V. vinifera* cv. 'PN40024' leaves over periods of 30min, 1h, and 4h to understand trends in gene expression critical to imbuing resistance. Further, systemic response, a critical defense response which primes unafflicted tissues prior to attack (Mamin et al., 2023), was also investigated.

Greater Conservation of Insect Herbivory Response Genes Across Time Points Observed in 'GREM4' Compared to 'PN40024'

Gene expression can alter within 15min of encountering a stimulus, such as simulated insect feeding (Zhang et al., 2021a; Srivastava et al., 2022; Lu et al., 2023), stress-related VOCs (Meents et al., 2019), mechanical wounding (Reymond et al., 2000; Glauser et al., 2009), or even simply touch or water (van Moerkercke et al., 2019). Nevertheless, in our study, large numbers of DEGs were conserved between time points with a greater number of DEGs identified in 'GREM4' compared to 'PN40024'. 65% of genes differentially expressed after 30min of herbivory were also differentially expressed at 1h and/or 4h in 'GREM4'. Whereas, in 'PN40024', only 27% of DEGs identified after 30min were also differentially expressed in at least one other time point. Similar to 'PN40024', some susceptible plants in other studies also exhibit conservation of DEGs between early (30min-1h) and late (3-4h) response to insect herbivory. In maize afflicted by fall armyworm (*Spodoptera exigua*), 41% of DEGs were conserved between 1h and 4h of herbivory (Tzin et al., 2017). Whereas, in rice, ~38% of up-regulated DEGs were conserved between 30min and 3h of rice leaf roller feeding (Zhuang et al., 2022). The majority of studies conducting comparative transcriptomic analysis upon insect herbivory

between a resistant and susceptible plant in a time course, which were also not a transgenic functional characterization study or strictly reporting qRT-PCR data, were found to be of greater time periods than employed in our study, ranging from > 6h to 15d (Broekgaarden et al., 2007; Li et al., 2016; Tetreault et al., 2019), thus preventing direct comparisons. Overall, these results indicate 'GREM4' exhibited greater consistency in the genes which were significantly differentially expressed across herbivory time points from 30min to 4h in response to Japanese beetle herbivory compared to 'PN40024'. It is possible this greater conservation of DEGs between time points positively impacts insect herbivory defense through a more consistent response.

A greater number of enrichments were also conserved across time points in 'GREM4' compared to 'PN40024'. Almost twice as many enriched pathways were identified in 'GREM4' compared to 'PN40024'. Some enrichments unique to 'GREM4' were 'flavonoid biosynthesis', 'sesquiterpenoid and triterpenoid biosynthesis', 'MAPK signaling', and others, which had known associations to insect herbivory defense and signaling, some of which are expanded upon below.

Some of the genes conserved between time points in 'GREM4' were implicated in phenylpropanoid biosynthesis, flavonoid biosynthesis, terpene biosynthesis, and phytohormone (JA, SA, ABA, ETH, and CK) signaling. These results are similar to those observed in maize and *Arabidopsis*, although both species were insect herbivory susceptible. In *Arabidopsis* upon diamond back moth (*Plutella xylostella*) feeding, genes implicated in pathogen response (*LOX*), phenylpropanoids (*CADL4* and *4CL3* (*4-*

coumarate-CoA ligase 3)), and JA (*AOC, OPCL1,* and *TAT3*) were up-regulated at both 1h and 4h (Ehlting et al., 2008) while in maize, under fall armyworm herbivory, DEGs with consistently increased expression from 1h to 24h were enriched in phenylpropanoid, JA, and ETH biosynthesis (Tzin et al., 2017). Flavonoids and terpenes were notably not observed in the two aforementioned studies. However, flavonoids have previously been observed in a study which reported expression at 4h, but did not assay 30min or 1h time points, in tea (Jing et al., 2024). In the study, many genes within the flavonoid pathway, including *F3H*, *CHS* (*Chalcone synthase*), and *FLS,* were up-regulated at 4h compared to 0h (Jing et al., 2024). Further, in cotton, some genes associated with defensive pathways, such as *PAL* and *CHS,* associated with phenylpropanoid and flavonoid biosynthesis respectively, were found to be consistently up-regulated, even after 24h, 2d, 3d, and 4d of feeding by chewing mouthpart insects (Dixit et al., 2020). In our study, phenylpropanoid biosynthesis gene *PAL1-4,* flavonoid biosynthesis genes *FLS2* and *FLS/F3H,* and JA biosynthesis gene *AOS1* were all up-regulated in both early and late response time points in 'GREM4', suggesting the importance of these genes and pathways in insect herbivory response, and likely, defense. Greater conservation of DEGs between time points was also observed in systemic leaves wherein over 24% of DEGs were conserved between at least two time points in 'GREM4' but only 8% were conserved in 'PN40024'.

Our findings, together with these examples, suggest that genes implicated in insect herbivory response and defense remain differentially expressed for extended periods of time upon insect herbivory in resistant or responsive plants.

Early and Late Response to Insect Herbivory Differ Between 'GREM4' and 'PN40024'

Next, we explicitly explored differences in numbers of genes, the genes themselves, and enrichments present in early (30min) and late (4h) responses to insect herbivory between 'GREM4' and 'PN40024'.

In exploring the number of total DEGs present, an increase, from early to late response, was observed in 'GREM4' herbivory samples resulting in the greatest total number of DEGs at 4h while a decrease was observed in 'PN40024' with the greatest number found at 30min. Examples in the literature, although using time scales greater than 0 to 4h, report great numbers of DEGs are observed, even in susceptible plants, over extended periods of time in response to insect herbivory, suggesting, at the least, the attack is perceived. Some examples include soybean (24h) (Wang et al., 2017), oilseed rape (*Brassica napus*) (12h, 24h, 2d, and 7d) (Sarosh and Meijer, 2007), white cabbage (*Brassica oleracea*) (6h, 24h, 2d, and 3d) (Broekgaarden et al., 2007), and *Arabidopsis* (6h and 24h) (Appel et al., 2014). Further, in resistant plants, the greatest number of DEGs were also typically observed at the terminal time point in the study, such as seen in resistant cotton after 48h of cotton bollworm (*Helicoverpa armigera*) feeding (Huang et al., 2015). Additionally, typically, a greater number of DEGs are observed in resistant compared to susceptible plants. In resistant to susceptible cotton in response to whitefly (*Bemisia tabaci*) feeding, resistant cotton exhibited greater than 1,000 more DEGs after 24h than susceptible; By 48h, the terminal time point, a greater number of DEGs was still observed (Li et al., 2016). Similar responses are seen in resistant versus susceptible

cotton (Dixit et al., 2020) and resistant versus susceptible soybean (Wang et al., 2017). Together, generally speaking, these studies suggest that increased numbers of DEGs are observed in resistant compared to susceptible plants, which, by the late response, was observed in our study.

Importantly, the impact of constitutive defense was not considered in the previous examples. Constitutive defense is a critical component of plant defense and may impact inducible responses, particularly in the regard that heightened constitutive defenses typically result in more mild inducible transcriptomic alterations when a stress is encountered (Rasmann et al., 2015; Whitehill et al., 2021; Zhang et al., 2022c; Lv et al., 2023). Previous work reported that, while DEGs identified when comparing basal expression (0h) between 'GREM4' and 'PN40024' were generally broadly distributed amongst biological functions and pathways, up-regulated DEGs in 'GREM4' were enriched in disease/plant-pathogen interaction-related genes and 'biosynthesis of secondary metabolites', which included terpene biosynthesis genes (Chapter 2). Both genes related to pathogen defense (Ralph et al., 2006; Huang et al., 2015; Zhou et al., 2020; Jan et al., 2022) and terpenes (Liu et al., 2020a; de Albuquerque Lima et al., 2021; Zavala-Gómez et al., 2021; Sun et al., 2022; Wang et al., 2023a) have known associations within damage response and insect herbivory defense suggesting heightened constitutive defense in 'GREM4' compared to 'PN40024'. Surprisingly, however, our results report 'PN40024', by the late response, exhibits a transcriptomic response more similar to that at 0h than was observed of the early response. As 'PN40024' does not appear as well-

positioned for constitutive insect herbivory defense as ‘GREM4’, this declining number of DEGs from 30min to 4h upon insect herbivory in ‘PN40024’, signifying a shift towards a transcriptomic state closer to that of 0h, may hinder defense.

Next, we reviewed differences in individual genes which were identified between early and late response and found greater numbers of genes associated with insect herbivory defense in ‘GREM4’. Some of the topmost differentially expressed genes in ‘PN40024’ in the early response were associated with insect herbivory defense including multiple genes implicated in disease defense and phytohormonal regulation, all of which were up-regulated. However, by the late response, many of the topmost genes were implicated in growth and reproductive processes. Conversely, in ‘GREM4’, while, in the early response, insect herbivory defense genes were up-regulated, and some were some of the topmost differentially expressed genes, the number of these genes were generally limited. However, by the late response, a large number of DEGs implicated in insect herbivory defense were identified in ‘GREM4’, including those related to phenylpropanoid biosynthesis such as *PAL1-1*. *PAL* is a key insect herbivory response and defense gene in many species, such as seen in rice. When comparing 3h of rice leaf roller herbivory to 30min in rice, phenylpropanoid biosynthetic pathway genes *PAL6, PAL7, 4CL6,* and *SHT1* were similarly up-regulated (Zhuang et al., 2022). *PAL* gene expression is also seen to increase upon oriental longheaded grasshopper (*Acrida chinensis*) herbivory in grapevine (Jia et al., 2022). *PAL* is also known to impact resistance. In a screen of 29 carrot (*Daucus carota*) accessions, *PAL1* and *PAL3*

expression generally correlated with insect herbivory resistance, with the highest expression observed in the two most resistant lines (Simlat et al., 2013). Multiple genes related to JA production, such as *(E)-2-methylbutanal oxime monooxygenase* (*CYP71E7*) (*Vitla_GREM4_6g74.45*), *AOS1,* and *Gretchen Hagan 3.1* (AKA – '*AVRPPHB susceptible 3.1'*) (*Vitla_GREM4_3g62.21*) were also observed as DEGs in late response in 'GREM4'. *AOS* is critical to JA accumulation *in planta* (Kongrit et al., 2007) and known to positively impact resistance. In rice, *AOS* expression was found to increase upon both striped stem borer (*Chilo suppressalis*) and brown planthopper (*Niaparvata lugens*) herbivory (Zeng et al., 2021). Silencing either *OsAOS1* or *OsAOS2* resulted in decreased accumulation of JA upon insect herbivory and increased herbivory damage from stem borers, compared to WT (Zeng et al., 2021). Similarly, increased expression was observed of *GmAOS* in resistant soybean compared to susceptible upon cotton worm (*Prodenia litura*) herbivory and, when *GmAOS* was over-expressed in tobacco (*Nicotiana tabacum*), increased resistance, heightened chymotrypsin inhibitor and peroxidase activity, and increased numbers of trichomes were observed (Wu et al., 2008). *AOS* expression was also found to increase in other plants, and upon other insect stressors, such as tea geometrid herbivory of tea (Jing et al., 2024) and in *V. vinifera* grapevine upon two-spotted spider mite (*Tetranychus urticae*) herbivory (Díaz-Riquelme et al., 2016). Terpene-related genes were also found to be some of the most up-regulated DEGs in the late response in 'GREM4' including genes such as *TPS14-1* and *Monoterpene synthase* (*MTPS*) (*Vitla_GREM4_12g74.32*). *TPS*, implicated in many steps in the

terpene biosynthetic process (Sun et al., 2022; Li et al., 2023b), when silenced in rice, lead to increased susceptibility to bird cherry oat aphid (*Rhopalosiphum padi*) compared to WT (Sun et al., 2017). Further, over-expressor plants were more resistant (Sun et al., 2017). In cotton, eight *GhTPS* genes exhibited up-regulation in response to concurrent cotton bollworm and small green plant aphid (*Apolygus lucorum*) herbivory (Huang et al., 2018). Further, tobacco over-expressing *GhTPS12* demonstrated increased resistance to bollworm and aphid herbivory compared to WT (Huang et al., 2018). Overall, genes implicated in phenylpropanoid, flavonoid, and terpene biosynthesis were identified in the late response of 'GREM4', but not in 'PN40024'. Up-regulation of these genes likely positively impact insect herbivory resistance in 'GREM4'.

Herbivory studies in 'GREM4' and 'PN40024' beyond 4h are needed to determine longer-term transcriptomic response to herbivory which would additionally allow for more direct comparisons between studies with larger timescales. Two questions such additional studies could answer include if the trend of increasing numbers of DEGs in 'GREM4' and decreasing numbers if 'PN40024' would continue or change and if different functions, pathways, or genes would be observed beyond 4h, such as perhaps those involved in damage repair. Further, studies exploring temporal expression over multiple time points between an insect herbivory resistant and susceptible cultivar/accession within the same study are needed for many species.

Systemic Response in ‘GREM4’ Primes Unafflicted Leaves via Signaling and Flavonoids for Insect Herbivory Defense in Contrast to ‘PN40024’

Systemic response is an important aspect of plant defense. In our study, when comparing systemic leaves to 0h across time points upon Japanese beetle herbivory, over 814 non-duplicated DEGs were identified in ‘GREM4’ compared to only 109 in ‘PN40024’. Hundreds to thousands of DEGs have been observed in distal, non-afflicted leaves in response to insect herbivory in other studies. However, most investigations have been completed over the course of many hours, or days, or methods deviated considerably from those in our study. Nonetheless, other studies shed some light on the context of responses in our study. Tea fed upon by tea geometrid (in which all time points of 3h, 6h, 9h, 12h, and 24h were pooled for RNA-seq) resulted in 558 total DEGs in systemic leaves (Zhou et al., 2020). In maize, leaves collected 2h after mechanical wounding and were subsequently treated with oriental armyworm (*Mythimna separata*) oral secretions exhibited 276 total DEGs (ul Malook et al., 2019). Two studies in *Arabidopsis,* one afflicted by one of four different insect pests for 6h and the other afflicted by cotton leafworm (*Spodoptera litura*) for 24h, resulted in 134-203 (Appel et al., 2014) and 2,885 DEGs (Xue et al., 2022), respectively. Together these studies support the finding that expression alterations are observed upon insect herbivory in systemic leaves.

Systemic responses have also been shown to provide heightened insect herbivory resistance in unafflicted tissues with key defensive pathways and genes implicated in

such responses. Examples exist across many species including maize (ul Malook et al., 2019, 2021), husk tomato (tomatillo) (*Physalis philadelphica*) (Meza-Canales et al., 2022), rice (Tong et al., 2023), *Arabidopsis* (Xue et al., 2022), and cotton (Mamin et al., 2023). In these studies, exposure to insect herbivory led to differential accumulation of metabolites and altered gene expression which were primarily characterized by heightened levels of gene expression, enrichment, or metabolite accumulation associated with terpenes, trypsin protease inhibitors, JA, signaling pathways, and benzoxazinoids. In our study, when combining all DEGs across time points, in 'GREM4' 16 functional enrichments were identified, none of which were identified in 'PN40024'. Some functional enrichments identified in 'GREM4' were similar to those reported in previous studies, such as those related to signaling (calcium ion signaling, 'lipid transport', 'MAPK signaling pathway') and phytohormone responses ('ethylene-activated signaling pathway'). However, other functional enrichments identified in 'GREM4' were not as widely identified in other studies such as 'defense response to fungus', acyltransferase-related terms, and 'peroxidase activity'. Meanwhile, enrichments in 'PN40024' were nearly exclusively related to protein remodeling, which were unlikely to impact defense. KEGG results supported ORA findings, and reported additional enrichment in signaling, flavonoid biosynthesis, stilbene biosynthesis, phenylpropanoids, and pathogen response genes in 'GREM4', none of which were observed in 'PN40024' (however, one of the three total enriched pathways in 'PN40024' was 'sesquiterpenoid and triterpenoid biosynthesis'). These enrichments in 'GREM4' are well supported by past studies in

which enrichment or induction of genes associated with phenylpropanoid biosynthesis, flavonoid biosynthesis, calcium-ion signaling, and lipid biosynthesis and trafficking pathways have been observed in plants such as wild pigeonpea (*Cajanus scarabaeoides*) (Malhotra et al., 2022), *Arabidopsis* (Appel et al., 2014; Toyota et al., 2018; Xue et al., 2022), tea (Zhou et al., 2020), and others (Schilmiller and Howe, 2005). Overall, these results suggest 'GREM4' exhibited multiple means of systemic signaling and a vast induction of functions and pathways with known insect repellent and insecticidal activities upon insect herbivory, which was not observed in 'PN40024'.

An illustration of systemic response resulting in heightened distal defense can be observed within our study. The *GLR* gene family is known to increase calcium ion concentrations *in planta* to propagate defense signals (Toyota et al., 2018) which are critical to resistance, JA production, MAPK signaling, and phenylpropanoid biosynthesis (Xue et al., 2022). In 'GREM4', four of 14 total *GLR2* gene family members of *GLR2* (*Vitla_GREM4_10g39.50*), *GLR2.1* (*Vitla_GREM4_4g89.48*), *GLR2.7* (*Vitla_GREM4_10g39.49*), and *GLR2.9-like* (*Vitla_GREM4_00g36034*) were identified as up-regulated DEGs exclusively in 'GREM4' systemic leaves after 30min or 1h of herbivory. Further, calcium ion signaling was exclusively enriched in 'GREM4' systemic leaves, and, by extension, likely played a role in the observed heightened systemic expression of defensive secondary metabolite genes thereby contributing to defense.

Comparisons between herbivory and systemic leaves directly revealed roughly 200 DEGs at 30min in 'GREM4', but, by 4h, this number declined to only five. This result suggests that, by 4h, the responses in both systemic and herbivory leaves were similar, but this response took time. Intriguingly, in 'PN40024', only five total DEGs were identified between herbivory and systemic leaves across all time points revealing transcriptomes of herbivory and systemic leaves were similar throughout 30min to 4h of feeding. Considering fewer DEGs with known connections to insect herbivory defense were expressed in 'PN40024' herbivory leaves compared to 'GREM4' as covered above, it reasons that systemic leaves sharing the response of herbivory leaves in 'PN40024' would exhibit similarly hypothetically meager insect herbivory defense. Conversely, considering the transcriptomic response to insect herbivory in 'GREM4' herbivory leaves in which genes associated with pathways with known insect repellent and insecticidal activities were identified, it reasons that systemic leaves nearly mirroring the response of herbivory leaves would exhibit a similarly hypothetically stout defense response in 'GREM4'.

While a systemic insect herbivory resistance challenge assay was not undertaken in this study, it is likely that increased expression of genes associated with defense signaling pathways and secondary metabolites impact insect herbivory resistance in 'GREM4'. Conducting a systemic insect herbivory resistance challenge assay is necessary to determine if insect herbivory indeed acts as a priming mechanism resulting

in decreased herbivory in unafflicted tissues in ‘GREM4’ and how this response may differ from that of ‘PN40024’ and impact overall resistance.

Flavonoid and Phenylpropanoid Biosynthesis Genes Were Exclusively Enriched in ‘GREM4’ Herbivory and Systemic Leaves

In our study, enrichment of functions and pathways related to flavonoids were exclusively enriched in ‘GREM4’. Flavonoids are widely recognized as insect herbivory defensive compounds in plants and are known to be insecticidal (Kariyat et al., 2019; Chatterjee et al., 2022; Chen et al., 2022; Zhang et al., 2022a). For example, flavonoids extracted from sorghum (*Sorghum bicolor*) were found to be insecticidal to fall armyworm and increased mortality was observed of armyworms that fed on maize lines overproducing flavonoids compared to WT lines (Chatterjee et al., 2022). Flavonoids have also been linked to herbivory resistance in rice wherein accumulations of flavonoids were significantly greater in a resistant compared to susceptible cultivar upon brown planthopper feeding (Zhang et al., 2022a). In grapevine, expression of genes associated with flavonoid biosynthesis increased in grapevine hybrid ‘Kyoho’ (*V. vinifera* x *V. labrusca*) when fed upon by oriental longheaded grasshopper along with increased flavonoid concentrations (Jia et al., 2022). These findings illustrate that genes implicated in flavonoid biosynthesis up-regulate upon insect herbivory and that flavonoids exhibit insecticidal activity and deter insect herbivory, increasing resistance. In our study, flavonoid biosynthesis genes, such as *FLS2* and *FLS/F3H,* were identified as DEGs in both herbivory and systemic leaves exclusively in ‘GREM4’ while, additionally, the

KEGG pathway of ‘flavonoid biosynthesis’ was exclusively enriched in ‘GREM4’ herbivory and systemic leaves. Further, flavonoid biosynthesis gene *F3H* (*Vitla_GREM4_4g210.29*) was identified as a candidate gene in ‘GREM4’ as it was one of the topmost differentially expressed genes upon insect herbivory. *FLS* and *F3H* have previously been shown to be implicated in insect herbivory resistance. When *F3H* was overexpressed in rice, significantly increased accumulation of flavonoids and decreased whitebacked planthopper (*Sogatella furcifera*) herbivory was observed (Jan et al., 2020). For *FLS,* in resistant pigeonpea, upon cotton bollworm herbivory, *FLS* was one of many flavonoid genes to be significantly up-regulated (Tyagi et al., 2022), a response also observed in cassava (*Manihot esculenta*) upon two-spotted spider mite herbivory (Chen et al., 2022), lending support to their hypothetical role in ‘GREM4’ resistance. Interestingly, when combining all down-regulated systemic DEGs across all time points in ‘PN40024’, ‘biosynthesis of secondary metabolites’ was enriched suggesting the process is down-regulated in ‘PN40024’ upon insect herbivory.

The phenylpropanoid pathway is specific to plants and is a critical upstream pathway, or creates derivatives implicated in, multiple secondary metabolites critical to plant defense (La Camera et al., 2004). These include SA, stilbenes, coumarins, lignins, and flavonoids (La Camera et al., 2004; Chang et al., 2019). The first three steps of the pathway are catalyzed by the enzymes PAL, cinnamic acid 4-hydroxylase (C4H), and 4CL, respectively (La Camera et al., 2004). In our study, *C4H* was identified as an up-regulated DEG exclusively in ‘GREM4’ 4h in herbivory samples. A previous study,

notably, had reported the *PAL1* gene family had 12 total members in 'GREM4' compared to only four in 'PN40024', an increase of eight genes (Chapter 2). In this study, five *PAL1* family members in 'GREM4' were significantly up-regulated upon insect herbivory (four of which were unique to 'GREM4') while none of the four *PAL1* genes in 'PN40024' were significantly differentially expressed. Herein, it was found that four *PAL1* gene family members displayed significant induction in systemic leaves, three of which were unique to 'GREM4' (*PAL1-4, PAL1-7,* and *PAL1-8*). One of these genes, *PAL1-7,* was only seen to uniquely respond in systemic leaves, as it was not differentially expressed at any time point in herbivory leaves. This suggests *PAL1-7* has evolved novel function in 'GREM4' as a systemic response-specific, but perhaps to other regulatory cues as well, *PAL1* gene and that regulation of this gene is unique from other gene family members. This example captures well the classical phenomenon of gene family expansion with subsequent specialization resulting in novel responses and functionalities of genes. In another study, oriental longheaded grasshopper feeding on *V. vinifera* x *V. labrusca* hybrid 'Kyoho' was found to result in significantly increased expression of genes associated with flavonoid and phenylpropanoid pathways, namely *flavonoid 3-monooxygenase* (*F3M*), *FLS*, and *PAL* (Jia et al., 2022). In cassava, DEGs in insect resistant lines were enriched in 'phenylpropanoid biosynthesis' and 'flavonoid biosynthesis' compared to susceptible lines and increased accumulations of phenylpropanoid and flavonoid pathway compounds were also identified upon two-spotted spider mite (*Tetranychus urticae*) herbivory (Chen et al., 2022). In cotton,

multiple genes, including *PAL* and multiple genes in the flavonoid biosynthetic pathway like *CHI* (*Chalcone isomerase*), *ANR* (*Anthocyanin reductase*) and *ANS* (*Anthocyanin synthase*), were even found to be consistently up-regulated after 24h, 2d, 3d, and 4d of continuous feeding by chewing mouthpart insects (Dixit et al., 2020). It is likely that increased expression of genes in the phenylpropanoid pathway, especially *PAL*, play an important role in providing reactants for downstream processes, especially the biosynthesis of flavonoids.

Acyltransferases Were Exclusively Enriched in 'GREM4' Herbivory and Systemic Leaves

Acyltransferase-related terms were the most consistently identified enrichment amongst up-regulated DEGs in 'GREM4'. In contrast, acyltransferase-related enrichment was never observed in 'PN40024' in any comparison. 'Acyltransferase activity' is widely implicated across plant processes (Darzi et al., 2018) and dictates transfer of acyl groups from one compound to another (EMBL-EBI, n.d.). Acyltransferase activity related to modification of secondary metabolites (D'Auria, 2006) is a plausible connection to insect herbivory resistance in 'GREM4' considering the important role of secondary metabolites in plant defense and the complementary enrichment of secondary metabolite functional and pathway terms identified in our study. One gene annotated as exhibiting acyltransferase activity, *Defective in cuticular ridges* (*DCR*) (*Vitla_GREM4_8g159.4*), a BAHD acyltransferase that incorporates 9_{10},16-dihydroxy-hexadecanoic acid onto cutin (Panikashvili et al., 2009), was implicated in all

three herbivory time points in ‘GREM4’. BAHD acyltransferases are a large sub-group of acyltransferases involved in the biosynthesis of many secondary metabolites, such as anthocyanin, wax, capsaicin, and methyl anthranilate. This result, along with the identification of candidate genes implicated in wax biosynthesis including *O-methyltransferase* (*Wax synthase/acyl-CoA:diacylglycerol acyltransferase*) (*WSD1*) (*Vitla_GREM4_12g40.2*) and *Mid-chain alkane hydrolase 1* (*MAH1*) (*Vitla_GREM4_14g270.32*), suggests cuticle or wax biosynthesis may have been up-regulated in ‘GREM4’ from 30min to 4h after herbivory initiation. Another BAHD, *Acylsugar acyltransferase 2* (*ASAT2*), that was involved in insecticidal compound formation in *Nicotiana benthamiana*, when knocked out in another study, resulted in a decrease in insecticidal acylsugars *in planta* (Marchant et al., 2020), and insect resistance, compared to WT plants (Feng et al., 2021). This example supports the importance of BAHDs in insect herbivory resistance. It is possible that acyltransferase genes play similar roles in ‘GREM4’ in modifying secondary metabolites for increased defense.

Conclusions

Overall, these findings provide evidence for leveraging the genetic diversity inherent in wild grapevines as a means to enhance herbivory resistance in cultivated varieties. In our transcriptomic study herein, ‘GREM4’ and ‘PN40024’ were found to exhibit differing temporal and systemic responses to Japanese beetle herbivory. While the number of DEGs from early (30min) to late (4h) response increased in ‘GREM4’, in ‘PN40024’, DEGs decreased. Genes and enrichments associated with functions and

pathways with known insect repellent and insecticidal activities were identified in ‘GREM4’ upon herbivory and in systemic response leaves such as terpenes, phenylpropanoids, and flavonoids. In ‘GREM4’ systemic leaves, signaling-related genes and enrichments were identified but were nearly completely absent in ‘PN40024’. Together, these results report a transcriptomic response in both herbivory-afflicted and systemic leaves which suggest a heightened capacity for insect herbivory defense in ‘GREM4’ compared to ‘PN40024’. This assertion requires validation from future metabolomic or functional studies. To aid in these investigations, lists of candidate genes are provided which we suggest are reasonable targets for functional studies. Testing the effect of knock-out of a selection of these genes on insect herbivory resistance can validate their role in defense and further elucidate the genetic underpinnings of the insect herbivory resistant phenotype observed in ‘GREM4’. The candidate genes, in addition to enrichment results, may also help guide metabolomic analyses toward classes of metabolites which may be found to be differentially accumulating upon insect herbivory. Gaining a better understanding of the insect herbivory defenses of ‘GREM4’ holds promise to improve insect herbivory resistance in susceptible *Vitis* species and cultivars through breeding and transgenesis, thereby diminishing losses in the field attributed to insect damage, ultimately contributing to increased food production and the improvement of growers' livelihoods.

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Availability of Data and Materials

The datasets presented in this manuscript can be found within the main text and the links provided for some tables. In addition, the raw transcriptomic data underlying the results of this manuscript are available at NCBI (https://www.ncbi.nlm.nih.gov/) via BioProject number PRJNA1070606, or by request. The genome annotation may be found on GitHub at https://github.com/cdixo/Vitis-labrusca-Version-2-Genome-Annotation.git . Custom codes used for analyses and creation of the updated genome annotation are also available on GitHub at https://github.com/cdixo/Inter-species-and-Herbivory-Publication.git . We also refer you to Chapter 2 for additional information.

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Figures

Figure 3.1 – Herbivory Time Course Study Experimental Design

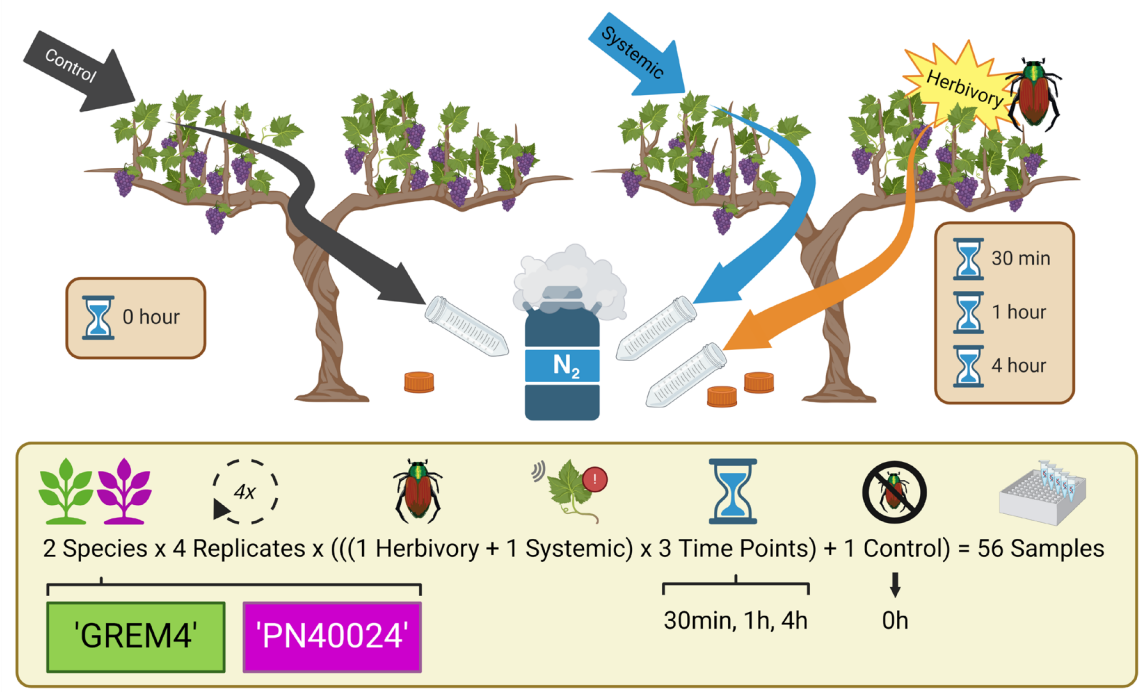
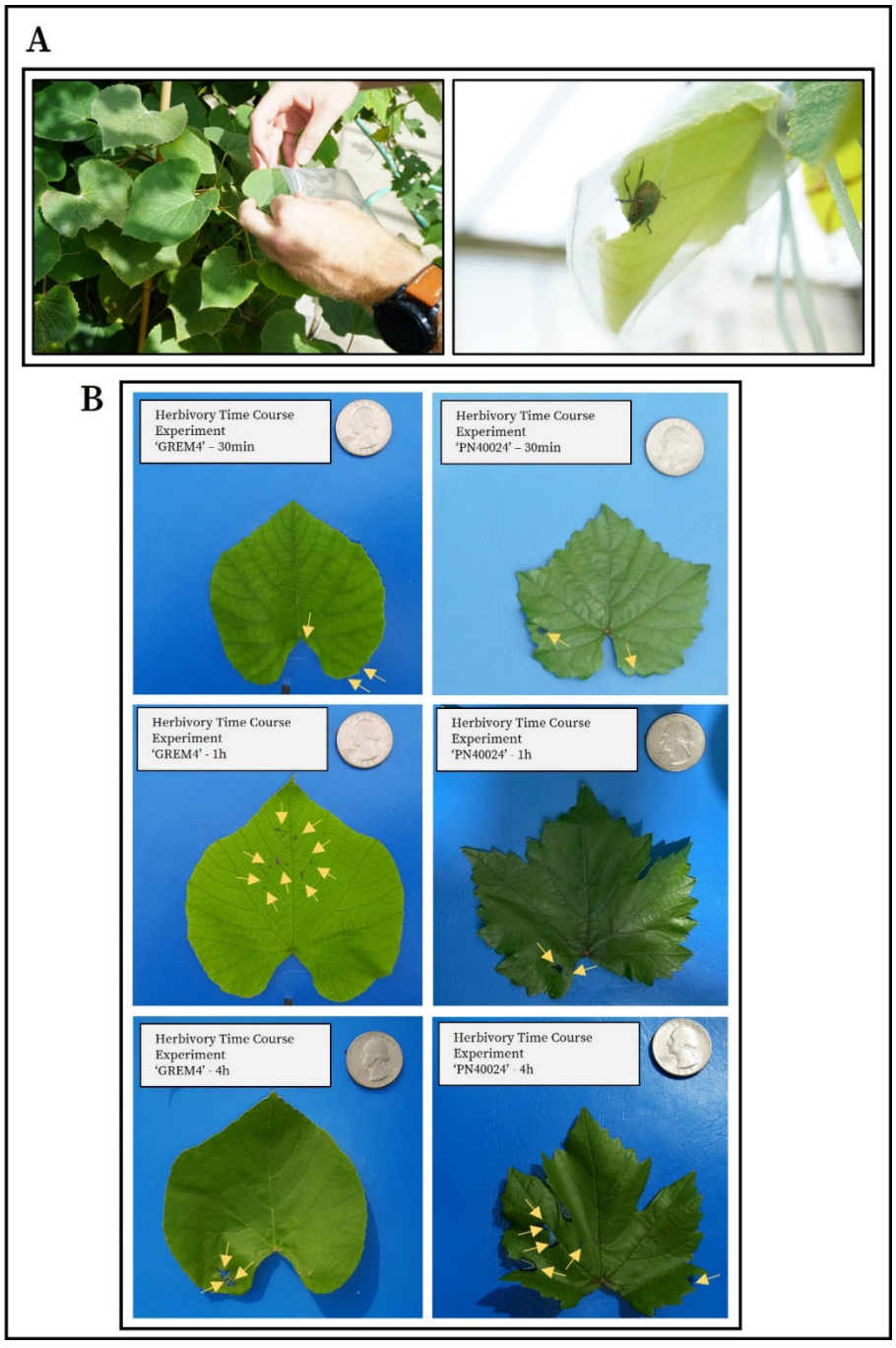


Figure 3.1 Caption – A depiction of the experimental design of the transcriptomic study. Herbivory, systemic, and 0h control tissue collection is depicted. 0h samples were collected before beetle placement while herbivory and systemic samples were collected concurrently after 30min, 1h, and 4h of herbivory by the beetle. Created with BioRender.com.

Figure 3.2 – Insect Herbivory Leaf Images



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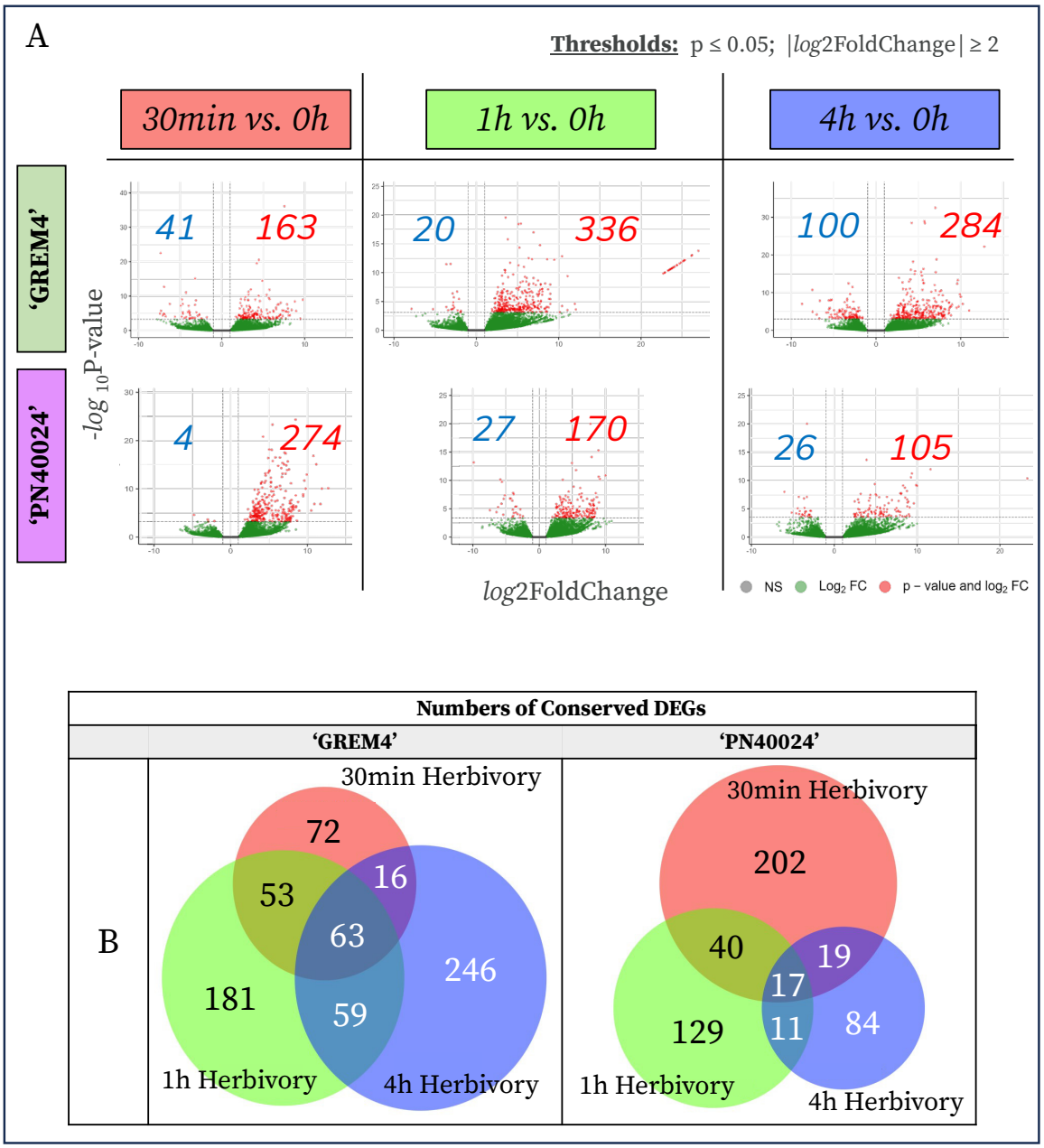
(Figure 3.2 continued)

Figure 3.2 Caption – Images from the herbivory time course study events which generated the insect-herbivory-afflicted leaf tissues which were sequenced for our transcriptomic study herein. A. Leftmost Picture – Placing a mesh bag over a grapevine leaf prior to placing a beetle within to start an experimental run. All leaves were mature and of the same size as they had to fit within the bag without folding. Rightmost Picture – A Japanese beetle in a bag during a feeding run. B. Representative images depicting damage from Japanese beetles in 'GREM4' and 'PN40024' leaves from the herbivory time course study. Images show feeding after 30min, 1h, and 4h. Arrows indicate locations of feeding damage.

Figure 3.3 – Numbers and Conservation of DEGs Between Direct Insect Herbivory Responses



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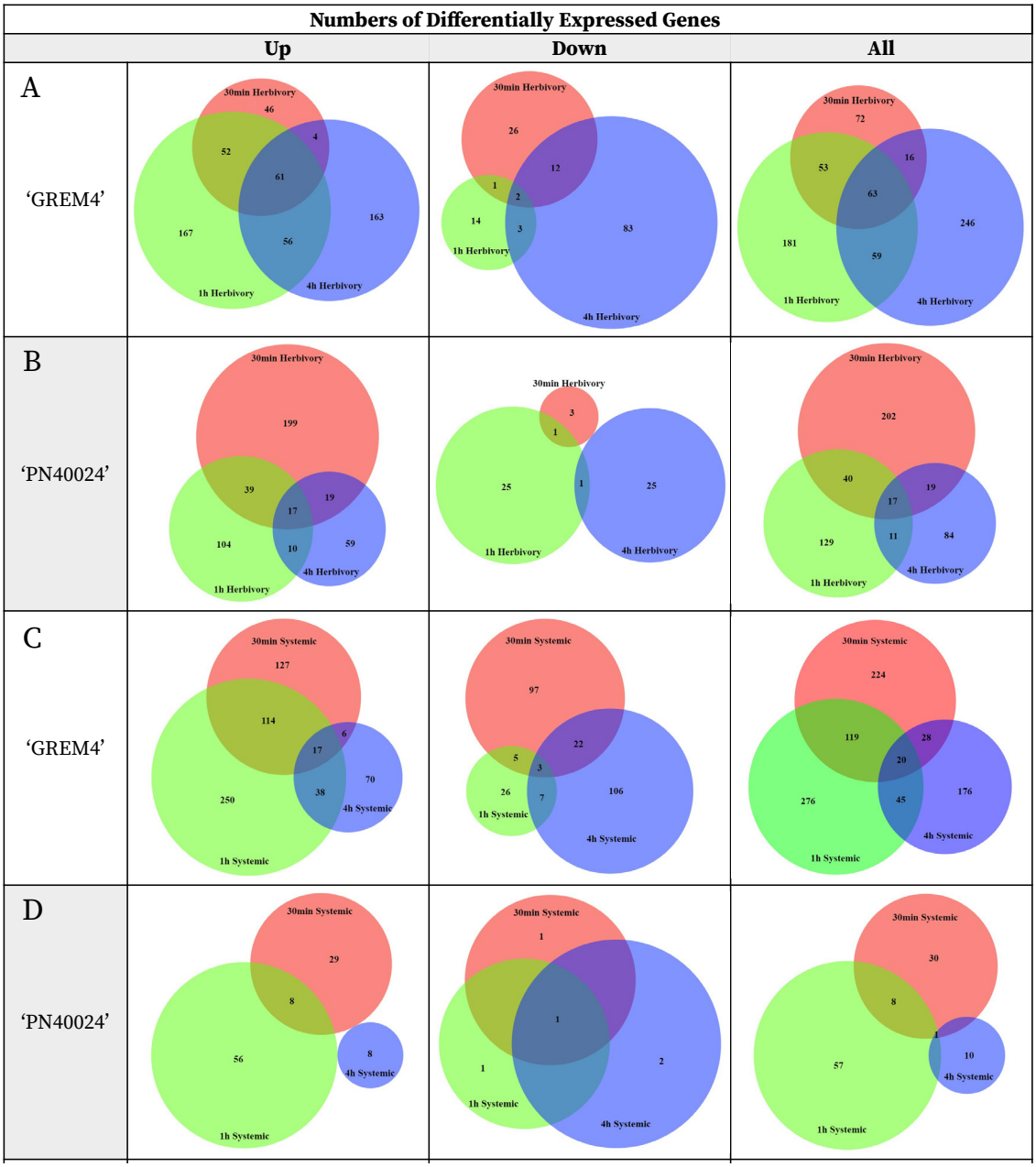
(Figure 3.3 continued)

Figure 3.3 Caption – Expression results from herbivory leaves after 30min, 1h, and 4h of Japanese beetle herbivory. A. Volcano plots depicting numbers of DEGs in 'GREM4' and 'PN40024' under insect herbivory when compared to 0h using a $|log2FoldChange| \geq 2$ and a p.adj value ≤ 0.05 to determine significance. Red and blue inlaid numbers report the numbers of up- and down-regulated DEGs, respectively. B. Venn diagrams depicting the number of overlapping and unique DEGs between 30min, 1h, and 4h of insect herbivory in 'GREM4' and 'PN40024', independently. Up- and down-regulated DEGs at each time point were combined for the overlap analysis depicted by these Venn diagrams.

Figure 3.4 – DEG Conservation (Venn Diagrams) In Transcriptomic Responses in Grapevine



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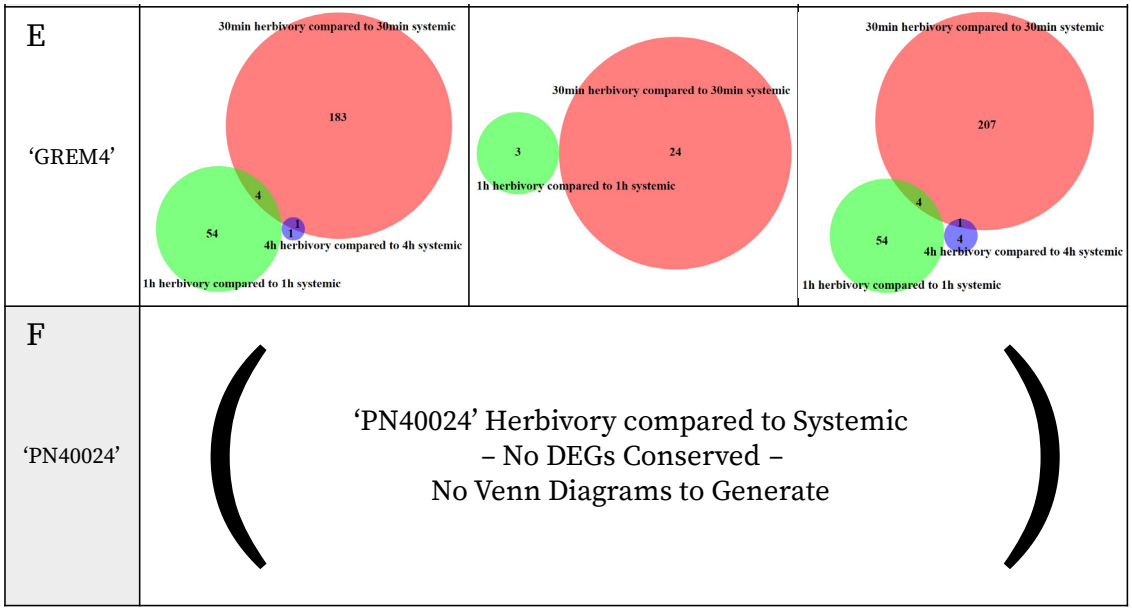
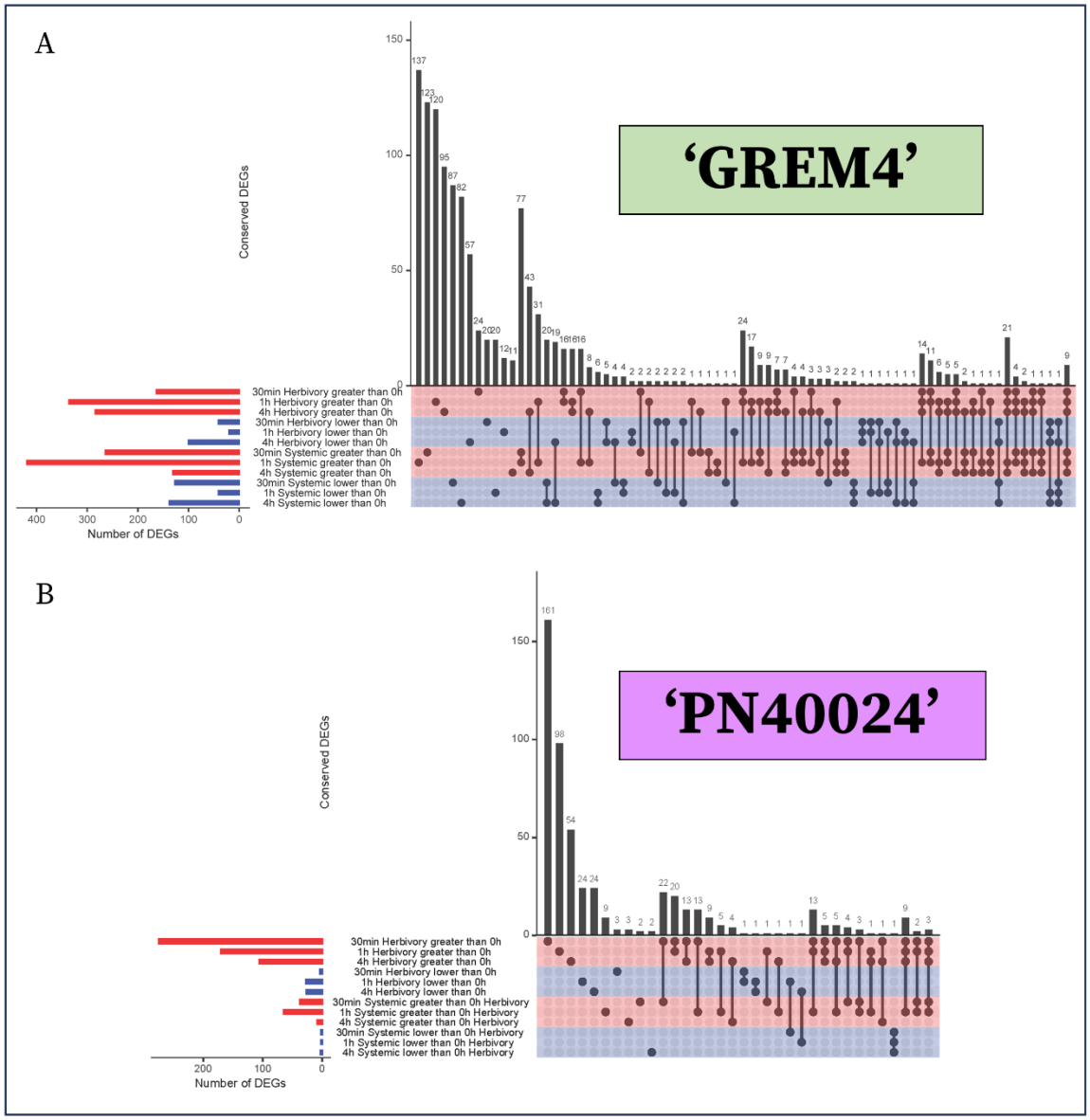


Figure 3.4 Caption – Venn diagrams depicting the number of overlapping and unique DEGs between groups of DEGs in various comparisons. Presented are Venn diagrams generated using up-regulated DEGs only ('Up'), down-regulated DEGs only ('Down'), and when all up- and down-regulated DEGs are combined ('All'). The 'All' comparison Venn diagram for A-D can also be found in other figures but are presented here as well for comparative purposes. Comparisons presented herein include herbivory compared to 0h (A & B), systemic compared to 0h (C & D), and herbivory compared to systemic (E & F). F. No DEGs were conserved between any time point for herbivory compared to systemic comparisons in 'PN40024', therefore, all DEGs were unique and no Venn diagrams were created.

Figure 3.5 – DEG Conservation (Upset Plot) In Transcriptomic Responses in Grapevine



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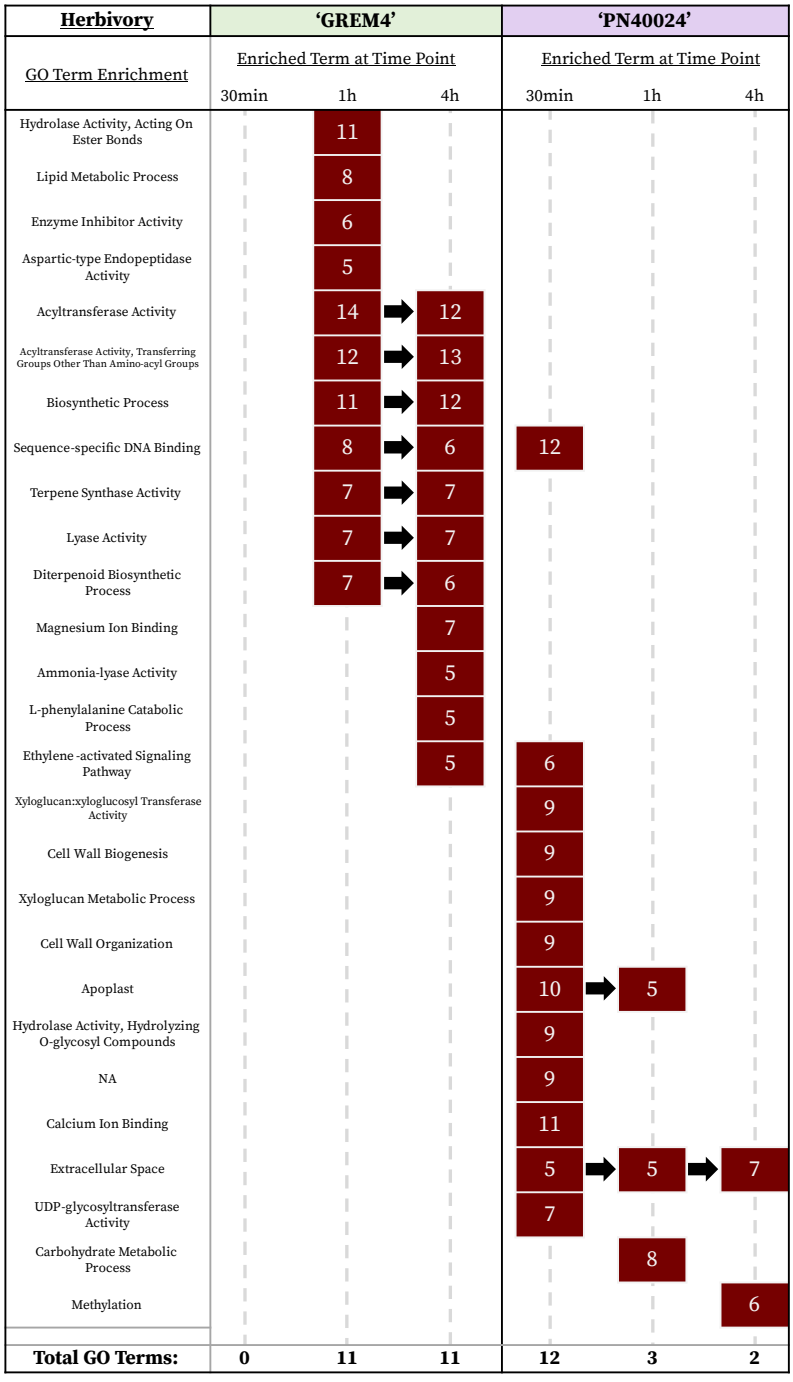
(Figure 3.5 continued)

Figure 3.5 Caption – UpSet plot illustrating the intersection (or overlap) among all sets of DEGs simultaneously. For this analysis, up- and down-regulated DEGs in herbivory and systemic responses were not combined and were further broken down by time point. Subsequently, these lists were simultaneously queried for overlaps, the results seen above. 'GREM4' (A) and 'PN40024' (B) comparisons were conducted independently. All comparisons were compared against 0h (control). Numbers of DEGs in each comparison (size of the lists of gene names) can be seen in the bottom left horizontal bar graph. Numbers of DEGs conserved, or unique, can be seen in the vertical bar graph, center. Single, unconnected dots in the matrix represent DEGs which were exclusively identified within only the noted comparison while dots connected by lines illustrate conservation of DEGs between the connected comparisons. Red indicates the set of genes is an up-regulated DEG dataset while blue indicates the gene set is a down-regulated DEG dataset. Graphic created via UpSetR.

Figure 3.6 – Herbivory Enrichment Graphic



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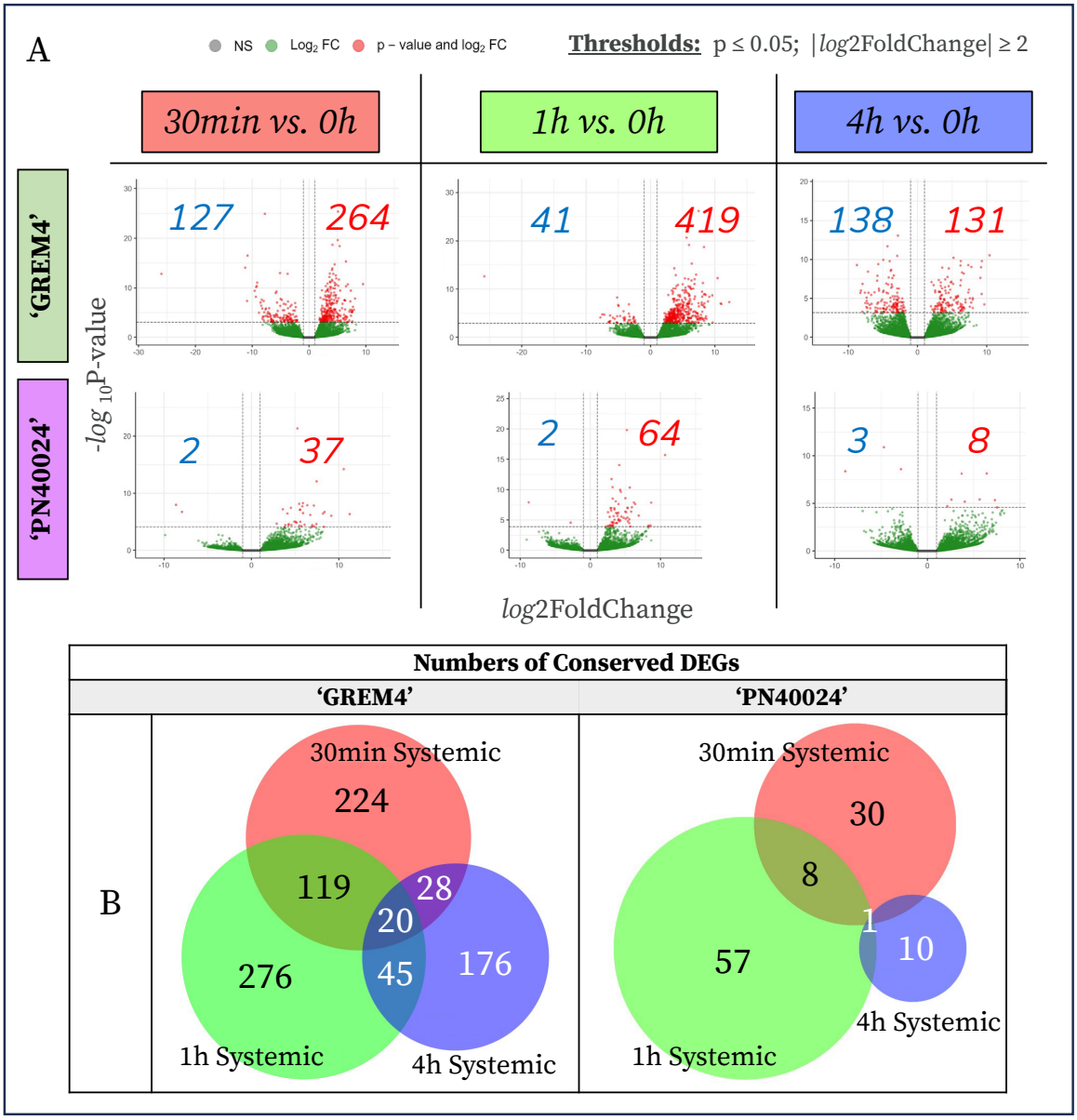
(Figure 3.6 continued)

Figure 3.6 Caption – Gene Ontology (GO) term functional enrichments in herbivory leaves in 'GREM4' and 'PN40024' at the time points 30min, 1h, and 4h. Significant enrichments were defined as p.adj ≤ 0.05 and if the number of implicated genes was ≥ 5 DEGs. A box to the right of the GO term indicates it was significantly enriched at the corresponding time point and species, both listed at the top of the column. The color of the box signifies if the list of DEGs from which the enrichment was identified were up- (maroon) or down-regulated (navy); The number within the box indicates the number of DEGs implicated in the enriched biological function. If an enrichment is found at more than one time point in a species, the boxes are connected with an arrow to indicate conservation between time points. If a box is not present for a given time point and functional enrichment, the function was not significantly enriched.

Figure 3.7 – Numbers and Conservation of DEGs Between Systemic Responses



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(Figure 3.7 continued)

Figure 3.7 Caption – Expression results from systemic leaves after 30min, 1h, and 4h of Japanese beetle herbivory. A. Volcano plots depicting numbers of DEGs in 'GREM4' and 'PN40024' in systemic leaves when compared to 0h using a $|log2FoldChange| \geq 2$ and a p.adj value ≤ 0.05 to determine significance. Red and blue inlaid numbers report the numbers of up- and down-regulated DEGs, respectively. B. Venn diagrams depicting the number of overlapping and unique DEGs in systemic leaves between 30min, 1h, and 4h of insect herbivory in 'GREM4' and 'PN40024', independently. Up- and down-regulated DEGs at each time point were combined for the overlap analysis depicted by these Venn diagrams.

Figure 3.8 – *PAL1* Gene Family Expansion Expression in Systemic Leaves

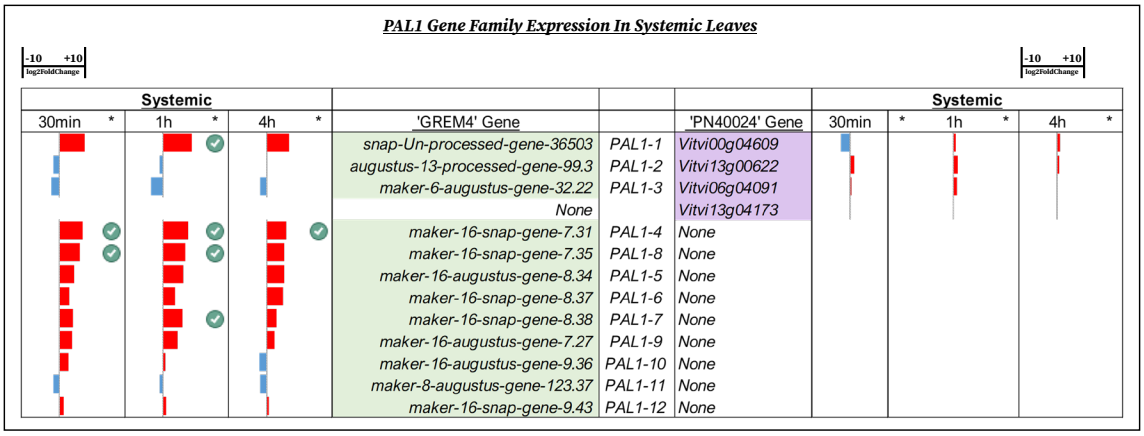
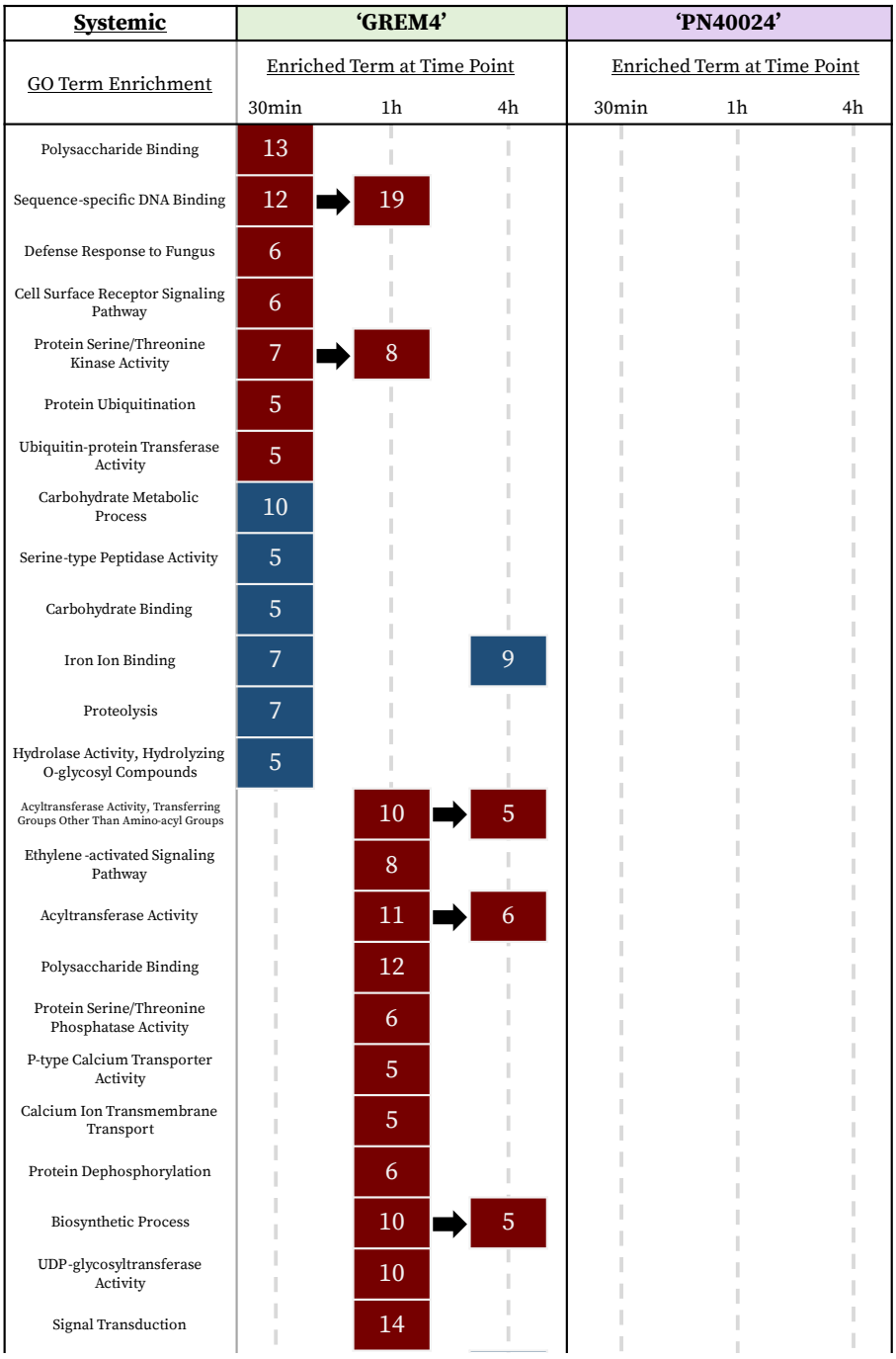


Figure 3.8 Caption – Expression of expanded (paralogous) gene family members in the *PAL1* gene family in systemic response leaves in 'GREM4' and 'PN40024'. All genes in the gene family are listed with expression depicted via horizontal bar graphs. Expression is reported via log2FoldChange where red denotes increased expression while blue denotes decreased. Green check marks indicate the change in expression was significant (p-adj ≤ 0.05).

Figure 3.9 – Systemic Enrichment Graphic



Continued…

(Figure 3.9 continued)

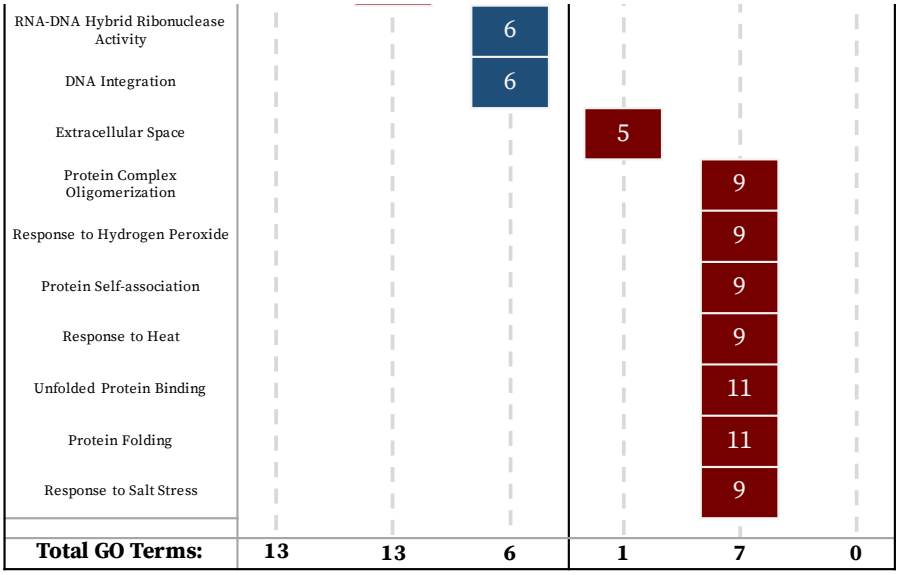
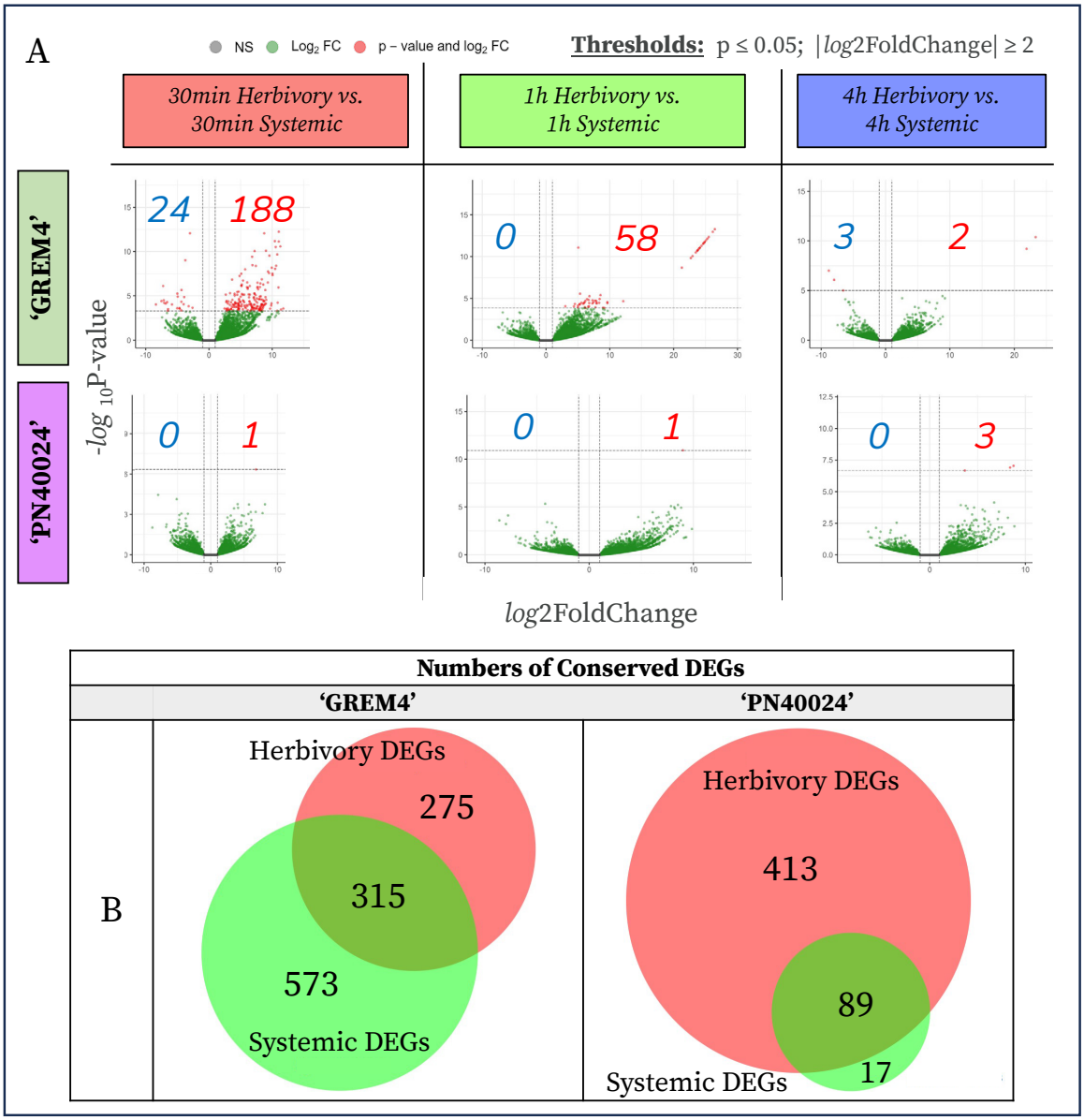


Figure 3.9 Caption – Gene Ontology (GO) term functional enrichments in systemic leaves in 'GREM4' and 'PN40024' at the time points 30min, 1h, and 4h. Significant enrichments were defined as p.adj ≤ 0.05 and if the number of implicated genes was ≥ 5 DEGs. A box to the right of the GO term indicates it was significantly enriched at the corresponding time point and species, both listed at the top of the column. The color of the box signifies if the list of DEGs from which the enrichment was identified were up- (maroon) or down-regulated (navy); The number within the box indicates the number of DEGs implicated in the enriched biological function. If an enrichment is found at more than one time point in a species, the boxes are connected with an arrow to indicate conservation between time points. If a box is not present for a given time point and functional enrichment, the function was not significantly enriched.

Figure 3.10 – Numbers and Conservation of DEGs Between Herbivory vs. Systemic Responses



Continued…

(Figure 3.10 continued)

Figure 3.10 Caption – Expression results from herbivory compared to systemic leaf comparisons after 30min, 1h, and 4h of Japanese beetle herbivory. A. Volcano plots depicting numbers of genes with significantly different expression between herbivory and systemic leaves in 'GREM4' and 'PN40024' at time points of 30min, 1h, and 4h. DEGs were defined using a $|\log2FoldChange| \geq 2$ and a p.adj value ≤ 0.05 to determine significance. Red and blue inlaid numbers report number of DEGs with greater, or lower, expression in herbivory compared to systemic leaves, respectively. B. Venn diagrams in 'GREM4' and 'PN40024' depicting the number of overlapping and unique DEGs between herbivory and systemic leaves when all up- and down-regulated DEGs across all time points were combined.

Tables

Table 3.1 – Expression Results for All Genes

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Table 3.2 – DEG Expression Results

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Table 3.3 – ‘GREM4’ Direct Herbivory Insect Herbivory Resistance Candidate Genes

Top Significantly Differentially Expressed ‘GREM4’ Genes Under Herbivory at Any Time Point[a]					
#	⇅	Biological Implication:	Abbreviated Gene Name:	Full Gene Name:	*V. labrusca* acc. ‘GREM4’ Gene:
1	↑	**Possible Oxidative Stress Signaling or Pathogen Defense**	*PRP4*	*Vitla_GREM4_3g23.32/23.45*	*Proline-rich protein 4*
2	↑	-	-	*Vitla_GREM4_6g212.27*	-
3	↑	**Biotic/Insect Herbivory Response**	*GELP*	*Vitla_GREM4_18g320.0*	*GDSL esterase/lipase*
4	↑	**Wax Biosynthesis; Plant Development**	*WSD1*	*Vitla_GREM4_12g40.2*	*O-methylransferase (Wax synthase/acyl-CoA:diacylglycerol acyltransferase)*
5	↑	**Fungal Defense; Glucosinolate Processing**	*BGLU16*	*Vitla_GREM4_13g317.61*	*Beta glucosidase 16*
6	↑	**Response to SA; Cell Wall Formation**	*GRP5-like 1*	*Vitla_GREM4_7g96.2*	*Glycine rich protein 5 -like 1*
7	↑	-	-	*Vitla_GREM4_14g4.6*	-
8	↑	**Response to SA; Cell Wall Formation**	*GRP5-like 2*	*Vitla_GREM4_7g95.10*	*Putative Glycine rich protein 5 -like 2*
9	↑	**Possible Pathogen or Abiotic Defense Response**	*GDSL1-like*	*Vitla_GREM4_18g181.27*	*GDSL esterase/lipase -like1*
10	↑	**JA Biosynthesis**	*AOS3*	*Vitla_GREM4_3g53.38*	*Allene oxide synthase 3*
11	↑	**Phytohormone Regulation; Antioxidant and Defense Metabolite Biosynthesis**	*CYP-like*	*Vitla_GREM4_15g170.54*	*Cytochrome P450 -like*
12	↑	**Terpene Biosynthesis**	*BAS isoform X2/CAMS1*	*Vitla_GREM4_10g108.60*	*Beta-amyrin synthase isoform X2 / Camelliol C synthase 1*
13	↑	**Phosphate Transport**	*PHO1-like 3*	*Vitla_GREM4_1g132.33*	*Phosphate 1-like 3*
14	↑	**Pectin Cell Wall Remodeling; Pathogen Resistance**	*PMEI25*	*Vitla_GREM4_13g203.16*	*Pectin methylesterase inhibitor 25*

Continued…

(Table 3.3 continued)

15	↑	**Lipid Biosynthesis, Proteolysis Protection, Signaling, and Biotic Sensing**	*TLC*	*Vitla_GREM4_18g109.56*	*TRAM/LAG1/CLN8 (TLC) lipid-sensing domain containing protein*
16	↑	**Disease Resistance; SAR and Ethylene Induction**	*GLIP1*	*Vitla_GREM4_19g86.34*	*GDSL esterase/lipase 1*
17	↑	**Plant Development**	*CFL1*	*Vitla_GREM4_5g189.22*	*Curly flag leaf 1*
18	↑	**Cell Wall and Cellulose Organization**	*GUN11*	*Vitla_GREM4_19g85.51*	*Endoglucanase 11*
19	↑	**Terpene Biosynthesis**	*CYP716A1*	*Vitla_GREM4_18g311.31*	*Cytochrome P450 monooxygenase, family 716, subfamily A, polypeptide 1 / Beta-amyrin 28-monooxygenase-like*
20	↑	**Primary and Secondary Metabolism**	*AAE6*	*Vitla_GREM4_4g100.5*	*Acyl-activating enzyme 6*
21	↑	**Fungal and Nematode Defense**	*GRP1-like*	*Vitla_GREM4_00g36202*	*Putative Glycine rich protein 1 -like 1*
22	↑	**Growth Regulation**	*GRF5*	*Vitla_GREM4_9g12.50*	*Growth regulating factor 5*
23	↑	**Flavonoid Biosynthesis**	*F3H*	*Vitla_GREM4_4g210.29*	*Flavanone 3-hydroxylase*
24	↑	**Disease Resistance; SAR and ETH Induction**	*GDSL-like*	*Vitla_GREM4_18g317.26*	*GDSL-like lipase/Acylhydrolase*
25	↑	-	-	*Vitla_GREM4_5g213.11*	-
26	↑	**Activates an Anthocyanin Repressor; Enhanced Lignin Biosynthesis**	*MYB306*	*Vitla_GREM4_1g129.23*	*MYB related protein 306*

Footnote: a. Candidate genes were selected by identifying any DEGs, in any time point of 30min, 1h, or 4h, with a $|\log2FoldChange| \geq 20$ and a $p.adj \leq 0.01$. All genes matching these parameters were up-regulated.

Table 3.4 – Top 10 DEGs Identified in Early and Late Herbivory Response in 'GREM4' and 'PN40024'

Top 10 DEGs Identified in Early and Late Herbivory Response in 'GREM4' and 'PN40024'[a]

	'GREM4'				'PN40024'			
	#	⇅	Biological Implication:	Abbreviated Gene Name[b]:	#	⇅	Biological Implication:	Abbreviated Gene Name[b]:
30min	1	↑	(BLAST Reports No Characterized Hits)	-	1	↑	Abiotic Stress Tolerance; Putative Insect Pest Response	*DREB1E-like*
	2	↑	Ethylene Biosynthesis and Response	*ABR1-like*	2	↑	Positive Regulator of Leaf and Root Cellular Expansion and Brassinosteroids	*EXO*
	3	↑	Cuticular Wax Formation	*MAH1*	3	↑	Ethylene Biosynthesis and Response	*ABR1-like*
	4	↑	Ethylene Response Factor; Wax Biosynthesis	*WIN1*	4	↑	Disease Response Transcription Factor; Ethylene Regulation	*ERF017-1*
	5	↑	Uncharacterized Acyltransferase	-	5	↑	Disease Response Transcription Factor; Ethylene Regulation	*ERF017-2*
	6	↑	Terpene Biosynthesis	*TPS14-1*	6	↑	Regulation of Insect Defense, General Defense, JA, and Wounding Response;	*TIFY5A/JAZ8*
	7	↑	Acyltransferase; Disease Resistance	*SPCL-like 45*	7	↑	Cell Wall and Xyloglucan Biogenesis	*XTH2*
	8	↑	(BLAST Reports No Characterized Hits)	-	8	↑	JA Response; Drought Tolerance and Root Architecture	*OPR11*
	9	↑	Inducible Defense Signaling	*CRK26*	9	↑	(BLAST Reports No Characterized Hits)	-
	10	↑	Associated with Increased SA; Pathogen Defense; Glucosinolate Transport	*UMAMIT14/ WAT1-related*	10	↑	(BLAST Reports No Characterized Hits)	-
4h	1	↑	SA/MeSA Regulation	*SAMT2*	1	↑	Pollen Grain and Germ Tube Growth; Stomatal Opening Regulation	*PLA2-ALPHA*
	2	↑	Cytochrome P450-associated; Similar Structure to Sunflower *AOS*	*CYP71E7*	2	↑	Ethylene Biosynthesis and Response	*ABR1-like*
	3	↑	Ethylene Biosynthesis and Response	*ABR1-like*	3	↑	Nodule Development in Roots	*ENOD2-1*
	4	↑	(BLAST Reports No Characterized Hits)	-	4	↑	(BLAST Reports No Characterized Hits)	-
	5	↑	Terpene Biosynthesis	*TPS14-1*	5	↑	Glutathione Biosynthesis; Negative Regulator of Salt and Drought Stress Signaling	*GSTU17*
	6	↑	Stilbene Biosynthesis	*STS1-4*	6	↑	Nodule Development in Roots	*ENOD2-2*
	7	↑	(BLAST Reports No Characterized Hits)	-	7	↑	Nodule Development in Roots	*ENOD2-3*
	8	↑	Cuticular Wax Formation	*MAH1*	8	↑	Methylglyoxal (Cytotoxic Development and Abiotic Stress Regulator) Detoxification	*GLYI7*
	9	↑	Phenylpropanoid Biosynthesis	*PAL1-1*	9	↑	Fungal Resistance; Response to ABA	*MYB78*
	10	↑	Transcription Factor	*bHLH167*	10	↑	Plant Immune Signaling to Insects and Pathogens	*LecRK VII.1*

Footnote: a. 10 DEGs (which were defined as genes exhibiting a |log2FoldChange| ≥ 2 and p.adj ≤ 0.05 by default) with the greatest |log2FoldChange| at 30min and 4h were identified in both 'GREM4' and 'PN40024'. b. '-' indicates that no functional annotation was identified for the gene via our annotation pipeline.

Table 3.5 – Gene Conservation Lists

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Table 3.6 – ORA GO Term Enrichments

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Table 3.7 – KEGG Pathway Enrichments

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Table 3.8 – ‘GREM4’ Systemic Response Insect Herbivory Resistance Candidate Genes

Top Significantly Differentially Expressed ‘GREM4’ Genes From Systemic Tissues at Any Time Point[a]					
#	⇅	Biological Implication:	Abbreviated Gene Name:	Full Gene Name:	*V. labrusca* acc. ‘GREM4’ Gene:
1	↓	**Cuticular Wax Formation; Plant Development**	*nsLTP*	*Vitla_GREM4_6g91.3*	*Non-specific lipid transfer protein*
2	↑	**Response to Mite Herbivory and ETH; Negative Regulation of Fungal Defense; Glucosinolate Biosynthesis**	*ERF9-like*	*Vitla_GREM4_7g208.54*	*ERF9-like*
3	↓	**Response to ABA, Drought, and Salt Stress**	*RD22*	*Vitla_GREM4_4g39.9*	*Responsive to desiccation 22*
4	↑	**(BLAST Reports No Characterized Hits)**	-	*Vitla_GREM4_8g102.2*	-
5	↓	**Cell Wall Modification; Plant Development**	*TPRP-F1*	*Vitla_GREM4_11g56.15*	*36.4 kDa proline-rich protein*
6	↑	**ETH Biosynthesis and Response**	*ABR1-like*	*Vitla_GREM4_18g199.44*	*ABA repressor 1-like*
7	↓	**Plant/Stigma/Flower Development**	*Stigma-specific STIG1-like protein 1*	*Vitla_GREM4_10g4.11*	*Stigma-specific STIG1-like protein 1*
8	↑	**Regulation of Insect Defense, General Defense, JA Response, and Wounding Response; Induced by MeJA;**	*TIFY5A/JAZ8*	*Vitla_GREM4_10g104.33*	*Conserved motif (TIF[F/Y]XG) containing protein 5a / Jasmonate-ZIM-domain protein 8*
9	↑	**SA/MeSA Regulation**	*SAMT2*	*Vitla_GREM4_00g36975*	*Salicylate carboxymethyl transferase 1*

Footnote: a. Candidate genes were selected by identifying any DEGs, in any time point of 30min, 1h, or 4h, with a |log2FoldChange| ≥ 10 and a p.adj ≤ 0.01 .

Chapter 4 - Comparative Metabolomic Analysis Reveals Differential Accumulation of Insect Herbivory Defensive Metabolites in Resistant *Vitis labrusca* and Susceptible *Vitis vinifera*

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Status: In Preparation

Abstract

Grapevine (*Vitis*) is a globally important crop. However, insect herbivory decreases yields. Japanese beetle (*Popillia japonica*) is an invasive polyphagous pest of economic importance in many grapevine growing regions. *Vitis labrusca* acc. 'GREM4' was previously reported to be resistant to Japanese beetles while *Vitis vinifera* cv. 'PN40024' was susceptible. While transcriptomic studies have illustrated that genes implicated in flavonoids, phenylpropanoids, and terpenes were up-regulated in 'GREM4' compared to 'PN40024', the metabolomic effect of these transcriptomic alterations is still unknown. To answer this question, we conducted a comparative, untargeted metabolomic study between resistant 'GREM4' and susceptible 'PN40024' under pre-herbivory (0h) and insect herbivory-afflicted (1h) conditions. Here, we report that insect herbivory defense metabolites, some known to be insecticidal, were identified at constitutively greater levels in 'GREM4' compared to 'PN40024' before commencement of herbivory. Then, upon insect herbivory, flavonoids were identified as the main class of enriched metabolite in both species' responses. A greater number of differentially accumulating metabolites were identified in 'PN40024' after 1h of herbivory compared 'PN40024' 0h compared to 'GREM4' after 1h of herbivory compared to 'GREM4' 0h. We hypothesize the stouter regime of constitutive defensive metabolites in 'GREM4' played an important role in the insect herbivory resistant phenotype thus necessitating less aggressive alteration of the metabolome upon insect herbivory, explaining the greater inducible response observed in 'PN40024'. Overall, our metabolomic findings support

previous transcriptomic studies in that constitutive defense and flavonoid, phenylpropanoid, and terpene defensive metabolites are important in conferring the insect herbivory resistance phenotype observed in *V. labrusca* acc. 'GREM4'.

Introduction

Grapevine is a crop of global importance (Ehrhardt et al., 2014; Food and Agriculture Organization of the United Nations, 2020; Hasanaliyeva et al., 2020; Gaeta and Corsinovi, 2024). Despite this, insect herbivory decreases yields necessitating frequent applications of insecticides. One such pest of grapevine is Japanese beetle (*Popillia japonica*) (Gu and Pomper, 2008; Johnson et al., 2010). Japanese beetles are a polyphagous pest which damage crops by skeletonizing leaves - consuming the photosynthetic tissue between leaf veins (The United States Department of Agriculture - Agricultural Research Service; and Fleming, 1972; Potter and Held, 2002; Shanovich et al., 2019). As decreased photosynthetic capacity can lead to decreased yields, understanding why some grapevine species are more resistant to insect herbivory is important.

Previous work has identified *Vitis labrusca* acc. 'GREM4' grapevine as resistant to Japanese beetle (*Popillia japonica*) herbivory while *Vitis vinifera* cv. 'PN40024' was found to be susceptible (Chapter 2). Transcriptomic studies identified genes associated with flavonoids, phenylpropanoids, terpenes, plant-pathogen interactions/disease resistance, and acyltransferases were significantly up-regulated or enriched in resistant

‘GREM4’ compared to susceptible ‘PN40024’ under beetle herbivory (Chapter 2 & Chapter 3). Despite these findings, it was still unknown how such changes in gene expression impacted the metabolomic profiles of ‘GREM4’ and ‘PN40024’ under insect herbivory.

Some metabolites can be categorized as chemical defensive compounds which protect the plant from biotic stressors by acting as deterrent, detrimental, or insecticidal agents. While chemical defensive compounds can vary between species, and even between populations under different environmental conditions, flavonoids, terpenes, and phenylpropanoids are three major classes which are widely observed. Flavonoids are a large class of plant secondary metabolites with a variety of biological functions which are found in the majority of plant species (Ibraheem et al., 2015; Men et al., 2022; Lv et al., 2023). Flavonoids are characterized by the presence of two benzene rings linked by a 3-carbon chain or by a pyrone ring (Zhang et al., 2021b). Flavonoids display insect herbivory resistant and insecticidal characteristics in a variety of plants (Malone et al., 2009; Kariyat et al., 2019; Zhang et al., 2022b, 2022c). Terpenes are the most abundant class of plant secondary metabolite, with over 25,000 constituents, which are subdivided into subclasses of sesquiterpenes, monoterpenes, diterpenes, triterpenes, tetraterpenes, and others, based on the number of isoprenes present in their structure (Li et al., 2023b). Terpenes are typically comprised of one to four five-carbon groups (Li et al., 2023b) and while some subclasses are vital signaling volatile organic compounds (Tamiru et al., 2017; Han et al., 2023) others have direct implications in resistance, and can also be

insecticidal (Cantrell et al., 2005; Phschiutta et al., 2017; Sun et al., 2017). The phenylpropanoid pathway is an important pathway in plants, which is upstream of many critical biosynthetic pathways including those of stilbenes, coumarins, flavonoids, lignin, and salicylic acid (SA) (La Camera et al., 2004). They are comprised of products stemming from the shikimate pathway and are named for coumaric acid, which is comprised of a six-carbon aromatic phenyl group with a three-carbon propene tail, which is an integral intermediate compound in the phenylpropanoid biosynthetic process (Yadav et al., 2020; Cesarino et al., 2022). Phenylpropanoids have been shown to be central to insect herbivory defense responses against a variety of pests due, in large part, to their prominent position upstream of many critical pathways including flavonoid, lignin, and SA biosynthesis (Van Eck et al., 2010; Capitani et al., 2013; He et al., 2019). Overall, flavonoids, terpenes, and phenylpropanoids are only a cross-section of metabolite classes associated with defense, nonetheless, they represent three key classes of compounds with known associations with insect herbivory defense and resistance.

Many previous studies have undertaken metabolomic analysis of grapevine berries (Ehrhardt et al., 2014; Du et al., 2021; Selli et al., 2023) and wines (Yin et al., 2024). However, few studies have explored the metabolome in other tissues (Eisenmann et al., 2019; Labois et al., 2020), the majority of which explored pathogen response and resistance mechanisms (Hong et al., 2012; Eisenmann et al., 2019; Jia et al., 2022). For example, a screen of 11 different grapevines from *V. vinifera, V. labrusca,* and four other species, found that species resistant to fungal pathogens constitutively accumulated

greater concentrations of flavonoids compared to susceptible species when conducting Pathway Analysis via MetaboAnalyst (Maia et al., 2020). Interestingly, catechin, a flavonoid, was a positive biomarker for susceptibility (Maia et al., 2020). Meanwhile, other flavonoids, such as quercetin, trifolin, and aureusidin 6-O-glucoside, were positive biomarkers for resistance, showcasing the complexity of unraveling resistance mechanisms (Maia et al., 2020). In other studies, gamma-aminobutyric acid and phenylpropanoids were found to be positively correlated with pathogen resistance in *V. rupestris* compared to susceptible *V. vinifera* (Brasili et al., 2021). Over the past decade, only a few studies have investigated how grapevines respond metabolically to insect herbivory. In *V. berlandieri* x *V. riparia* hybrid grapevine roots under phylloxera (*Daktulosphaira vitifoliae*) herbivory, terpenes, aromatics, and other metabolites, were increased in headspace collections surrounding roots of afflicted, compared to control, plants (Lawo et al., 2011). *V. vinifera* x *V. labrusca* hybrid grapevine under oriental longheaded grasshopper (*Acrida chinensis*) herbivory was found to exhibit increased accumulation of malic acid, wax inducer (WIN), chitinase (CHI), bHLH transcription factor (bHLH-t), cinnamoyl-CoA reductase, cis-4-hydroxy-D-proline, proline, and D-(+)-Maltose upon herbivory (Jia et al., 2022). Most notably, increased accumulations of flavonoid biosynthetic enzymes flavonol synthase/flavanone 3-hydroxylase (FLS/F3H) and phenylpropanoid phenylalanine (PAL) were identified in grasshopper afflicted leaves compared to controls (Jia et al., 2022). While these studies provide insight into some

grapevine species, previous studies to date have not explored the metabolism underlying insect herbivory resistance in *V. labrusca.*

In this manuscript, we conducted a comparative, untargeted metabolomics study under Japanese beetle herbivory to identify metabolites which differentially accumulated in resistant *Vitis labrusca* acc. 'GREM4' compared to susceptible *Vitis vinifera* cv. 'PN40024'. The metabolomes of leaves collected at 0h from 'GREM4' and 'PN40024' were compared to determine constitutive differences before feeding began. Additionally, leaf samples after 1h of herbivory were also assayed, and compared to 0h within each respective species, to determine inducible metabolomic changes in response to insect herbivory. These inducible metabolomic alterations were also compared between species. Overall, this study provides insight into metabolites and metabolic classes which may play a role in conferring insect herbivory resistance in 'GREM4' which could aid future breeding efforts to decrease insect damage in grapevine, and perhaps, other crops.

Materials and Methods

Plant Materials

Cuttings from *Vitis labrusca* acc. 'GREM4' (PI-588583) and *Vitis vinifera* cv. 'PN40024' (DVIT-908) grapevine were acquired from the United States Department of Agriculture at Geneva, NY and Davis, CA, respectively, in 2019 (Prins and Agricultural Research Service - United States Department of Agriculture, 2018; Grape Genetics Research Unit, 2020). 'PN40024' was selected due to its role as the *V. vinifera* reference

cultivar/reference genome since 2007 (Jaillon et al., 2007). 'GREM4' was selected due to the availability of a reference genome sequence (Li and Gschwend, 2023), its resistance to pathogens (suggesting broad fitness in its local environment (Cadle-Davidson, 2008)), and resistance to Japanese beetle herbivory (Chapter 2). Both species were propagated from cuttings and grown in greenhouses at The Ohio State University, Columbus, OH USA under 16hr light:8hr dark and a temperature of ~>10°F than ambient for Ohio, USA. At time of experimentation, the plants were two- to three-year-old rooted vegetative plants. Experiments took place from the end of August through mid-September 2021.

Insect Collections

Popillia japonica (Japanese beetles) were collected from The Ohio State Waterman Agricultural and Natural Resources Laboratory, Columbus OH, USA from the end of August through mid-September 2021. Beetles were collected using "Spectracide Bag-A-Bug Japanese Beetle Trap2" pheromone traps (Spectrum Brands, 2023) in a soybean field which had not been sprayed with insecticides. Beetles were kept in a 16.5 x 16.5 x 30in 'bug dorm' (Educational Science, 2019) within a growth chamber overnight and semi-starved (one small *V. vinifera* leaf provided to prevent death due to starvation or dehydration) and were used for experiments the following day. The growth chamber was set to a 16hr light:8hr dark cycle at 25ºC and 21ºC, respectively.

Herbivory Study

Tissue samples used in this study were portions of the same leaves which were used for the previously undertaken transcriptomic experiments (Chapter 2 & Chapter 3). A detailed description of the time course experiment from which the 1h samples were derived can be seen as previously described in Chapter 2 – Materials and Methods. Briefly, one Japanese beetle was placed in a transparent, mesh bag which was then placed over one mature attached leaf of either 'GREM4' or 'PN40024'. Beetles were permitted to directly feed upon the leaf for 30min, 1h, or 4h (experimental groups), while no herbivory occurred on 0h leaves (control group) which were collected before feeding began. To avoid confounding transcriptomic and metabolomic responses *in planta* due to the removal of a leaf, 0h samples were collected from a different plant than herbivory leaves. For metabolomic analyses, 1h was chosen as the sole experimental time point.

Leaves were immediately placed inside 50mL conical tubes and plunged into liquid nitrogen at completion of a run to preserve the tissue for metabolomic analysis (and previously undertaken transcriptomic analyses). Samples were stored in liquid nitrogen for less than 1h and subsequently sub-divided into two samples - one used for RNA isolation (Chapter 2 & Chapter 3) and the other used for metabolomics studies. The leaf samples were then stored at -80ºC until use.

Metabolite Extraction

The extraction procedure employed was adapted from the procedures of a grapevine-specific extraction method (Wang et al., 2020) and a rice (*Oryzae sativa*)

extraction method (Dias et al., 2022) which had both been successfully employed at the Laboratory for the Analysis of Metabolites of Plants (LAMP) (The Ohio State University, Columbus, OH, USA) previously.

Leaf tissue which remained from the previous transcriptomic analysis, on average 1,400mg of tissue, was used for metabolite extraction two years after collection. All following steps occurred on liquid nitrogen (LN) until otherwise noted. Frozen leaves were coarsely double ground with a Pyrex glass rod in a 15mL sample tube prior to aliquoting between 78 to 236mg of leaf tissue (139mg on average) into pre-weighed 2mL centrifuge tubes containing one 4mm diameter and one 2mm diameter ball bearing (for use in following grinding step). Homogenization was achieved by grinding using a Qiagen TissueLyser II (QIAGEN, 2024), which had aluminum sample blocks pre-chilled in LN, at 20hz for two 40sec cycles. The high trichome density of 'GREM4' leaves made grinding these leaves challenging. Therefore, additional grinding was employed as needed for these tissues. Ultimately, all samples were confirmed to be a homogenous fine powder before advancing to the next step.

A methanol extraction buffer, which contained an internal standard of 2µL of 5ng/µL d^3-TRP (deuterated tryptophan), was added to each sample at a ratio of 500µL MeOH:100mg tissue, which, on average, was 695µL per tube. At this point, all further steps occurred on ice until otherwise noted. Samples were then removed from LN and nutated, horizontally, in a 40°F walk-in freezer for 30min, flipping them halfway through. Samples were then spun down for 20min at 15,000 RCF within the same 40°F

walk-in freezer. The supernatant was then immediately transferred to new 1.7mL centrifuge tubes. Samples then rested at 4°C overnight. Samples were centrifuged the following morning at 16,100 RCF (max speed) in a 40°F walk-in freezer for 40min to pellet particulates in the solution which precipitated out overnight which were identified predominantly in 'GREM4' samples (which were presumed to be shredded vestiges of trichomes due to the known high trichome density in 'GREM4' and the challenges the trichomes posed in grinding the samples). Samples were then filtered using Agilent Captiva Econofilter 13mm diameter by 0.2µm pore size nylon filters (Agilent, 2024b) using a syringe to apply air pressure. Samples were then directly submitted to the Campus Chemical Instrument Center (CCIC) (Campus Chemical Instrument Center - The Ohio State University, 2024) and ran immediately upon receipt.

LC-MS/MS Procedure

Untargeted metabolomic analysis at the CCIC was conducted via LC-MS/MS on a Thermo Scientific Exploris 480 Orbitrap mass spectrometer (Thermo Fisher Scientific, 2024c) with LC separation on a Poroshell 120 SB-C18 (2 x 100mm, 2.7µm particle size) column (Agilent, 2024a) on a Vanquish UHPLC system (Thermo Fisher Scientific, n.d.). The gradient consisted of solvent A, H2O with 0.1% formic acid, and solvent B, MeOH with 0.1% formic acid, at a 200µL/min flow rate with an initial 2% solvent B with a linear ramp to 90% solvent B at 15 minutes, up to 95% solvent B for 1 minute, and back to 2% solvent B at minute 17 and equilibration of 2% solvent B until minute 30 while at 40ºC. 10µL was injected for each sample at a mass range of 80 to 1200 m/z at an

orbitrap resolution of 60,000 and a 60% RF lens with 1 microscan and 100ms maximum injection time, with a Thermo HESI Source in positive (3,500 V) mode, sheath gas at 40, aux gas at 8, sweep gas at 1, ion transfer tube at 275°C, and a vaporizer temperature at 320°C. Data dependent analysis was performed at a 0.6sec cycle between full scan and MS/MS scans with a 2sec dynamic exclusion window with HCD fragmentation performed at 15%, 35%, and 80% normalized collision energies at 30,000 resolution, an isolation window of 1.5, and a 54ms maximum injection time.

Each sample group ('GREM4' 0h, 'GREM4' 1h insect herbivory, 'PN40024' 0h, and 'PN40024' 1h insect herbivory) had four biological replicates, of which, each was injected twice as two technical replicates. This resulted in each sample being run through the LC-MS/MS two times resulting in 32 total injections (4 groups x 4 biological replicates x 2 technical replicates = 32 injections).

Metabolomic Data Processing and Statistical Analysis

Compound Discoverer 3.3 SP2 software (Thermo Fisher Scientific, 2024a) (CD) was used to analyze the LC-MS/MS data. A custom untargeted workflow for filtering and analyzing the dataset was employed based on previously created workflows present at the CCIC and can be found at the permanent repository hosted on "figshare" at https://figshare.com/s/95ea878b3f5153bac962 . A listing of all metabolites which passed the filtering regime may be seen in full as Table 4.1. Principle component analysis (PCA), box and whisker plot, and heatmap analyses were conducted and visualized within CD on the resulting metabolites. The PCA was generated using the following

parameters – Normalization: On; Group: By Condition; Data Source: Compounds; Center and Scaling: On. The box and whisker plot was generated using the following parameters – Normalization: On; Log-transformation of data: On; Group: By Condition; Data Source: Compounds. The heatmap was generated using the following parameters - Distance Function: Euclidean; Linkage Method: Complete; Scale: Applied Before Clustering; Normalization: On.

From the filtered list of metabolites generated above, differentially accumulated metabolites (DAMs) were identified by filtering the list further by the threshold of $|\log2FoldChange| \geq 1$ and a p-value of ≤ 0.05, as per common parameters in the field (Li et al., 2023a; Ojeda-Rivera et al., 2023), within CD (see previous figshare link for additional information). To assign putative names to these DAMs, the following databases/tools were employed within CD in the following order: mzCloud (HighChem LLC and Thermo Fisher Scientific, 2024), an online spectra library with fragmentation patterns using reference quality standards for > 2.8M metabolites, first assigned naming if a match was found; If no likely match was found, predicted compositions next calculated hypothetical chemical formulas based on the spectra/molecular weight of the metabolite, in many cases settling on one best match; Based on the chemical formula option(s), ChemSpider (Royal Society of Chemistry 2024, 2024) next assigned naming by cross-referencing the chemical formula(s) against a chemical structural database of > 59M known chemicals; In the event ChemSpider identified multiple possible candidates, or if multiple chemical formula options were proposed by predicted compositions, mzLogic

(Thermo Fisher Scientific, 2024b) used a combination of KEGG (Kanehisa and Goto, 2000), HMDB (Wishart et al., 2022), BioCyC (Karp et al., 2019), and PubChem (Kim et al., 2023) data in parallel to make the best assignment based on molecular weight, predicted composition, and alignment of closely matching spectra. Most compounds were assigned a reliable identification based on these variety of filters and database integrations.

Assignment of metabolic classes to metabolites was ascribed by a manual investigation of individual metabolites via KEGG, PubChem, ChEBI (Hastings et al., 2015), MetaCyc (Karp et al., 2019), LIPID MAPS (Conroy et al., 2024), NPAtlas (Van Santen et al., 2022), mzCloud, ChemSpider, and, if necessary, a literature search. Ultimately, KEGG BRITE's hierarchical classification of metabolites was used as the template for classifying all metabolites. Over 300 metabolites, covering the 50 increased and 50 decreased DAMs with the greatest |log2FoldChange| for each of the four comparisons (overlap between DAMs identified between lists necessitated only completing ~300 to cover the top 50 up and down for each comparison), were curated and manually checked using this methodology.

To identify metabolic pathways enriched in lists of DAMs, the Pathway Analysis tool within MetaboAnalyst 6.0 (Ewald et al., 2024) (MA) was employed implementing the following parameters - Visualization: Scatter Plot; Enrichment Method: Hypergeometric Test; Topology Analysis: Relative-betweeness Centrality Theory; Reference Metabolome: Use all compounds in the selected pathway library; Pathway

Library: Arabidopsis (KEGG). Only those enrichments with a p-value ≤ 0.05 and a pathway impact of ≥ 0.10 were considered significant.

Overlap (conservation) analysis to identify DAMs conserved and unique between comparisons and create Venn diagrams was conducted via BioVenn (Hulsen et al., 2008), BioInfoRx (BioInfoRx, 2023), and molbiotools (Molbiotools, 2023).

Results

In order to identify the metabolomic differences between insect herbivory resistant *Vitis labrusca* acc. 'GREM4' and susceptible *Vitis vinifera* cv. 'PN40024' against Japanese beetle herbivory, an untargeted metabolomic experiment was conducted via LC-MS/MS in positive ionization mode. Experimental (1h herbivory) and control (0h) groups were analyzed for each species, resulting in four total groups.

Variability and Broad-Scale Differences Between Responses

First, we conducted a data integrity check on the metabolomic dataset of each injection to ensure data was satisfactory for downstream analyses. Technical replicates were satisfactorily precise, as their dots in multivariate PCA were either completely, or very close to being completely, overlapping (Figure 4.1A). Additionally, the PCA also illustrated that 'GREM4' and 'PN40024' samples separately clustered, indicating metabolomic differences between the two cultivars, even before herbivory began (0h), as well as at 1h of herbivory. Differences between treatments within each species were also observed, although 'GREM4' under herbivory was more variable than 'PN40024'. Next,

biological replicates were reviewed and found to be reasonably precise in respect to one another, since none of the average concentrations of metabolites in each injection fell outside the upper or lower quartiles of any other injection within the same biological group (Figure 4.1B). When reviewing concentrations of individual metabolites between samples, differences in metabolite concentrations were found to exist between 'GREM4' 0h, 'GREM4' 1h insect herbivory, 'PN40024' 0h, and 'PN40024' 1h insect herbivory samples illustrated by the hierarchical clustering of the injections along the top of the x-axis and coloration of the heatmap (Figure 4.1C).

Overall, these results confirm that technical and biological replicates are precise with respect to one another, and thus, can be employed for downstream analysis. Further, differences in metabolomes exist between species and between experimental and control treatments.

Constitutive (0h) Comparison of Metabolomes

When identifying differentially accumulating metabolites (DAMs) ($|log2FoldChange| \geq 1$; $p.value \leq 0.05$) between 'GREM4' 0h and 'PN40024' 0h, 226 metabolites had significantly greater levels in 'GREM4' compared to 'PN40024' while 112 had significantly lower levels (Figure 4.2). A listing of all identified DAMs from all comparisons conducted within this manuscript may be seen in Table 4.2. The top 15 DAMs, sorted by log2FoldChange, with the most heightened and most lowered levels in 'GREM4' 0h compared to 'PN40024' 0h may be found as Table 4.3.

The top 50 DAMs, of 226 total, with the most heightened levels in ‘GREM4’ 0h compared to ‘PN40024’ 0h were identified and the greatest number of these metabolites were classified as flavonoids (14), while terpenoids (4), phenylpropanoids (4), and organic heterocyclic compounds (4) were tied for the next classes with the greatest number of DAMs (Figure 4.3A). In the top 50 DAMs, of 112 total, with the most lowered levels in ‘GREM4’ 0h compared to ‘PN40024’ 0h (i.e. - metabolites with the most heightened level in ‘PN40024’ compared to ‘GREM4’) the classification with the greatest number of DAMs was flavonoids (5), while second most was tied between lipids (4) and terpenoids (4) (Figure 4.3B).

‘GREM4’ 1h Insect Herbivory compared to ‘GREM4’ 0h

Next, we explored the alteration in the metabolome upon insect herbivory in resistant ‘GREM4’ by comparing the metabolome at 1h of Japanese beetle herbivory compared to 0h. 1h was selected as the experimental time point in the study as we hypothesized 1h would permit observation of the effects of alterations in the early response (roughly 30min after herbivory initiation) as well as some alterations related to the transition to late response (4h) (Chapter 3). 31 metabolites had significantly increased accumulations in ‘GREM4’ at 1h compared to 0h while 56 had significantly decreased (Figure 4.2). The top 15 DAMs, sorted by log2FoldChange, with the most increased and most decreased accumulation in ‘GREM4’ 1h compared to ‘GREM4’ 0h may be found as Table 4.3.

When reviewing all 31 DAMs with increased accumulation at 1h compared to 0h in ‘GREM4’, the classification with the greatest number of DAMs was flavonoids (11). Five other metabolic classes had one metabolite (Figure 4.4A). In DAMs with decreased accumulation from 0h to 1h in ‘GREM4’, of which, all 56 DAMs were assayed, the greatest number of metabolites were identified in the classification of flavonoids (8), the second most was terpenoids (6), while third was lipids (5) (Figure 4.4B).

To determine the metabolic pathways which were significantly enriched amongst DAMs with increased or decreased concentrations in ‘GREM4’ from 0h to 1h, a Pathway Analysis was conducted via MA. All 31 DAMs with increased accumulation from 0h to 1h in ‘GREM4’ were analyzed and the only significant enrichment was ‘flavone and flavanol biosynthesis’ (Figure 4.5A & Table 4.4) wherein five DAMs (metabolites) analyzed were identified in the pathway (Figure 4.5B). As the enrichment had a pathway impact score of 38%, this statistic reports that 38% of pathway products were hypothesized to differentially accumulate due to the alterations of the provided metabolites (DAMs) in the pathway. For enrichment of metabolites which decreased from 0h to 1h in ‘GREM4’, too few metabolites had associated KEGG pathways for enrichment analysis to be conducted.

<u>‘PN40024’ 1h Insect Herbivory compared to 'PN40024’ 0h</u>

Next, we explored the alteration in the metabolome upon insect herbivory in susceptible ‘PN40024’ by comparing the metabolome at 1h of Japanese beetle herbivory to 0h. 96 metabolites had significantly increased accumulations in ‘PN40024’ at 1h

compared to 0h while 24 had significantly decreased (Figure 4.2). The top 15 DAMs, sorted by log2FoldChange, with the most increased and most decreased accumulation in 'PN40024' 1h compared to 'PN40024' 0h may be found as Table 4.3.

The top 50 DAMs, of 96 total, with increased accumulation at 1h compared to 0h in 'PN40024' were identified and the classification with the greatest number of DAMs was flavonoids (20), the second most was amino acids (9), while third was tied between alkaloids (4), organic hydroxy compounds (4), and phenylpropanoids (4) (Figure 4.4A). In DAMs with decreased accumulation from 0h to 1h in 'PN40024', of which all 24 were assayed, the greatest number of DAMs were identified in the classification of flavonoids (4), while second was terpenoids (3), while four classes were tied for third most with one each (Figure 4.4B).

Pathway Analysis via MA was next undertaken. When analyzing the top 50 DAMs with increased accumulation from 0h to 1h in 'PN40024' after sorting by log2FoldChange, three significantly enriched pathways were identified (Figure 4.6A). These three pathways were 'phenylalanine metabolism' (42% pathway impact), 'flavone and flavonol biosynthesis' (35%), and 'flavonoid biosynthesis' (10%). Only two DAMs (metabolites) analyzed were identified in the flavone and flavonol biosynthesis pathway in 'PN40024' (Figure 4.6B) compared to five in 'GREM4' in the same pathway (Figure 4.5B). For enrichment of metabolites which decreased from 0h to 1h in 'PN40024', too few metabolites had associated KEGG pathways for enrichment analysis to be conducted.

‘GREM4’ 1h Insect Herbivory compared to ‘PN40024’ 1h Insect Herbivory

Next, we explored metabolomic differences of ‘GREM4’ and ‘PN40024’ after 1h of insect herbivory by comparing their metabolomes directly (‘GREM4’ 1h compared to ‘PN40024’ 1h). 182 metabolites had significantly greater accumulations in ‘GREM4’ at 1h compared to ‘PN40024’ at 1h while 142 had significantly lower accumulations (Figure 4.2). The top 15 DAMs, sorted by log2FoldChange, with the most heightened and most lowered accumulation in ‘GREM4’ 1h compared to ‘PN40024’ 1h may be found as Table 4.3.

The top 50 DAMs, of 182 total, with the most heightened accumulation in ‘GREM4’ compared to ‘PN40024’ at 1h were identified and the greatest number of these metabolites were classified as flavonoids (16), while second most was lipids (4), while third was tied between phenylpropanoids (3) and terpenoids (3) (Figure 4.3A). In the top 50 DAMs, of 142 total, with most lowered accumulation in ‘GREM4’ compared to ‘PN40024’ at 1h, flavonoids (8) were again the greatest classification, second was amino acids (4), and third most was tied between lipids (3) and terpenoids (3) (Figure 4.3B).

Overlap Analysis Between Comparisons

Next, we wanted to determine which DAMs were conserved, or unique, to individual time points or species to identify metabolites which may impact insect herbivory resistance. All lists of conserved or unique DAMs throughout this manuscript may be seen in Table 4.2. First, we compared DAMs identified between all comparisons (‘GREM4’ 0h compared to ‘PN40024’ 0h, ‘GREM4’ 1h compared to ‘GREM4’ 0h,

‘PN40024’ 1h compared to ‘PN40024’ 0h, and ‘GREM4’ 1h compared to ‘PN40024’ 1h) (Figure 4.7A). When DAMs with greater or lower accumulation within each comparison were combined, it was found that the number of DAMs in many intersections in the Venn diagram ranged between 1–30 DAMs. However, 185 total DAMs were conserved between ‘GREM4’ 0h compared to ‘PN40024’ 0h and ‘GREM4’ 1h compared to ‘PN40024’ 1h suggesting a high degree of conservation between the two inter-species comparisons between the 0h and 1h time points.

To parse apart these conservations further, we next conducted comparisons between intra- and inter-species comparisons.

Intra-species Comparisons

First, we conducted an overlap analysis between ‘GREM4’ 1h compared to ‘GREM4’ 0h and ‘PN40024’ 1h compared to ‘PN40024’ 0h metabolomic responses to determine metabolites which were unique or conserved in the inducible response to 1h of insect herbivory in one species or the other, or both.

When separating out DAMs with increased accumulation or decreased accumulation and then comparing between comparisons (four total lists), no conservation was observed between increased accumulation DAMs in one comparison with decreased accumulation DAMs in the other. We next compared only increased to increased and decreased to decreased DAMs across comparisons. Of 96 DAMs with increased accumulation at 1h compared to 0h in ‘PN40024’, after removing DAMs with duplicated names, 87 remained (Figure 4.2). Of these 87 DAMs, 72 (83%) were unique to

‘PN40024’ while the remaining 15 (17%) were conserved with ‘GREM4’; 13 (46%) were unique to ‘GREM4’ which had 28 DAMs after removing duplicated names (Figure 4.2 & Figure 4.7B). Of 24 DAMs with decreased accumulation at 1h compared to 0h in ‘PN40024’, after removing DAMs with duplicated names, 20 remained (Figure 4.2). Of these 20 DAMs, 7 (35%) were unique to ‘PN40024’ while the remaining 13 (65%) were conserved with ‘GREM4’; 32 (71%) were unique to ‘GREM4’ which had 45 DAMs after removing duplicated names (Figure 4.2 & Figure 4.7C). A list of DAMs with increased accumulation in either ‘GREM4’ 1h compared to ‘GREM4’ 0h or ‘PN40024’ 1h compared to ‘PN40024’ 0h separated out by those conserved or unique between the species may be seen as Table 4.5. A list of DAMs with decreased accumulation may be seen as Table 4.6.

In ‘GREM4’, the top three metabolites which exhibited the greatest increased accumulation from 0h to 1h that were also found to be unique to ‘GREM4’ were isoamylamine, trifolin (kaempferol-3-O-galactoside), and kaempferol. Meanwhile, the top three metabolites which exhibited the greatest increased accumulation from 0h to 1h that were unique to ‘PN40024’ were 4',7-dimethoxy-2,2,4-trimethyl-Delta(3)-isoflavan, benzoxiquine, and argininosuccinic acid (L-Argininosuccinic acid) (Table 4.5). The top three metabolites which exhibited the greatest increased accumulation from 0h to 1h that were conserved between ‘GREM4’ and ‘PN40024’ were 2,6-xylidine (2,6-Dimethylaniline), 2-aminonicotinic acid, and 5,7-dihydroxy-2-(4-hydroxy-3-

methoxyphenyl)-3-{[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}-4H-chromen-4-one.

Inter-species Comparisons

Next, we conducted an overlap analysis between 'GREM4' 0h compared to 'PN40024' 0h and 'GREM4' 1h compared to 'PN40024' 1h metabolomic responses to determine how the metabolomes of 'GREM4' and 'PN400024' altered over time, in respect to one another.

When separating out DAMs with heightened accumulation or lowered accumulation and then comparing between comparisons (four total lists), no conservation was observed between heightened accumulation DAMs in one comparison with lowered accumulation DAMs in the other. We next compared only heightened to heightened and lowered to lowered DAMs across comparisons. Of 226 DAMs with heightened accumulation in 'GREM4' compared to 'PN40024' at 0h, after removing DAMs with duplicated names, 161 remained (Figure 4.2). Of these 161 DAMs, 46 (29%) were unique to the 0h comparison while 115 (71%) were conserved with 1h; 26 (18%) were unique to the 1h comparison which had 141 DAMs after removing duplicated names (Figure 4.2 & Figure 4.7D). Of 112 DAMs with lowered levels in 'GREM4' compared to 'PN40024' at 0h, after removing DAMs with duplicated names, 102 remained (Figure 4.2). Of these 102 DAMs, 15 (15%) were unique to the 0h comparison while 87 (87%) were conserved with 1h while 39 (31%) were unique to the 1h comparison which had 126 DAMs after removing duplicated names (Figure 4.2 & Figure 4.7E). A list of DAMs

with heightened accumulation in either 1h or 0h inter-species comparisons separated out by those conserved or unique between the time points may be seen as Table 4.7. A list of DAMs with lowered accumulation may be seen as Table 4.8.

The top three metabolites which exhibited the most heightened accumulation in 'GREM4' compared to 'PN40024' that were conserved between 0h and 1h, when sorted by summed log2FoldChange values between the two comparisons, were neochlorogenic acid ((1r,3R,4s,5S)-4-{[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}-1,3,5-trihydroxycyclohexane-1-carboxylic acid), chromene, and 6-methoxyflavanone (6-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one) (Table 4.7).

Discussion

Discussion Background

Previous studies report *Vitis labrusca* acc. 'GREM4' grapevine was resistant to *Popillia japonica* (Japanese beetle) herbivory compared to *Vitis vinifera* cv. 'PN40024' (Chapter 2). Further investigation revealed genes implicated in chiefly flavonoids, phenylpropanoids, and terpenes were uniquely implicated in the insect herbivory response in 'GREM4' compared to 'PN40024' (Chapter 2 & Chapter 3). Additionally, constitutive and temporal transcriptomic differences were observed between the two species (Chapter 2 & Chapter 3). Using untargeted metabolomic analysis via LC-MS/MS, we identified differences in metabolomic profiles between 'GREM4' and 'PN40024' under insect herbivory for 1h. Key differences between species were

identified which supported previous transcriptomic findings and suggest potential mechanisms imparting resistance.

Across all comparisons, DAMs with greater accumulations were overwhelmingly flavonoids, while phenylpropanoids and terpenes were also identified. As it is known that flavonoids (Kumar and Yadav, 2017; Kariyat et al., 2019; Chatterjee et al., 2022; Zhang et al., 2022a; Lv et al., 2023), phenylpropanoids (Moing et al., 2003; Capitani et al., 2013; Dixit et al., 2020; Wang et al., 2022), and terpenes (Sun et al., 2017; Liu et al., 2020a; Wang et al., 2023a) can be insecticidal or are implicated in insect herbivory resistance, increased accumulation of these metabolites likely plays a role in conferring resistance. DAMs with decreased accumulations were often of mixed metabolomic classes. However, flavonoids were the only classification generally conserved amongst DAMs with the greatest decreased accumulations.

Broad Conservation of DAMs Observed Between 0h and 1h in Inter-species Comparisons

Overlap analysis of inter-species comparisons (‘GREM4’ 0h compared to ‘PN40024’ 0h and ‘GREM4’ 1h compared to ‘PN40024’ 1h) revealed most DAMs, whether heightened or lowered in accumulation in ‘GREM4’ compared to ‘PN40024’, were conserved from 0h to 1h. Of DAMs with heightened accumulation, 71% of ‘GREM4’ 0h compared to ‘PN40024’ 0h DAMs and 82% of ‘GREM4’ 1h compared to ‘PN40024’ 1h DAMs were conserved between the comparisons. Similar results were reported for DAMs with lowered accumulation. These results report that the majority of

the differences observed in metabolite concentrations of significantly differentially accumulating metabolites between ‘GREM4’ and ‘PN40024’ at 0h were still observed after 1h of insect herbivory. This result suggests that the majority of DAMs, when comparing relative from one species to the other, did not experience significantly altered accumulation upon insect herbivory. However, metabolomic alterations were indeed identified which are discussed in the following sections. As it relates to this overlap analysis, it is worth noting that, since, in these comparisons, the responses were calculated relative from one species to the other, it is possible that changes in metabolite concentrations could have occurred from 0h to 1h in both species, but these changes were similar in both species which ultimately still resulted in reporting of a significant difference between the two metabolites at 1h regardless as, relative to one another, the difference in accumulation did not change.

Metabolites Linked to Insect Herbivory Resistance Constitutively Accumulate, and Perpetuate in, ‘GREM4’ at Greater Levels than ‘PN40024’

The metabolic classification with the greatest number of associated metabolites at the basal (0h) and 1h inter-species responses, with 14 and 16 metabolites identified in this class, respectively, was flavonoids - the greatest classification by more than 10 metabolites in each. When reviewing the top 15 DAMs with the greatest increased accumulation in ‘GREM4’ compared to ‘PN40024’ in inter-species comparisons, six flavonoids were identified between the 0h and 1h response, the most of any classification. At 0h, their summed log2FoldChange was 14.22, while by 1h, they rose to 26.80.

Flavonoid 6-methoxyflavanone and flavonoid/phenylpropanoid-related metabolite vanilloyl glucose (1-O-vanilloyl-beta-D-glucose) were identified in both inter-species comparisons at 0h and 1h in the top 15 DAMs with the greatest increases in 'GREM4' compared to 'PN40024'. Literature searches revealed relatively little is known about either metabolite, outside of biomedical explorations (Akbar et al., 2020; Barragán-Zarate et al., 2022). Aside from being known to accumulate in specific plant species (Jiang et al., 2021; Csorba et al., 2022), and specifically in *V. vinifera* berries in the case of vanilloyl glucose (Du et al., 2021), neither appear to have previously been implicated in insect herbivory resistance. However, vanilloyl glucose accumulation is known to significantly increase in response to fungal pathogen tan spot (*Pyrenophora tritici-repentis*) in susceptible wheat (*Triticum aestivum*) (Ferreira et al., 2024). While the impact of these metabolites on fungal resistance or susceptibility are not yet known, when considering the results herein with findings from the aforementioned fungal study, we hypothesize vanilloyl glucose and 6-methoxyflavanone are implicated in biotic stress response. Considering flavonoids are known to impact fungal (Nicholson et al., 1987; Lo et al., 1999; Ibraheem et al., 2015) and insect resistance (Kumar and Yadav, 2017; Kariyat et al., 2019; Chatterjee et al., 2022; Zhang et al., 2022a; Lv et al., 2023), it is plausible such metabolites could play a role in conferring insect herbivory resistance. As such, future functional studies could reduce vanilloyl glucose or 6-methoxyflavanone accumulation *in planta* to observe impacts on insect herbivory resistance to test this hypothesis.

Beyond flavonoids, other classes of metabolites with known implications in insect herbivory resistance were also more greatly accumulated in 'GREM4' compared to 'PN40024' at both 0h and 1h. These include phenylpropanoids and terpenes. Identification of these metabolites supports previous transcriptomic findings in which, while thousands of genes were differentially expressed, genes implicated in secondary metabolite biosynthesis were enriched in DEGs with greater expression in 'GREM4' compared to 'PN40024', even before insect herbivory began, at 0h (Chapter 2). Genes implicated in this enrichment were predominantly associated with terpene, carotenoid, phenylpropanoid, and flavonoid biosynthesis (Chapter 2). Considering the integral role of constitutive defense in conferring resistance to insect herbivory, such as seen in Sitka spruce (*Picea sitchensis*) (Whitehill et al., 2021), wheat (*Triticum aestivum*) (Lv et al., 2023), alfalfa (*Medicago sativa*) (Zhang et al., 2022c), and other plants (Weinblum et al., 2021), and considering constitutive defense is typically wrought by complex networks of genes from many biosynthetic pathways (Rasmann et al., 2015; Paudel et al., 2019), it is reasonable that metabolites from multiple classes exhibited differential accumulation under basal conditions and these metabolites likely play a role this foundational and complex defense.

The phenylpropanoid pathway is directly upstream of the lignin and flavonoid biosynthesis pathways, thus, it is integral to multiple key plant processes (La Camera et al., 2004; Chang et al., 2019). Previous studies found five gene family members belonging to a critical phenylpropanoid pathway gene, *Phenylalanine ammonia-lipase 1*

(*PAL1*), were up-regulated upon Japanese beetle herbivory in 'GREM4' while no up-regulation of gene family members was observed in 'PN40024' (Chapter 2). Additionally, previous studies in *V. vinifera* x *V. labrusca* hybrid grapevine 'Kyoho' fed upon by oriental longheaded grasshopper exhibited similar up-regulation of *PAL1* and also exhibited significantly increased accumulation of PAL (Jia et al., 2022). Phenylpropanoids were also found to be significantly enriched in resistant compared to susceptible wheat (*Triticum aestivum*) attacked by orange wheat blossom midge (*Sitodiplosis mosellana*) (Wang et al., 2022) and in resistant versus susceptible cotton (*Gossypium hirsutum*) against cotton bollworm (*Helicoverpa armigera*) and cotton leafworm (*Spodoptera litura*) (Dixit et al., 2020). While these studies in resistant plants report inducible phenylpropanoid accumulation and pathway gene up-regulation, not constitutive, it is conceivable that constitutive presence of phenylpropanoids prior to insect herbivory could equally result in resistance. Phenylpropanoid neochlorogenic acid was one of the top 15 DAMs with the most heightened accumulation in 'GREM4' compared to 'PN40024' at both 0h and 1h in our study. Neochlorogenic acid is known to confer resistance to fungal pathogens (Ji et al., 2021; Li et al., 2023c), but, seemingly few studies have explored insecticidal activity of neochlorogenic acid and were all conducted in peach. In a *Prunus persica* peach cultivar resistant to Mediterranean fly (*Ceratitis capitata*), heightened accumulations of neochlorogenic acid were identified compared to a susceptible variety (Capitani et al., 2013). Similarly, a wild species, *Prunus davidiana,* with known insect resistance, was found to exhibit higher accumulation of

neochlorogenic acid compared to cultivated, susceptible, *P. persica* (Moing et al., 2003). Alike the flavonoids, it is likely that phenylpropanoids, especially neochlorogenic acid, play an important role in the insect herbivory resistance of 'GREM4' compared to 'PN40024', but further study is necessary to confirm this hypothesis.

Terpenes 8-geranylesculetin and 4-(4-hydroxy-2,6,6-trimethyl-3-{[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}cyclohex-1-en-1-yl)butan-2-one were identified in the top 15 DAMs with the most heightened accumulation in 'GREM4' compared to 'PN40024' in both 0h and 1h, as well. To our knowledge, no studies have referenced either metabolite, although, 8-geranylesculetin is known to be a product of a reaction which converts geranyl diphosphate and esculetin to a diphosphate and 8-geranylesculetin (Kyoto Encyclopedia of Genes and Genomes, 2024). While further research is necessary to further elucidate these compounds, terpenes are known to impact insect resistance in many plants including tea (*Camellia sinensis*) (Liu et al., 2020a), rice (Sun et al., 2017), maize (*Zea mays*) (Wang et al., 2023a), and others. Therefore, it is possible these metabolites also play a similar role.

Taking into consideration all inter-species comparison results at 0h and 1h, we hypothesize that the insect herbivory resistance observed in 'GREM4' compared to 'PN40024' is due in large part to constitutive accumulation of insect herbivory defensive metabolites *in planta* which afford immediate defense, and perhaps, deterrence, against insect predators. Many of these metabolites were further found to also be DAMs at 1h reporting the altered accumulation perpetuated even after insect herbivory. While

flavonoids likely play the largest role in heightened insect herbivory resistance in ‘GREM4’, other classes such as phenylpropanoids and terpenes also play an important role. Specifically, flavonoids 6-methoxyflavanone and vanilloyl glucose, phenylpropanoid neochlorogenic acid, and terpenes 8-geranylesculetin and 4-(4-hydroxy-2,6,6-trimethyl-3-{[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}cyclohex-1-en-1-yl)butan-2-one likely play important roles in conferring the heightened insect herbivory resistance observed of ‘GREM4’ considering their increased accumulation compared to ‘PN40024’ under both basal and 1h of herbivory conditions.

Greater Inducible Insect Herbivory Metabolomic Response Revealed in ‘PN40024’

Intra-species comparisons (‘GREM4’ 1h compared to ‘GREM4’ 0h and ‘PN40024’ 1h compared to ‘PN40024’ 0h) exploring temporal responses revealed significantly altered accumulation of many metabolites from 0h to 1h in both species. In ‘PN40024’, 87 metabolites increased in accumulation while 20 metabolites decreased from 0h to 1h. In ‘GREM4’, 28 metabolites increased while 45 decreased. Additionally, the majority (83%) of DAMs with increased accumulation from 0h to 1h in ‘PN40024’ were not identified in the ‘GREM4’ 1h compared to ‘GREM4’ 0h comparison. Conversely, ‘GREM4’ exhibited a greater number (32 compared to seven) of unique DAMs with decreased accumulation from 0h to 1h compared to ‘PN40024’. Together, these findings report the response to insect herbivory in ‘PN40024’ is both more responsive and is comprised of different metabolites than observed in ‘GREM4’.

While, at the outset, this result was unexpected, when considering the hypothetically stout constitutive insect herbivory defenses exhibited by 'GREM4' compared to 'PN40024', which is reported by transcriptomic data from Chapter 2 and supported by metabolomic data herein, it is reasonable that lesser induction of metabolites would be observed in 'GREM4'. According to current theory, a plant constitutively well prepared (adapted) for defense against insect herbivory need not drastically alter the composition of the metabolome when the stress is encountered, as doing so would unnecessarily expend finite energetic resources when defense is already adequate (Karban and Baldwin, 1997; Agrawal et al., 2010; Rasmann et al., 2015). Conversely, susceptible 'PN40024' (Dami et al., 2005; Smith, 2005; Dami, 2007) (Chapter 2), which does not appear constitutively well-positioned for defense compared to 'GREM4' (Chapter 2), would be expected to undergo a more pronounced metabolic alteration upon attack in an attempt to exact defense. Indeed, when heightened constitutive defense is observed in plants, less inducible responses are often observed. For instance, a study which subjected *Arabidopsis* lines with variable insect herbivory resistance and glucosinolate accumulation to herbivory by both the Egyptian cotton leafworm (*Spodoptera littoralis*) and the cabbage butterfly (*Pieris brassicae*) found lines with higher levels of constitutive defense displayed diminished inducible responses, while those with lower constitutive defense exhibited enhanced inducible responses (Rasmann et al., 2015). Likewise, the same inverse relationship was observed in a screen of 77 plant species upon Egyptian cotton leafworm feeding (Kempel et al., 2011). As a

final example, a resistant tomato (*Solanum lycopersicum*) cultivar, prior to any herbivory by two-spotted spider mite (*Tetranychus urticae*), exhibited greater expression of multiple genes associated with insect herbivory resistance, including five *TPS* genes, which resulted in constitutively greater accumulation of eight terpenes, compared to a susceptible cultivar (Weinblum et al., 2021). A review of all constitutively differentially expressed genes revealed enrichment in phenylpropanoid and terpenoid biosynthesis in the resistant cultivar (Weinblum et al., 2021). It is notable, however, that, upon spider mite herbivory, both species exhibited inducible alterations in expression and metabolite accumulation (Weinblum et al., 2021). In general, the inducible responses observed in this study wherein 'PN40024' exhibited a greater inducible response compared to 'GREM4' appear consistent with our current understanding of the inverse relationship between constitutive and inducible defense considering the hypothetically enhanced constitutive insect herbivory resistance observed in 'GREM4'.

Increased Kaempferol Accumulation is Observed in 'GREM4' Under Both Constitutive and Insect Herbivory Conditions

To illustrate how metabolomic responses differed between species and time points, overlap analyses were conducted which identified DAMs unique or conserved between intra-species comparisons. Overlap analysis of these intra-species comparisons revealed 11 total metabolites with increased accumulation uniquely in 'GREM4'. Five of these 11 metabolites had known classifications comprising four flavonoids and one terpene. Notably, kaempferol was identified as one of the four flavonoid DAMs, and further, was

found to be exclusively identified in ‘GREM4’ in both intra- and inter-species comparisons. Previous studies have shown kaempferol exhibits insect repellent and insecticidal characteristics. When flavonoid biosynthesis gene *Flavone 3-hydroxylase* (*F3H*) was overexpressed in rice, heightened resistance to white-backed planthopper (*Sogatella furcifera*) was observed (Jan et al., 2020). Kaempferol was found to have increased in the overexpressed compared to wild type plants and was further found to be insecticidal (Jan et al., 2020). Similar responses, illustrating resistance or the insecticidal nature of kaempferol, have been observed against other insect pests in soybean treated with exogenous kaempferol (Stec et al., 2021), 375 cowpea (*Vigna unguiculata*) lines in which heightened levels of kaempferol were correlated with increased herbivory resistance (Togola et al., 2020), and in an insecticidal lead screening study in which kaempferol ingested by insects increased mortality (Su et al., 2018). In our study, kaempferol significantly increased in accumulation from 0h to 1h in ‘GREM4’ and was the metabolite with the greatest heightened accumulation (5.22 log2FoldChange) which was unique to ‘GREM4’ 1h compared to ‘PN40024’ 1h. Kaempferol was previously noted to increase in grapevine upon UV-B exposure (Berli et al., 2010). However, no previous work in grapevine pertaining to its role in insect herbivory could be identified. These findings suggest kaempferol is a prime metabolite for further investigation as it bears known insecticidal characteristics and accumulated at much greater concentrations in ‘GREM4’ compared to ‘PN40024’.

Flavonoids Increased in Accumulation Temporally in Both ‘GREM4’ and ‘PN40024’

Defense against insect herbivory typically is not only impacted by constitutive defenses but also those initiated by attack – inducible defenses. While inducible defenses were reviewed in previous sections, a deeper exploration of this temporal response to determine which metabolites increased or decreased significantly from 0h to 1h in each species warrants exploration.

Flavonoids were the sole class identified in the top 15 DAMs with the greatest increase in accumulation from ‘GREM4’ 0h to ‘GREM4’ 1h. These flavonoids in ‘GREM4’ included trifolin, kaempferol, quercetin (isoquercetin), 5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-{[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}-4H-chromen-4-one, and quercetin 3-O-rhamnoside-7-O-glucoside. Kaempferol was discussed previous above, while quercetin has also been shown to be insecticidal, or associated with resistance, in rice (Jan et al., 2020), cowpea (Togola et al., 2020), and tea (Jing et al., 2024). Further, quercetin has been found to be insecticidal in an insecticide lead study (Su et al., 2018). Together, the six DAMs classified as flavonoids in ‘GREM4’ accounted for a total log2FoldChange of 19.59. Meanwhile, of the top 15 most increased DAMs in ‘PN40024’, only two flavonoids were identified accounting for a total log2FoldChange of 7.44 – metabolomic class amino acids was actually the greatest with three total metabolites. When comparing a pathway found to be enriched in both the ‘GREM4’ 1h compared to ‘GREM4’ 0h and the ‘PN40024’ 1h compared to ‘PN40024’ 0h temporal responses, flavone and flavanone biosynthesis, a greater number of

implicated metabolites could be identified in the ‘GREM4’ enrichment compared to the ‘PN40024’ enrichment, as illustrated by the KEGG pathway map, and was also much more significant. Overall, while a greater number of flavonoids were identified in ‘PN40024’, these flavonoids were not some of the most significantly differentially accumulating metabolites, as was seen in ‘GREM4’. Flavonoids with clear connections to insect herbivory resistance were identified as some of the topmost increased accumulation DAMs in ‘GREM4’ from 0h to 1h, likely resulting in heightened defense. Additional studies are necessary to functionally test the importance of some of these top flavonoids, such as quercetin or kaempferol, to validate their impact on insect herbivory resistance in grapevine.

DAMs with lower accumulation from 0h to 1h in the intra-species analysis also reported flavonoids as being the classification with the greatest number of DAMs. However, DAMs were more evenly distributed amongst a variety of metabolomic classifications which included terpenoids, lipids, phenylpropanoids, and hydrocarbons. Regardless, the finding that flavonoids were the most implicated metabolic class of both DAMs with increased and decreased accumulation over time is reasonable considering pools of reactants, intermediates, and products of reactions upstream, within, and downstream of biosynthetic pathways are not infinite. While some metabolites may increase in accumulation in a given pathway, it is likely that others will decrease as a result, as they are consumed in reactions, or alternative branches of the pathway are favored, as is often observed in other systems (Chen et al., 2022; Wang et al., 2022; Xue

et al., 2022; Lv et al., 2023). It should not be surprising, therefore, that some metabolites classified as flavonoids are also found to decrease upon insect herbivory.

Conclusions

Untargeted metabolomic analysis revealed differences in metabolite accumulation between resistant *V. labrusca* acc. 'GREM4' and susceptible *V. vinifera* cv. 'PN40024' at 0h and after 1h of *Popillia japonica* (Japanese beetle) herbivory. Critically, some of the most differentially accumulating metabolites had known connections to insect resistance. Basal comparisons revealed constitutively greater accumulations of metabolites classified as flavonoids, phenylpropanoids, and terpenoids in insect herbivory resistant 'GREM4' compared to susceptible 'PN40024'. Flavonoids 6-methoxyflavanone and vanilloyl glucose, phenylpropanoid neochlorogenic acid, and terpenoids 8-geranylesculetin and 4-(4-hydroxy-2,6,6-trimethyl-3-{[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}cyclohex-1-en-1-yl)butan-2-one were some of the metabolites with the greatest increased accumulation in 'GREM4' compared to 'PN40024' and likely impact insect herbivory defense. Further, upon Japanese beetle herbivory, flavonoids were the predominantly identified class of metabolites and increased in both species, but a greater number were identified in 'PN40024'. Flavonoid kaempferol was exclusively significantly differentially accumulated in 'GREM4' compared to 'PN40024' and is a prime candidate for conferring defense as it is known to be insecticidal. Overall, this study reports that 'PN40024' has a greater inducible

metabolomic response after 1h of Japanese beetle feeding than ‘GREM4’. However, far stouter constitutive defenses, even compared to the inducible response observed of ‘PN40024’, are present in ‘GREM4’ which include metabolites in classes with known insect herbivory defensive activities including flavonoids, phenylpropanoids, and terpenes. This heightened basal defense likely explains the less drastic inducible defensive response observed in ‘GREM4’ compared to ‘PN40024’ upon herbivory. It is likely the heightened constitutive defenses identified in ‘GREM4’ play a role in the observed insect herbivory resistance phenotype of ‘GREM4’ but follow up functional studies are necessary to truly reveal the correlation between specific insect herbivory defensive metabolites and defense in ‘GREM4’.

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Availability of Data and Materials

The datasets presented in this article can be found within the main text and at the permanent repository hosted by figshare at https://figshare.com/s/95ea878b3f5153bac962 .

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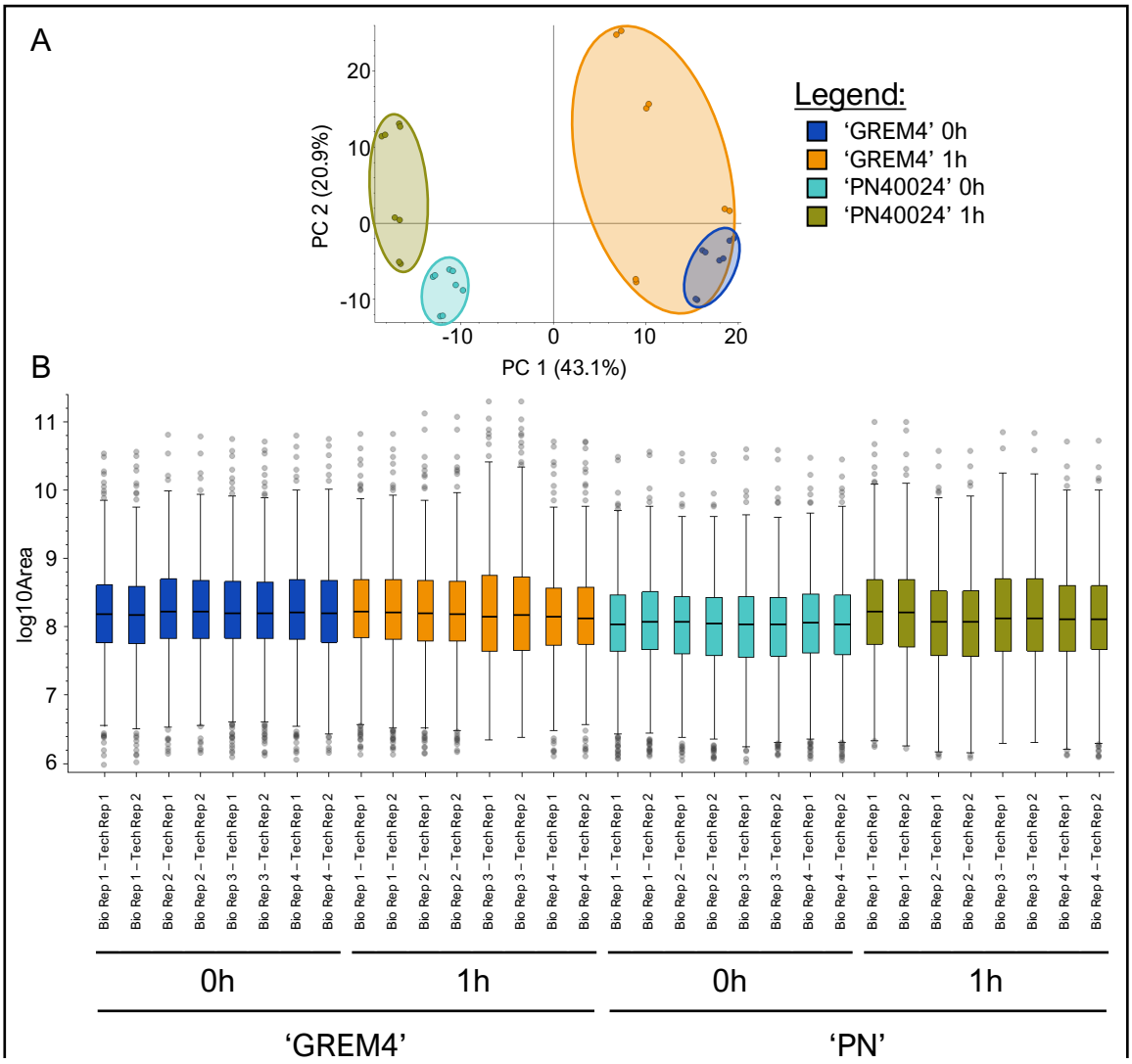
Figures

Figure 4.1 – Sample Variability Overview



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(Figure 4.1 continued)

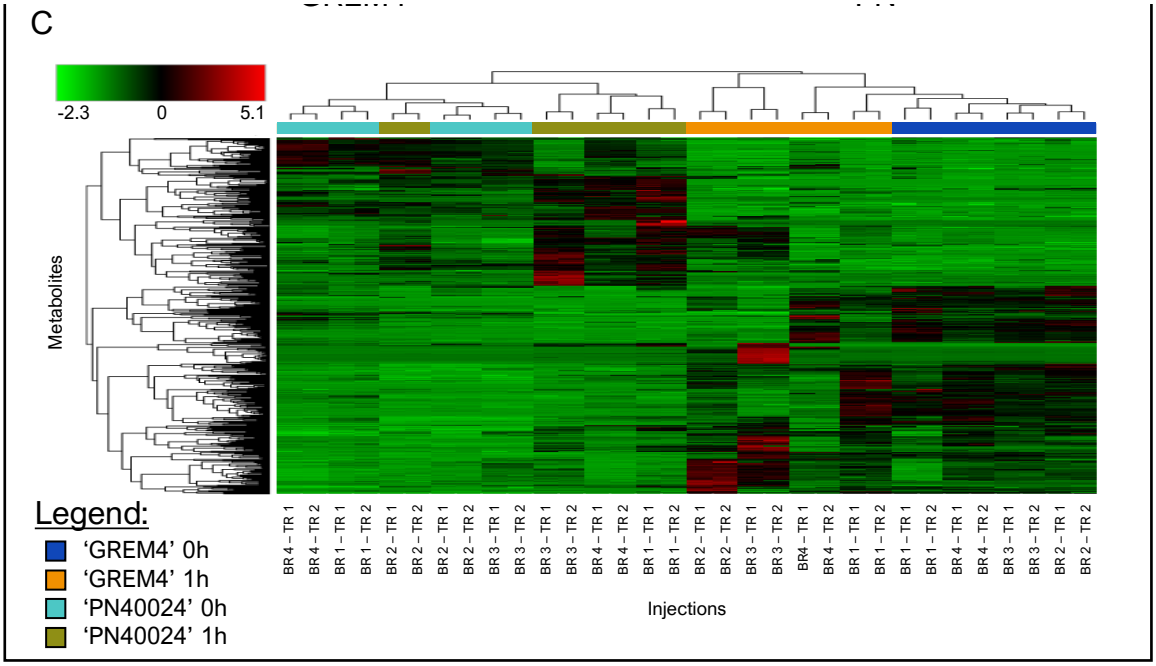


Figure 4.1 Caption – Graphics illustrating the variability and general accumulation trends of the metabolites identified within the samples. A. PCA plot of metabolomic compositions of injections. Each sample was injected twice resulting in eight total points per color (condition). B. Box and whisker plot displaying the average metabolite concentrations (areas under the peaks) for all injections. 'Log10Area' is the log10 value of the integrated area under the curve used to determine metabolite concentration. C. Heatmap illustrating differences in metabolite concentrations (areas under the peaks) for all injections. Naming on the x-axis is abbreviated wherein 'BR' = 'biological replicate' and 'TR' = 'technical replicate'. Coloration depicts the concentration of the metabolite in

Continued…

(Figure 4.1 continued)

the injection. Black is average (as determined against the overall concentrations of all metabolites in the entire dataset, and then normalized to 1), green represents below average, and red represents above average. All graphics were generated using Thermo Compound Discoverer.

Figure 4.2 – Numbers of DAMs in All Comparisons

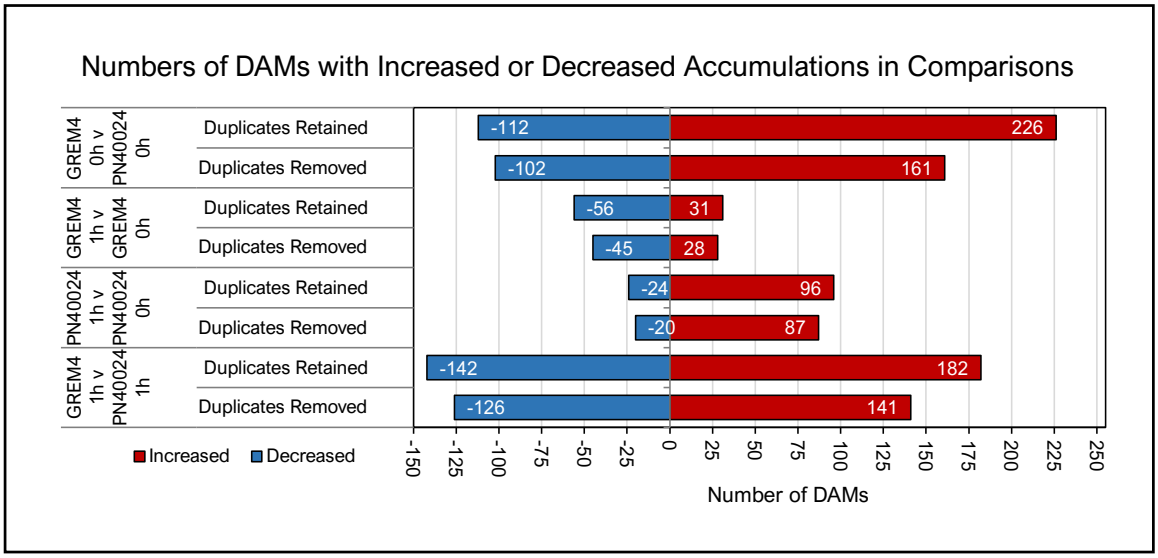
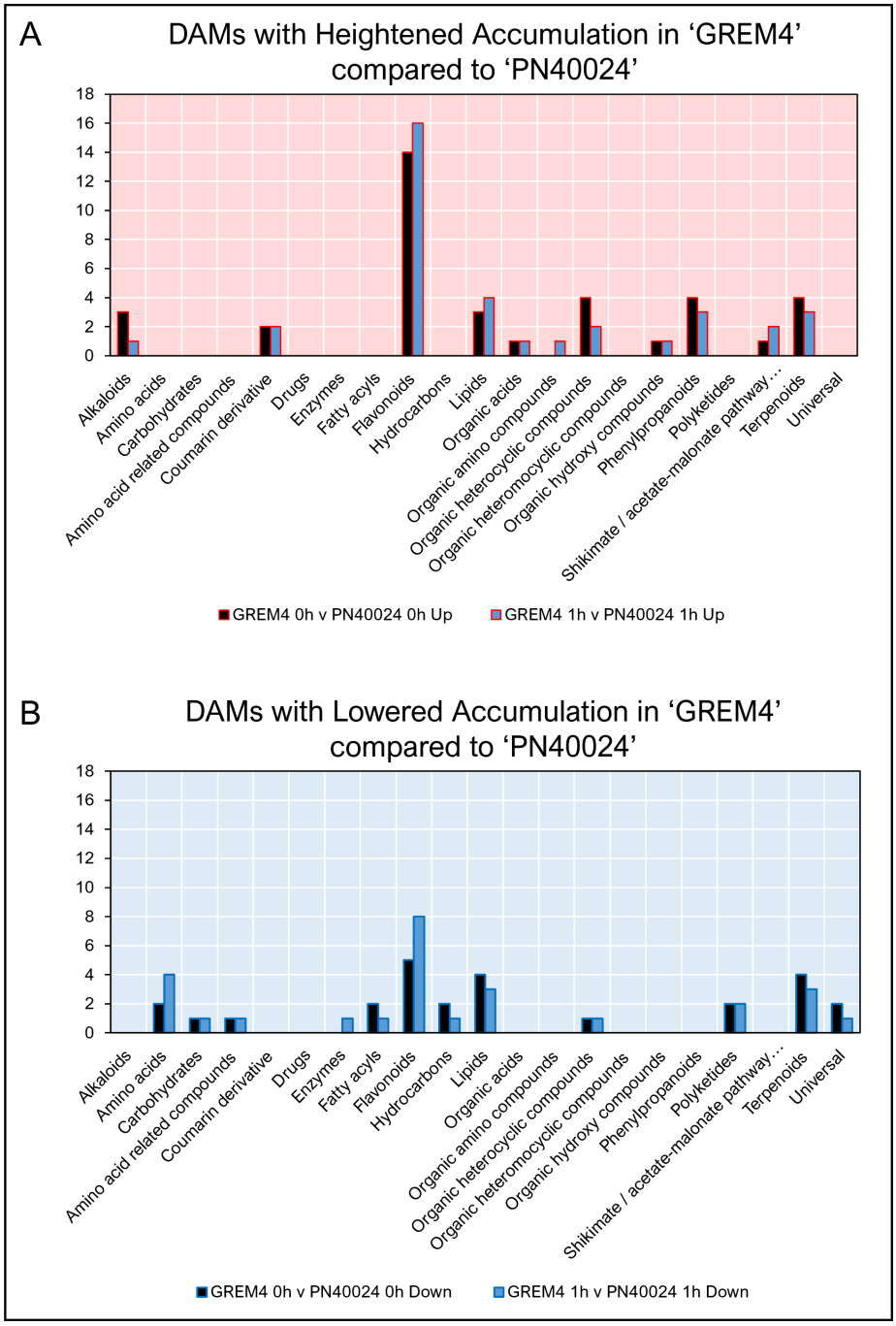


Figure 4.2 Caption – Numbers of DAMs in comparisons which were increased or decreased in accumulation. Numbers when both DAMs with duplicated names were removed or retained are presented. Red bars indicate DAMs which exhibited increased/heightened accumulation within the comparison while blue bars indicate DAMs which exhibited decreased/lower accumulation.

Figure 4.3 – Metabolite Classification Breakdown from Inter-species Comparisons

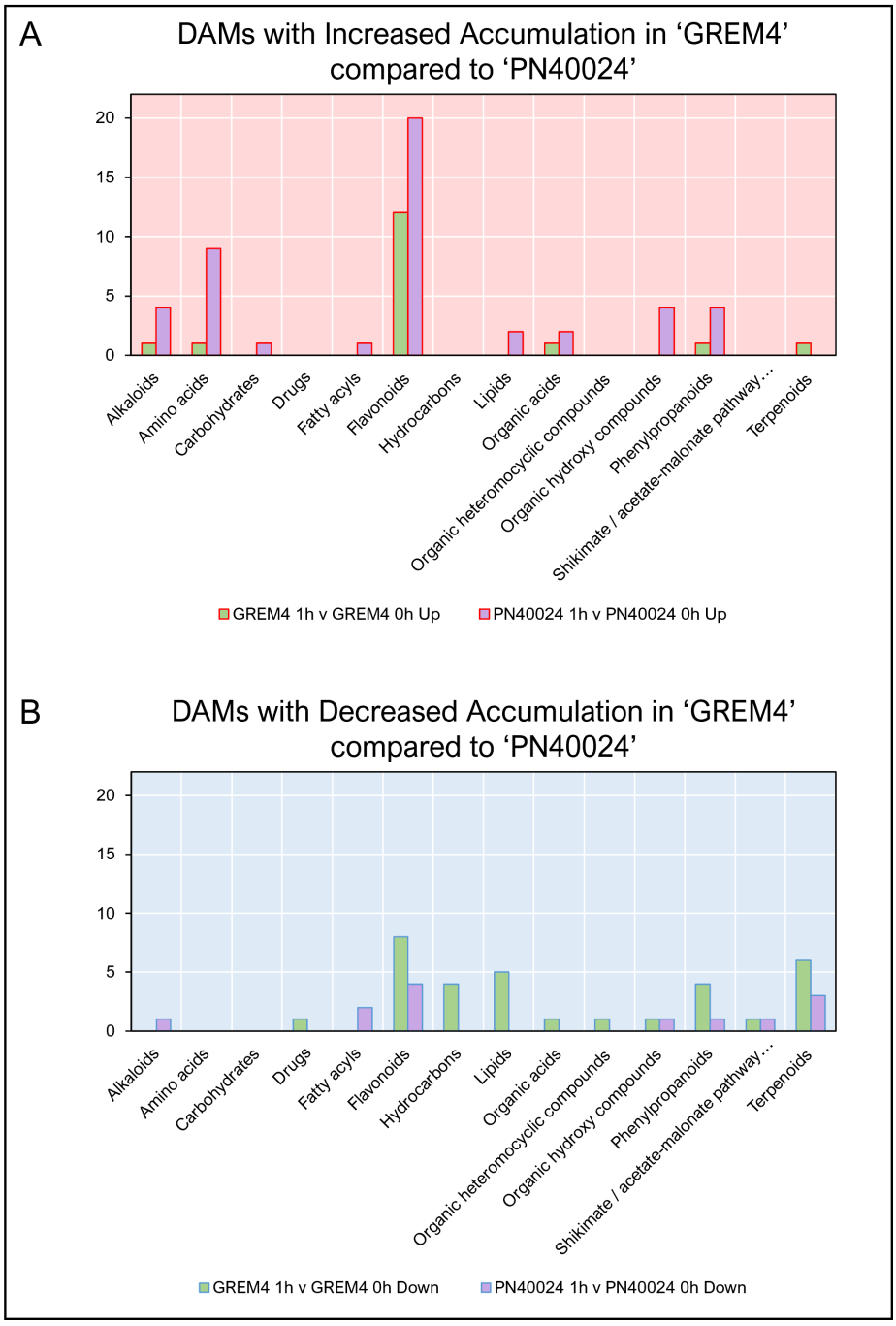


Continued…

(Figure 4.3 continued)

Figure 4.3 Caption – Numbers of metabolites associated with metabolic classifications from inter-species comparisons between ‘GREM4’ and ‘PN40024’. Black bars represent numbers of DAMs from 0h compared to 0h comparisons. Blue bars represent numbers of DAMs from 1h compared to 1h comparisons. A. DAMs with greater accumulation in ‘GREM4’ compared to ‘PN40024’. B. DAMs with lower accumulation in ‘GREM4’ compared to ‘PN40024’.

Figure 4.4 – Metabolite Classification Breakdown from Intra-species Comparisons



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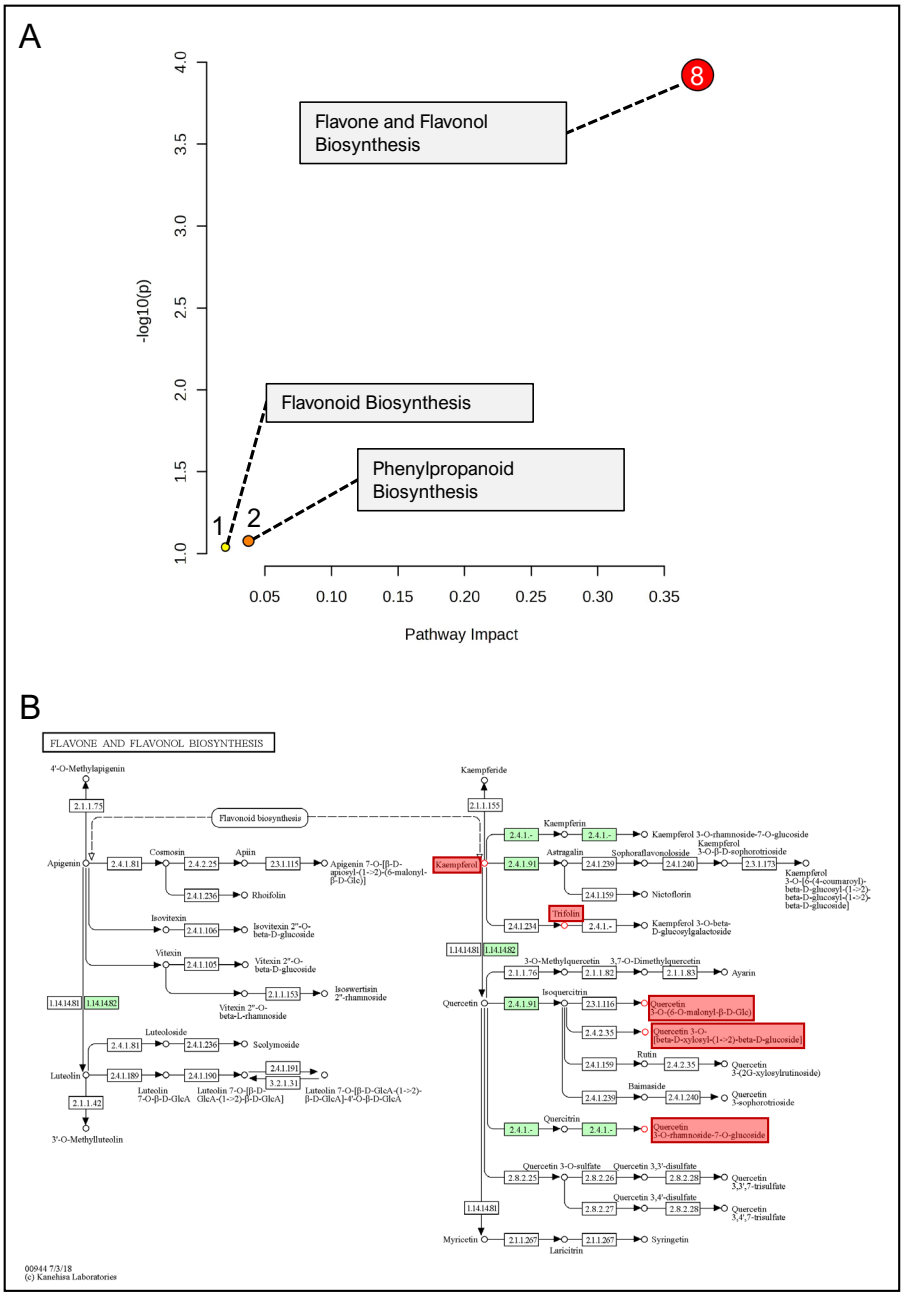
(Figure 4.4 continued)

Figure 4.4 Caption – Numbers of metabolites associated with metabolic classifications from intra-species comparisons between 1h and 0h. Green bars represent numbers of DAMs from 'GREM4' comparisons. Purple bars represent numbers of DAMs from 'PN40024' comparisons. A. DAMs with greater accumulation in 1h compared to 0h. B. DAMs with lower accumulation in 1h compared to 0h.

Figure 4.5 – ‘GREM4’ 1h compared to ‘GREM4’ 0h Pathway Enrichment Analysis Results



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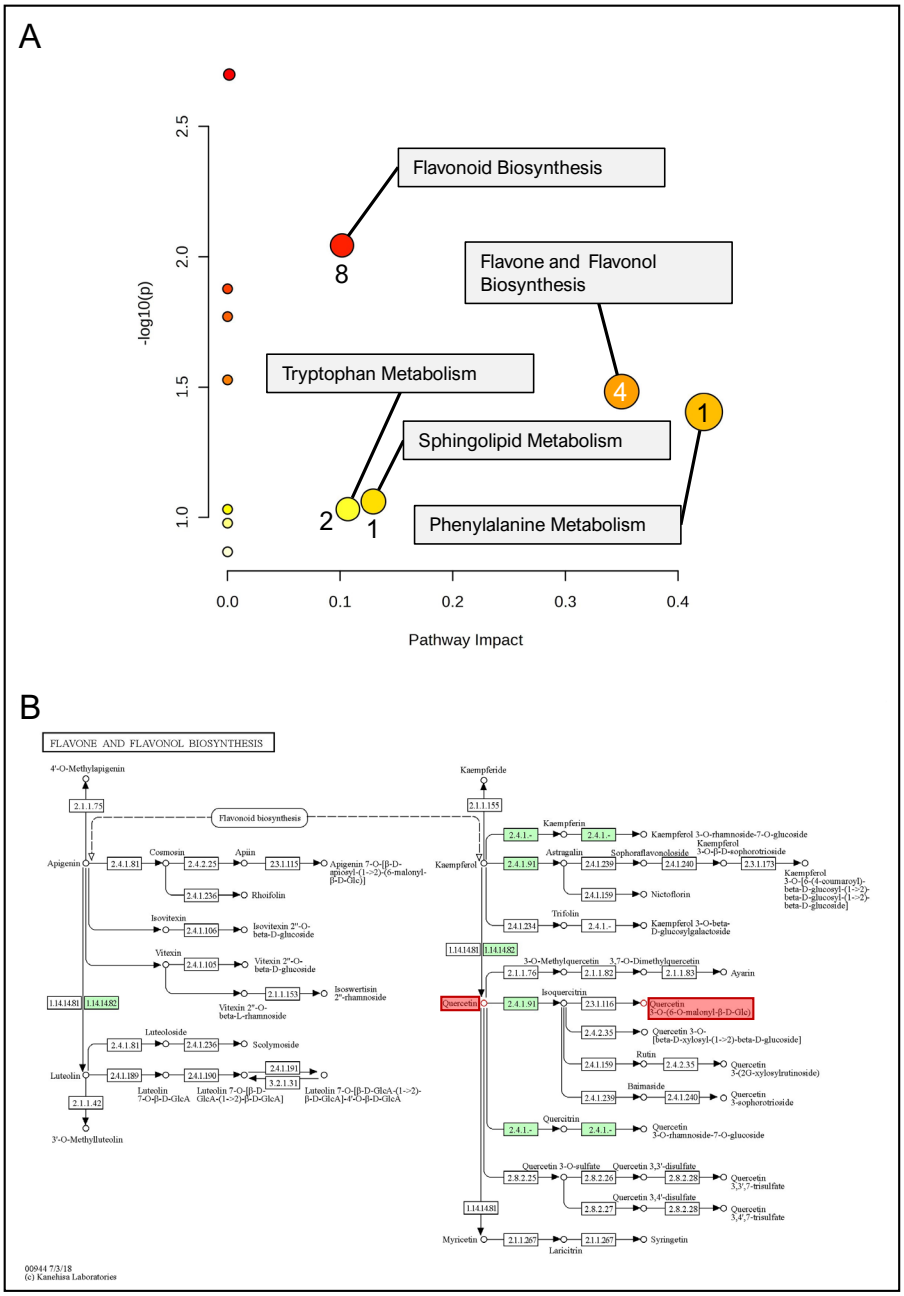
(Figure 4.5 continued)

Figure 4.5 Caption – Pathway Analysis results revealing biological pathways which were significantly enriched in DAMs with greater accumulation in ‘GREM4’ 1h compared to ‘GREM4’ 0h. A. Pathway Analysis enrichment dot plot. Pathway Analysis was conducted via MetaboAnalyst 6.0. Size of dot (small to large) positively correlates to pathway impact while color (yellow to red) correlates with low to high internal statistical significance. Numbers within, or adjacent to, dots relay the total number of top 50 DAMs, sorted by |log2FoldChange|, in the comparison which were assigned the noted KEGG pathway term in the KEGG Pathway Database. Enrichments were only deemed significant if a p-value of ≤ 0.05 and a pathway impact of ≥ 0.10 was reported for the pathway. B. The flavone and flavonol biosynthesis pathway diagram from KEGG is presented with the metabolites implicated as DAMs with increased accumulation from 0h to 1h in ‘GREM4’ highlighted in red.

Figure 4.6 – ‘PN40024’ 1h compared to ‘PN40024’ 0h Pathway Enrichment Analysis Results



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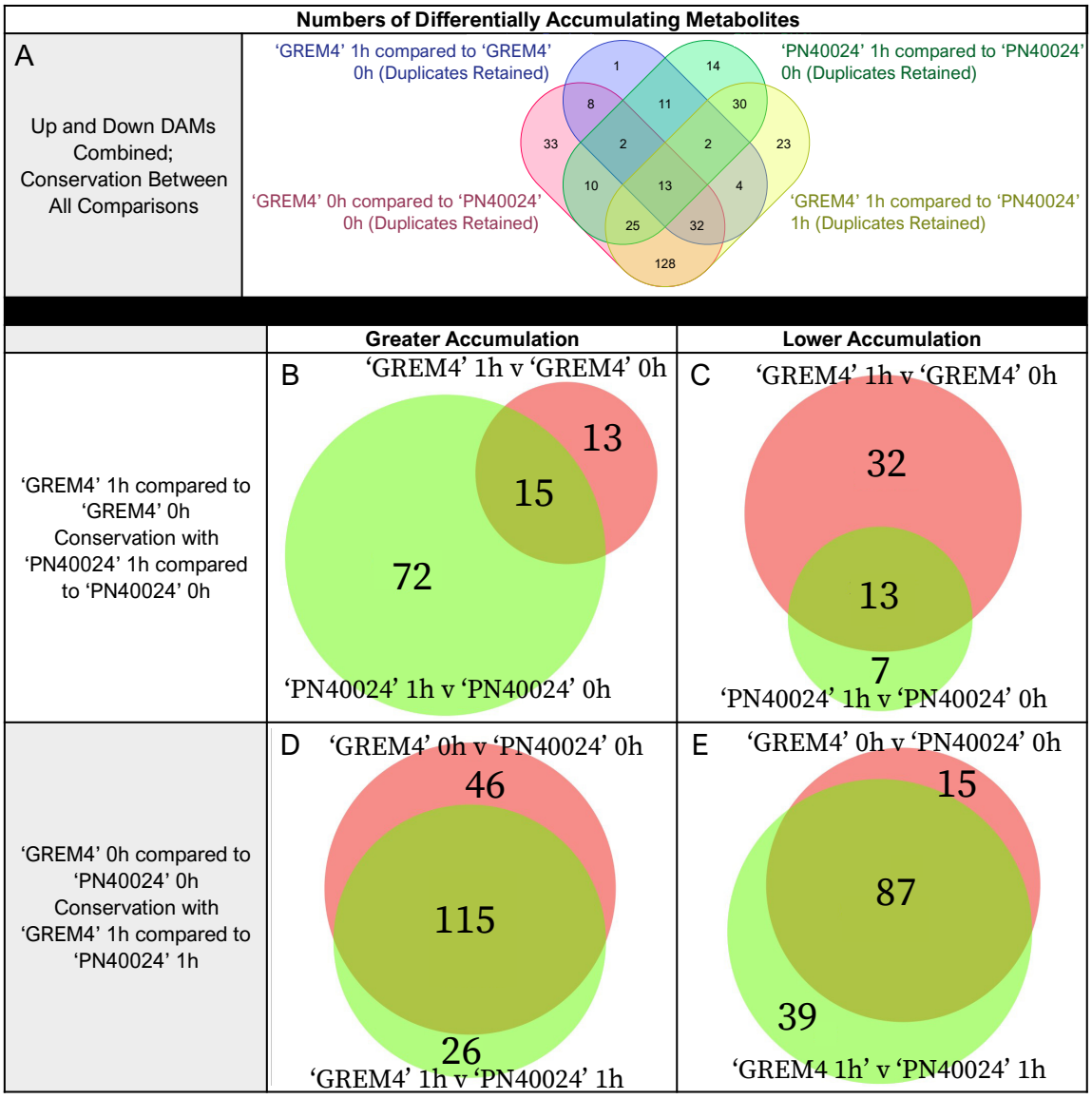
(Figure 4.6 continued)

Figure 4.6 Caption – Pathway Analysis results revealing biological pathways which were significantly enriched in DAMs with greater accumulation in ‘PN40024’ 1h compared to ‘PN40024’ 0h. A. Pathway Analysis enrichment dot plot. Pathway Analysis was conducted via MetaboAnalyst 6.0. The size of the dot (small to large) positively correlates to pathway impact while the color of the dot (yellow to red) correlates with low to high internal statistical significance. Numbers within, or adjacent to, dots relay the total number of top 50 DAMs, sorted by log2FoldChange, in the comparison which were assigned the noted KEGG pathway term in the KEGG Pathway Database. For our purposes, enrichments were only deemed significant if a p-value of ≤ 0.05 and a pathway impact of ≥ 0.10 was reported for the pathway. A dot without a number indicates their pathway impact was ≤ 0.01. B. The flavone and flavonol biosynthesis pathway diagram from KEGG is presented with the metabolites implicated as DAMs with increased accumulation from 0h to 1h in ‘PN40024’ highlighted in red.

Figure 4.7 – DAM Conservation Between Comparisons



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(Figure 4.7 continued)

Figure 4.7 Caption – Venn diagrams depicting DAMs which are unique or conserved between metabolomic comparisons. A. Conserved and unique DAMs between all comparisons (intra- and inter-species) when combining increased or decreased accumulating DAMs within each comparison. DAMs with the same name (duplicated names) were retained for these comparisons B. Conserved and unique DAMs between 'GREM4' 1h compared to 'GREM4' 0h and 'PN40024' 1h compared to 'PN40024' 0h which had increased in accumulation from 0h to 1h in their respective species. C. Conserved and unique DAMs between 'GREM4' 1h compared to 'GREM4' 0h and 'PN40024' 1h compared to 'PN40024' 0h which had decreased in accumulation from 0h to 1h in their respective species. D. Conserved and unique DAMs between 'GREM4' 0h compared to 'PN40024' 0h and 'GREM4' 1h compared to 'PN40024' 1h which had greater accumulation in 'GREM4' compared to 'PN40024' at their respective time points. E. Conserved and unique DAMs between 'GREM4' 0h compared to 'PN40024' 0h and 'GREM4' 1h compared to 'PN40024' 1h which had lower accumulation in 'GREM4' compared to 'PN40024' at their respective time points. All graphics were made with molbiotools or BioVenn.

Tables

Table 4.1 – All Metabolites Identified

File available at the permanent repository hosted by figshare at https://figshare.com/s/95ea878b3f5153bac962 .

Table 4.2 – DAM Conservation Lists

File available at the permanent repository hosted by figshare at https://figshare.com/s/95ea878b3f5153bac962 .

Table 4.3 – Top 15 Up and Down DAMs in Each Comparison

File available at the permanent repository hosted by figshare at https://figshare.com/s/95ea878b3f5153bac962 .

Table 4.4 – Pathway Analysis Enrichment Results

File available at the permanent repository hosted by figshare at https://figshare.com/s/95ea878b3f5153bac962 .

Table 4.5 – DAMs with Greater Accumulation at 1h compared to 0h in Intra-species Comparisons

DAMs with Greater Accumulation at 1h compared to 0h in Intra-species Comparisons*			
Groups:	**Metabolite:**		
	Brief Name[b]:	**Full Name[b]:**	**Classification[c]:**
Conserved In 'GREM4' and 'PN40024'[a]	2,6-Xylidine	2,6-Dimethylaniline	-
	2-Aminonicotinic acid	-	-
	-	5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-{[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}-4H-chromen-4-one	Flavonoids
	Quercetin 3-O-malonylglucoside	3-{[(2R,3S,4S,5R,6S)-6-{[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl]oxy}-3,4,5-trihydroxyoxan-2-yl]methoxy}-3-oxopropanoic acid	Flavonoids
	SCHEMBL6119167	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[[(2S,3R,4S,5S)-3,4,5-trihydroxyoxan-2-yl]oxy}-4H-chromen-4-one	Flavonoids
	Cochliophilin A	9-Hydroxy-6-phenyl-8H-[1,3]dioxolo[4,5-g]chromen-8-one	Flavonoids
	Isoleucine	-	Amino acids
	Caftaric acid	-	Organic acids
	Caffeic acid	-	Phenylpropanoids
	Piperidine	Azacyclohexane	Alkaloids
	4-Methylumbelliferone hydrate	Hymecromone hydrate	Flavonoids
Unique to 'GREM4'	Isoamylamine	-	-
	Trifolin	Kaempferol-3-O-galactoside	Flavonoids
	Kaempferol	-	Flavonoids
	-	3-{[(2S,3R,4S,5R,6R)-3,5-dihydroxy-6-(hydroxymethyl)-4-{[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy}oxan-2-yl]oxy}-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one	-
	-	2-(3,4-Dihydroxyphenyl)-3-(D-glucopyranosyloxy)-5-hydroxy-4-oxo-4H-chromen-7-yl D-glucopyranoside	-
	Quercetin 3-O-rhamnoside-7-O-glucoside	2-(3,4-dihydroxyphenyl)-5-hydroxy-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-3-[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxychromen-4-one	Flavonoids
	-	3-{[(2S,3R,4S,5S,6R)-4,5-dihydroxy-6-(hydroxymethyl)-3-{[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}oxan-2-yl]oxy}-2-(3,4-dihydroxyphenyl)-5-hydroxy-7-methoxy-4H-chromen-4-one	-
	Isoquercetin	Quercetin-3β-D-glucoside	Flavonoids
	-	5-(5,7-Dihydroxy-3-methoxy-4-oxo-4H-chromen-2-yl)-2-hydroxyphenyl beta-D-xylopyranoside	-
	Unknown-287	-	-
	Sesquiterpene lactone	-	Terpenoids

Continued…

(Table 4.5 continued)

Unique to 'PN40024'[d]	-	4',7-dimethoxy-2,2,4-trimethyl-Delta(3)-isoflavan	Flavonoids
	Benzoxiquine	-	Organic heterocyclic compounds
	Argininosuccinic acid	L-Argininosuccinic acid	Amino acids
	1-Caffeoyl-4-deoxyquinic acid	-	Phenylpropanoids
	Unknown Alkaloid-168	-	Alkaloids
	Unknown-484	-	-
	Proline	-	Amino acids
	N-Coumaroyl-L-aspartic acid	-	Amino acids
	Naringeninchalcone	-	Flavonoids
	Trigonelline	-	Alkaloids
	Jasmone	cis-jasmone	Fatty Acyls
	Xanthurenate	Xanthurenic acid	-
	-	7-Hydroxy-3-(4-methoxyphenyl)-4-propylcoumarin	Organic heterocyclic compounds
	Threonine	L-Threonine	Amino acids
	Lactamide	2-Hydroxypropanamide	Organic hydroxy compounds
	Isorhamnetin 4'-O-glucuronide	2-Methoxy-4-(3,5,7-trihydroxy-4-oxo-4H-chromen-2-yl)phenyl beta-D-glucopyranosiduronic acid	Flavonoids
	-	Indoxyl glucuronide	-
	-	Unknown-376	-
	-	5-hydroxy-2-(4-hydroxyphenyl)-6-methoxy-7-{[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}-4H-chromen-4-one	-
	Fraxetin	-	Phenylpropanoids
		Plus an additional 52 metabolites...	

Footnotes: *. DAMs with no name or an uncertain name were omitted from the table. a. As these DAMs were conserved in both species, the |log2FoldChange| values of the DAM in both species were combined, then, this summed log2FoldChange value was used for sorting, which occurred from greatest log2FoldChange to least. b. '-' denotes that no broadly used synonym was identified for the metabolite. c. '-' denotes that a metabolic classification could not be determined or the metabolite was not one of the 300 hand-curated metabolites with deeper annotation. d. Only the top 20 DAMs by |log2FoldChange| are presented as there were 72 total.

Table 4.6 – DAMs with Lower Accumulation at 1h compared to 0h in Intra-species Comparisons

DAMs with Lower Accumulation at 1h compared to 0h in Intra-species Comparisons*			
Groups:	**Metabolite:**		
	Brief Name[b]:	**Full Name[b]:**	**Classification[c]:**
Conserved In 'GREM4' and 'PN40024'[a]	Unknown-145	-	-
	Chromene	-	Flavonoids
	Unknown-291	-	-
	-	N-Acetyl-1,6-anhydro-Î²-muramic acid	-
	-	3,4,5-Trimethoxyphenyl acetate	Organic hydroxy compounds
	5,6,7-Trimethoxy-2-(2,3,4-trimethoxybenzylidene)indan-1-one	(2E)-5,6,7-trimethoxy-2-[(2,3,4-trimethoxyphenyl)methylidene]-3H-inden-1-one	-
	trans-3,3',5,5'-tetrahydroxy-4'-methoxystilbene	5-[(E)-2-(3,5-dihydroxyphenyl)ethenyl]-2-methoxybenzene-1,3-diol	Shikimate / acetate-malonate pathway derived compounds
	Choline sulfate	Choline O-Sulfate	-
	Sinapoylglucose	1-O-Sinapoyl-beta-D-glucose	Phenylpropanoids
	1-Methyl-4-(1-methyl-2-propenyl)-benzene	Benzene, 1-methyl-4-(1-methylethyl)-2-(1-propen-1-yl)-	Terpenoids
	beta-Ionone	EN0350000	Terpenoids

Continued…

(Table 4.6 continued)

Unique to 'GREM4'	Unknown-507	-	-
	Myristicin	4-methoxy-6-(prop-2-en-1-yl)-2H-1,3-benzodioxole	Phenylpropanoids
	3,4-Methyleneazelaic acid	-	Lipids
	2-(2E)-2-Octen-1-ylcyclopentanone	(E)-2-(2-Octenyl)cyclopentanone	-
	Methyl (E)-2-dodecenoate	-	Lipids
	-	4-{3-[3-(2,5-Cycloheptadien-1-ylidene)-1-propen-1-yl]-2-oxiranyl}butanal	-
	Acetyl tributyl citrate	Tributyl citrate acetate	Organic acids
	6-Methoxyflavanone	6-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one	Flavonoids
	(R)-Bitalin A	1-[2-(3-Hydroxy-1-propen-2-yl)-2,3-dihydro-1-benzofuran-5-yl]ethenone	-
	COSMENE	-	Hydrocarbons
	Apocynin	Acetovanillone	Drugs
	2-ETHOXYNAPHTHALENE	.beta.-Naphthol ethyl ether	-
	-	3-[(2H-1,3-benzodioxol-5-yl)methyl]-4-[(3,4-dimethoxyphenyl)methyl]oxolan-2-one	
	Heptylbenzene	4-Heptylphenol	Lipids
	Triptolide	-	Terpenoids
	p-Xylene	Ethylbenzene	Hydrocarbons
	ERIODYCTOL	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3,4-dihydro-2H-1-benzopyran-4-one	Lipids
	(-)-Rosmadial	7-Hydroxy-6-isopropyl-3',3'-dimethyl-2-oxospiro[1-benzofuran-3,1'-cyclohexane]-2',4-dicarbaldehyde	Organic heteromocyclic compounds
	8-Geranylesculetin	8-[(2E)-3,7-Dimethyl-2,6-octadien-1-yl]-6,7-dihydroxy-2H-chromen-2-one	Terpenoids
	Hexylbenzene	-	-
	(-)-epicatechin-3'-O-glucoside	(1xi)-1,5-Anhydro-1-[(2R,3R)-2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-3,4-dihydro-2H-chromen-6-yl]-D-glucitol	Flavonoids
	TDN	1,1,6-Trimethyl-1,2-dihydronaphthalene	-
	Methyl cinnamate	-	-
	Methyl (2E,6Z)-2,6-dodecadienoate	Methyl (2E,6Z)-dodeca-2,6-dienoate	Lipids
	-	1-(5-Acetyl-2-hydroxyphenyl)-3-methyl-1-butanone	-
	p-cymenene	1-Methyl-4-(prop-1-en-2-yl)benzene	Hydrocarbons
	Benzylideneacetone	4-Phenyl-3-buten-2-one	Terpenoids
	MCAL	4-Methoxycinnamaldehyde	-
	4'-Hydroxy-5,7-dimethoxyflavan	4-(5,7-Dimethoxy-3,4-dihydro-2H-chromen-2-yl)phenol	Flavonoids
Unique to 'PN40024'	Unknown Alkaloid-313	-	Alkaloids
	Phenylethyl primeveroside	2-(2-phenylethoxy)-6-{[(3,4,5-trihydroxyoxan-2-yl)oxy]methyl}oxane-3,4,5-triol	-
	Unknown Nitrogenous Compound-471	-	-
	2S-Amino-tridecanoic acid	-	Fatty acyls
	11-Aminoundecanoic acid	-	Fatty acyls

Footnotes: *. DAMs with no name or an uncertain name were omitted from the table. a. As these DAMs were conserved in both species, the |log2FoldChange| values of the DAM in both species were combined, then, this summed log2FoldChange value was used for sorting, which occurred from greatest log2FoldChange to least. b. '–' denotes that no broadly used synonym was identified for the metabolite. c. '–' denotes that a metabolic classification could not be determined or the metabolite was not one of the 300 hand–curated metabolites with deeper annotation.

Table 4.7 – DAMs with Greater Accumulation at 1h compared to 0h in Inter-species Comparisons

DAMs with Greater Accumulation at 1h compared to 0h in Inter-species Comparisons*			
Groups:	**Metabolite:**		
	Brief Name[b]:	**Full Name[b]:**	**Classification[c]:**
Conserved In Both 'GREM4' 0h compared to 'PN40024' 0h and 'GREM4' 1h compared to 'PN40024' 1h[a,d]	Neochlorogenic acid	(1r,3R,4s,5S)-4-{[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}-1,3,5-trihydroxycyclohexane-1-carboxylic acid	Phenylpropanoids
	Chromene		Flavonoids
	6-Methoxyflavanone	6-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one	Flavonoids
	Glycine derivative-21		Lipids
	8-Geranylesculetin	8-[(2E)-3,7-Dimethyl-2,6-octadien-1-yl]-6,7-dihydroxy-2H-chromen-2-one	Terpenoids
	-	4-(4-hydroxy-2,6,6-trimethyl-3-{[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}cyclohex-1-en-1-yl)butan-2-one	Terpenoids
	Vanilloyl glucose	1-O-vanilloyl-beta-D-glucose	-
	-	3-{[(2S,3R,4S,5S,6R)-4,5-dihydroxy-6-(hydroxymethyl)-3-{[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}oxan-2-yl]oxy}-2-(3,4-dihydroxyphenyl)-5-hydroxy-7-methoxy-4H-chromen-4-one	-
	Tributyl citrate acetate	Acetyl tributyl citrate	Organic acids
	Coumarin derivative-23	-	Coumarin derivative
	2,4-Diacetylphloroglucinol	1-(3-acetyl-2,4,6-trihydroxyphenyl)ethan-1-one	Organic hydroxy compounds
	10,2'-Dihydroxy-4',5'-methylenedioxy-isoflav-8-ene-7-one	(3R)-4a-Hydroxy-3-(6-hydroxy-1,3-benzodioxol-5-yl)-2,3,4,4a,5,6-hexahydro-7H-chromen-7-one	Flavonoids
	-	7-Hydroxy-3-(4-methoxyphenyl)-4-propylcoumarin	Organic heterocyclic compounds
	1-Caffeoyl-4-deoxyquinic acid	-	Phenylpropanoids
	Unknown-387	-	-
	(-)-epicatechin-3'-O-glucoside	(1xi)-1,5-Anhydro-1-[(2R,3R)-2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-3,4-dihydro-2H-chromen-6-yl]-D-glucitol	Flavonoids
	Isorhamnetin	-	Flavonoids
	-	(9aR,9bS)-9a-Hydroxy-6,9-dimethyl-3-methylene-3,3a,4,5,9a,9b-hexahydroazuleno[4,5-b]furan-2,7-dione	-
	-	5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-{[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}-4H-chromen-4-one	Flavonoids
	Alpha-ionone	EN0525000	Terpenoids
		Plus an additional 95 metabolites...	

Continued…

(Table 4.7 continued)

Unique to 'GREM4' 0h compared to 'PN40024' 0h[e]	Trigonelline	-	Alkaloids
	Unknown-507	-	-
	Methyl (E)-2-dodecenoate	-	Lipids
	N-Acetylnorvaline	-	-
	-	3,6,7-Trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-8aH-chromen-5-yl hexopyranosiduronic acid	-
	ERIODYCTOL	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3,4-dihydro-2H-1-benzopyran-4-one	Lipids
	-	1-(5-Acetyl-2-hydroxyphenyl)-3-methyl-1-butanone	-
	Sphinganine	-	Lipids
	Nitrosoguvacoline	-	-
	Taxifolin	-	Flavonoids
	Unknown-145	-	-
	Flavokawain A	-	-
	Diphenyl carbonate	-	-
	-	(+)-3'-hydroxylarreatricin	-
	-	N-COUMAROYL-L-ASPARTIC ACID	Amino acids
	-	7-(2-Chloroethyl)theophylline	-
	-	(3E)-4-(3-Hydroxy-1H-inden-2-yl)-2-oxo-3-butenoic acid	-
	Myristicin	4-methoxy-6-(prop-2-en-1-yl)-2H-1,3-benzodioxole	Phenylpropanoids
	Indoxyl glucuronide	-	-
	Methylone	-	-
		Plus an additional 26 metabolites...	Flavonoids
Unique to 'GREM4' 1h compared to 'PN40024' 1h	Kaempferol	-	Flavonoids
	Unknown Nitrogenous Compound-471	-	-
	-	2-(3,4-Dihydroxyphenyl)-3-(D-glucopyranosyloxy)-5-hydroxy-4-oxo-4H-chromen-7-yl D-glucopyranoside	-
	Unknown Alkaloid-313	-	Alkaloids
	N-Undecanoylglycine	-	Lipids
	1-Octylpyrrolidin-2-one	-	Organic amino compounds
	N-lauroylglycine	-	Lipids
	Catechin gallate	Epicatechin gallate	Flavonoids
	11-Aminoundecanoic acid	-	Fatty acyls
	2S-Amino-tridecanoic acid	-	Fatty acyls
	Phenylethyl primeveroside	2-(2-phenylethoxy)-6-{[(3,4,5-trihydroxyoxan-2-yl)oxy]methyl}oxane-3,4,5-triol	-
	Methyl (E)-2-dodecenoate	-	Lipids
	Unknown-287	-	-
	(±)-Malic Acid	-	Organic acids
	-	Bis(methylbenzylidene)sorbitol	-
	-	N-Acetyl-1,6-anhydro-Ĩ²-muramic acid	-
	-	(4E)-5-Hydroxy-4-{(2E,4E,6R)-1-hydroxy-6-[(3R,4R,6R)-6-hydroxy-1,4,8-trimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl]-4-methyl-2,4-heptadien-1-ylidene}-2,4-dihydro-3H-pyrrol-3-one	-
	Bicine	-	-
	Sesquiterpene lactone	-	Terpenoids
	Citral	-	Terpenoids
	-	4-[(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)methylidene]-3-methyl-1-phenyl-4,5-dihydro-1H-pyrazol-5-one	-
	Unknown-291	-	-
	2,4-Dimethylbenzaldehyde	-	-

Footnotes: *. DAMs with no name or an uncertain name were omitted from the table. a. As these DAMs were conserved in both species, the |log2FoldChange| values of the DAM in both species were combined, then, this summed log2FoldChange value was used for sorting, which occurred from greatest log2FoldChange to least. b. '-' denotes that no broadly used synonym was identified for the metabolite. c. '-' denotes that a metabolic classification could not be determined or the metabolite was not one of the 300 hand-curated metabolites with deeper annotation. d. Only the top 20 DAMs by |log2FoldChange| are presented as there were 115 total. e. Only the top 20 DAMs by |log2FoldChange| are presented as there were 46 total.

Table 4.8 – DAMs with Lower Accumulation at 1h compared to 0h in Inter-species Comparisons

DAMs with Lower Accumulation at 1h compared to 0h in Inter-species Comparisons*			
Groups:	**Metabolite:**		
	Brief Name[b]:	**Full Name[b]:**	**Classification[c]:**
Conserved In Both 'GREM4' 0h compared to 'PN40024' 0h and 'GREM4' 1h compared to 'PN40024' 1h[a,d]	-	5-hydroxy-2-(4-hydroxyphenyl)-6-methoxy-7-{[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}-4H-chromen-4-one	-
	Astilbin	-	Polyketides
	-	4-Hydroxy-2-(7-hydroxy-6-methoxy-4-oxo-4H-chromen-3-yl)-5-methoxyphenyl hexopyranoside	-
	Unknown-259	-	-
	-	1-(4-benzhydrylpiperazino)-3-[4-(1,2,3-thiadiazol-4-yl)phenoxy]-2-propanol	-
	Neodiosmin	5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxo-4H-1-benzopyran-7-yl 2-O-(6-deoxy-alpha-L-mannopyranosyl)-beta-D-glucopyranoside	Flavonoids
	Syringetin	-	Flavonoids
	Quercetin 3-O-rhamnoside-7-O-glucoside	2-(3,4-dihydroxyphenyl)-5-hydroxy-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-3-[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxychromen-4-one	Flavonoids
	Norlichexanthone	-	Polyketides
	-	(3E)-4-(3-Hydroxy-1H-inden-2-yl)-2-oxo-3-butenoic acid	
	Taxifolin	Dihydroquercetin	Flavonoids
	-	(4S)-4-hydroxy-3,5,5-trimethyl-4-[(1E)-3-{[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}but-1-en-1-yl]cyclohex-2-en-1-one	-
	Quercetin 3-O-malonylglucoside	3-{[(2R,3S,4S,5R,6S)-6-{[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl]oxy}-3,4,5-trihydroxyoxan-2-yl]methoxy}-3-oxopropanoic acid	Flavonoids
	g-Butyrobetaine	4-(Trimethylammonio)butanoate	-
	Methyl valerate	YV7750500	Lipids
	2,6-Xylidine	2,6-Dimethylaniline	-
	Phosphoric acid	-	Universal
	beta-L-Oleandropyranose	2,6-Dideoxy-3-O-methyl-L-arabino-hexopyranose	-
	Cyanogenic glucoside-520	-	Amino acid related compounds
	1-Isopropenyl-3-isopropylbenzene	-	-
		Plus an additional 67 metabolites…	
Unique to 'GREM4' 0h compared to 'PN40024' 0h	-	3-{[(2S,3R,4S,5R,6R)-3,5-dihydroxy-6-(hydroxymethyl)-4-{[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy}oxan-2-yl]oxy}-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one	-
	Tsibulin 1	4-Octyl-1,3-cyclopentanedione	-
	Jasmone	cis-jasmone	Fatty Acyls
	beta-Ionone	EN0349999	Terpenoids
	Resveratrol	-	Shikimate
	-	(3S)-3-Methyl-5-[(1S,8aR)-2,5,5,8a-tetramethyl-4-oxo-1,4,4a,5,6,7,8,8a-octahydro-1-naphthalenyl]pentanoic acid	-
	8-hydroxy-deoxyguanosine	-	-
	NP-008999	-	-
	-	(3E)-4-(4-Hydroxy-2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one	-
	Guanine	-	-
	-	Methyl 2,3-dihydro-3-hydroxy-2-oxo-1H-indole-3-acetate	-
	Isoleucine	-	Amino acids
	-	3',4'-Dihydroxyphenylacetone	-
	Hexyl 2-furoate	-	-

Continued…

(Table 4.8 continued)

Unique to 'GREM4' 1h compared to 'PN40024' 1h[e]	-	4',7-dimethoxy-2,2,4-trimethyl-Delta(3)-isoflavan	Flavonoids
	Argininosuccinic acid	L-Argininosuccinic acid	Amino acids
	Benzoxiquine	-	Organic heterocyclic compounds
	Jasmone	cis-jasmone	Fatty Acyls
	Isorhamnetin 4'-O-glucuronide	2-Methoxy-4-(3,5,7-trihydroxy-4-oxo-4H-chromen-2-yl)phenyl beta-D-glucopyranosiduronic acid	Flavonoids
	Threonine	L-Threonine	Amino acids
	Lactamide	2-Hydroxypropanamide	Organic hydroxy compounds
	Proline	-	Amino acids
	L-Glutamic acid	-	Amino acids
	Flavin mononucleotide (FMN)	-	-
	NP-013210	-	-
	L-Glutathione (reduced)	-	-
	L-(+)-Aspartic acid	-	-
	L-Aspartic acid	-	Amino acids
	3-Methyl-1-phenyl-2-butene	-	-
	Vitamin C	-	Vitamin and cofactors
	N-Acetyl-L-glutamic acid	-	-
	5-Pentylresorcinol	-	-
	Butopyronoxyl	-	-
	-	(2E,4Z)-2-Hydroxy-6-(2-hydroxyphenoxy)-6-oxo-2,4-hexadienoic acid	-
		Plus an additional 19 metabolites...	

Footnotes: *. DAMs with no name or an uncertain name were omitted from the table. a. As these DAMs were conserved in both species, the |log2FoldChange| values of the DAM in both species were combined, then, this summed log2FoldChange value was used for sorting, which occurred from greatest log2FoldChange to least. b. '-' denotes that no broadly used synonym was identified for the metabolite. c. '-' denotes that a metabolic classification could not be determined or the metabolite was not one of the 300 hand-curated metabolites with deeper annotation. d. Only the top 20 DAMs by |log2FoldChange| are presented as there were 87 total. e. Only the top 20 DAMs by |log2FoldChange| are presented as there were 39 total.

Chapter 5 - Conclusions and Future Directions

Grapevine is a crop cultivated across the globe for its great taste, high nutritional quality, and antioxidants (Hasanaliyeva et al., 2020). Grapes can be enjoyed in many forms, from table grapes to jellies to juices and wines. As such, exploring mechanisms to enhance insect herbivory resistance in grapevine is an undertaking with wide-reaching implications.

Insect pests are one of many biotic stressors affecting grapevine yields, Japanese beetles (*Popillia japonica*) being chiefly among them (Gu and Pomper, 2008; Johnson et al., 2010). While *Vitis labrusca*, a wild grapevine native to northern North America, is known to be resistant to fungal pathogens (Cadle-Davidson, 2008), resistance to insect pests was previously unknown outside of studies exploring hybrids containing *V. labrusca* in their breeding backgrounds (Mercader and Isaacs, 2003), and other wild grapevines (Gu and Pomper, 2008; Naegele et al., 2020), which had shown increased resistance in a field setting. Conversely, *Vitis vinifera* grapevine, which is native to Europe, is highly susceptible to pathogens and insect pests (Moio and Etievant, 1995; Dami et al., 2005; Smith, 2005; Dami, 2007; Cadle-Davidson et al., 2011; Qiu et al., 2015).

Our results reported that *V. labrusca* grapevine accession 'GREM4' exhibited insect herbivory resistance compared to *Vitis vinifera* cultivar 'PN40024' in both choice and no-choice herbivory studies over varying periods of time. While these results were observed in a greenhouse setting, additional studies are necessary to determine if the observed insect herbivory resistance is translatable to the field. Moreover, additional experimentation with various insect species, particularly those from diverse feeding guilds, is warranted. Previous studies suggest that insect herbivory defense and resistance can be tailored to specific feeding types, and in some instances, may even be specific to particular genera or species (Appel et al., 2014; Zeng et al., 2021).

High densities of trichomes were identified on leaves of 'GREM4' compared to 'PN40024'. Upon testing, beetles were found to prefer to feed on the low compared to high trichomes density sides of leaves, although the total leaf area lost to herbivory was not significant. Trichome density imparting insect herbivory resistance has been observed in many crops previously including wheat (Singh et al., 2021), *Datura stramonium* (Valverde et al., 2001), soybean (de Queiroz et al., 2020), and have even been found to specifically aid in defense against Japanese beetles in grapevine (Dami et al., 2005; Johnson et al., 2010). In our study, when trichome densities were not significantly different from one another, a greater leaf area was still damaged in 'PN40024' compared to 'GREM4' suggesting other factors, in addition to trichomes, played a role in conferring the resistance. It is possible other morphological adaptations may play a role in the insect herbivory resistant phenotype of 'GREM4' beyond

trichomes, such as increased sclerophylly. Deeper exploration of the morphological and associated physiological adaptations which may impact insect herbivory resistance in *Vitis*, employing 'GREM4' as a known insect herbivory resistant ideotype to compare against, could allow for interrogation of this question.

As trichomes were not the sole factor imparting resistance, a comparative transcriptomic study was undertaken. An exploration of constitutive expression, at 0h before any feeding, reported thousands of differentially expressed genes (DEGs) which included numerous genes with increased expression in 'GREM4' compared to 'PN40024' related to terpenes, carotenoids, phenylpropanoids, and flavonoids. Considering terpenes (Sun et al., 2017; Liu et al., 2020a; Wang et al., 2023a), flavonoids (Kumar and Yadav, 2017; Kariyat et al., 2019; Chatterjee et al., 2022; Zhang et al., 2022a; Lv et al., 2023), and phenylpropanoids (Moing et al., 2003; Capitani et al., 2013; Dixit et al., 2020; Wang et al., 2022) are known to be insect repellent or insecticidal, and, as it is known that constitutive expression of genes related to defense increases resistance (Weinblum et al., 2021; Whitehill et al., 2021; Lv et al., 2023), it is likely these genes play a role in conferring heightened insect herbivory resistance. When reviewing inducible defenses, the number of DEGs increased from 30min to 4h in 'GREM4' but decreased for 'PN40024'. Some of the topmost differentially expressed genes at 4h in 'PN40024' were related to defense but others were related to growth and reproduction which likely do not aid in defense. Meanwhile, in 'GREM4', by 4h, many DEGs related to defense were significantly up-regulated including *PAL1-1*, *AOS1,* and *TPS14-1* – genes with known

implications in insect herbivory defense (Wu et al., 2008; Sun et al., 2017; He et al., 2019). These results suggest that 'GREM4' exhibited an increasing defense response over time while 'PN40024' exhibited a decreasing response.

An important consideration, when interpreting transcriptomic and metabolomic (-omic) data, is that many factors ultimately impact the -omic state of a plant. As such, transcriptomic and metabolomic states *in planta* are composites of a confluence of many factors which alter gene expression and metabolite production. Some considerations include abiotic factors such as water stress (Mickky et al., 2020), time of day (Schmelz et al., 2003), or UV irradiation (Escobar-Bravo et al., 2019), biotic factors such as fungal infection (Maia et al., 2020), presence of VOCs (Meents et al., 2019), or tissue damage (Reymond et al., 2000; Glauser et al., 2009), as well as other seemingly minor factors such as if leaves are hit with rain droplets (van Moerkercke et al., 2019). Therefore, the responses elucidated in these studies should be replicated under varying conditions. For instance, conducting similar studies on *V. labrusca* during the veraison stage could provide insight into how the response might vary as the plant progresses throughout the growing season and could inform management practices or breeding decisions. One highly important aspect of this paradigm of differential response which depends upon the interaction of various abiotic and biotic factors is insect feeding type. As insects from different feeding guilds are known to elicit unique transcriptomic responses *in planta* (Appel et al., 2014; Erb and Reymond, 2019; Mostafa et al., 2022), and considering studies herein exclusively employed Japanese beetles, a rasping mouthpart feeder, it is

critical to conduct a comparative transcriptomic and/or metabolomic study, similar to that presented here, with a piercing-sucking mouthpart feeding type insect to determine if similar responses are observed upon attack by a different feeding guild and how such unique, or conserved, responses may alter defense.

Systemic response, another critical aspect of defense, was investigated and over 1,100 DEGs were identified in 'GREM4' across 30min, 1h, and 4h time points while only 116 total DEGs were identified in 'PN40024'. Some of the enrichments identified in systemic response leaves of 'GREM4' included pathogen defense response and signaling-related terms, meanwhile, only a few enriched terms were identified for 'PN40024' and were mostly related to abiotic stress response and protein folding. These results suggest that a more responsive inducible transcriptomic response is observed in 'GREM4' compared to 'PN40024' and involved a variety of signaling mechanisms including calcium ion signaling, lipid transport, and MAPK signaling. As many plants with heightened insect herbivory resistance in systemic tissues due to priming exhibit heightened gene expression in primed systemic tissues compared to unprimed controls (ul Malook et al., 2019, 2021; Meza-Canales et al., 2022; Xue et al., 2022; Mamin et al., 2023; Tong et al., 2023), it reasons that heightened transcriptomic response in unafflicted leaves reported herein may result in the same heightened resistance. For this reason, an intriguing future study could explore if systemic leaves in 'GREM4', after being primed by insect herbivory in distal leaves, exhibit heightened insect herbivory resistance compared to non-primed 'GREM4' leaves.

An untargeted comparative metabolomic study was next undertaken to investigate metabolomic differences upon beetle herbivory in ‘GREM4’ and ‘PN40024’ and follow up on transcriptomic responses observed in our previous work. Prior to any herbivory, constitutively significantly greater accumulation of multiple metabolites related to the metabolic classes of flavonoids, phenylpropanoids, and terpenes were identified in ‘GREM4’ compared to ‘PN40024’ which likely positively impacted insect herbivory resistance in ‘GREM4’ considering the known implications of these metabolic classes in insect defense. Upon herbivory, inducible alterations in the metabolomes between the two species revealed greater numbers of significantly differentially accumulating metabolites (DAMs) in ‘PN40024’ compared to ‘GREM4’. While this result was intriguing at first, in many plants an inverse relationship exists between constitutive defenses and inducible defenses wherein plants with heightened constitutive defenses often possess diminished inducible defense while plants with low constitutive defense often undergo more aggressive inducible alterations (Karban and Baldwin, 1997; Agrawal et al., 2010; Rasmann et al., 2015). We hypothesize that this phenomenon explains the lesser inducible response observed in ‘GREM4’ compared to ‘PN40024’ as ‘GREM4’ constitutively produces a hypothetically robust defense which is not observed in ‘PN40024’. It is also possible that the altered expression of DEGs in ‘GREM4’ did not yet impact the metabolome at 1h to the same degree as was seen in ‘PN40024’ at 1h, as it is known that ‘PN40024’ exhibited a greater total number of DEGs at 30min compared to ‘GREM4’. A notable metabolite, neochlorogenic acid, a phenylpropanoid

which is shown to accumulate in greater concentrations in insect herbivory compared to susceptible peach (Moing et al., 2003; Capitani et al., 2013), was one of the topmost DAMs with a greater accumulation in 'GREM4' compared to 'PN40024' at both 0h and 1h. Meanwhile, another notable metabolite, kaempferol, a flavonoid which is implicated in heightened insect herbivory resistance (Su et al., 2018), was found to be one of the most increased DAMs from 0h to 1h upon herbivory and was unique to 'GREM4'. Overall, the constitutive metabolomic profile of 'GREM4' appeared to provide a base level of insect herbivory defense which was then augmented through inducible induction of further insect herbivory pathways resulting in heighted accumulation of metabolites with known connections to insect herbivory resistance. Meanwhile, 'PN40024' did not appear to exhibit strong constitutive defense which resulted in greater inducible response but, even still, with fewer overall metabolites associated with defense compared to 'GREM4'.

To aid in future studies, we have reported candidate genes and metabolites which we hypothesize play an important role in conferring the insect herbivory resistance observed in 'GREM4'. These candidates were either exclusively identified in, or were highly differentially expressed or accumulating, in 'GREM4'. These candidates should be the subjects of future follow-up studies in which knock-out or over-expression of the genes can confirm or reject their importance in conferring the insect herbivory resistant phenotype when transgenic plants are challenged by an insect pest. Similarly, genes implicated in the production (biosynthetic pathways) of metabolite candidates can be

subjected to functional studies as well. Metabolites themselves can additionally be isolated from plant tissues and employed in artificial diet assays to determine their insecticidal activity. Findings of genes, functions, pathways, and metabolites implicated in conferring resistance in 'GREM4' compared to 'PN40024' will likely have the greatest immediate value in *Vitis* for use in guiding breeding and transgenesis programs. These findings, however, may also be applied to other crops, especially those of particular pathways or functions, and could even potentially lay the groundwork for metabolites to become insecticides, whether biological or synthetically engineered to improve activity or durability.

Taking into account our studies holistically, our findings offer comprehensive insights into insect herbivory defense in *V. labrusca* and *V. vinifera,* and perhaps in *Vitis* more broadly. The canonical insect herbivory defensive paradigm (Hettenhausen et al., 2015; Bigeard and Hirt, 2018; Johns et al., 2021; Zhang and Zhang, 2022; Bender and Zipfel, 2023) follows a relatively conserved process in which elicitor molecules, either in the form of herbivory associated molecular patterns (HAMPs) or damage associated molecular patterns (DAMPs), bind with receptors on the cell wall surface (Figure 5.1). This binding causes the receptor, which is anchored in the cell membrane and possesses a component which protrudes into the cytoplasm, to alter conformationally or phosphorylate another molecule within the cell which conveys a signal that the receptor has been activated (Bender and Zipfel, 2023). This signal is then typically conveyed downstream by subsequent phosphorylation of intermediate proteins, which, most

frequently, results in activation of the mitogen activated phosphorylation signaling cascade (MAPK signaling cascade) in which subsequent rounds of phosphorylation result in an increasingly amplified signal which ultimately result in one of two cell-state altering processes (Bigeard and Hirt, 2018). The first option is the modification of proteins resulting in altered activity, stability or degradation, conformation, or a targeting signal. These modifications are known as post-translational modifications (PTMs) and include methylation, acylation, phosphorylation, and ubiquitination which modify the functionality of the protein resulting in an altered cellular state (response) (Zhang and Zeng, 2020). The second option entails alteration of transcription via activation of transcription factors (TFs) which activate or repress transcription of targeted genes, a process most well understood in *Arabidopsis* (Bigeard and Hirt, 2018). Altering transcription of genes can result in production of novel proteins that may aid in insect resistance or perhaps repress expression of genes which encode proteins that repress biotic-stress-responsive processes under homeostatic conditions. Overall, this phenomenon is referred to as signal transduction and delineates the process through which an external signal is recognized by the cell, leading to subsequent alterations in cellular processes that culminate in defensive responses.

Cellular signals are diverse in their signaling methods and functions. Cellular signals are not restricted to only biotic stress responses, but rather, such signals consistently propagate throughout the plant and are integral in growth, development, and abiotic stress response as well (Johns et al., 2021; Zhang and Zhang, 2022). A variety of

signals conveying these responses have been observed in plants including phytohormones, peptides, metabolites, RNAs, ions (ion fluxes), ROS, transmembrane electrical potential differences, and sugar mass flow, all of which pass from cell to cell via the apoplastic, symplastic, or vasculature pathways (aside from electrical potential differences which are wrought via electric potentials of the membranes themselves) (Johns et al., 2021).

Our transcriptomic findings herein provide insight into the current paradigm of effector reception, signal transduction, and its impact on defense response and resistance in grapevine. Notably, a response consistent with the current paradigm in other plants (Hettenhausen et al., 2015; Bigeard and Hirt, 2018; Johns et al., 2021; Zhang and Zhang, 2022; Bender and Zipfel, 2023) was observed in resistant 'GREM4' in which indicators of successful perception of effectors, activation of signal transduction pathways, and downstream PTM and expression-altering processes resulted in induction of defense response pathways and resistance. Specifically, genes which were significantly differentially expressed in 'GREM4' were related to Mg^{+} and Na^{2+} ion signaling, phytohormones (specifically JA, SA, and ETH), signal transduction, MAPK signaling, and sequence-specific DNA binding. These genes likely played roles in propagating signals which induced expression of genes in pathways known to impact insect herbivory defense such as wax biosynthesis, terpenes, flavonoids, and phenylpropanoids. Acyltransferases (and PTMs in general) also likely play an integral role in resistance in

‘GREM4’ and/or defense response as acyltransferases were exclusively enriched in ‘GREM4’ compared to ‘PN40024’ in herbivory leaves.

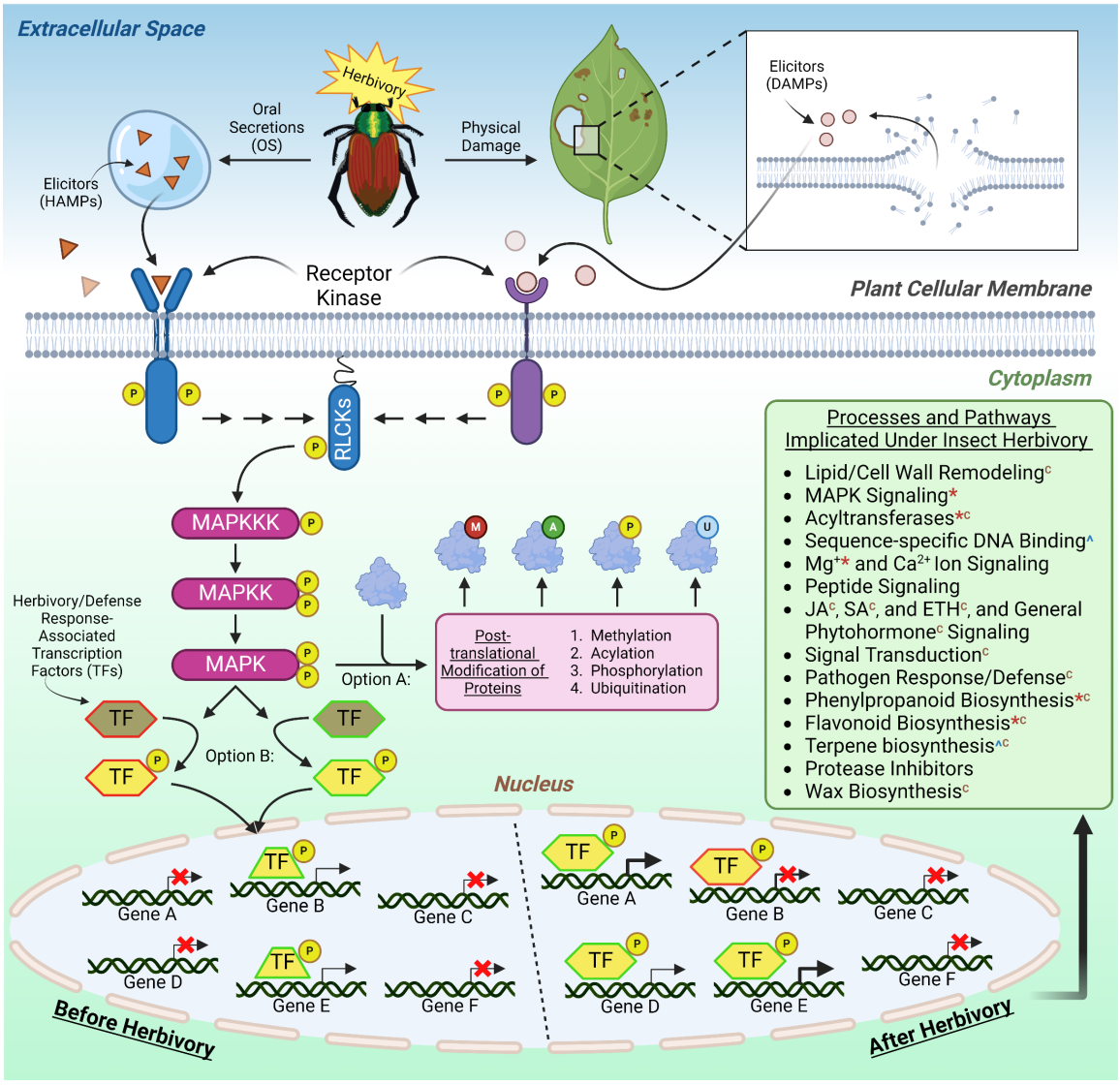
Based on the collective findings of all studies conducted in this dissertation, we propose one of the primary biological factors distinguishing resistance in ‘GREM4’ from susceptibility in ‘PN40024’, aside from trichome density, is signaling reception and transduction upon insect herbivory. Overall, our findings suggest ‘GREM4’ exhibited a greater activation of signaling transduction upon insect herbivory compared to ‘PN40024’ as many genes and enrichments related to such processes, including MAPK signaling, Mg^{+} ion signaling, and acyltransferases, were exclusively identified in ‘GREM4’ which, in turn, likely resulted in greater induction of defensive processes and pathways downstream imbuing defense as was observed in our studies. Combining these results with the greater than 1,100 DEGs identified in systemic leaves which included genes related to Ca^{2+} signaling, cell surface signal receptors, iron ion signaling, ubiquitination activity, acyltransferases, and many other functions directly implicated in signal transduction, our results point to reception of effectors and subsequent signal transduction as playing a large role in conferring the insect herbivory resistance found in ‘GREM4’ compared to the relative lack of such genes and enrichments identified in ‘PN40024’, especially in systemic response leaves. Together, PTMs and altered expression of defense genes in both herbivory and systemic leaves appear critical to defense in ‘GREM4’. The comparative studies conducted herein between ‘GREM4’ and

'PN40024' provide further support to the canonical insect herbivory defense paradigm in plants and supports the critical role of signal transduction in effective *in planta* defense.

As insect pests decrease yields globally, identification of insect herbivory resistance genes, pathways, and metabolites are critical to advancing crop protection goals required to feed the globe's increasing population. This dissertation reports that *V. labrusca* acc. 'GREM4' is resistant to Japanese beetle herbivory and describes key genes, pathways, and metabolites which likely impart the resistance. If genes or metabolites are found to impact insect herbivory resistance after testing via functional studies or artificial diet assays, respectively, these learnings can help decrease yield losses, increase growers' bottom lines, and enhance food security.

Figures

Figure 5.1 – General Insect Herbivory Response Signaling Paradigm and the Processes/Pathways Identified in Dissertation Studies



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(Figure 5.1 continued)

Figure 5.1 Caption – General herbivory defense response paradigm and the components/pathways identified in the dissertation study. DAMPs are damage associated molecular patterns while HAMPs are herbivory associated molecular patterns. OS are oral secretions from the feeding Japanese beetles. An 'M', in a red circle represents methylation, an 'A' in a green circle represents acylation, a 'P' in a yellow circle represents phosphorylation, while a 'U' in a blue circle represents monoubiquitination. Transcription factors (TFs) in the shape of a trapezoid represent TFs from another response *in planta* while diamond shaped TFs represent TFs related to herbivory or defense response. Genes within the nucleus and their associated transcriptional states represent multiple outcomes of insect herbivory-related TF binding and impacts on transcription – An untranscribed gene becomes transcribed (Gene A & D) (note levels of transcription are greater for Gene A than Gene D as different genes experience different responses); A gene transcribed via an activating TF from another biological process is repressed by a TF associated with insect herbivory (Gene B); A gene untranscribed remains untranscribed (Gene C & F); A gene transcribed via an activating TF from another biological process is instead now activated by a TF associated with insect herbivory which increases transcription of the gene beyond levels observed prior to herbivory (Gene E). The 'Processes and Pathways Implicated Under Insect Herbivory' box reports processes or pathways which were identified in 'GREM4' via…

Continued…

(Figure 5.1 continued)

…transcriptomic results in herbivory leaves. Tags on specific processes or pathways relay the following - * = Unique to 'GREM4' compared to 'PN40024'; ^ = Greater numbers of DEGs in 'GREM4' compared to 'PN40024'; [C] = A candidate gene was identified in 'GREM4' associated with this process or pathway. Created with BioRender.com.

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Bibliography encompasses all citations within the entire dissertation.

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