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Variation in Induced Responses of *Datura wrightii* to Herbivore Attack: Plasticity of Volatile Organic Compound Emissions and Gene Expression Across Genotypes, Ontogeny, and a Single Attack

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ABSTRACT OF THE DISSERTATION

Variation in Induced Responses of *Datura wrightii* to Herbivore Attack: Plasticity of Volatile Organic Compound Emissions and Gene Expression Across Genotypes, Ontogeny, and a Single Attack.

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Due to their sessile nature, plants must alter their phenotype to respond to environmental stressors. Herbivory is the most ubiquitous ecological interaction, and plants have consequently evolved an extensive array of traits with which to mitigate fitness lost to herbivores. The response to herbivory typically involves deploying suites of chemical resistance traits, known as "inducible" traits, and altering metabolic processes, all of which are generated through large-scale restructuring of the plant transcriptome mediated by a small set of phytohormones. Despite evidence of genetic and ecologically-driven variation in induced responses, very little is known about the proximal mechanisms responsible for variation in chemical resistance traits. Using herbivore-induced volatile organic compound (VOC) emissions, which act as a within-plant signal of damage and indirect resistance trait, as markers of induced resistance, this dissertation explores the transcriptional basis of variation in phenotypic plasticity in the undomesticated perennial *Datura wrightii*. Using a non-targeted approach, RNA-Seq

analyses were used to track differences in gene expression (1) over the course of a single, continuous herbivore attack in the laboratory, (2) at various intervals over the course of two growing seasons under field conditions, and (3) across inbred genetic lines grown under field conditions. The results of these studies indicate that transcriptional variation in damaged plants across time, ontogeny, and genotypes is far greater than VOC emissions would suggest. The overall number of genes induced was highly variable with respect to plant ontogeny and genotype, and showed no relation to VOC emissions. Instead, multivariate analyses show that phytohormone-related gene activity predicts VOC emissions, and has increased power to do so when phytohormone pathways are included that ostensibly have no relation to resistance induction. Taken together, these results suggest that a smaller number of "upstream" signaling molecules regulate VOC emissions independently of other, large-scale changes to the transcriptome following herbivore attack. Such a pattern implies that natural selection may act on components of the induced response individually, but contemporary variation among populations is likely the result of diffuse selection.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter One: A General Introduction to the Study of Induced Responses to Arthropod Herbivory

Due to their sessile nature, plants must alter their phenotype to respond to environmental stressors. Herbivory is one of the most ubiquitous ecological interactions, and plants have consequently evolved an extensive array of tools with which to mitigate fitness lost to herbivores (Karban and Baldwin 1997). The research community in the field of chemical ecology is particularly interested in chemical traits employed by plants and their effects on arthropod communities (Raguso *et al*. 2015). These traits may be present in the leaf constitutively, or alternatively, may increase in abundance after herbivore attack, in which case they are termed "inducible".

Plants use several types of chemical traits to resist herbivory. Toxic compounds function to prevent unadapted herbivores from feeding on the plant (Ehrlich and Raven 1964). Other compounds reduce herbivory by making the plant less palatable. Antidigestive compounds, such as protease inhibitors, impede the ability of the herbivore midgut to process leaf matter once it has been consumed. Antinutritive compounds, on the other hand, limit the nutritional value of the leaf itself (Duffey and Stout 1996). All of these compounds are found within plant tissues because their mode of action centers on consumption by the herbivore. Traits that impact the herbivore itself are termed "direct" resistance traits. Direct traits may be either inducible or constitutively expressed, with this distinction depending on the compound and particular species involved.

In contrast, volatile organic compounds (VOCs) are secondary metabolites emitted by leaves following attack by herbivorous arthropods and serve several important ecological

functions. VOCs have been shown to act as within-plant signals in lima bean (*Phaseolus lunatus*; Heil and Silva-Bueno 2007) and hybrid poplar (*Populus deltoides* x *nigra*; Frost *et al.* 2008), priming defenses in nearby tissue to produce a greater and more rapid response if they are subsequently attacked. Both con- and heterospecific plants in close proximity to emitters are also capable of eavesdropping on volatile signals, although this is an incidental outcome of VOC emissions and likely to be maladaptive for the emitter (Karban *et al.* 2014). Because VOCs can only function as within-plant informational signals if they reliably appear after attack has begun, VOCs are an inducible trait; although many plants also emit low levels constitutively (e.g. Hare 2010).

Plant volatiles are also an important source of information for arthropods. Because herbivore attack greatly increases the emission of VOCs, olfactory cues inform carnivorous arthropods and parasitoids of the location of potential prey or hosts and allow them to focus their searching behaviors at the source of emission (Dicke and Sabelis 1988; Dicke *et al*. 1990; Turlings *et al.* 1993). The efficacy of information-based indirect resistance has been demonstrated under field conditions, where the experimental release of authentic compounds led to a 4.9-7.5-fold increase in predation of hornworm (*Manduca sexta*; Sphingidae) eggs by the generalist hemipteran *Geocoris pallens* on *Nicotiana attenuata* (Kessler and Baldwin, 2001). Evidence for an adaptive benefit of attracting natural enemies through herbivore-induced VOCs remains circumstantial at best, however, and true tests of Darwinian fitness are lacking (Hare 2011; Kessler and Heil, 2011). Because natural enemies are capable of associative learning, release of any specific blend from plants may be less important than prior experience with successful

oviposition opportunities (Allison and Hare, 2009). In addition, although VOCs have repellant effects on some herbivorous insects or reduce oviposition of herbivore eggs (Kessler and Baldwin 2001; Laothawornkitkul *et al.* 2008), VOCs can also attract herbivores (Heil 2004), sometimes in a sex-specific manner (Ballhorn *et al*. 2013), or have no effect at all (Reisenman *et al.* 2013).

A central goal of chemical ecology is to explain the abundance and variation of chemical traits that mediate relationships between organisms. For plant-insect interactions, several ideas have been proposed to explain the distribution of chemical traits in an evolutionary context. Ehrlich and Raven (1964) posited that novel chemical traits that reduce herbivory might proliferate rapidly in plant populations and lead to adaptive radiation, followed by a return of pressure as herbivores adapt to these new traits. This pattern of coevolution implies a strong phylogenetic signal of resistance traits with primarily qualitative effects on arthropod communities. Feeny (1976) proposed that the resistance traits employed by plants should depend on the apparency of the plant, or the likelihood of attack based on the reliability of being discovered by herbivores. Within this framework, plants under greater threat would invest more in traits that provide robust defense, whereas plants that might escape herbivory through ephemerality would be predicted to invest in less costly defenses that provide limited protection in the case of discovery. As some have pointed out, apparency is not a trait intrinsic to the plant, but rather a byproduct of the interaction between the plant and its associated herbivore community (Schuman and Baldwin 2015). Spatial variations in herbivore communities and other environmental pressures are thought to impose variable selective

pressures on plant traits, leading to differences in defense phenotypes among individuals and populations (Thompson 1999a; Thompson 1999b; Zangerl and Berenbaum 2003).

At the organismal level, the distribution and investment in chemical traits is assumed to be limited by the fitness cost of expressing that trait. Costs of resistance can be either metabolic allocation costs, in that investment in resistance detracts from other processes such as growth or reproduction, or ecological costs if resistance traits reduce fitness through effects on the plant's biotic or abiotic environment by, for instance, reducing pollinator visitation or attracting additional herbivores (Herms and Mattson 1992; Strauss *et al*. 2002; Kessler and Heil 2011). These resistance traits occur despite the costs because of the selection pressure imposed by herbivores: specifically, the frequency of attack and the reduction of plant fitness imposed by herbivory in the absence of these traits. Within an individual plant, organs may vary with regard to their vulnerability to attack, the cost of defending them, and their contribution to plant fitness, and these variations may further depend on the developmental state of the organ. Together, these axioms comprise the optimal defense theory (ODT), developed as a framework for predicting the investment in defenses across tissues and ontogeny (McKey 1974).

These theories are complicated somewhat by inducible resistance traits, a feature of plant systems that became evident after many of the earlier works had been published (Green and Ryan 1972). The ability to express resistance traits in a tissue-specific manner in response to external factors might be expected to release plants from constraints implicit to the foundational theories of plant-insect interactions. Most

importantly, inducible resistance traits may reduce costs when herbivores are absent, although the latency of resistance manifestation may leave tissue vulnerable (Karban and Myers 1989; Zangerl 2003; Walters and Heil 2007). The ability to respond dynamically to biotic and abiotic threats should also reduce the diffusion of selective pressures somewhat, so long as plants are able to reliably coordinate effective responses. Induced resistance, however, has not been formally incorporated into theoretical frameworks that seek to explain variation in chemical traits, except to note that it should reduce these constraints.

Although many theories seek to explain why variation in chemical traits exists among plants, very little is known about the proximal mechanisms responsible for variation in chemical resistance traits. The inducible resistance traits of plants are an ideal system for studying the synthesis of chemical traits as well as potential constraints. Eliciting phenotypic changes is as simple as placing an herbivore on a leaf, and as such the timing, duration, and intensity of attack can all be manipulated with relative ease. Because elicitation is under experimental control, plants can be left undamaged to serve as clear baselines against which to compare the induced expression of genes and traits. The degree of control afforded by plant-insect systems make them a highly valuable model system for studying the genetic regulation of phenotypic traits.

Using next-generation gene sequencing (NGS) analyses, the activity of genes can be measured simultaneously across the entire transcriptome. Unlike trait-based analyses that require prior hypotheses about which traits might be important, NGS uses a non-targeted approach that may reveal unanticipated results (Baldwin *et al.* 2001). Tracking gene

expression across manipulative experiments that cause variation in the induced response can reveal expression patterns underlying variation in traits. Coupled with information about gene ontology, the relationships between expression patterns and induced traits can begin to take shape. Because of the overwhelming number of genes involved in the induced response (e.g. Tzin *et al*. 2015), and the possibility that traits may vary due to unique proximal mechanisms depending on the cause of the variation, it is imperative that variation is studied under a variety of causal conditions.

Many studies of induced responses are conducted under laboratory conditions, to minimize environmental effects and experimental error. Because plants must integrate their responses to perceived stimuli using a network of shared pathways, many responses at the transcriptional and phenotypic level are context-dependent (Kant and Baldwin, 2007). Studies comparing resistance of plants under laboratory and field conditions found that induced responses in field-grown plants are more difficult to detect at least in part because they appear to show signs of increased resistance due solely to environmental conditions (Schmidt and Baldwin 2006). Several studies have demonstrated effects on induced resistance traits caused by various conditions found in natural environments, including ultraviolet light quality (Izaguirre *et al*. 2007; Demkura *et al*. 2010; Kruidhof *et al*. 2012), wind (Cippolini 1997), and competition with nearby plants (Schmidt and Baldwin 2006; Kigathi *et al*. 2012; Izaguirre *et al*. 2013). Studying induced responses under laboratory conditions may be invaluable for studying many aspects of the biochemical and molecular responses at a very fine, manipulative scale, but this approach can only produce limited inferences about the evolved response to

herbivores. Although environmental effects may seem to obscure patterns of induced resistance, any natural selection by herbivores that has shaped contemporary induced responses has necessarily occurred in natural environments (Kant and Baldwin 2007). Plants must cope with multiple stressors and adjustments to their phenotype are constrained by their ability to respond to each simultaneously. For these reasons, it is crucial that studies of large-scale patterns of induced responses take place under field conditions, in order to account for the constraints involved in responding to herbivory.

It is currently unclear how the many biochemical pathways involved in precipitating the induced response contribute to the broad range of dynamic responses possible. By characterizing the plant responses under conditions that reveal variation, the relationship between the regulatory molecular elements and the phenotypic traits elicited can be mapped. With the *Datura wrightii* Regel (Solanaceae) system, it is possible to study variation in induced traits that occurs due to multiple factors in an undomesticated system, using herbivores with which the plant has naturally evolved.

Prior ecological studies have characterized the VOC emissions emitted by *D. wrightii* in response to herbivory, and identified substantial variation, as well as the effects of variation on the herbivore and natural enemy community. Under laboratory conditions, fifth-generation inbred lines originating in populations located across southern California showed substantial variation in the quantity of VOC emissions released after attack by *Lema daturaphila* (Hare 2007). This suggests that considerable standing genetic variation can be found among natural populations. Extending the sampling across multiple time points reveals a temporal component, as well. *Datura wrightii* responds to

24 hours of feeding by *L. daturaphila* by releasing a blend of VOCs that reach peak emission by two days after the start of feeding (Hare and Sun 2011a). Individual compounds, however, reached their maxima at different times after the initial period of damage, and emissions declined steadily thereafter (Hare and Sun 2011a).

When grown in the field, *D. wrightii* plants are highly inducible during the vegetative growth stages early in the growing season, but plants that have reached the reproductive stage cease to emit volatiles in response to herbivory (Hare 2010). When present, the quantity of VOC emission is positively correlated with predation by *Geocoris pallens*, the dominant predator in this system and a generalist that feeds on eggs and young larvae of many insects. The efficacy of VOCs in attracting *G. pallens* is independent of genotypic differences in the composition of blends (Hare and Sun 2011b).

The characteristics of the *D. wrightii* system make it amenable for study of the variations in induced responses to herbivory. This species is native to southern California and can be studied in a natural setting using native herbivore species. Within southern California populations, *D. wrightii* exhibits two leaf trichome phenotypes (van Dam *et al*. 1999). The "velvety" phenotype is densely covered by short, non-glandular trichomes, whereas the "sticky" phenotype is less densely covered with glandular trichomes that secrete esters of glucose and aliphatic acids. The trichome phenotype is governed by a single locus and is inherited in a Mendelian fashion; the allele for the sticky phenotype is dominant over that of the velvety phenotype (van Dam *et al*. 1999). Although the two trichome morphs are not associated with any difference in VOC emissions (Hare 2007),

velvety plants tend to have higher reproductive output in the first two years compared to sticky plants, which tend to grow larger (van Dam *et al*. 1999).

The development of fifth-generation inbred lines allows the studies presented here to minimize experimental error by controlling for genetic differences between plants, including differences at the trichome locus. The prior characterization of specific VOCs emitted following herbivory provides a robust suite of traits to measure across treatments. For these studies, a reference transcriptome was developed based on the tomato genome, described in detail in the first chapter, which allowed for monitoring of all genes involved in the response to herbivory. Using this system, several types of variation in particular were examined.

First, the deployment of resistance traits is not instantaneous; instead, traits appear at different times after initiation of attack and have similarly characteristic relaxation times. Among VOCs, for instance, GLVs are released rapidly as a result of damage to plant cells and are among the earliest components of the blend to appear (e.g. Allmann and Baldwin 2010). Terpenoid compounds, however, are typically synthesized following damage and appear after GLVs (e.g. Turlings *et al.* 1998). The first data chapter of this dissertation (Chapter 2) traces VOC emissions over the time course of feeding by herbivores, coupled with gene expression analyses at sampling points throughout the attack. This approach allows for a relatively fine-scale analysis of the relationship between VOCs and regulatory elements, as well as characterizing the response dynamics as the plant is attacked.

The second data chapter (Chapter 3) examines variation in induced responses due to ontogenetic changes in the plant. Using meta-analyses, Barton and Koricheva (2010) found that, in forbs, resistance typically switches from inducible to constitutive expression in mature plants. This change often occurs in spite of the continued presence of herbivores, and affects a wide range of traits (Boege and Marquis 2005). The ontogenetic decline in inducibility can affect both direct and indirect resistance traits. Although many stresses tend to vary seasonally, ontogeny affects all plants that successfully reproduce, and all plant responses to biotic and abiotic stresses necessarily occur within an ontogenetic context. This persistent influence of ontogeny and the pervasiveness of its effects suggest that the mechanisms responsible for associated changes in inducibility are likely to be well-established among natural populations. This reliability also creates a repeatable treatment regime with which to examine the interaction between the induced herbivore response and developmental processes that may compete for resource allocation and hormone signaling. This chapter examines the responses of *D. wrightii* at multiple time points across two growing seasons, to identify patterns in gene expression underlying ontogenetic changes in herbivore-induced VOC emissions.

Finally, the third data chapter (Chapter 4) of this dissertation focuses on the effect of genetic variation on the induced response. Genetic variation of traits within a population is a fundamental characteristic of biological systems and a prerequisite for evolution by natural selection. Considerable evidence of variation in VOC emissions among undomesticated populations has accumulated (Hare 2011). Little is known about the

proximal mechanisms responsible, however, and elucidating the basis of such variation can assist in developing hypotheses to explain why variation occurs and how selection on phenotypes operates.

By studying the variation in induced responses to herbivory across multiple scales and factors, this body of work represents a robust exploration of the nature of variation in phenotypic plasticity. The mechanisms responsible for producing variation may be common to different ecological or genetic conditions, or may be unique to each. Broadly, the general patterns of gene expression may indicate that a small number of upstream genetic elements (e.g. signaling molecules) leading to extensive downstream changes, or differences may be localized to downstream processes involved in the biosynthesis of traits. By utilizing non-targeted molecular approaches that can identify changes to the entirety of the transcriptome, these studies are able to identify broad patterns of changes in gene expression and relate those changes to phenotypic plasticity.

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Chapter Two: Expression Pattern of Defense-Related Traits Is Not Correlated with Continuous Herbivore Damage in *Datura Wrightii*

Introduction

As sessile organisms, plants respond to environmental stressors through remarkable phenotypic plasticity. The response to herbivory typically involves deploying suites of chemical resistance traits, known as "inducible" traits, and altering metabolic processes, all of which is generated through a large-scale restructuring of the plant transcriptome mediated by a small set of phytohormones. Induced resistance traits may function directly by impeding the ability of the herbivore to feed on the plant or by repelling it. Alternatively, plants also employ indirect resistance traits such as volatile organic compounds (VOCs) that are attractive to carnivorous and parasitoid arthropods (hereafter "natural enemies).

Induced responses are generated through widespread changes in the expression of the plant genome, involving as many as 10% of all genes (Reymond *et al*. 2004; Appel *et al*. 2014). The extent of the chemical and metabolic changes associated with the response to herbivory are difficult to characterize and often vary due to species of the herbivore (Rodriguez-Saona *et al*. 2010) and plant involved (Schmidt *et al.* 2005), as well as a range of abiotic conditions (Gouinguené and Turlings 2002; Schmidt and Baldwin 2006). Despite context-dependent variations in the induced response, several general patterns have emerged.

Transcripts associated with a range of signaling molecules are differentially regulated during the induction process. The organization of the herbivory response generally centers around several phytohormones and their associated pathways (Pieterse *et al*.

2009), in particular the jasmonic acid (JA) and salicylic acid (SA) pathways (Ali and Agrawal 2012; Thaler *et al*. 2012). In addition, the role of other phytohormones in modulating these responses increasingly appears to be critical (reviewed in Robert-Seilaniantz *et al.* 2011 and Erb *et al*. 2012). Because phytohormones precipitate the phenotypic changes associated with induction, transcripts associated with hormone biosynthesis are expected to be among the first to differ in expression, along with transcription factors, kinases, and other signaling molecules that are needed to convey the signal (Wu and Baldwin 2009).

Processes associated with growth, and investment in photosynthetic capacity in particular, are typically suppressed in damaged leaves (Reymond *et al*. 2004; Bilgin *et al*. 2010; Rodriguez-Saona *et al.* 2010). This phenomenon is consistent with the growth-defense tradeoff paradigm, which states that because available resources are limited, plants must allocate them exclusively to either growth- or defense-related processes (Herms and Mattson 1992). Recent studies, however, have suggested that suppression of primary metabolic processes may instead be adaptive. *Nicotiana attenuata* with a silenced ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase) activase gene, responsible for activating the major carbon fixation protein, showed an impaired ability to elicit hormones in response to herbivory, suggesting that photosynthetic mechanisms serve additional functions (Mitra and Baldwin 2008). Shutting down photosynthesis may be adaptive because it denies nutrients, such as carbon or root-derived nitrogen, to attacking herbivores, or at least prevents the likely loss of those nutrients if they are not shunted away from the vulnerable leaf (Schwachtje *et al.* 2006; Gomez *et al.* 2010).

Furthermore, resource sequestration during attack aids in compensatory regrowth of tomato after simulated herbivory and treatment with *Manduca sexta* regurgitant compared to mechanical damage alone, and may represent an adaptation that allows plants to tolerate defoliation by herbivores (Orians *et al.* 2011; Korpita *et al.* 2014).

Transcription of genes coding for secondary metabolites generally appear to follow the phenotypic patterns of secondary metabolite induction. Tomato plants induced by Lepidopterans upregulated genes for protease inhibitors (*PI*s) and polyphenol oxidase (*PPO*), as well as defense-related enzymes and precursor molecules (Rodriguez-Saona *et al.* 2010; Chung and Felton 2011). Expression of serine and cysteine PIs increased to a greater degree, however, when tomato plants were attacked by *M. sexta* compared to Colorado potato beetle (*Leptinotarsa decemlineata*; Coleoptera), although several other *PI* genes were similarly induced (Chung and Felton 2011). Challenging *N. attenuata* and *Solanum nigrum* with *M. sexta* larvae caused both to increase transcription of some genes associated with two types of VOCs, terpenoids and green leaf volatiles (GLV), but differential regulation of a far greater number of genes was unique to one plant species or the other (Schmidt *et al.* 2005). Although significant progress has been made in identifying genes relevant to the production of plant VOCs and other resistance traits, less is known about how these genes are expressed after herbivory (Danner *et al.* 2011; Falara *et al.* 2011).

Herbivore-induced VOCs are secondary metabolites emitted by leaves following attack by herbivorous arthropods and serve several important ecological functions. VOCs have been shown to act as within-plant signals in lima bean (*Phaseolus lunatus*;

Heil and Silva-Bueno 2007) and hybrid poplar (*Populus deltoides* x *nigra*; Frost *et al.* 2008), priming defenses in nearby tissue to produce a greater and more rapid response if they are subsequently attacked. Both con- and heterospecific plants in close proximity to emitters are also capable of eavesdropping on volatile signals. A meta-analysis of 48 studies showed this phenomenon can occur in a multitude of species, as well as in both laboratory and field settings (Karban *et al*. 2014).

Plant volatiles are also an important source of information for arthropods. Because herbivore attack greatly increases the emission of VOCs, olfactory cues inform natural enemies of the location of potential prey/hosts and allow them to focus their searching behaviors at the source of emission (Dicke and Sabelis 1988; Dicke *et al*. 1990; Turlings *et al.* 1993). Through either innate recognition of cues or associative learning, numerous species of natural enemies have demonstrated the ability to discern odors emitted by plants that were damaged by suitable host herbivores compared to non-hosts (Hare and Allison 2009). Although VOCs have repellant effects on some herbivorous insects or reduce oviposition of herbivore eggs (Kessler and Baldwin 2001; Laothawornkitkul *et al.* 2008), VOCs can also attract herbivores (Heil 2004), have no effect at all (Reisenman *et al.* 2013), and can be sex-specific (Ballhorn *et al.* 2013).

In total, over 1700 unique floral and vegetative VOCs have been identified from 90 plant families (Knudsen and Gershenzon 2006). VOCs are emitted as complex blends of compounds that may include GLVs, terpenoids, benzenoids, indoles, and aliphatics, depending on the plant species. VOCs are produced from four major pathways that branch from primary metabolism: the shikimate, the methylerythritol phosphate,

mevalonic acid, and lipoxygenase pathways (Dudareva *et al.* 2013). These pathways are not independent of one another, and the production of any one compound may compete or correlate with the production of others (Dudareva and Pichersky 2000; Hare 2011; Dudareva *et al.* 2013). Geranyl pyrophosphate, for instance, is a precursor used by all monoterpenes, while farnesyl pyrophosphate is involved in the synthesis of sesquiterpenes. The specific relationships between compounds are not always straightforward. In maize, a single terpene synthase enzyme is responsible for production of a specific ratio of nine sesquiterpene volatiles (Schnee *et al.* 2006). Terpene synthases isolated from *Populus trichocarpa*, however, produced both mono- and sesquiterpenes products when heterologously expressed in *Escherichia coli* (Danner *et al.* 2011). Because of these complex relationships, studies aiming to characterize variation in volatile emissions must account for the relationships between blend components.

Although many studies of herbivore-induced VOCs rely on "snapshots" of volatile blends collected at specific time points following herbivory, the temporal dynamics of the various pathways produce complex emission patterns. GLVs are produced through the octadecanoid pathway when C_{18} fatty acids are cleaved to form various C_6 derivatives (Dudareva *et al.* 2013). These compounds are released rapidly as a result of damage to plant cells and are among the earliest components of the blend to appear. Terpenoid compounds, however, are typically synthesized following damage and appear after GLVs. In maize (*Zea mays*), for example, emission of several C_6 compounds peaks immediately after damage and declines substantially after one hour. In contrast, induced terpenoid emission is not detectable until after the first hour following damage, with peak

emission occurring during the next 4-12 hours, depending on the compound (Turlings *et al.* 1998). Studies that do not explicitly account for temporal dynamics of emissions may therefore underestimate particular components of the blend.

The damage treatment used to induce the plant in many studies of VOCs has generally consisted of either a short interval of exposure to an herbivore (Halitschke *et al.* 2000; Reymond *et al.* 2000; Hare and Sun 2011b), or a single instance of mechanical damage followed by application of herbivore oral secretions (Turlings *et al.* 1998; Mitra and Baldwin 2008; Skibbe *et al.* 2008). Because the pattern of damage to plants has been shown to affect induced traits, it is important to explicitly consider the ecological relevance of the damage treatment imposed (Mithöfer *et al.* 2005; Hilker and Meiners 2010). Short bursts of damage may be relatively uncommon for larval stages of insects that feed extensively prior to pupation. There is evidence that suggests specialist larvae may tend to remain on induced leaves longer than generalists, which benefit from periodically moving to distal, uninduced leaves (Perkins *et al.* 2013).

Regardless of the specialization of the herbivore, the plant response may have evolved in the context of extended periods of attack, and studies that have used this type of damage treatment have reached important conclusions. Compared to maize, the induced response in cotton (*Gossypium hirsutum*) is slower to deploy but generally similar, with GLVs appearing directly after damage and terpenes reaching peak emission roughly 24 hours after the initiation of feeding (Loughrin *et al.* 1994). Continuous attack by *Spodoptera exigua* over 60 hours revealed the emission of induced terpenoid compounds follows a diurnal emission pattern, abating each night and returning the

following day, likely due to the need for photosynthetic metabolic processes to produce precursor compounds. Interestingly, cotton also stores some terpene compounds in specialized gland structures on the leaf surface (Elzen *et al.* 1985). These compounds are released upon damage to the gland, resulting in continual emission of the stored compounds as long as glands continue to rupture. The release of GLVs and stored glandular compounds remains relatively consistent throughout day and night while herbivores are feeding, and may be the exception to the diurnal pattern of emission that proves the rule.

Continuous attack by spider mites induces a temporally staggered response in tomato plants, wherein PIs are induced after one day of damage, but VOCs do not significantly increase until three days after the infestation begins (Kant *et al.* 2004). This study also used microarrays to examine the expression of some 400 genes preselected for their likely involvement in the induction process. Ultimately, only 60 and 67 of those genes were differentially regulated after one and four days of damage, respectively, which were the only time points assessed. This study provides insight into the kinetics of the induced response, although the conclusions that can be drawn from two time points are limited.

Further study is needed to characterize how plants have evolved to utilize resistance traits during an attack, as well as the molecular mechanisms that precipitate this response. By tracking the deployment of resistance traits and gene expression over the course of a sustained attack, phenotypic traits can be mapped to underlying regulatory elements. With the *Datura wrightii* Regel (Solanaceae) system, it is possible to study the time

course of induced resistance in an undomesticated system, using herbivores with which the plant has naturally evolved.

Datura wrightii is native to the southwestern United States. Within southern California populations, *D. wrightii* exhibits two leaf trichome phenotypes, and aspects of the ecology and genetics of the trichome dimorphism have been described elsewhere (van Dam and Hare 1998; van Dam et al. 1999). The "velvety" phenotype is densely covered by short, non-glandular trichomes, whereas the "sticky" phenotype is less densely covered with glandular trichomes that secrete esters of glucose and aliphatic acids. The trichome phenotype is governed by a single locus and is inherited in a Mendelian fashion; the allele for the sticky phenotype is dominant over that of the velvety phenotype (van Dam et al. 1999).

The herbivore community that attacks *D. wrightii* consists of approximately 5-10 species depending on location and season (Elle and Hare 2000). *Lema daturaphila*, the most damaging herbivore, is a multivoltine chrysomelid that feeds on both sticky and velvety morphs of *D. wrightii* throughout the growing season as both larva and adult. Damage by *L. daturaphila* increases emission of up to 20 compounds. The blend is composed of GLVs, mono- and sesquiterpenes, with (*E*)-β-caryophyllene, (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11- tridecatetraene (hereafter TMTT), (*E*)-4,8-dimethyl-1,3,7- nonatriene (hereafter DMNT), and β-selinene typically comprising the bulk of the emitted compounds (Hare 2010; Hare and Sun 2011a; Hare and Sun 2011b). Among several genetic lines of *D. wrightii* assayed, VOC production increases 3.9 to 16.2 times after attack, and the relative abundance of individual

compounds varies, with the most abundant compound, (*E*)-β-caryophyllene, comprising 17 to 59% of the blend. This variation has been shown to be heritable; the trichome phenotype has no effect on volatile production (Hare 2007).

When grown in the field, *D. wrightii* plants are highly inducible during the vegetative growth stages early in the growing season, but plants that have reached the reproductive stage cease to emit volatiles in response to herbivory (Hare 2010). Predation of *L. daturaphila* eggs by *Geocoris pallens*, the dominant predator in this system and a generalist that feeds on eggs and young larvae of many insects, was positively correlated with the quantity of VOC emissions (Hare and Sun 2011a). The efficacy of VOCs in attracting *G. pallens* is independent of genotypic differences in the composition of blends, although predation is higher on velvety plants.

Under laboratory conditions, *D. wrightii* responds to 24 hours of feeding by *L. daturaphila* by releasing a blend of VOCs that reach peak total emission by two days after the start of feeding (Hare and Sun 2011b). Consistent with results from other species, the GLV (*Z*)-3-hexenyl acetate reaches peak emission one day after the start of damage, one day earlier than other compounds. (*E*)-β-ocimene, (E,E)-α-farnesene, and DMNT peaked after two days and subsequently declined continuously. Only (*E*)-β-caryophyllene and TMTT emission persisted longer before declining. Induction due to damage from *M. sexta*, rather than *L. daturaphila*, led to a smaller quantity of emission, but had no significant qualitative effect on the blend (Hare and Sun 2011b).

The goal of the present study was to assess the response of *D. wrightii* to continuous feeding that is representative of what this species might encounter under natural

conditions. Specifically, we asked how herbivore-induced VOCs emissions were deployed over time, and whether the emission pattern was proportional to the amount of damage sustained. We also asked how the underlying genome responds to continuous damage, using an untargeted, transcriptome-wide approach and sampling at a range of time points. In order to monitor gene expression, we assembled the *D. wrightii* transcriptome by aligning *D. wrightii* transcripts to the tomato genome.

Methods

Basic sampling design

The study consisted of two groups of plants: one that was damaged continuously for the duration of the experiment (the treatment group) and one that was left undamaged (the control group). Continuous damage is an appropriate treatment for studying induced responses in this system because it replicates the natural feeding behavior of *Lema daturaphila* larvae, the dominant herbivore in southern California *D. wrightii* populations.

From the two groups of plants, we collected VOC emissions, calculated the amount of leaf area consumed by herbivores in the treatment group, and measured the differential expression of genes. In order to follow the time course of the induced response, data were collected at several time points, including before damage began (0-hour time point), as well as 12, 24, 48, 72, 96, 120, and 144 hours after the initiation of damage. Because tissue is collected destructively for both gene expression and leaf consumption analyses, a subset of plants was removed from the experiment for this

purpose at each time point. Therefore, although the same number of plants were involved in the analysis of gene expression and tissue consumption at each time point, VOCs were collected from a smaller number of remaining plants at each subsequent time point.

Plants

This study used *Datura wrightii* from the MVV6 line, which has been shown to increase volatile production substantially in response to herbivore attack (Hare, 2007). All plants were fifth-generation backcross sibs, and expected to be at least 98.4% similar. Three germinated seedlings were planted in each pot, spaced evenly, to form a single biological replicate. Plants were grown in a greenhouse equipped with high-pressure sodium lamps providing supplemental illumination for 14 hr/d so that midday light intensities averaged 1250 ± 39 μmol m^{-2} s^{-1} PAR illumination at plant height (Hare and Sun, 2011a). Greenhouse temperatures ranged between 15° and 35°C. Plants were maintained under these conditions for approximately 3-4 months until they had produced 8-10 true leaves. Plants were transferred to a laboratory growth room with 14:10 photophase:scotophase, 26°C day and 20°C night temperatures with light intensity of 293 (± 29) μmol m^{-2} s^{-1} PAR illumination at plant height provided by a 1000 watt metal halide light (Hare and Sun, 2011a). Each of three blocks consisted of 16 biological replicates, divided equally between treatment and control.

Induction treatment

For the induction treatment, a single third-instar *Lema daturaphila* larva was placed on the youngest, full-sized leaf on each plant and allowed to feed continuously until plants were sampled for gene induction. The larvae were enclosed on the focal leaf using organdy cages. Larvae were monitored daily for progression to the fourth instar, and were replaced as needed with fresh third instar larvae to maintain continuous plant damage. Control plants received organdy cages without larvae. *Lema daturaphila* used in this study were reared on greenhouse-grown *D. wrightii* in an insectary; this stock population was periodically supplemented with field-collected individuals to minimize effects of inbreeding.

Tissue collection

Focal leaves from one induced and one control replicate were destructively sampled before any plants were damaged by larvae, and at 12, 24, 48, 72, 96, 120, and 144 hours after the initiation of feeding. All sampling times were between 9-10 a.m., with the 12-hour collection at 9 p.m, which was included in the photoperiod. Prior to collection, larvae were removed from the leaf using a paintbrush. The focal leaf from each plant was severed using a razor blade treated with RNAse Away (Invitrogen, Carlsbad, CA, USA) and three discs were excised near recent feeding sites using a 10-mm diameter cork borer. One disc from each of the three plants within each biological replicate were pooled in a microcentrifuge tube and immediately frozen in liquid nitrogen. Two additional discs from each leaf were used to create pooled backup samples

in the same manner. Remaining leaf tissue was scanned alongside a ruler and digitized to determine the amount of leaf tissue removed by larvae using Scion Imaging Software for Windows ver. A.4.0.3.2 (Scion Corp., Frederick, MD, USA). Discs excised for tissue collection were not included in the tissue removal analysis. Replicates were removed from the experiment following tissue collection.

Volatile collection and analysis

Volatiles were collected non-destructively from biological replicates using a push-pull collection system described previously (Hare, 2007). This configuration prevented any airborne contamination between damaged and undamaged plants. Briefly, aeration chambers were constructed from polyester cooking bags (unprinted 45 x 55 cm, Terinex, Bedford, England), placed over replicates, and secured around the pot using a rubber band. Intake and exhaust ports were created in the chambers using gastight fittings (Swagelok, San Diego, CA, USA). Airflow was controlled with flow meters (Aalborg, Orangeburg, NY, USA). Compressed air was filtered through activated charcoal and flowed to the chamber at a rate of 500 mL/min through PTFE tubing. Air flowing out of the chamber passed through a glass trap containing Super-Q (25 mg, Alltech, State College, PA, USA), an adsorbent used to collect volatiles, and subsequently through PVC tubing. At each collection time point, traps were wrapped in aluminum foil and frozen at -20° C for extraction later that day.

Volatiles were eluted from the traps with 150 µl of CH_2Cl_2 containing 4 ng/µl of n-bromoheptane (Sigma-Aldrich) as an internal standard into autosampler vials with 250-

µl glass inserts, and vials were sealed with crimp caps and PTFE-lined rubber septa. Samples were analyzed by gas-liquid chromatography. Peaks were quantified in units of ng g^{-1} leaf (dry wt.) hr^{-1} using Agilent ChemStation® software based on comparison of the peak height of each VOC component with that of the internal standard (Hare and Sun 2011b)

Next generation sequencing

RNA was extracted from leaf tissue using Qiagen RNEasy Plant Mini Kit following manufacturer instructions. RNA concentrations were confirmed by NanoDrop spectrophotometer prior to final concentration and integrity check using Agilent 2100 Bioanalyzer. RNA-Seq libraries were prepared using NEBNext Ultra RNA Library Prep Kit for Illumina according to manufacturer recommendations. Six individual samples were multiplexed per lane using compatible index primers and sequenced using an Illumina HiSeq 2000 sequencing system using single-end 50-bp reads.

Transcriptome assembly

To assemble the transcriptome, RNA was collected from individual leaves of six *D. wrightii* plants from the MVV6 line that were separate from the remainder of the study and sequenced as above. Three of these plants were induced by 24 hours of feeding by *L. daturaphila* and three were left undamaged. Leaf tissue was excised using a razor blade, frozen in liquid nitrogen, and stored at -80°.

After trimming the index primers from each read, the resulting sequence data was aligned to the tomato genome (version 2.40; The Tomato Genome Consortium, 2012) using MosaikAligner, with the following parameters: hash size of 15, maximum mismatch of 4, and a gap open penalty of 15. SNPs and INDELs were identified using SamTools mpileup and called with bcftools in order to digitally edit variants into the tomato genome (Li *et al*. 2009; Li *et al*. 2011). Following the edits, exon boundary positions were updated to reflect the net change in basepairs.

MapMan (Thimm *et al*. 2004) was used to tentatively assign gene ontology (GO) annotations from the tomato genome to *D. wrightii* genes based on their sequence location, as well as guide pathway-based analysis. We predicted open reading frames and amino acid sequences for putative terpene synthase genes using ExPASy SIB bioinformatics resource portal (http://web.expasy.org/translate/; Artimo *et al*., 2012). The resulting protein sequence was used in BLAST searches of the tomato genome using the Sol Genomics Network (www.solgenomics.net; Fernandez-Pozo et al., 2014) to more accurately assign ontology terms to these genes and identify genes that may have differing functionality compared to homologous genes in tomato.

Gene expression

To analyze the time course of gene expression, RNA was extracted from one sample of ground leaf tissue from each of the pooled replicates and sequenced as above. Sequencing data was aligned against the *D. wrightii* reference transcriptome with Mosaik, using a maximum mismatch threshold of four (Lee *et al*. 2014). The edgeR

package in R was used to compare gene expression in the treatment and control groups using the exact test for the negative binomial distribution (Robinson and Smyth, 2007; Robinson and Smyth, 2008; Robinson *et al*. 2010; McCarthy *et al*. 2012; Zhou *et al*. 2014). Gene expression greater than $\pm$2 log-fold changes compared to controls were considered differentially expressed using a false discovery rate of <0.05 (Reymond *et al*. 2004).

RNA-Seq data was validated using quantitative PCR (qPCR) for the following genes, identified by their sequence similarity to annotated tomato genes: *hydroperoxide lyase 1* (*HPL1*), *phenylalanine ammonia lyase* (*PAL*), *terpene synthase 12* (*DwTPS12*), *lipoxygenase 2* (*LOX2*), and *RuBPCase small chain 2B* (*RUB*), with *actin* (*ACT*) and *ubiquitin* (*UBI*) chosen as reference genes. Primers were designed using Primer3 (Koressaar *et al.*, 2007; Untergasser *et al.*, 2012) and assayed for efficiency in triplicate with cDNA templates diluted over five orders of magnitude (Table 2.1). Melting curves for each primer used in this study produced a single, sharp peak.

To create cDNA, total RNA extracted during the procedure described above was first treated with RQ1 DNase (Promega, Chicago, IL, USA) to remove any remaining DNA. DNase was inactivated and cDNA was synthesized using oligo(dT) primer (Promega), SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), dNTPs (Sigma Aldrich, St. Louis, MO, USA), and RNase inhibitor (Promega). qPCR was performed in triplicate using SYBR Green master mix (Bio-Rad, Los Angeles, CA, USA) and 96-well optical plates (Bio-Rad) in a Bio-Rad iQ5 Real-Time Detection System, with PCR conditions as follows: denaturation at 95°C for 3 min, followed by 40

cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 60 s, and a melting curve that showed each experimental sample yielded a single, sharp peak at the amplicon's melting point.

Expression values were calculated using $2^{-\Delta\Delta Ct}$ to find the log-fold change in expression in the treatment group (Pfaffl 2001). Comparisons between the two reference genes, *ACT* and *UBI*, showed highly similar relative expression patterns across samples. Values reported in the final analysis used *ACT* as the reference. It should be noted that a small number of qPCR analyses are based on fewer than 3 biological replicates due to a shortage of available tissue for RNA extraction. The affected analyses are indicated below.

Statistical analysis

Tissue consumption was only assessed when biological replicates were removed from the experiment at each time point. Because quantification of VOC emission is dependent upon leaf mass, it was necessary to extrapolate leaf mass for replicates at time points prior to their removal. Leaf areas at sampling times prior to tissue collection were estimated by measuring the total leaf area removed, dividing by the amount of time since the start of herbivory, and cumulatively subtracting the quotient from the total leaf area. Preliminary work determined that the relationship between fresh leaf area and dry leaf mass was linear (R^2=0.7662) and fresh leaf area could be converted to dry mass using the equation ([leaf area]*0.0004)+0.0483). Estimated leaf area at each time point was

converted to dry weight and used to determine VOC quantities. A regression analysis was used to check if the rate of tissue consumption was linear and similar across blocks.

VOC emissions were $\log_{10}(x+1)$ transformed to ensure normality of errors and accommodate samples for which no volatiles were detected. In addition to using the total emission quantity, the data were subjected to a principal components analysis (PCA) in order to explore variation in subsets of the total blend. For the PCA, smaller number of uncorrelated components were created based on the covariance matrix of the individual compound quantities. The factor loadings show the contribution of each original variable to the new component, and factor scores for the new components are calculated for the original observations.

Total VOC emissions principal components, and leaf consumption were analyzed using a repeated measures analysis of covariance (ANCOVA) in JMP Pro 11, with 1) treatment, time, and their interaction as fixed effects; 2) block as a random effect; and 3) biological replicate nested within block as the repeated subject. Leaf consumption was not included as a covariate because it increased continuously, conflating it with time. Decomposition of the interaction between treatment and time allowed us to determine at which time points treatment caused significant differences in VOC emission.

Heatmaps were used to visualize patterns of gene expression across time points. Using the ComplexHeatmap package in R (Ver. 3.3.1), separate analyses were conducted on the expression of all DEGS detected throughout the time course, the 16 terpene synthases, and genes putatively involved in biosynthesis of phytohormones, including jasmonic acid (JA), salicylic acid (SA), and ethylene (ET), auxin (IAA), and abscisic acid

(ABA). Hierarchical clusters were used to group genes and time points according to similarity of expression profiles using the default settings of the package.

Results

Tissue consumption

Damage increased consistently over time on treatment plants (R^2=0.937, $F_{7,80}$=127.56, p<0.0001; Fig. 2.1). *L. daturaphila* larvae consumed an average of 44.62 ±4.58 mm^2/day, representing 6.9 ±0.94% of initially available leaf tissue per day.

VOC emissions

Prior to the start of the experiment, plants assigned to the treatment group did not emit more total VOCs than those in the control group (time point 0; $F_{1,149}$=0.23, p=0.63; Fig. 2.2). Overall, damage by *L. daturaphila* larvae induced an average 31-fold increase in total VOCs emitted across all time points ($F_{1,56}$=56.00, p<0.0001). The effect of damage was modulated by the time since damage was initiated ($F_{7,155}$=7.42, p<0.0001), with emission increasing 2.2 fold over control plants by 12 hours ($F_{1,158}$=4.61, p=0.0333), 3 fold by 24 hours ($F_{1,170}$=22.02, p<0.0001), and between 17 and 112 fold from 48 to 120 hours, partly due to variation in the control plant emission (all p<0.0001), before decreasing to 5 fold by 144 hours ($F_{1,178}$=4.28, p=0.0401).

Seventeen unique compounds were detected in the headspace of *D. wrightii*. DMNT, (*E*)-β-ocimene, (*Z*)-3-hexenyl acetate, and (*E*)-β-caryophyllene comprised the majority of the blend, with the remainder composed of *trans*-2-hexanal, *cis*-3-hexen-1-ol,

trans-2-hexen-1-ol, limonene, linalool, methyl salicylate, α-bergamotene, geranyl acetone, α-humulene, β-selenine, α-farnesene, and TMTT. The results of the PCA of VOCs show that the first component (PC 1) accounts for 59% of the variation in the data, PC 2 accounts for 16.5%, PC 3 for 7%, PC 4 for 4%. Additional components make up the remainder of the variation but will not be considered further.

The four compounds that make up the bulk of the blend, DMNT, (*E*)-β-ocimene, (*Z*)-3-hexenyl acetate, and (*E*)-β-caryophyllene, loaded strongly and positively on PC 1 (Table 2.2), suggesting that this variable represents the major quantitative variation in the dataset and can be considered a general marker of induction. PC 1 was significantly affected by the damage treatment ($F_{1,67}$=87.06, p<0.0001, Fig. 2.3a) and the interaction between treatment and time ($F_{7,156}$=43.92, p<0.0001). The effect of treatment on PC 1 was significant at all time points after and including 24 hours after initiation of damage (all p≤0.0008), and levels off after 48 hours. Unlike the analysis of the total VOC blend, treatment did not differ significantly affect PC 1 at 12 hours ($F_{1,133}$=1.89, p=0.17).

PC 2 primarily represents two compounds, *trans*-2-hexanal and *trans*-2-hexen-1-ol, that loaded positively on this variable. Although PC 2 did vary over time ($F_{7,158}$=8.29, p<0.0001; Fig. 2.3b), there was no significant effect of treatment ($F_{1,64}$=0.38, p=0.54) or treatment by time ($F_{1,158}$=0.54, p=0.80). Generally, both damaged and undamaged plants produced blends richer in PC2 at 24 hours, before it steadily declined, with a non-significant increase in the treatment group at 144 hours ($F_{1,179}$=0.91, p=0.34).

PC 3 contrasts production of (*E*)-β-caryophyllene and, to a lesser extent, (*Z*)-3-hexenyl acetate with production of linalool, DMNT, limonene, and (*E*)-β-ocimene.

Although the effect of treatment was not significant ($F_{1,63}$=0.0055, p=0.9413; Fig. 2.3c), the effect of time ($F_{7,157}$=9.16, p<0.001) and the treatment by time interaction ($F_{7,157}$=4.79, p<0.001) were both highly significant. Decomposing the interaction reveals that while PC 3 scores for control plants didn't differ over time ($F_{7,158}$=1.78, p=0.0957), the scores for the treatment group did ($F_{7,153}$=11.88, p<0.0001). Three time points in particular showed significant differences in the scores for PC 3: 24 hours ($F_{1,165}$=13.74, p=0.0003), 48 hours ($F_{1,172}$=8.58, p=0.0039), and 120 hours ($F_{1,187}$=5.57, p=0.0183).

PC 4 shows positive loadings for linalool, limonene, (*E*)-β-caryophyllene, (*E*)-β-ocimene and methyl salicylate and negative loadings for DMNT and (*Z*)-3-hexenyl acetate. Overall, the fixed effects of treatment, time, and their interaction were all nonsignificant (all p>0.26; Fig. 2.3d), however a decomposition of the interaction showed that there was a significant effect of treatment at 48 hours ($F_{1,186}$=4.41, p=0.037).

The individual compounds responsible for time by treatment significance in the analysis of each principal component were identified by examining the time course of emission of those compounds with the highest factor loading scores. Unsurprisingly, the emissions of DMNT, (*E*)-β-ocimene, (*Z*)-3-hexenyl acetate, and (*E*)-β-caryophyllene, the four compounds that loaded most strongly on PC 1, all showed major increases in emission from damaged plants for the majority of the study (Fig. 2.4). For (*E*)-β-caryophyllene and (*Z*)-3-hexenyl acetate, however, this increase did not begin until 48 hours after damage started, unlike DMNT and β-ocimene, both of which showed higher increases at 24 hours. By 144 hours, only (*E*)-β-caryophyllene remained induced, and PC 1 no longer differs significantly from controls. For PC 3, the significance at 24 and 48

hours is mainly driven by increases in linalool, limonene, DMNT, and β-ocimene, all of which loaded negatively, while (*E*)-β-caryophyllene had not yet reached peak emissions. Over the remainder of the time course, however, the blend from damaged plants became relatively richer in (*E*)-β-caryophyllene, while limonene and linalool were no longer induced. The difference in PC 4 seen at 48 hours may be explained by the induced emission of DMNT and the relatively low emission of limonene at that time point.

To summarize the induced emissions, linalool, MeSA, α-humulene, and TMTT were restricted to the middle of the timecourse, reaching peak emissions in the 48-72 hour collections. (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene DMNT and α-farnesene were induced somewhat consistently from 48 to 120 hours, while *cis*-3-hexen-1-ol, (*E*)-β-caryophyllene, α-bergamotene, and geranyl acetone peaked in the 96 and 120-hour collections. Induced limonene, β-selenine and α-pinene emissions were inconsistent and detected sporadically.

Transcriptome assembly

Across the six samples, a total of 49,860,481 reads were aligned to the tomato genome, an average of 8,310,080 ($\pm$ 3,062108) per sample, representing 31.9$\pm$0.63% of the total reads. Including both SNPs and indels, the assembly of the *D. wrightii* transcriptome shows 580,388 variants compared to the tomato genome. Once the tomato genome was altered to account for these, the resulting *D. wrightii* reference transcriptome consisted of 781,611,657 basepairs. This figure represents an overall increase in size from the 760-Mb tomato genome (Tomato Genome Consortium 2012).

Sixteen terpene synthases were found to be differentially expressed during the time course of induction (see below). *TPS* genes had at least 29x coverage, with an average of 171.3 coverage across all samples, providing sufficient read depth to make inferences about sequence divergence from tomato (Sims *et al*. 2014). Large open reading frames were detected in the amino acid sequences predicted for each gene, suggesting that none are obvious pseudo-genes (Table 2.3). Protein to protein BLAST searches of the tomato genome reveal that non-synonymous substitutions are present in all but two of the sixteen sequences, and the gene with the greatest proportion of variants, a *TPS37*-like gene, had substitutions in 9.5% of its amino acid positions.

Gene expression

Across all samples, MosaikAlign reported an average of 27.29% (±1.60) unique and 27.39% (± 3.69) non-unique alignments. The remainder (45.31% ± 2.26) were filtered out and not included in analyses. Plants from each treatment group sampled prior to the start of the experiment showed no differential expression of genes, indicating there was no systematic bias between the two groups. Differential expression of genes was detectable 12 hours after the start of damage, as well as at every time point sampled thereafter (Fig. 2.5). In terms of total number of differentially expressed genes (DEGs), there were 39 at 12 hours, 929 at 24 hours, and expression peaked at 48 hours with 3,399 genes before declining to 727 at 96 hours after damage began. There was a strong bias towards increased expression of genes at each of those time points, although as many as 31.8% of DEGS were downregulated at 48 hours.

Breaking down expression by functional categories assigned by MapMan showed that a large portion of genes were involved in protein metabolism, with 372 genes induced and 153 suppressed at 48 hours (Fig. 2.6). The next largest groups included cell maintenance, transcription and transport. The relative proportion of upregulated to downregulated genes was remarkably consistent across groups and time points, with the notable exception of photosynthesis-related genes. A disproportionate number of photosynthesis genes were suppressed from 24 to 96 hours, with the largest number occurring at 48 hours. Overall, there was a higher proportion of suppressed genes at the 96-hour time point compared with the 24-hour sample. Correspondingly, most gene groups had more suppressed genes at 96 hours. Notably, however, genes involved in secondary metabolism, biotic/abiotic stress, and transcription showed relatively fewer suppressed genes at 96 hours.

In the analysis that included DEGs detected across all time points, 3,790 genes in total (see supplemental table 1), the number of DEGs at each time point varied sufficiently that hierarchical clustering grouped time points according to the number of genes that were not differentially regulated (Fig. 2.7a). At the 96-hour time point, expression had begun to relax and most closely resembled the 12-hour time point, when induction was ramping up. Although the 24- and 96-hour time points had a roughly similar number of DEGs (929 and 727, respectively), only 364 were differentially expressed at both time points. Meanwhile, 2,491 genes were uniquely expressed at the 48-hour time point, and only 390 DEGs detected in this study were not induced at 48 hours. Of these 390, 267 were only detected at 24 hours and 112 were unique to 96

hours. No genes were unique to the 12-hour sample. Taken together, these results suggest that, aside from the 12-hour sample, the genes differentially expressed at each time point were predominately unique to that time point.

Looking specifically at genes putatively assigned to phytohormone biosynthesis, six of the 39 DEGs detected in the 12-hour sample are involved in JA signaling, and two are assigned to the ET pathway (Fig. 2.7b). Out of the eight genes that were expressed at the 12-hour time point, none were unique to that time. The 24-hour sample, however, was enriched in genes from the full range of phytohormone pathways, including ABA, IAA, BR, SA, and an even greater number from both ETH and JA. Of the 94 genes included in the analysis, 83 were differentially expressed at 48 hours, and 47 were unique to that time. The number of DEGs assigned to each hormone pathway peaked at 48 hours, although by this point roughly half of the genes assigned to the IAA pathway were suppressed. While other hormone pathways had relaxed at 96 hours to the point where they resembled expression at 24 hours, the JA pathway remained largely consistent; the number of upregulated genes was nearly identical to 24 and 48 hours, and it was the only hormone pathway that contained no suppressed genes at any time point. The hierarchical clustering analysis showed that the 24- and 96-hour time points were most similar. In total, 25 genes were commonly expressed at both the 24- and 96-hour time points, 47 were expressed at neither, and the remaining 22 were uniquely expressed at one of the two time points. Overall, the ethylene pathway is most represented among phytohormone pathways, with 32 unique genes differentially regulated across all time points. The bulk

of these, however, are only differentially expressed at the 48-hour time point. The JA pathway, in contrast, had the most consistent expression across time points.

None of the 16 genes included in the analysis of terpene synthases were differentially expressed 12 hours after the initiation of herbivory (Fig. 2.7c). At 48 hours, all 16 genes showed changes in regulation. Fourteen showed higher expression levels, while two genes were suppressed. These suppressed genes were not induced or suppressed at any other time point, and their predicted products, copalyl diphosphate and cycloartenol, were not detected in *D. wrightii* headspace. The 24- and 96-hour time points were again the most similar according to the cluster analysis, with eight genes upregulated at both time points, the predicted products of which include limonene, D-limonene, β-ocimene, (E,E)-α-farnesene,(-)-ent-kaurene, nerolidol, linalool, and one undetermined product. Four genes, *TPS3*, *TPS4*, *TPS5*, and *TPS37* were induced at 24 hours but not 96, and are predicted to produce camphene or tricyclene, β-phellandrene, linalool, and nerolidol or β-ocimene, respectively. *TPS38*, possibly responsible for α-bergamotene production, was the only TPS gene induced at 96 hours but not 24.

Confirmation of RNA-Seq by qPCR

Quantitative PCR was used to compare the expression of five genes that were identified by RNA-Seq analysis as being differentially expressed at various time points throughout the study. Expression of *HPL1* analyzed by qPCR corroborated the RNA-Seq data qualitatively, showing that expression by damaged plants was increased at all time points relative to the control (Fig. 2.8a). qPCR results differed quantitatively from those

of the RNA-Seq analysis, showing relatively lower expression at 12 hours, but greater expression at 24 and 48 hours. *LOX2* expression measured by qPCR showed relatively lower and greater expression at 12 and 96 hours, respectively, when compared to RNA-Seq analysis, but the values for 24 and 48 hours were very similar (Fig. 2.8b). Note that the qPCR value at 12 hours is representative of two biological replicates rather than three, due to a lack of amplification across all technical replicates. This may partially account for the large error term associated with the 12-hour value. *PAL* expression was very similar across both techniques, with the exception of greater expression measured by qPCR at 24 hours (Fig. 2.8c). *RUB* was downregulated at 48 hours according to both techniques (Fig 2.8d). At the remaining time points, qPCR reported values below the threshold of significance, while RNA-Seq showed values that were above the threshold but statistically non-significant. The 96-hour value for RUB is based on only two biological replicates due to a lack of amplification in one of the biological replicates. Both techniques agreed on qualitative changes in expression of *TPS*, although qPCR reported slightly lower expression at 24 and 48 hours (Fig. 2.8e). The 12-hour TPS value for qPCR only represents one biological replicate due a lack of amplification in several technical replicates and a shortage of backup tissue. Similarly, the 96-hour sample only represents 2 biological replicates.

Discussion

Our results indicate that continuous damage by *L. daturaphila* larvae does not induce a proportional or sustained response in *D. wrightii* in terms of VOC production or gene

transcription. Despite increasing levels of damage over time, total emission of volatiles reached a maximum 48 hours after initiation of damage and remained relatively consistent for the remainder of the sampling times, with some individual compounds tapering off over time. The response of the *D. wrightii* transcriptome to continuous damage showed a similar pattern, consisting of a very distinct step-wise increase over the first 48 hours, before declining at 96 hours.

Continuous damage elicits a prolonged emission response

The greatest increases in emission were found in terpenes, including DMNT, (*E*)-β-ocimene, and (*E*)-β-caryophyllene, and one GLV, (*Z*)-3-hexenyl acetate. These compounds have been collected from the headspace of *D. wrightii* previously, in both the lab and the field, and are known to increase in response to herbivore damage (Hare 2007; Hare 2010; Hare and Sun 2011a). Each of these compounds were produced in very low amounts until 24-48 hours of feeding had occurred, at which point emission increased considerably. Emission of DMNT, (*E*)-β-ocimene, and (*Z*)-3-hexenyl acetate increased rapidly before leveling out and eventually declining at 144 hours, while (*E*)-β-caryophyllene steadily increased over the course of the entire sampling period. The remaining compounds followed unique and less distinct time courses.

In contrast to the current study using a continuous damage treatment, *D. wrightii* damaged by *L. daturaphila* for 24 hours showed an attenuated response (Hare and Sun 2011b). In that study, total volatile emission peaked 2-3 days after the start of damage, before declining precipitously until the conclusion of the study on day 8. In agreement

with the present study, the same compounds were found to be most abundant, with the addition of (E,E)-α-farnesene, although the abbreviated exposure to damage was associated with a brief emission maximum 2-4 days after the start of damage followed by a distinct decline. Studies in other systems have found similar patterns of emission, although the temporal variation in emission is highly dependent on the plant species. When challenged with a single instance of mechanical damage and *Spodoptera littoralis* regurgitant, maize responded with distinct emission maxima of several compounds followed by relaxation within the span of 12 hours (Turlings *et al*. 1998). In Norway spruce (*Picea abies*), a single application of MeJA induced emissions of mono- and sesquiterpenes that peaked 24-48 hours after application before declining over the next five to six days. Interestingly, accumulation of terpenes in pine needles peaked 15 days after treatment, suggesting that these compounds were still being synthesized but not volatilized (Martin *et al*. 2003)

Few studies have tracked emissions at multiple time points during prolonged bouts of insect herbivory. In tomato, continuous infestation of spider mites (*Tetranychus urticae*) induced emission of four compounds, including methyl salicylate, *trans*-β-ocimene, *trans*-nerolidol, and linalool, that steadily increased over five days of sampling (Kant et al. 2004). On the other hand, cucumber (*Cucumis sativus*) plants respond to *T. urticae* damage with a peak in induced emission of DMNT, (*E*)-β-ocimene and (E,E)-α-farnesene at 72 hours after the start of infestation, and only (*Z*)-3-hexenyl acetate continually increased over the 168 hour sampling time (Mercke *et al*. 2004). Black Poplar (*Populus nigra*) challenged with 48 hours of damage by gypsy moth larvae (*Lymantria dispar*)

emitted several compounds that showed similarly-sized emission peaks during the two diurnal sampling periods while damage was occurring. Once herbivore damage was terminated, emissions of several compounds ceased immediately or within 24 hours, and only α-pinene persisted until sampling ended at 96 hours after the start (McCormick *et al*. 2014). Taken together, these results suggest that ongoing damage leads to prolonged VOC emissions. It is unclear if studies showing continual increases in emission of particular compounds were too brief to capture the emission peak and subsequent decline, or whether the emission was positively correlated to leaf damage. Continual emissions could be possible through either continual *de novo* synthesis of emitted compounds or through mechanical disruption of VOC storage structures as insects feed (e.g. Elzen *et al*. 1985). In *D. wrightii*, no such structures have been identified, and all VOCs are presumed to be synthesized *de novo* upon induction.

VOCs have mainly been studied for their ecological role in attraction of arthropod predators, and it has been established that herbivore-induced VOCs emitted by *D. wrightii* are used as foraging cues by *Geocoris pallens,* the dominant predator in southern California populations of *D. wrightii*. Although VOC emissions that last throughout the herbivore attack may help to attract predators, *G. pallens* feed on eggs and neonates of *L. daturaphila* and other species and are physically unable to feed on *Lema* that have matured past the first instar. Therefore, attraction of these predators may provide little benefit to plants attacked by older larvae, and are unlikely to have driven the evolution of such a trait. Adult *L. daturaphila* also feed on *D. wrightii*, appearing to sample leaves by chewing small pits prior to ovipositing on the underside of younger leaves. Prior field

studies have demonstrated a positive association between VOCs emitted in response to feeding by adults and egg predation by *G. pallens* (Hare and Sun 2011a). Although the feeding pattern and the extent of damage inflicted by adults differ from the treatment used in this study, VOC emissions that persist through the duration of attack by adults could function to attract *G. pallens* while herbivore eggs are present, prior to hatching. Since adult *L. daturaphila* are highly mobile and frequently move between leaves, it's unclear whether a persistent, induced response would result from the natural feeding behavior of adults, or if such a response would increase egg predation rates. It is possible that other predators that visit *D. wrightii* may provide a benefit, although Lacewing larvae (*Chrysoperla spp*), Lynx spiders (*Habronattus pyrrithrix*), and Damsel bugs (*Nabis americoferus* or *alternatus*) are present in low abundances in the *D. wrightii* system and are not likely to have a large influence on herbivore populations (Gassmann and Hare 2005).

The pattern of VOC emissions observed in this study may be better explained as a within-plant signal. VOCs have been observed to function as a within-plant signal in several species (Karban *et al*. 2006; Heil and Silva-Bueno 2007; Frost et al. 2008). It is generally assumed that such signals benefit the plant when mobile herbivores are likely to move between leaves on the same plant. Although vascular tissue is able to transmit a damage signal between leaves, this method is limited by the plant's orthostichy, or vascular architecture (Orians 2005; Choi *et al*. 2016). Airborne signals, however, are able to reach leaves that are vascularly disconnected but in close spatial proximity. Two types of responses have been noted in tissue that receives a damage signal. In some

species, such as maize seedlings, leaves will induce resistance traits upon receiving a signal, producing an increase in resistance metabolites (Engelberth *et al*. 2004). In addition, maize seedlings will prepare to produce resistance traits, a phenomenon called priming. When primed leaves are subsequently damaged, the response is of a greater magnitude and produced more quickly than in unprimed leaves. In some species, such as lima bean or poplar, priming is the only response upon receipt of airborne signals (Heil and Silva Bueno 2007; Frost *et al*. 2008).

Producing a within-plant signal proportional to the amount of damage may not provide the best protection to the plant. Hypothetically, leaves emitting less of a signal in response to attack by very young larvae may poorly prepare nearby tissue for attack if those larvae increase their rate of feeding exponentially as they mature. On the other hand, producing a larger plume in response to greater damage may induce or prime leaves that are farther away and at less risk of attack. Such a scenario might impose a metabolic cost in both the emitter leaf investing carbon in unnecessary signaling compounds, as well as in receiver leaves that prepare for attacks when they are not at risk. It may also be advantageous to limit the emission of VOCs to prevent competing plants from eavesdropping on airborne signals and gaining an advantage by preparing for possible future attack (Heil and Adame-Álvarez 2010). Emission of a steady plume throughout the duration of feeding by an herbivore may be adequate to reliably signal to nearby tissue at risk of attack while minimizing ecological and metabolic costs. Similarly, the relaxation of induced resistance traits when herbivory ceases (Hare and Sun 2011b; McCormick *et al*. 2014) may reduce the likelihood that nearby leaves receive

a signal to prepare resistance if the herbivore is no longer present. Receipt of erroneous signals may be costly to the plant if resistance traits divert limited metabolic resources from growth and reproduction, although the cost of producing VOCs does not appear to be particularly demanding (Kessler and Heil 2011).

Because inducible traits appear to be largely co-regulated and rely on shared metabolic pathways, the behavior of one may be best explained by the constraints or benefits of others. Activation of the methylerythritol pathway (MEP), for instance, is responsible for production of volatile monoterpenes, but also produces a host of non-volatile carotenoids and diterpenes that may play important roles in direct resistance against herbivores (Dudareva *et al*. 2013). The persistent emission of VOCs may be a byproduct of the continued investment into metabolic pathways producing direct resistance traits that have a greater role in mitigating herbivore damage. As a corollary, the relaxation of emissions after cessation of herbivory seen in several studies could be a cost-saving measure driven by the relatively greater cost of other traits to which VOC emissions are metabolically linked. In addition to other resistance mechanisms, the induced response is typically accompanied by a suppression of photosynthesis (Arimura *et al*. 2000; Hermsmeier *et al*. 2001; Bilgin *et al*. 2010; but see Schmidt *et al*. 2005 and Halitschke *et al*. 2011), and plants that remain in the induced state after herbivores have dispersed or died are likely to suffer both primary and secondary metabolic costs.

Terpene synthase gene sequences are conserved but expression is not

The assembly of the reference transcriptome revealed that the estimated 21 million years of divergence between *D. wrightii* and tomato (Sarkinen *et al*. 2013) has resulted in a somewhat larger transcriptome for *D. wrightii*. Among the 16 terpene synthases found to be inducible in this study, only two showed no non-synonymous amino acid substitutions compared to tomato terpene synthases. Of those that showed non-synonymous substitutions, nine had changes to fewer than 2% of their predicted amino acids, although two genes showed differences of 7.4% (*TPS25*-like) and 9.5% (*TPS37*-like). It's unclear what effect these differences have on the resulting enzymes' abilities to synthesize their putative products. *TPS25* is predicted to produce β-ocimene and *TPS37* is predicted to produce nerolidol and linalool. Although nerolidol was not detected in this study, β-ocimene and linalool both were. The presence of these compounds, however, may be attributed to the action of other genes with the same predicted products and much lower rates of substitutions (e.g. *TPS5*, *TPS39* for linalool and an undesignated β-ocimene synthase). Such redundancies make it difficult to attribute the presence of a particular compound to any one gene, and evaluations of the functionality of genes are merely speculative without further *in vitro* testing or gene silencing.

In *D. wrightii*, the major increase in VOC emission from damaged plants coincided with the first detection of induced terpene synthase genes at the 24-hour time point. Although most *TPS* genes were induced by 24 hours, two additional genes were activated in the 48-hour sample, and several showed higher expression levels. The two

genes that were suppressed in the 48-hour sample, putatively coding for copalyl diphosphate synthase (CDS) and cycloartenol synthase, were not differentially expressed at any other time point, and it is unclear what function their suppression may serve. Unlike other *TPS* genes monitored in this study, neither of these genes are expected to contribute to VOC production. Downregulation of *CDS* may reduce available substrate for GA synthesis, although curiously an (-)-ent-kaurene synthase that converts copalyl diphosphate into the first committed precursor for GA synthesis was induced from 24 to 96 hours after herbivory (Hedden and Thomas 2012). This contradiction might indicate that *CDS* is a "gatekeeper" gene that controls access to rate-limiting substrates in order to regulate GA synthesis. Several other GA-synthesis-related and GA-responsive genes were suppressed throughout the time course, supporting the hypothesis that the reduction in gene activity is part of a coordinated response to herbivory. Although the substrate of *CDS* activity, geranylgeranyl diphosphate, is also required for carotenoid and chlorophyll production, enzymes that produce those compounds draw from a separate substrate source and the downregulation of *CDS* therefore cannot be explained as a diversion of resources to production of those products (Hedden and Thomas 2012). The reduced expression of cycloartenol synthase may similarly reflect a necessary change in the priority of phytosterol synthesis, although it is less clear how this fits into the broader picture of plant resistance. Regardless of the reasons, suppression of both of these genes was short-lived.

The remaining *D. wrightii* TPS genes detected in this study were induced in response to herbivory. Consistent with the pattern of volatile emissions, more TPS genes

were differentially expressed at 24 and 48 hours, when emissions were increasing and reaching their zenith, than at 96 hours, when several compounds were decreasing in abundance. Although the majority of compounds predicted to be synthesized by induced *D. wrightii* TPS genes were detected, the products of *TPS3* and *TPS4* were not. Previous studies detected both *TPS3* and *TPS4* activity exclusively in the stem, root and immature fruit of tomato plants, where TPS3 converted geranyl diphosphate (GPP) into the monoterpenes camphene and tricyclene, and TPS4 produced *β*-phellandrene (van Schie *et al*. 2007; Falara *et al*. 2011). Here, *TPS3* and *TPS4* were both detected in the leaf, although none of those compounds were detected in the *D. wrightii* headspace. Both genes code for monoterpene synthases that are generally capable of producing a range of products in *Salvia officinalis* (Croteau *et al*. 1988) and *Abies grandis* (Bohlmann *et al*. 1999), so it is possible that these enzymes are involved in producing one or more of the various monoterpenes detected in this study.

Other genes also showed differences in tissue specificity from tomato. The *D. wrightii TPS33*-like and *TPS38* genes were both absent from leaf tissue in tomato, and *TPS25* and *TPS27* were not found to be inducible in any tomato organs, yet all were inducible in *D. wrightii* leaves. Finally, *TPS39* was detectable in young trichomes on tomato leaves, but not in the leaves themselves (Falara *et al*. 2011). Here, we did not distinguish between trichomes and leaves. *D. wrightii* trichomes are not known to have any function in VOC production or emission and VOC emissions are independent of the two trichome phenotypes found in southern California (Hare 2007), so the detected *TPS39* activity is assumed to be localized to the leaf tissue. Although this study detected

differential expression of more putative terpene synthase genes than have been shown to be inducible in tomato, several corresponded to genes that have not been given a *TPS* designation and may have gone overlooked in previous studies. Additionally, ten tomato TPS genes expressed in leaves as reported by Falara *et al*. (2011) were not induced in *D. wrightii* leaves. In sum, only four *TPS* genes expressed in tomato leaves and leaf trichomes were induced in this study: *TPS5*, *TPS37*-like, *TPS39*, and *TPS40*.

Regulatory changes that alter tissue specificity of gene expression can create novel phenotypes. It is currently unclear what effect, if any, the changes in TPS activity between tomato and *D. wrightii* might have. Several VOCs emitted from tomato (e.g. β-phellandrene, β-myrcene, and numerous others; Kant *et al.* 2004; van Schie *et al*. 2007) are not found in the headspace of *D. wrightii*, but more work is necessary to determine whether the absence of these compounds is due to *TPS* gene activity. Because VOCs mediate interactions between multiple trophic levels, including both beneficial and deleterious relationships for the plant, changes to the site of activity of terpene synthase genes can have important evolutionary implications (e.g. Dudareva *et al*. 1996). Variations in the pool of inducible terpene synthases in leaves are likely to impact interactions with herbivores and their natural enemies.

Because the dominant natural enemy in the *D. wrightii* system is a generalist that appears to respond to quantitative differences in VOC emissions regardless of blend composition, however, the differences are not likely to due to selection by natural enemies, at least in the *D. wrightii* system (Hare and Sun 2011a). Herbivores, on the other hand, can have strong influences on plant fitness by inflicting damage on plant

tissues contributing to or necessary for reproduction. VOCs that affect the incidence, duration, or intensity of herbivore attack through attraction or repulsion of herbivores are likely to have a strong influence on the fitness landscape. Predicting the effects of VOCs on herbivores is very challenging; although VOCs can repel potential herbivores (Kessler and Baldwin 2001; Laothawornkitkul *et al*. 2008; Reisenman *et al*. 2012; Ballhorn *et al* 2013b), they can also act as foraging cues to others (Bolter *et al*. 1997; Schütz *et al*. 1997;), sometimes in a sex- and dose-dependent fashion (Ballhorn *et al*. 2013). Ultimately, the consequences, if any, of inducible expression of additional *TPS* genes in the leaves of *D. wrightii* depends on the specific compounds produced and their effects on organisms that are able to perceive these emissions. Alternatively, the suite of *TPS* genes deployed following herbivory may be the result of neutral selection if those genes are redundant or non-functional, or if the contribution of individual compounds to the resistance phenotype is subsumed by a greater importance of the overall blend. As more data concerning solanaceous *TPS* genes becomes available, the additional phylogenetic context will allow for more informed hypotheses regarding the evolution of *TPS* genes in both tomato and *D. wrightii*.

Alternatively, such widespread differences in resistance traits may be result from the domestication of tomato. Selection for desirable characteristics such as yield and produce quality have likely had a range of unintended effects on resistance traits (Falara *et al*. 2011), as the need for pest management would indicate. There is currently insufficient genomic and phylogenetic information available to draw accurate conclusions about the effects of domestication on *TPS* gene activity. Although there is

no doubt as to the value of studying tomato's response to herbivores for ecological and economic purposes, the traits mediating the interaction are likely far removed from the evolutionary context in which they evolved. Studying resistance traits and their underlying genetic basis in undomesticated relatives of crop species therefore represents an important step toward improving the pest resistance of tomato and other crops (Blauth *et al.* 1998).

Herbivory elicits a complex response from phytohormone pathways

It has been well established that the JA pathway is principally responsible for the induced response to chewing herbivores (Farmer and Ryan 1990; Farmer *et al*. 1992; Howe 2004) and, when applied exogenously as MeJA, is sufficient to induce resistance traits in *D. wrightii* (Hare and Walling 2006; Hare 2007). The results of the present study indicate an early and prolonged response of transcripts involved in JA signaling, supporting the hypothesis that JA represents the core pathway responsible for the induced response to chewing insects.

The SA pathway, typically seen as a response to phloem-feeding insects and pathogens, acts antagonistically to JA in a number of systems (Thaler *et al*. 2012). The relatively minimal response of the SA pathway suggests it is not heavily involved in the induced response observed here. It should be noted, though, that some SA biosynthetic genes were induced in the 48-hour sample, and this was followed by a burst of methyl salicylate captured in the 72-hour volatile collection.

ET has been identified as an important modulator of the JA-based response and functions to suppress the SA response in *N. attenuata* and *A. thaliana* (Diezel *et al*. 2009; Leon-Reyes *et al*. 2009; Leon-Reyes *et al*. 2010). Studies of both cultivated and undomesticated species show ET is individually insufficient to produce a typical induced response, yet ET-insensitive plants are incapable of deploying their full induced response following MeJA application or when challenged by herbivores (reviewed in von Dahl and Baldwin 2007). The early appearance and preponderance of ET-related transcripts in the present study suggest that it could play an important role in *D. wrightii* as well.

Recently, the actions of other hormones in the induced response has gained attention, although their specific roles in the response to herbivory are not well understood. IAA, BR, and ABA have been extensively studied for their activities regulating traits nominally unrelated to resistance, and so the induction of these pathways following attack by herbivores begs the question of whether their induced changes reflect resistance strategies related to these established functions, secondary functions regulating resistance traits, or are simply incidental "side effects" of induction. The mechanical damage inherent to attack by chewing herbivores may necessitate responses that function to maintain the physiological integrity of the plant.

ABA's involvement in the response to abiotic stress, and drought in particular, has led some to hypothesize that it may also help to mitigate water loss when leaf surfaces are breached by chewing herbivores (Erb *et al*. 2012). Through unknown mechanisms, however, ABA has also been found to affect biosynthesis of JA and influence expression of downstream JA-responsive transcripts (Adie *et al*. 2007;

Bodenhausen and Reymond 2007), as well as alter the relationship between JA and SA (Thaler and Bostock 2004).

The deployment of resistance traits concomitantly reduces growth and primary metabolism (Herms and Mattson 1992), and IAA levels, known to stimulate division and growth of plant cells (Teale *et al*. 2006), have been observed to decrease in *N. tabacum* (Thornburg and Li 1991) and *N. attenuata* (Onkokesung *et al*. 2010) following wounding treatments. *N. attenuata* plants that are impaired in their ability to produce both JA and ET fail to show typical decreases in IAA concentration in local leaf tissue following herbivory and also experience cell regrowth at the site of wounding that is absent in wild type plants, leading the authors of that study to hypothesize that the negative relationship between JA/ET and IAA allows plants to shift resources away from growth and into defense (Onkokesung *et al*. 2010). Downstream products of the IAA pathway also modulate resistance to pathogens more directly through their effect on cell wall characteristics (Ding *et al*. 2008) and SCF-mediated ubiquitination in addition to their antagonistic relationships with JA, SA, and ET (Llorente *et al* 2008). Effective resistance against pathogens typically requires different means than those plants use to mitigate damage from herbivores, but these findings suggest that the IAA's contribution to plant resistance may be multifaceted.

Overall response pattern

In total, *D. wrightii* responded to herbivory by differentially expressing 3,778 unique genes. The minimal response of 39 DEGs at 12 hours escalated incrementally to

3,399 DEGs by 48 hours before declining to 727 in the 96-hour sample. The number of genes that responded to herbivory represents roughly one tenth of the estimated genome, a figure in line with other studies (Appel *et al*. 2014). Far more genes were induced than suppressed by herbivory, suggesting an active response from the plant, although roughly one third of the DEGs in the 48-hour sample were suppressed.

The response at 48 hours includes differential expression of a broad array of genes involved in the resistance and stress response, but a far greater number that deal with metabolism, transcription and various other infrastructural processes. Most of the differential regulation measured at this time point was fleeting, however, with the majority of genes showing baseline levels of transcription at both the preceding and subsequent time points. The lack of temporal proteomic data in plant response studies makes predictions about the persistence of gene products very difficult; resulting proteins remain active for an undetermined length of time after the gene itself has returned to baseline expression levels. The relative persistence of genes with established functions in the resistance response, such as JA and terpene biosynthesis, through most time points in the present study suggest that heightened expression may be necessary for continual effect, and, conversely, suggests that the effectual activity of most genes responding to herbivory may be as short lived as their differential expression.

Brief activity periods may be sufficient for signaling molecules or transcription factors to precipitate downstream reactions, but it is difficult to explain the transient suppression of photosynthesis in this way. Current hypotheses suggest that growth-related processes slow in response to herbivory as a diversion of finite resources toward

defensive compound production (Herms and Mattson 1992), or as a way of withholding nutritive resources from herbivores (Mitra and Baldwin 2008), possibly in an effort to increase reserves for later regrowth (Orians *et al*. 2011; Korpita *et al*. 2014). The latter hypothesis would seem untenable in this system, due to the brevity of the effect. The optimal strategy might be to withhold resources early on during attack to discourage newly-arrived herbivores and hamper the growth of newly-hatched larvae, or, if the goal is to minimize resource losses, plants should increasingly suppress photosynthesis as feeding continues if attackers are likely to be larvae that remove increasing amounts of leaf tissue as they mature. Here, neither approach is taken; suppression is primarily limited to the 48-hour time point and returns nearly to baseline levels by 96 hours, leaving only a short period in which resources would likely be withheld from attackers.

In accordance with the growth-defense tradeoff paradigm, decreases in photosynthesis gene activity may primarily function to reduce investment in the massive quantities of ribulose-1,5-biphosphate carboxylase (RuBPCase) normally required for carbon fixation (Ellis 1979), and instead diverting those resources into secondary metabolite production. Accordingly, the suppression of photosynthesis genes coincides with a substantial change in expression of genes involved in protein metabolism. Although roughly 150 of these genes are suppressed, more than twice that number are induced, suggesting a widespread reorganization of the *D. wrightii* proteome. Little is known about the proteomic response to herbivory in *D. wrightii*, although much of the shift likely supports production of secondary metabolites that are known to be metabolically costly (Zangerl *et al.* 1997; Zavala *et al*. 2004). In addition to VOCs, *D.*

wrightii is known to synthesize PIs, a range of phenolic compounds, and polyphenol oxidase (Hare and Walling 2006).

Perhaps most surprising was the very low number of DEGs at the 12-hour time point, none of which were unique to that sample. The expectation is that the early response should be enriched in transcripts responsible for herbivore or damage perception, as well as those involved in phytohormone biosynthesis and signal transduction (Walling 2000; Wu and Baldwin 2009). In this study, few genes putatively assigned to hormone biosynthesis pathways were induced after 12 hours of damage, and no transcription factors were induced. One possible explanation is that the expected early burst of activity occurred prior to the 12-hour sample, and reports from other systems support this hypothesis. Expression and relaxation of transcription for inducible signaling kinases occurs within the first three hours in *N. attenuata* treated with wounding and oral secretions from *M. sexta* (Wu *et al*. 2008). In the same system, WRKY transcription factors necessary for eliciting the induced response are primarily active within the first two hours after treatment (Skibbe *et al*. 2008). In a more extreme example, continuous aphids feeding on maize induced the largest response, including some 1,000 DEGs, only two hours after the start of attack, followed by a marked decline at 24 hours before increasing again at 48 and 96 hours (Tzin *et al*. 2015). Similarly, more than 1,000 genes in cotton showed altered expression after only two hours of damage by whiteflies or aphids, with that number remaining relatively constant at 24 hours (Dubey *et al*. 2013).

Alternatively, the lack of response at 12 hours in the present study may simply reflect differences in the induced response. Variations in the timing of plant responses to herbivory are well documented across species. Maize is very quick to emit volatiles when damaged, with much of the response occurring within 12 hours (Turlings *et al*. 1998). Given that *D. wrightii* has scarcely begun to emit induced volatiles in this timeframe, it is reasonable to expect that the time course of gene expression will also differ substantially from that of maize. The low point observed at 24 hours in the maize time course still showed roughly 300 DEGs, far more than the 39 observed at 12 hours in the present study, and a much larger fraction of the total number of DEGs observed (Tzin *et al.* 2015). *D. wrightii* may simply be slow to respond rather than in a lull at that time point, although further studies will be needed to characterize the earliest responses to herbivore damage in *D. wrightii*. Qualitatively similar patterns of expression were seen in *Arabidopsis* (Kuśnierczyk *et al*. 2008; Kerchev *et al*. 2013), barley (*Hordeum vulgare*; Gutsche *et al*. 2009) and tomato (Coppola *et al*. 2013) infested with aphids, with expression ramping up at earlier time points before peaking at 2-3 days after the start of damage. Interestingly, although each of these studies showed that the plant response to aphids primarily involved the SA pathway rather than the JA pathway, a typical difference in responses to phloem-feeding and chewing insects, the overall pattern of response was somewhat similar and suggests that signaling and response dynamics may have more in common than metabolite and phytohormone analyses would indicate.

As much of the plant-insect literature would attest, specific results of interaction studies are sometimes dependent on the species of herbivore involved, with feeding guild

having the greatest influence (e.g. Agrawal 2000; De Vos *et al.* 2005; Chung and Felton 2011; Ali and Agrawal 2012; Foyer *et al* 2015). In contrast to the previously mentioned study involving aphids, tomato plants infested with spider mites responded with an equal number of DEGs one and four days after infestation, with differences only found in the magnitude of expression of those genes. It should be noted, however, that this study used a microarray and was limited in the pool of genes assayed (Kant *et al.* 2004). Sorghum damaged by greenbug aphids (*Schizaphis graminum*) upregulated SA-dependent pathogenesis resistance genes as soon as six hours after infestation, far more rapidly than exogenous applications of SA. The authors suggest that the aphids may have evolved to elicit this response in an effort to antagonize a more effective JA response, which was limited to the early time points (Zhu-Salminen *et al*. 2004). Five pathogen and herbivore attackers with different modes of attack each induced a unique phytohormone response time course in *A. thaliana*. Despite several of the attackers inducing JA-intensive responses, the resulting gene expression profiles differed substantially (De Vos *et al*. 2005). In contrast to these and other studies, *D. wrightii*'s VOC emissions differed only quantitatively, rather than qualitatively, when attacked by *L. daturaphila*, *Manduca sexta*, or the piercing-sucking mirid bug *Tupiocoris notatus* (Hare and Sun 2011b). Although differences likely exist in *D. wrightii*'s response to various attackers at the transcriptional level, the similarity of the VOC responses suggests these differences may be small.

On the other hand, the influence of genotypic variation in the plant response has received less attention with the widespread use of standardized isogenic lines, although some data is available from agriculture. Two studies using domesticated soybean

(*Glycine max*) have demonstrated that host genotype influences the pattern of gene expression. Wang *et al*. (2014) found that the expression pattern of several resistance-related genes followed very different time courses in two soybean genotypes damaged by *Spodoptera litura*, and furthermore that the pattern of expression closely reflected the level of resistance as measured in feeding assays. In a separate study, Prochaska *et al*. (2015) found that feeding by aphids induced more genes in a resistant soybean genotype than in a susceptible line. Interestingly, however, more genes were differentially expressed after 15 days of feeding than after five, a far slower reaction than observed after *S. litura* feeding. Resistant and susceptible lines of sorghum induced by aphids showed more differences in gene expression than the resistant line did compared to uninduced plants from the same line, indicating that substantial intraspecific differences in gene expression may be commonplace (Park *et al*. 2006). Future studies will need to explicitly account for genetic variation in both plant and herbivore populations in order to characterize the breadth of plant responses and the importance of variation in resistance traits to plant fitness.

Overall, the induced response was highly complex and not proportional to the amount of damage sustained. VOC emissions reached a peak 48 hours after damage started, before emissions leveled out. At the transcriptional level, herbivore attack elicited a pulse of differential gene expression that peaked 48 hours after damage and then subsided. This pattern suggests a regulatory mechanism preventing additional herbivore damage from eliciting further response from the plant. This study also reveals that the transcriptional reorganization following attack is best described as a shifting

pattern of genes that are differentially regulated and relax relatively quickly, rather than showing sustained changes in expression. Future studies will benefit from taking a more holistic look at induced responses to elucidate gene activity, rather than focusing on particular time points after attack.

Table 2.1. List of genes and their associated primers analyzed by qPCR to confirm RNA-Seq results.

Gene	Abbreviation	Gene ID	Forward (5'→3')	Reverse (3'→5')
Hydroperoxide lyase 1	*HPL1*	Solyc07g049690	AAATGTGGTGGCGGTTCTG	GGCACAAACACGCATATCTC
Phenylalanine ammonia lyase	*PAL*	Solyc09g007900	GCTCCACCTACCCTTTGATG	TCGTCCTCGAAAGCTACAATC
Terpene synthase	*TPS*	Solyc03g006550	GGGATGCAAAGAACCTTGAT	TGGCAACATCACACACAAGA
Lipoxygenase 2	*LOX2*	Solyc01g006540	GGTGATGGAGTTGGAGAAAGA	GGACGGAGTAAACGGTGTTG
RuBPCase small chain 2B	*RUB*	Solyc02g063150	TCCCGTTACCAAGAAGAACAAC	AGCAAGGAACCCATCCACTT
Actin	*ACT*	Solyc04g071260	TCTTCCAGCCATCCTTGATT	TGACCCACCACTAAGCACAA
Ubiquitin	*UBI*	Solyc12g099030	TCCACCTTGTCCTTCGTCTC	CCTCTGAACCTTGCCAGAA

Figure 2.1. Mean (±SE) cumulative leaf area removed in millimeters squared by third-instar *Lema daturaphila* caged on individual *Datura wrightii* leaves over a period of 144 hours.

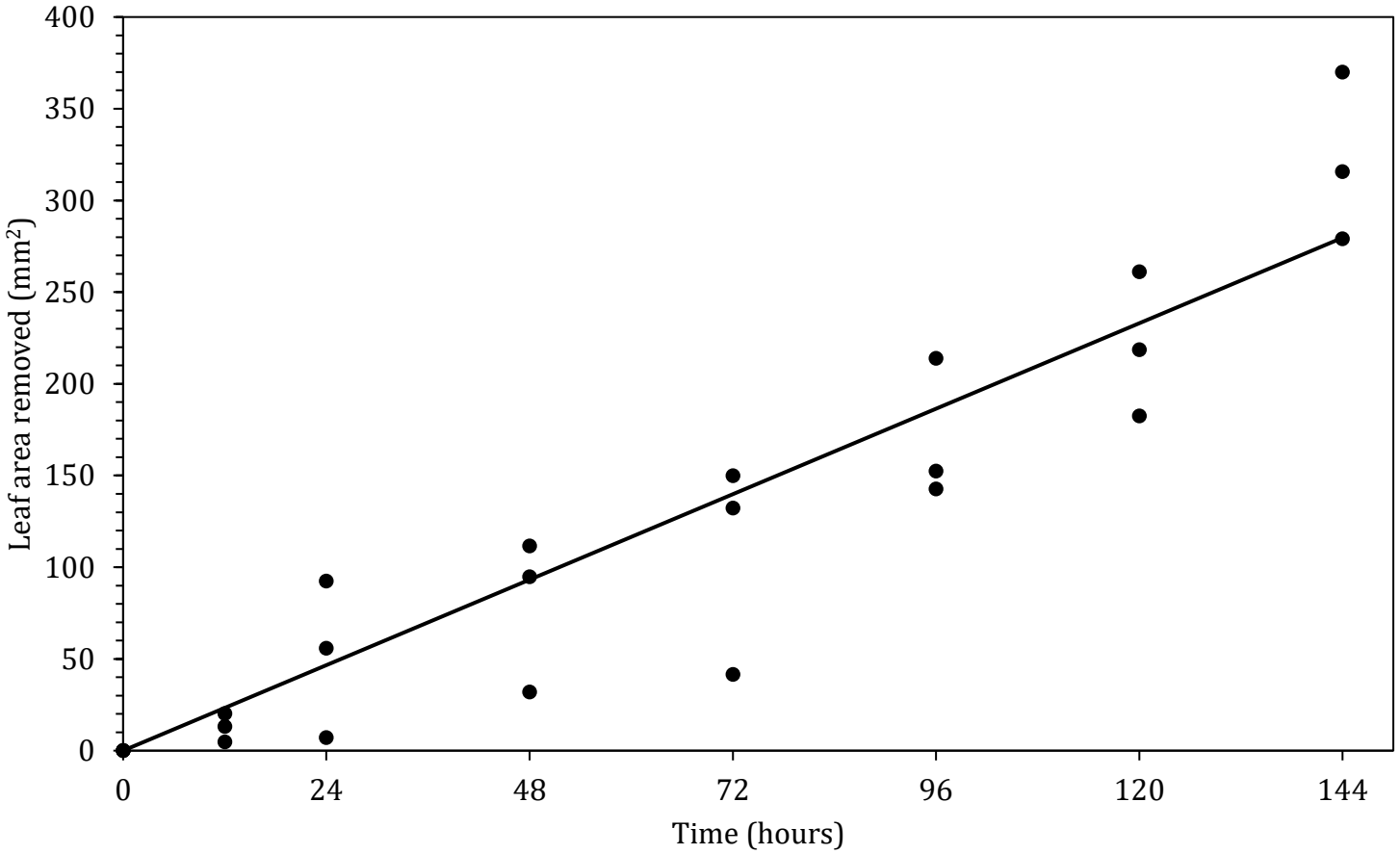


Figure 2.2. Mean (±SE) $\log_{10}$ total VOC emissions by hour and treatment. Closed circles represent plants continuously damaged by *L. daturaphila*; open circles represent undamaged control plants.

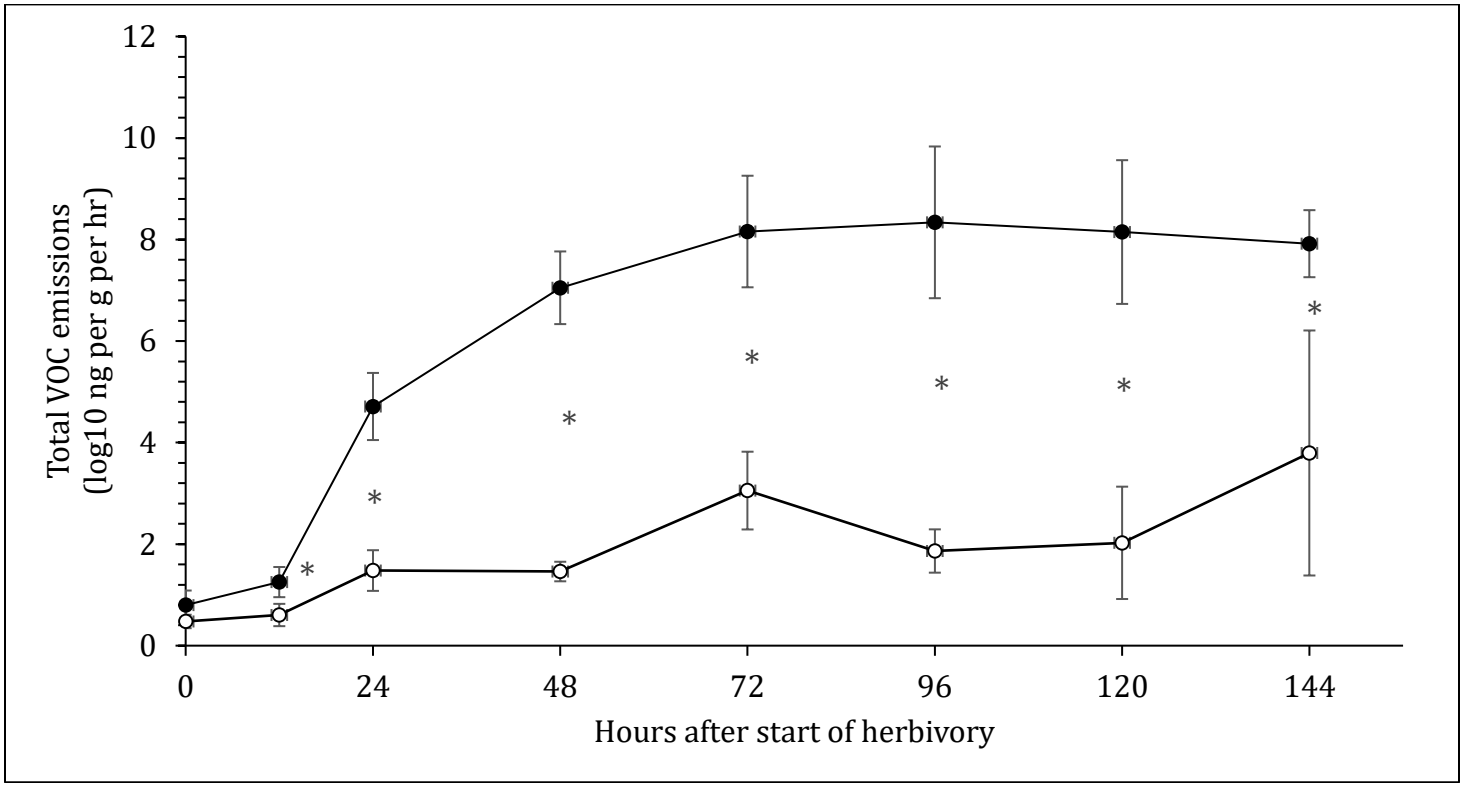


Table 2.2. List of VOCs included in a principal components analysis, as well as their factor loadings toward each individual component. The percent of the variation explained by each PC is listed at the top.

	PC1	PC2	PC3	PC4
Percent of variation	59.1	16.5	6.9	4.2
Compound				
trans-2-hexanal	-0.03	0.80	0.03	-0.09
cis-3-hexen-1-ol	0.08	0.05	0.13	0.10
trans-2-hexen-1-ol	0.05	0.58	0.07	0.08
α-pinene	0.01	0.00	-0.02	0.05
Hexenyl acetate	0.31	0.04	0.21	-0.17
Limonene	0.07	0.10	-0.21	0.44
(*E*)-β-ocimene	0.40	-0.01	-0.17	0.30
Linalool	0.07	0.01	-0.40	0.57
DMNT	0.74	0.00	-0.37	-0.40
Methyl salicylate	0.03	-0.01	-0.04	0.18
β-caryophyllene	0.40	-0.07	0.74	0.35
α-bergamotene	0.06	0.00	0.09	-0.05
Geranyl acetone	0.01	0.00	0.02	-0.02
α-humulene	0.00	0.00	0.01	-0.01
β-selenine	0.02	0.04	-0.01	0.11
TMTT	0.00	0.00	0.01	0.00
α-farnesene	0.06	-0.01	0.11	0.01

Figure 2.3. Mean (±SE) factor scores for principal components 1-4 for *Datura wrightii* plants damaged continuously by *Lema daturaphila* (closed circles) or left undamaged (open circles).

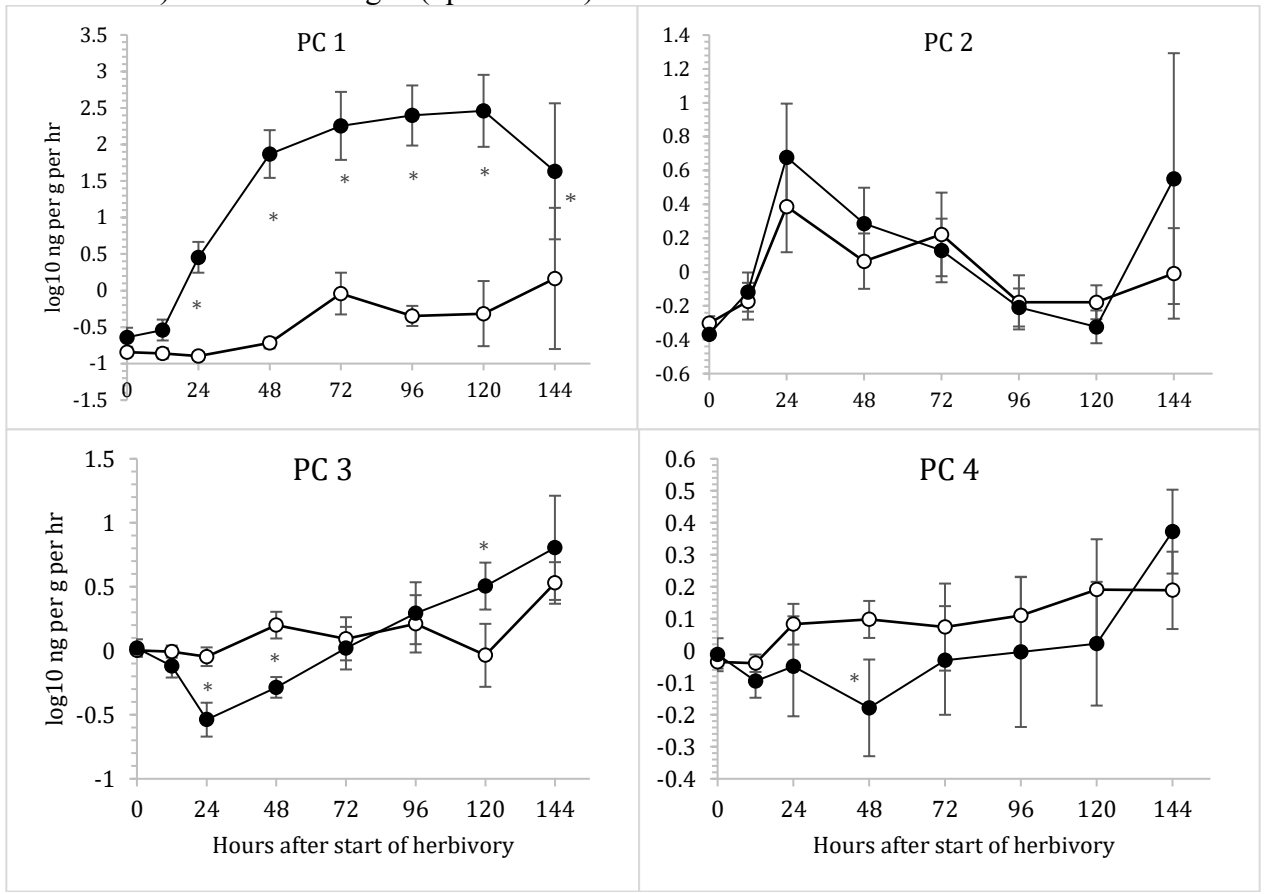


Figure 2.4. Emission of DMNT, β-ocimene, (*Z*)-3-hexenyl acetate, (*E*)-β-caryophyllene, limonene, linalool, and methyl salicylate by *D. wrightii* damaged continuously by *L. daturaphila*. Mean (± standard errors) in $\log_{10}$ ng g^{-1} (dry wt.) leaf hr^{-1} are shown.

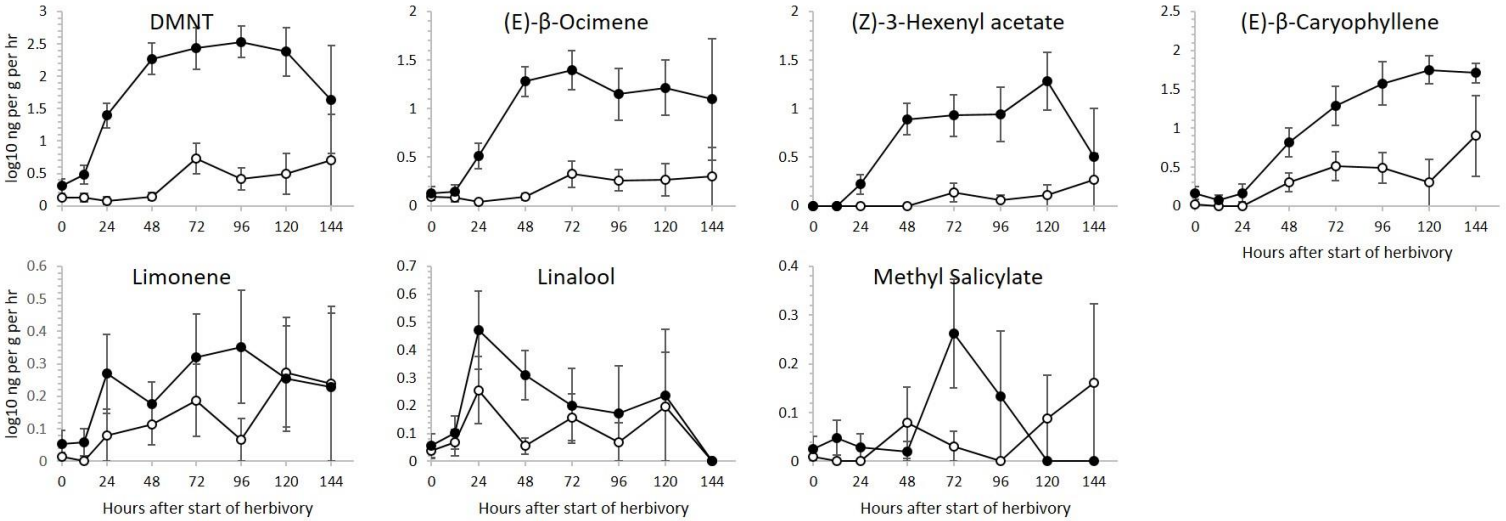


Table 2.3. A list of terpene synthase genes identified as being differentially expressed during the time course of induction in *Datura wrightii*. Nucleotide sequences from the *D. wrightii* transcriptome were used to predict amino acid sequences and open reading frames, and the resulting sequences were compared to the tomato proteome. Predicted products for each gene are from the reference listed.

Datura wrightii gene	Tomato ortholog gene ID	Tomato homolog	Predicted amino acid length	Non-similar amino acid substitutions from tomato	Expect (E) value	Main predicted product	Reference
DwTPS1	Solyc00g154480		521	7	0	Unknown terpenoid	Fernandez-Pozo et al. 2015
DwTPS3	Solyc01g101190	*SlTPS33*-like	665	19	e-141	α-humulene / (-)-(*E*)-β-caryhophyllene	Falara *et al.*, 2012
DwTPS4	Solyc01g105870	Identical to S*lTPS3*	607	0	0	Camphene / tricyclene	Falara et al., 2012
DwTPS5	Solyc01g105880	Nearly identical to SlTPS4	590	0	0	β-phellandrene	Van Schie et al. 2007
DwTPS6	Solyc01g105890	SlTPS5-like	609	2	0	Linalool	Falara et al., 2012
DwTPS7	Solyc01g105910		131	0	2E-90	D-limonene	Fernandez-Pozo et al. 2015
DwTPS8	Solyc02g079840	SlTPS38-like	538	4	0	α-bergamotene	Falara et al., 2012
DwTPS9	Solyc02g079890	SlTPS25-like	122	5	5E-67	β-ocimene	Falara et al., 2012
DwTPS10	Solyc02g079900		56	2	4E-32	(*E*,*E*)-α-farnesene	Fernandez-Pozo et al. 2015
DwTPS11	Solyc02g079910	SlTPS27-like	160	2	4E-67	Limonene	Falara et al., 2012
DwTPS12	Solyc03g006550		621	8	0	(-)-*ent*-kaurene	Fernandez-Pozo et al. 2015
DwTPS13	Solyc03g007730		562	3	0	β-ocimene	Fernandez-Pozo et al. 2015
DwTPS17	Solyc06g084240	Truncated SlTPS40-like	206	2	2E-44	Copalyl diphosphate	Schilmiller et al 2009
DwTPS18	Solyc10g005390	Nearly identical to SlTPS39	563	0	0	Nerolidol / linalool	Falara et al., 2012
DwTPS19	Solyc10g005410	Truncated SlTPS37-like	137	11	7E-26	Nerolidol / β-ocimene	Falara et al., 2012
DwTPS22	Solyc12g006530		761	0	0	Cycloartenol	Fernandez-Pozo et al. 2015

Figure 2.5. Number of genes differentially expressed by continuously damaged plants at several time points. Black bars above the horizontal line indicate the number of genes that with increased expression; grey bars below the line indicate suppressed genes.

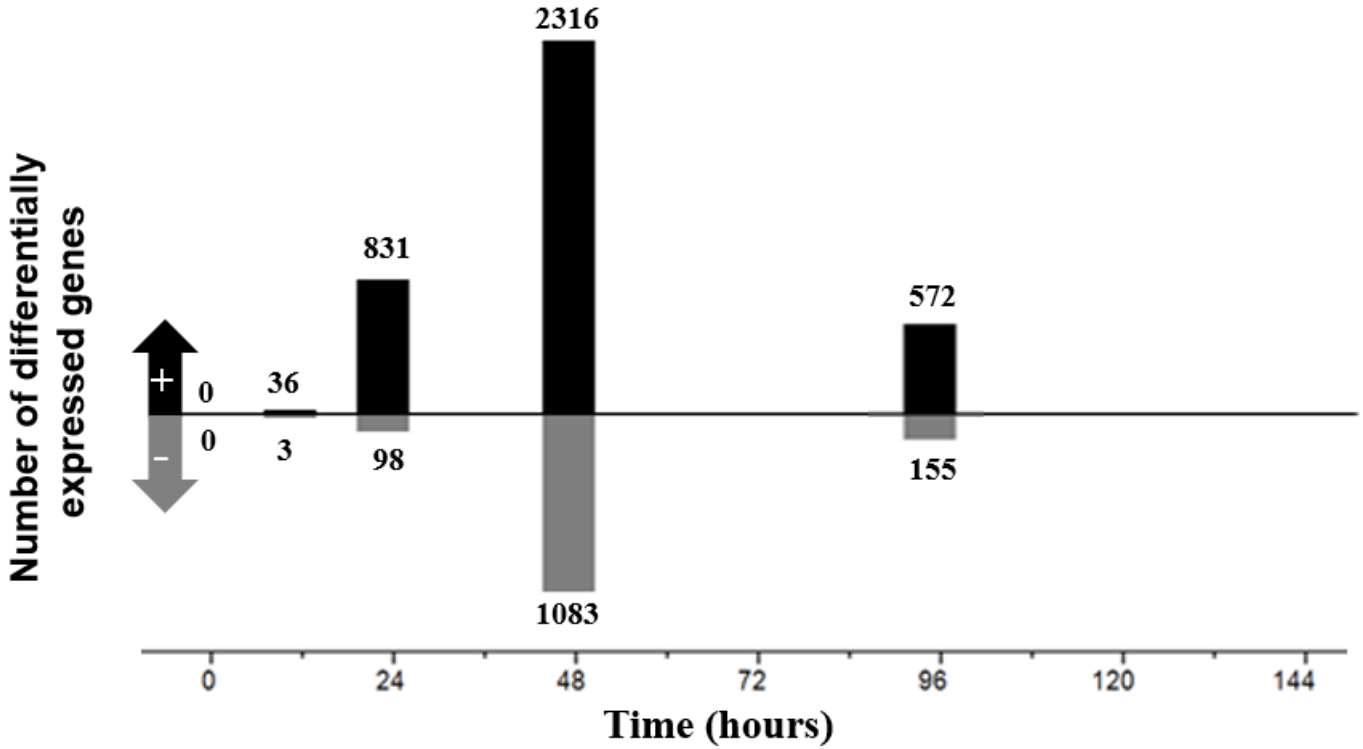


Figure 2.6. Number of genes differentially expressed at each time point according to functional group assigned by MapMan. Left, genes that were upregulated relative to controls. Right, genes that were downregulated compared to controls.

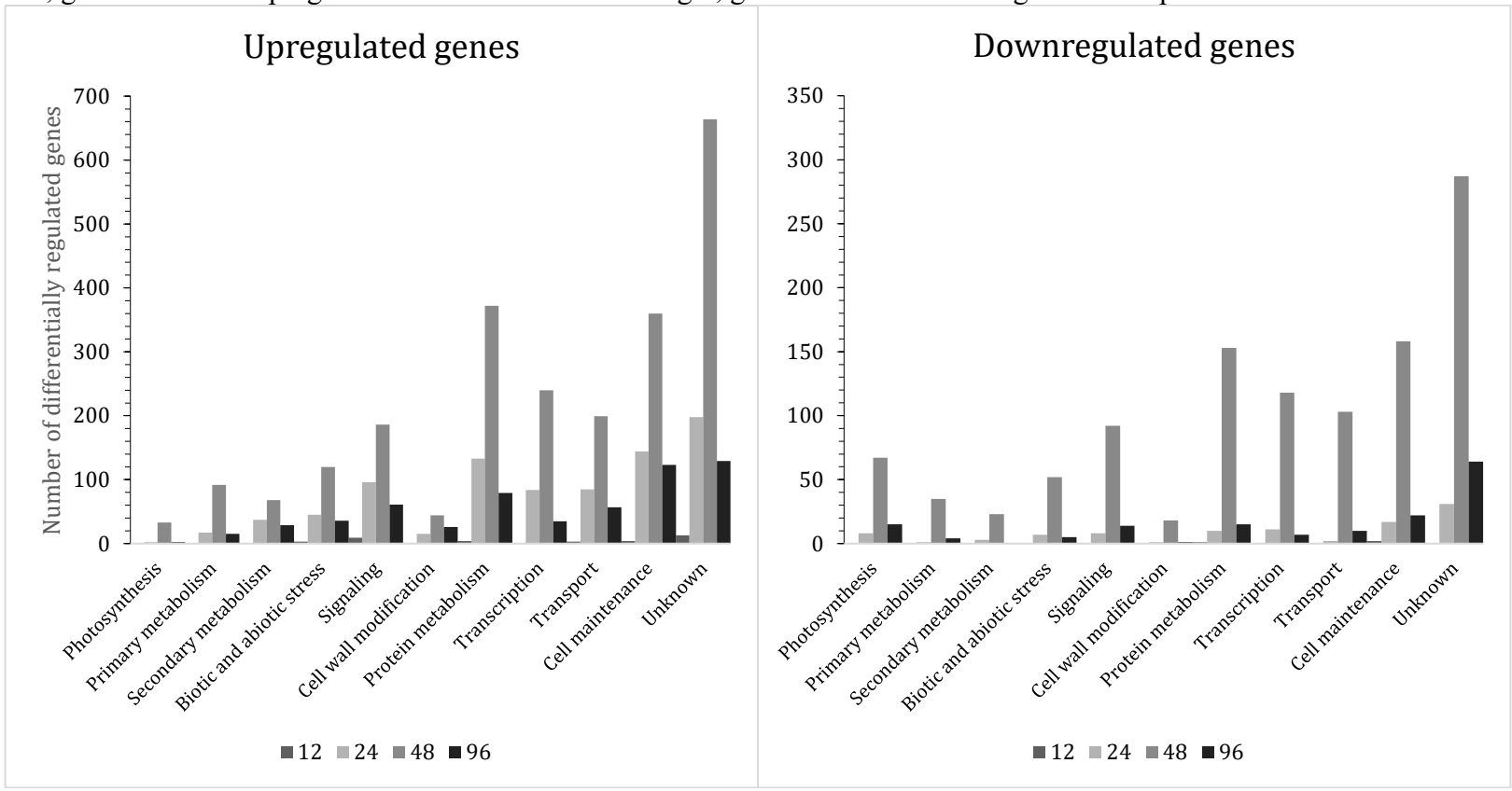


Figure 2.7. Heatmap of genes induced by continuous feeding, with hierarchical clusters showing similarity of genes (rows) and time points (columns). (a) All 3,790 differentially expressed genes detected throughout the study. (b) Expression of 94 genes putatively assigned to biosynthesis of phytohormones, including jasmonic acid, salicylic acid, ethylene, abscisic acid, and auxin. (c) Expression of 17 terpene synthases. Relative to controls, red cells indicate increased expression of genes, and green indicates suppression.

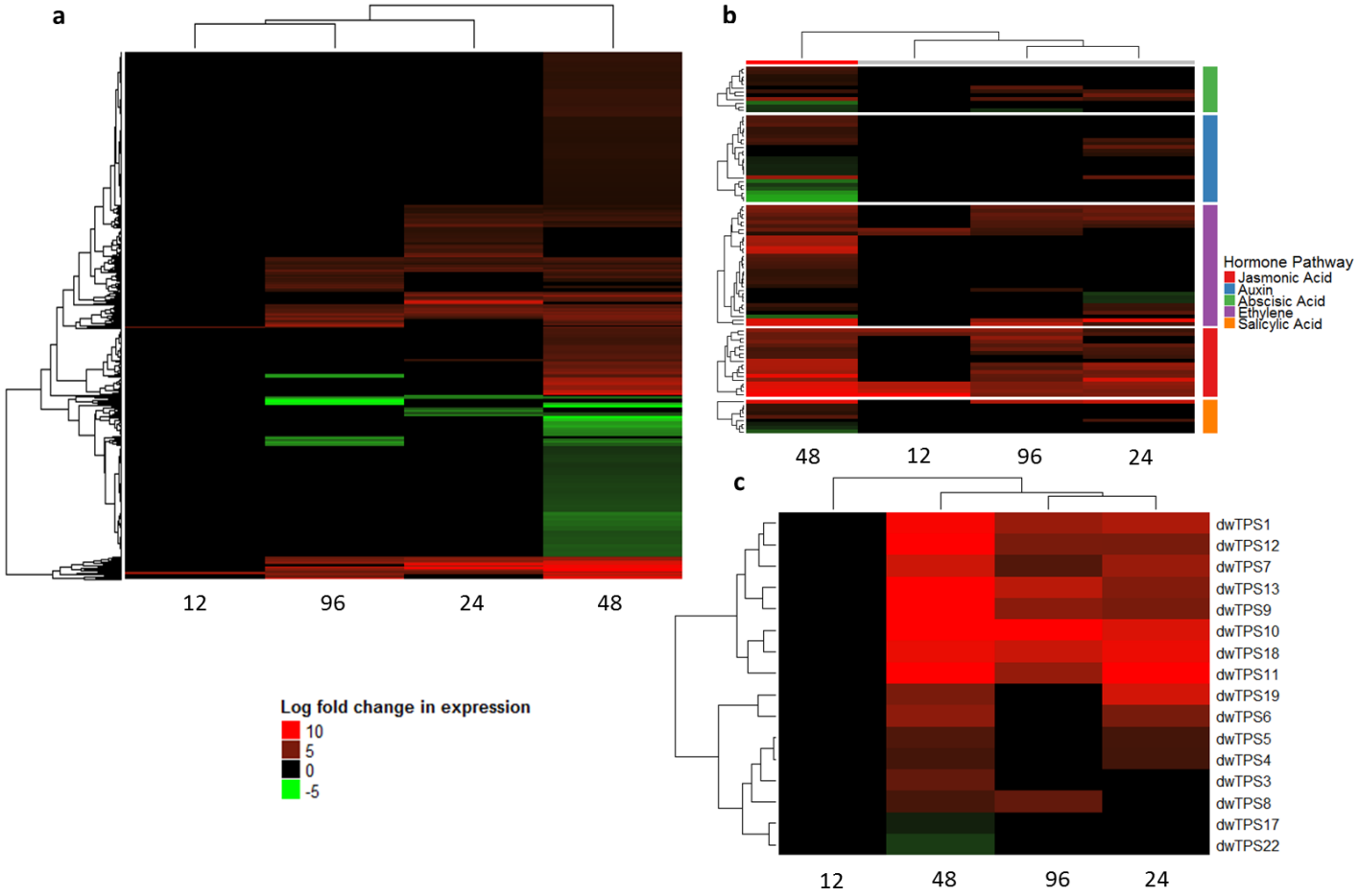
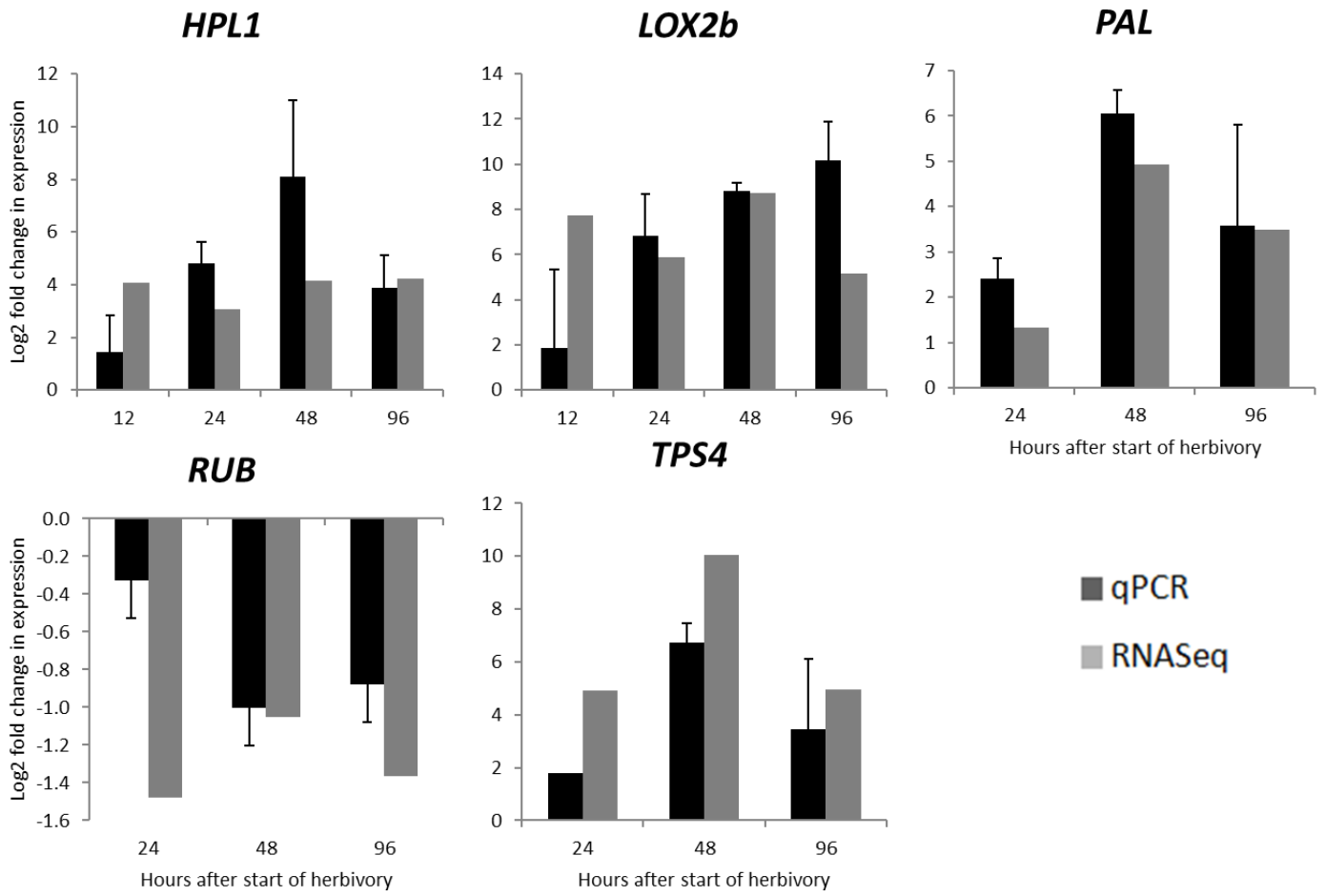


Figure 2.8. Comparison of gene expression patterns at various time points using quantitative PCR (qPCR) and RNA-Seq for plants continuously damaged by *Lema daturaphila*. Dark bars represent mean (±SE) qPCR analyses. Light bars show RNA-Seq results. Some qPCR values reflect fewer than three biological replicates (see text for details).



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Chapter Three: Production of Herbivore-Induced Volatile Emissions Is Constrained Seasonally in the Field but Induced Transcriptional Changes are Highly Variable

Introduction

The challenges that plants face change as they progress from juvenility to maturity, as do the traits that they have evolved to mitigate fitness loss due to external threats. Plants possess myriad chemical traits that reduce the effects of herbivory by arthropods. The manner in which these traits are deployed, however, often changes as plants age. Using meta-analyses, Barton and Koricheva (2010) found that, in forbs, resistance typically switches from inducible to constitutive expression in mature plants. This change often occurs in spite of the continued presence of herbivores, and affects a wide range of traits (Boege and Marquis 2005).

Resistance traits employed by plants can be categorized as either direct or indirect. Direct resistance traits are those that deter, poison, or inhibit the feeding of herbivores (Karban and Baldwin 1997). Although compounds employed to inhibit feeding by herbivores often share common modes of action, the specific nature of the compounds employed often varies between species, with some, for example toxins, showing a strong phylogenetic component (Ehrlich and Raven 1964; Berenbaum 1983). Indirect resistance mechanisms, such as volatile organic compounds (VOCs) and extra-floral nectary (EFN) secretions, may reduce the herbivore load on the plant by increasing the local abundance of carnivorous and parasitoid arthropods, hereafter referred to as "natural enemies" (Kessler and Heil 2011). EFNs accomplish this by offering nutritive subsidies attractive to predatory arthropods that opportunistically feed on herbivores that

they find on the plant. VOCs, on the other hand, seem to have evolved as a within-plant damage signal that increases preparedness in leaves proximal to tissue experiencing an attack (Karban *et al*. 2006; Heil and Silva Bueno 2007; Frost *et al*. 2008). Natural enemies can co-opt herbivore-induced VOCs emitted from damaged tissue as cues that indicate the presence of potential prey or hosts, and consequently increase their searching behavior near the emission source. Although these VOCs have the potential to reduce the abundance of herbivores on a plant (e.g. Kessler and Baldwin 2001), evidence of a corresponding increase in fitness for the emitting plant is still lacking (Kessler and Heil 2011). Therefore, natural enemies may be under selective pressure to learn or innately respond to VOCs (Allison and Hare 2009; Gols *et al.* 2012), but there is no evidence that VOC emissions from plants are necessarily under pressure to convey information to natural enemies.

The ontogenetic decline in inducibility can affect both direct and indirect resistance traits. In young *Nicotiana sylvestris*, inducibility of nicotine, a toxic alkaloid, declines in individual leaves as they age, but also declines in the whole plant as it begins reproduction (Ohnmeiss and Baldwin 2000). Protease inhibitors are ontogenetically constrained in *N. attenuata* and appear to be carefully regulated: unopened flowers contain the highest levels, but quickly decline to low levels once the flowers open. In leaves of the plant, constitutive levels of PIs vary according to source-sink relationships, and inducibility declines once they reach the flowering stage (van Dam *et al* 2001). In tomato, several markers of resistance, including protease inhibitors, polyphenol oxidase, lipoxygenase, and peroxidase, all declined in inducibility as plants approached

reproductive maturity (Stout *et al*. 1996). Field-grown tomato plants sprayed with methyl jasmonate showed a high degree of variability in their response but protease inhibitors and polyphenol oxidases generally declined as plants aged (Thaler *et al*. 1996). Among indirect resistance traits, inducibility of volatile organic compounds has been shown to decline with advanced ontogeny in *Datura wrightii* Regel (Solanaceae; Hare 2010; Hare and Sun 2011a) and in soybean (Rostás and Eggert 2008). That such disparate resistance traits are similarly affected in different species suggests that this shift is reflective of a constraint common to many plants, or perhaps a shared evolutionary history.

Theories developed to explain intraspecific variation of plant resistance traits may help to explain ontogenetic variation. The trade-off between herbivore defense and the cost of expressing those traits in the absence of herbivores may drive expression toward an "optimal" configuration as the probability of herbivore attack changes over the life of the plant; such an outcome may lead to defense expression that follows the pattern of herbivore incidence (McKey 1974). On the other hand, the relative costs of resistance and the variable impact of herbivory, including effects of different types of attackers, are likely to produce uneven selective pressures on resistance phenotypes throughout the ontogenetic stages of plants (Boege and Marquis 2005). The expression of resistance traits at certain stages may therefore be variable due to weak or diffuse selection on resistance at that stage, or may be the result of selection for trait expression at a different stage of life. Apart from these general theories of the evolution of plant resistance, the role of developmental constraint must be considered. Plants have evolved a limited set of hormones with which to integrate external stimuli and coordinate internal responses

(Pieterse *et al*. 2009), and ontogenetic shifts in resistance may reflect limitations in the ability of plants to regulate multiple pathways simultaneously.

Costs of resistance can be either metabolic allocation costs, in that investment in resistance detracts from other processes such as growth or reproduction, or ecological costs if resistance traits reduce fitness through effects on the plant's biotic or abiotic environment by, for instance, reducing pollinator visitation or attracting additional herbivores (Herms and Mattson 1992; Strauss *et al*. 2002; Kessler and Heil 2011). These resistance traits occur despite the costs because of the selection pressure imposed by herbivores: specifically, the frequency of attack and the reduction of plant fitness imposed by herbivory in the absence of these traits. Within an individual plant, organs may vary with regard to their vulnerability to attack, the cost of defending them, and their contribution to plant fitness, and these variations may further depend on the developmental state of the organ. Together, these axioms comprise the optimal defense theory (ODT), developed as a framework for predicting the investment in defenses across tissues and ontogeny (McKey 1974).

Inducible resistance traits may reduce costs when herbivores are absent, although the latency of resistance manifestation may leave tissue vulnerable (Karban and Myers 1989; Zangerl 2003; Walters and Heil 2007). A common hypothesis used to explain the ontogenetic decline in inducibility centers around the shift in relatively greater cost of producing resistance traits in young plants (Barton and Koricheva 2010). Juvenile plants typically lack the significant energy reserves available to older plants, and those that unnecessarily invest in resistance may suffer compounding reductions in growth that lead

to decreased fitness. Costs are only saved, however, when herbivory is variable (Zangerl 2003), suggesting that the preponderance of inducible traits early in the season is consonant with the prediction that herbivory may be less predictable in young, less apparent plants (Feeny 1976).

Declines in inducibility in older plants may not necessarily reflect an increased investment in constitutive resistance traits. Although increases in resistance occur over the season in some species, others may increasingly rely on tolerance of herbivory rather than, or in addition to, resistance (Boege and Marquis 2005; Boege *et al*. 2007). Herbivore tolerance is defined as the ability of plants to maintain fitness despite tissue loss (Stowe *et al*. 2000). While some species appear to actively sequester resources upon damage to aid in compensatory regrowth (Orians *et al*. 2011), costs incurred by tolerant plants in the absence of herbivory are evidence of ongoing differences in growth and resource allocation throughout the life of the plant (Stowe *et al*. 2000). Additionally, plants that have accumulated resources sufficient for reproduction may gain no fitness benefit from investing in resistance in leaves that are attacked. The loss of these leaves may elicit no response, and incur no fitness loss to the plant. Therefore, although tolerance may involve an active response on the part of the plant, it may also be a passive process depending on the potential contribution of the damaged tissue to the fitness of the plant.

Induced responses to herbivory are coordinated by complex networks of signaling molecules. The response to biotic attack is primarily mediated by the jasmonic acid (JA) and modulated by ethylene (ET) (Adie *et al*. 2007; Pieterse *et al*. 2009). Jasmonic acid is

produced from linoleic acid precursors through the octadecanoid pathway, and concentrations of this hormone increase substantially after attack by chewing herbivores and necrotrophic pathogens (Staswick 2008). ET is incapable of eliciting a resistance response on its own, but significantly modifies the JA response (Adie *et al*. 2007; Onkokesung *et al*. 2010). Salicylic acid (SA), a phenolic compound derived from the shikimic pathway, is a negative regulator of JA (Pieterse *et al*. 2009). SA is commonly induced by biotrophic pathogens and phloem-feeding herbivores, although this latter case may involve adaptive suppression of effectual JA-mediated resistance by the herbivore (e.g. Zarate *et al*. 2007)

Although JA regulates the response to herbivory, this pathway is also involved in the regulation of a wide range of functions, including photosynthesis, senescence, and reproduction (Balmer and Mauch-Mani 2012). ET plays a major role in fruit ripening and senescence, germination, cell elongation, and various other functions (Chae and Kieber 2005). SA is similarly involved in several functions related to development and physiology, including seed germination, seedling establishment, respiration, stomatal closure, and reproduction (Vlot *et al*. 2009).

While JA, SA, and ET are principal regulators of resistance expression that have additional "housekeeping" functions, several other hormones that are well-known for their regulation of growth and physiological processes have been found to influence induced resistance as well. Abscisic acid (ABA), well-studied for its role in abiotic stress responses, affects the biosynthesis of JA and can influence downstream expression of JA-responsive transcripts (Bodenhausen and Reymond 2007). ABA's influence over

induced resistance may stem from a prioritization of stress responses; damage by herbivores threatens the physiological integrity of the plant in some of the same ways that abiotic stresses can, and ABA-mediated mitigation of harm caused by, for instance, water loss, may provide a greater benefit to the plant than producing resistance traits. Additionally, levels of auxin (IAA), a major regulator of division and growth of plant cells, decrease locally in both *N. tabacum* (Thornburg and Li 1991) and *N. attenuata* (Onkokesung *et al*. 2010) in response to wounding by herbivores. This relationship may underlie the classic growth-defense trade-off hypothesis (Herms and Mattson 1992), allowing for adaptive prioritization of resources to facilitate responses to environmental stimuli.

In addition to herbivore attack, plants face numerous external threats to which they must adjust their phenotype. Although plant phenotypes are remarkably plastic, they are inherently limited in their ability to respond to multiple stimuli simultaneously due to shared biosynthetic mechanisms, cross-talk between a limited number of hormones, and possible deleterious effects of responses against stresses other than those that elicited the response (Rasmussen *et al*. 2013). The specific traits that are induced following elicitation by herbivores are dependent on inter- and intra-specific variation of the host plant (Degen *et al*. 2004; Schmidt *et al*. 2005; Hare and Walling 2006; Wu *et al*. 2008; Delphia *et al*. 2009; Schuman *et al*. 2009), the species of herbivore (Chung and Felton 2011; Ali and Agrawal 2012), the abiotic environment (Schmidt and Baldwin 2006; Kruidhof *et al*. 2012; Rasmussen *et al*. 2013), and the ontogenetic stage of the plant (van Dam *et al*. 2001; Boege and Marquis 2005; Barton and Koricheva 2010; Hare and Sun

2011a). Because the underlying network of phytohormones govern the induced response, it is expected to vary based on the hormonal context in which the response is elicited. In other words, the specific response induced by chewing herbivores, mediated primarily by JA and ET, may depend on physiological and developmental processes as well as biotic and abiotic stimuli if those phenomena affect concentrations of hormones that impact the biosynthesis and signaling of JA and ET.

Although many stresses tend to vary seasonally, ontogeny affects all plants that successfully reproduce, and all plant responses to biotic and abiotic stresses necessarily occur within an ontogenetic context. This persistent influence of ontogeny and the pervasiveness of its effects suggest that the mechanisms responsible for associated changes in inducibility are likely to be well-established among natural populations. This reliability also creates a repeatable treatment regime with which to examine the interaction between the induced herbivore response and developmental processes that may compete for resource allocation and hormone signaling.

In the *D. wrightii* system, VOC emissions increase substantially after herbivore attack during vegetative growth stages, but are no longer inducible once flowering has begun (Hare 2010; Hare and Sun 2011a). Inducibility of *D. wrightii* can be partially restored by cutting the plant back to the root crown and allowing it to resprout; in this case, regrowth was accompanied by other markers of juvenility, supporting the assertion that ontogenetic change drove the shift in traits (Hare 2010). In *N. attenuata*, flowering attenuates the increases of JA and ET normally associated with herbivory, but the potential for these hormone bursts returns quickly after removal of the flower (Diezel *et*

al. 2011). Although hypotheses regarding the causal mechanisms can be extrapolated from these two studies, the nature and extent of the effects on the induced pathway reconfiguration are largely unknown. Because underlying transcriptional networks are ultimately responsible for generating plant phenotypes, studies of transcriptomics are useful for conducting large-scale surveys of induced changes to plant pathways. The pleiotropic nature of many regulatory elements dictates that traits may often be related in unpredictable ways, such as the strong correlation between JA and only three out of twenty induced VOCs in *N. attenuata* (Schuman *et al*. 2009). Selection on any one trait governed by a regulatory element has the potential to indirectly apply selective pressure on every other trait governed by that same element (Doebley and Lukens, 1998). Therefore, determining how regulatory elements influence phenotypic variation in traits is necessary to understand how plants integrate responses to multiple stimuli, and the potential for selection to influence those responses (Anderson and Mitchell-Olds 2011).

With the *D. wrightii* Regel (Solanaceae) system, it is possible to study the ontogenetic variation in induced traits in an undomesticated system, using herbivores with which the plant has naturally evolved. The scarcity of reference transcriptomes for non-model organisms make *D. wrightii* one of the few systems in which molecular tools can be used to measure induced responses in their evolutionary context. The perennial life history of this species, coupled with prior studies demonstrating the return of inducible VOCs in plants cut back to the root crown (Hare 2010), provide a unique opportunity to study the effects of ontogeny and multiple growth seasons on the transcriptomic regulation of inducible traits.

Within southern California populations, *D. wrightii* exhibits two leaf trichome phenotypes, and aspects of the ecology and genetics of the trichome dimorphism have been described elsewhere (van Dam and Hare 1998; van Dam et al. 1999). The "velvety" phenotype is densely covered by short, non-glandular trichomes, whereas the "sticky" phenotype is less densely covered with glandular trichomes that secrete esters of glucose and aliphatic acids. The trichome phenotype is governed by a single locus and is inherited in a Mendelian fashion; the allele for the sticky phenotype is dominant over that of the velvety phenotype (van Dam et al. 1999).

The herbivore community that attacks *D. wrightii* consists of approximately 5-10 species depending on location and season (Elle and Hare 2000). *Lema daturaphila*, the most damaging herbivore, is a multivoltine chrysomelid that feeds on both sticky and velvety morphs of *D. wrightii* throughout the growing season as both larva and adult. Damage by *L. daturaphila* increases emission of up to 20 compounds. The blend is composed of GLVs, mono- and sesquiterpenes, with (*E*)-β-Caryophyllene, (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, (E,E)-4,8,12-trimethyl-1,3,7,11- tridecatetraene (hereafter TMTT), (*E*)-4,8-dimethyl-1,3,7- nonatriene (hereafter DMNT), and β-selinene typically comprising the bulk of the emitted compounds (Hare 2010; Hare and Sun 2011a; Hare and Sun 2011b). Among several genetic lines of *D. wrightii* assayed, VOC production increases 3.9 to 16.2 times after attack, and the relative abundance of individual compounds varies, with the most abundant compound, (*E*)-β-Caryophyllene, comprising 17 to 59% of the blend. This variation has been shown to be heritable and the trichome phenotype has no effect on volatile production (Hare 2007).

Under laboratory conditions, *D. wrightii* responds to 24 hours of feeding by *L. daturaphila* by releasing a blend of VOCs that reach peak total emission by two days after the start of feeding (Hare and Sun 2011b). Consistent with results from other species, the GLV (*Z*)-3-hexenyl acetate reaches peak emission one day after the start of damage, one day earlier than other compounds. (*E*)-β-ocimene, (E,E)-α-farnesene, and DMNT peaked after two days and subsequently declined continuously. Only (*E*)-β-Caryophyllene and TMTT emission persisted longer before declining. Induction due to damage from *M. sexta*, rather than *L. daturaphila*, led to a smaller quantity of emission, but had no significant qualitative effect on the blend (Hare and Sun 2011b).

When grown in the field, *D. wrightii* plants are highly inducible during the vegetative growth stages early in the growing season, but plants that have reached the reproductive stage cease to emit volatiles in response to herbivory (Hare 2010). When present, the quantity of VOC emission is positively correlated with predation by *Geocoris pallens*, the dominant predator in this system and a generalist that feeds on eggs and young larvae of many insects (Hare and Sun 2011a). The efficacy of VOCs in attracting *G. pallens* is independent of genotypic differences in the composition of blends, although predation is higher on velvety plants.

The goal of the present study is to characterize ontogenetic changes in pathways associated the induced response of *D. wrightii* over the course of two growing seasons. We accomplish this by measuring the expression levels of genes across the transcriptome, volatile emissions, and the growth and survival of herbivore larvae. Specifically, we asked the following questions: (1) How does herbivore induced gene expression vary

across a single growing season? (2) How do patterns of gene expression vary between years? (3) Based on prior knowledge of transcriptional mechanisms involved in elicitation and biosynthesis of induced resistance traits, how reliably can subsets of genes be used to predict ontogenetic decline in induced VOC emissions? (4) Finally, how do changes in inducibility affect herbivore performance?

Methods

Overview

To characterize ontogenetic changes in expression, all plants used in this study were germinated and planted simultaneously, but sampled at different times throughout the growing season. In each sampling period, one focal leaf and three leaves forming a terminal group on another branch of each plant were individually damaged by two *L. daturaphila* adults. Control plants were left undamaged. At each time period, three types of data were collected. VOCs were collected from individual plants immediately before and after 24 hours of treatment. Following the post-treatment VOC collection, the focal leaves from which VOCs were excised for gene expression analyses. Finally, ten freshly-hatched first instar *L. daturaphila* larvae were transferred to the terminal group of leaves to measure the effect of induced resistance on larval performance.

Plants

Plants used in this study were from the MVV6 line, which has been shown to greatly increase volatile emissions following herbivory (Hare 2007). The MVV6 line

was developed by backcrossing heterozygous sticky progeny to their original velvety pollen parent for five generations. Because the trichome phenotype is controlled by a single gene, crossing heterozygotes with the velvety recessive homozygotes results in an expected 1:1 ratio of sticky to velvety offspring. The method of backcrossing within the line results in sibs that are expected to be 98.4% similar after five generations at loci other than the trichome locus (Hare and Sun 2011a).

Seedlings were allowed to germinate and grow under greenhouse conditions until they had 8-10 leaves and could be transferred to the field. The greenhouse was equipped with high-pressure sodium lamps providing supplemental illumination for 14 hr/d so that midday light intensities averaged 1250±39 μmol m−2 s−1 PAR illumination at plant height. Greenhouse temperatures ranged between 15° and 35°C (Hare and Sun 2011a).

Plants were transplanted to the field on 7 May, 2013. Prior to planting, the field was treated with a pre-emergence herbicide (Trifluralin at the rate of 668 ml/ha) to suppress natural weed growth. Furrows were irrigated for 24 hours directly before and after transplanting to allow plants to establish after normal winter rains had ceased. Plants were irrigated one final time on 14 May, 2013, one week after planting. There was no rainfall during the 2013 growing season, typical of sage scrub in southern California. Plants were arranged in blocks of four individuals. Half the individuals within each block had the sticky phenotype, and half were velvety. Plants within blocks were 0.76 m from their nearest neighbor. Blocks were a minimum of 1.5 m from any other block. In southern California, the prevailing winds are from the west, and control plants were planted at least 3 m west (upwind) of treatment plants in order to prevent any possible

induction or priming due to airborne damage signals (Kruidhof *et al*. 2012). The experimental protocol involved collecting multiple samples from each plant at different time points, however each plant was only subjected to this regime once per season. Because *D. wrightii* is a perennial, the experiment was repeated in the second year after plants had resprouted.

Treatment

Sampling was conducted during four time periods each year for two years. Sampling began on 28 May, 24 June, 1 August, and 10 September during the 2013 growing season, and 29 April, 12 June, 24 July, and 21 August in 2014. Plants were treated with biweekly applications of acephate (Orthene 97, Valent Chemical Co., Walnut Creek, CA, USA 1.2 g/L) in water to prevent natural herbivory. Acephate treatment was withheld on plants for two weeks prior to sampling to allow herbivores to feed and resumed afterwards.

Young, fully-expanded leaves were chosen on each of 32 plants at the start of each sampling period. Two adult *L. daturaphila* were caged on focal leaves of the treatment group using mesh bags. Control plants received empty mesh bags. Additionally, these treatments were replicated on another terminal branch of each plant; a leaf of similar age to the initial focal leaf, as well as two adjacent, fully-expanded leaves each received individual cages, with the damage or control treatment matching that of the focal leaf. Insects were caged on leaves for 22.5 hours and then cages and insects were removed. *L. daturaphila* used in this study were reared on greenhouse-grown *D. wrightii*

in an insectary; this stock population was periodically supplemented with field-collected individuals to minimize effects of inbreeding.

Volatile collection and analysis

VOCs were collected non-destructively from all focal leaves immediately prior to treatment and one day later after treatment was terminated, using a collection system described previously (Hare 2010). Briefly, aeration chambers were constructed from polyester cooking bags (unprinted 45 x 55 cm, Terinex, Bedford, England), placed over individual leaves, and secured using a twist tie. Intake and exhaust ports were created in the chambers using gastight fittings (Swagelok, San Diego, CA, USA). Two 12-V portable air pumps (Model # MOA- P125-JH, Gast Manufacturing, Benton Harbor, MI, USA) powered by a 12-V deep-cycle marine battery, created a "push-pull" airflow scheme regulated by flow meters (Aalborg, Orangeburg, NY, USA) to ensure a constant rate of 1.0 L/min for each leaf. Air was filtered through activated charcoal and flowed to the chamber through PTFE tubing (4.6-mm O.D.). Air flowing out of the chamber passed through a glass trap containing Super-Q (25 mg, Alltech, State College, PA, USA), an adsorbent used to collect volatiles, and subsequently through PVC tubing. VOCs were collected from 16 plants concurrently, with two 90-minute collections per day occurring from 9-10:30 and 11:30-13:00. Plants included in each collection were divided equally between control and treatment groups. At the end of each collection, traps were wrapped in aluminum foil, returned to the laboratory, and frozen at -20° C for extraction later that day.

Volatiles were eluted from the traps with 150 µl of CH_2Cl_2 containing 4 ng/µl of n-bromoheptane (Sigma-Aldrich) as an internal standard into autosampler vials with 250 µl glass inserts, and vials were sealed with crimp caps and PTFE-lined rubber septa. Samples were analyzed by gas-liquid chromatography as described in Hare and Sun (2011b). Peaks were quantified in units of ng g^{-1} leaf (dry wt.) hr^{-1} using Agilent ChemStation® software based on comparison of the peak height of each VOC component with that of the internal standard.

Gene expression

Gene expression was measured in the leaves 24 hours after the initiation of damage. Immediately following collection of the 24-hour VOC sample, focal leaves were excised and flash frozen in liquid nitrogen. To accommodate the requirements for robust expression analyses, leaf samples from all four plants in each block were pooled to form one biological replicate, leaving four replicates per treatment level.

RNA was extracted from leaf tissue of pooled replicates using Qiagen RNEasy Plant Mini Kit following manufacturer instructions. RNA concentrations were confirmed by NanoDrop spectrophotometer prior to final concentration and integrity check using Agilent 2100 Bioanalyzer. RNA-Seq libraries were prepared using NEBNext Ultra RNA Library Prep Kit for Illumina according to manufacturer recommendations. Six individual samples were multiplexed per lane using compatible index primers and sequenced using an Illumina HiSeq 2000 sequencing system using single-end 50-bp reads.

After trimming the index primers from each read, sequencing data was aligned against the *D. wrightii* reference transcriptome (described in the previous chapter) with Mosaik, using a maximum mismatch threshold of four (Lee *et al* 2014). The edgeR package in R was used to compare gene expression in the treatment and control groups using the exact test for the negative binomial distribution (Robinson and Smyth 2007; Robinson and Smyth, 2008; Robinson *et al*. 2010; McCarthy *et al*. 2012; Zhou *et al* 2014). Gene expression greater than $\pm$2 log fold changes compared to controls were considered differentially expressed using a false discovery rate of <0.05 (Reymond *et al* 2004).

RNA-Seq data was validated using quantitative PCR (qPCR) for the following genes, identified by their sequence similarity to annotated tomato genes: *hydroperoxide lyase 1* (*HPL1*), *phenylalanine ammonia lyase* (*PAL*), *terpene synthase 12* (*DwTPS12*), *lipoxygenase 2* (*LOX2*), and *RuBPCase small chain 2B* (*RUB*), with *actin* (*ACT*) and *ubiquitin* (*UBI*) chosen as reference genes. Primers were designed using Primer3 (Koressaar *et al.*, 2007; Untergasser *et al.*, 2012) and assayed for efficiency in triplicate with cDNA templates diluted over five orders of magnitude (Table 3.1). Melting curves for each primer used in this study produced a single, sharp peak.

To create cDNA, total RNA extracted during the procedure described above was first treated with RQ1 DNase (Promega, Chicago, IL, USA) to remove any remaining DNA. DNase was inactivated and cDNA was synthesized using oligo(dT) primer (Promega), SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), dNTPs (Sigma Aldrich, St. Louis, MO, USA), and RNase inhibitor (Promega). qPCR

was performed in triplicate using SYBR Green master mix (Bio-Rad, Los Angeles, CA, USA) and 96-well optical plates (Bio-Rad) in a Bio-Rad iQ5 Real-Time Detection System, with PCR conditions as follows: denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 60 s, and a melting curve that showed each experimental sample yielded a single, sharp peak at the amplicon's melting point. Expression values were calculated using $2^{-\Delta\Delta Ct}$ to find the log fold change in expression in the treatment group(Pfaffl 2001).

Bioassay

A feeding bioassay was used to determine the effect of seasonal changes in induced resistance on larval performance, as measured by mass at eclosion and survival to adulthood. This portion of the study involved the three leaves on each plant treated identically to the focal leaves. *L. daturaphila* eggs masses were collected from the insectary and transferred to undamaged *D. wrightii* plants maintained in isolation. Eggs were refrigerated at 4°C as needed to delay hatching and ensure that a sufficient number were available at the start of the bioassay. Preliminary work showed that this has no effect on the growth or survival of the larvae. Only first-instar larvae that hatched the morning of the start of the bioassay were used; this timing is easily discernible because larvae produce reliable amounts of damage on host leaves.

Larvae were transferred to the field by excising the leaves on which they hatched and transporting them in petri dishes within a chilled cooler. Aggregations of larvae were pared down to approximately ten per leaf section by removing excess larvae or dividing

leaf tissue with a razor blade. Larvae used in the study were never moved or manipulated directly, as doing so might cause injury. Leaf sections containing aggregations were affixed to the underside of the terminal leaf, located centrally among the three included in each treatment, and held in place using a rubber washer affixed to a spring hair clip. The cluster of three leaves was enclosed in a larger mesh bag to prevent natural predation but allow larvae to move freely between the three leaves. Because young larvae are particularly vulnerable, mortality occurred after handling and exposure to direct sunlight during the afternoon, despite efforts to minimize both. For this reason, larvae were recounted the following morning and plants with egregious mortality were removed from the study. Larvae had all moved to the leaf by this time, so leaf clips were removed.

All larvae were collected and transferred to the laboratory when approximately half of the larvae in the bioassay had reached the 4^{th} and final instar. Larvae from each plant were housed in ventilated plastic containers containing a live branch taken from that same plant, allowing them to continue feeding as needed. When appropriate conditions are reached, *L. daturaphila* larvae drop to the ground and encase themselves in soil litter to pupate. To facilitate collection of pupae, containers were lined with loose sand that could be safely sifted. Pupae were transferred to smaller containers, absent food sources, where they were monitored daily for emergence, at which time they were weighed.

Statistics

VOC emissions rates were calculated based on the weight of the leaves after they were flash frozen. Dry leaf weight was determined by dividing frozen tissue weight by

3.079, a figure derived by comparing the proportional difference in weight loss when leaves are oven-dried or flash frozen in liquid nitrogen. VOC emissions were $\log_{10}(x+1)$ transformed to ensure normality of errors and to accommodate samples for which no volatiles were detected. Total VOC emissions were analyzed using a mixed model analysis of variance (ANOVA) in JMP Pro 11, with treatment, sampling period, and their interaction as fixed effects; block and plant nested within block were random effects. Decomposition of the interaction between treatment and sampling period allowed us to determine at which time points treatment caused significant differences in VOC emission. VOC data collected prior to treatment was analyzed separately from post-treatment data to ensure that plants were uninduced and no bias existed in the groups; these data were not included in the final analysis of post-treatment data.

Principal components analyses (PCAs) were used to explore the relationship between gene expression and VOC emission. Gene expression and VOC emission data have important structural differences that make comparative analyses difficult. Although the samples originated from the same set of plants, the number of effective biological replicates differed. Rigorous analyses of gene expression require pooling of tissue from multiple plants to form a single biological replicate; therefore, although VOC emissions were collected from each plant individually for a maximum of 32 replicates in each sampling period, gene expression produced a single value for each gene at each sampling period. Furthermore, unlike VOC emissions data, gene expression is a relative value based on the difference between the treatment and control sample, and all control samples are effectively assigned a value of zero. To accommodate these differences, VOC

emission data were transformed into a relative value: for each individual compound, the average emission of control plants in each sampling period was subtracted from the average of the treatment group, producing a single value. Both relative VOC emissions data and specific gene groups were separately subjected to PCAs, creating fewer, uncorrelated variables based on the correlation matrix of individual compounds and genes, respectively. The factor loadings show the contribution of each original variable to the new component, and factor scores for the new components are calculated for the original observations. In order to assess the contribution of subsets of genes with presumed roles in induced plant resistance, PCAs were conducted on terpene synthase genes and various combinations of hormone biosynthesis genes, including jasmonic acid; jasmonic acid and ethylene; jasmonic acid, ethylene, abscisic acid, and salicylic acid; and, finally, genes related to biosynthesis of all phytohormone genes. Principal components (PCs) derived from these analyses were then regressed against both the total induced emissions, as well as PCs of the VOC emissions.

For the bioassay, survival was calculated as a proportion on a per-plant basis to account for small differences in the number of initial larvae. Survival and mass at eclosion of larvae in the bioassay were analyzed separately using mixed model ANOVAs. Fixed effects included treatment, sampling period, and their interaction; random effects were plant nested within sampling period.

Results

Volatile emissions

Fifteen unique compounds were detected across both years of the study. Quantitatively, the greatest emission rates were from limonene, linalool, and methyl salicylate, in that order, followed by intermediate levels of *trans*-2-hexenal, hexenyl acetate, DMNT, and *cis*-3-hexen-1-ol. Lesser quantities of nerolidol, (*E*)-β-caryophyllene, (*E*)-β-ocimene, α-farnesene, β-selenine, geranyl acetone, *trans*-2-hexen-1-ol, and (E,E)-4,8,12-trimethyl-1,3,7,11- tridecatetraene were also detected. Pre-treatment VOC collections varied by sampling period ($F_{7,217}$=3.71, p=0.0008), but no difference was found between treatment groups ($F_{1,217}$=2.21, p=0.1387), and the interaction was insignificant ($F_{7,217}$=1.16, p=0.32). The random effect of block did not significantly improve the model and was dropped from the final analysis. With no evidence of bias between plants in the two treatment groups, these data were excluded from further analyses.

Trans-2-hexen-1-ol and TMTT were not detected in the 24-hour sample, leaving only 13 compounds detected in the headspace of post-treatment plants. These collections show significant effects of treatment ($F_{1,208}$=22.94, p<0.0001; Fig. 3.1) and sampling period ($F_{7,208}$=10.21, p<0.0001), as well as a significant interaction between the two effects ($F_{7,208}$=9.83, p<0.0001). The random block effect was not included in this model, either. Decomposing the interaction shows that treatment groups only differed significantly in sampling periods 1 and 2 of the first year ($F_{1,208}$=41.98, p<0.0001 and

$F_{1,208}$=53.58, p<0.0001, respectively), while all other sampling periods were highly non-significant *(*all p>0.77).

Gene expression

In sum, 6,017 unique genes were differentially expressed over the course of the five sampling periods (see supplemental table 2). The first two sampling periods showed widespread changes in gene expression; 2,517 genes were upregulated and 767 downregulated in the first period, while 1,922 were upregulated and 628 downregulated in the second period (Fig. 3.2). In contrast, only 75 and 19 were upregulated and downregulated, respectively, in the final sampling period of the first year. The second year of the study showed a somewhat dissimilar trend; although VOCs were not inducible at any time point in the second year, samples from the earliest time point showed 468 genes with increased expression and 687 that were suppressed. Plants from the final time point had a similar number of DEGs to inducible samples from the first year: 2,087 genes were induced and 602 were suppressed.

There was, however, little overlap between expression in inducible samples (Fig. 3.3). Although the early sampling periods from the first year had similar numbers of DEGs overall, only 1,298 genes were induced in both samples, whereas 3,141 were uniquely upregulated in one sample or the other. Similarly, for genes suppressed in these samples, only 378 showed reduced activity in both, limiting suppression of 1,017 genes to one sample or the other. Despite these widespread differences, no genes showed opposing expression patterns between the two samples.

Comparing the first sampling period of the first year and the final sampling period of the second year produces similar results. Only 737 genes were commonly induced, and only 209 were suppressed in both samples, leaving 3,867 and 1,160 genes uniquely induced and suppressed, respectively. Among these samples, however, 126 genes were induced in one sample and suppressed in the other.

DEGs were separated into functional categorizations based on assigned pathways identified using MAPMAN (Thimm *et al*. 2004). This analysis shows that most induced changes in expression were involved in cellular maintenance, transcription, protein modification, and signaling (Fig. 3.4, top). Predictably, periods 1,2, and 8 all had far more genes assigned to each category than samples 4 and 5, simply due to the overwhelming number of DEGs in those samples overall. Additionally, there is a remarkable consistency in the proportion of genes in each category across samples. The only minor exception to this pattern is a relatively higher proportion of induced stress-related genes in period 5, although the number of DEGS was still less than in other samples. Overall, the similar proportions of DEGs across samples is surprising, given the number of unique genes detected in each sample. In other words, different sets of genes were induced in plants from each sampling period, for the most part, but the proportion of those genes involved in various functions changed very little.

Suppression of genes was somewhat more consistent across categories, although cell maintenance was the largest category by far (Fig. 3.4, bottom). Several notable differences stand out compared to the breakdown of induced genes. Photosynthesis-related genes were heavily suppressed in samples 1, 2, and 8, whereas very few were

induced in any samples. Although sampling period 8 showed consistent parity with periods 1 and 2 across most categories, the number of suppressed genes related to stress was roughly twofold higher in period 8. Only sampling period 5 showed a greater number of genes that were suppressed rather than induced; the number of suppressed genes from this sample was roughly equivalent in most categories to the genes suppressed in periods 1, 2, and 8. In several categories, cell maintenance, signaling, transport, primary metabolism, and cell wall modification, period 5 showed more suppressed genes than any other period. Notably, however, period 5 suppressed far fewer genes related to photosynthesis than other time points.

Of particular interest are the terpene synthases, a subset of genes involved in secondary metabolism. Six *TPS* genes expressed in this study were not identified as inducible in *D. wrightii* in the previous chapter of this dissertation (Table 3.2). The predicted amino acid sequences coded by three of these genes appear to be severely truncated, with sequences less than half the length of their tomato homologs. The other three unique to this study putatively code for a undecaprenyl pyrophosphate synthase, a triterpene sterol synthase, and an α-humulene/(-)-(*E*)-β-caryophyllene synthase, each with less than 4% of their sequences non-synonymously substituted compared to tomato homologs.

A cluster of eight terpene synthase genes were induced in periods 1, 2, and 8, the three samples that showed widespread changes in gene expression (Fig. 3.5), putatively coding for synthases of β-ocimene, (E E)-alpha-farnesene, limonene, nerolidol, linalool, and one unknown terpene synthase. The remaining nine genes showed variable

expression, with none represented in more than two samples. The only terpene synthase downregulated during this study, the truncated α-humulene/(-)-(*E*)-β-caryophyllene synthase, was suppressed in periods 1 and 2. No terpene synthase genes were differentially regulated at the end of the first year in period 4, and only one, an β-ocimene synthase, was induced in period 5.

The pattern of phytohormone expression is similar to that of terpene synthases and genes at large: although the overall numbers of up- and down-regulated genes was similar in periods 1, 2, and 8, only a small number of those genes were commonly regulated across time points (Fig. 3.6). Period 4 had very little phytohormone gene activity, while period 5 showed extensive suppression of genes related to most hormones. The jasmonic acid and ethylene pathways were among the largest phytohormone groups represented. In periods 1, 2, 4, and 8, these gene groups were predominately induced; in period 5, no JA genes were induced while four were suppressed, and the twice as many ethylene genes were suppressed as induced. Auxin-related genes were also well-represented, with periods 1, 2, and 5 showing significant suppression in addition to induction, and period 8 favoring only induction. Thirteen SA-regulated genes were induced in period 8, roughly twice the number found in periods 1 and 2.

Gene expression is calculated based on the relative expression of two experimental groups, rather than absolute values, and changes across sampling periods could potentially be explained by changes in baseline expression values of genes in control plants. To explore this possibility, two comparisons were made: control samples from periods 1 and 4, as well as 4 and 8. Period 1 control samples showed higher

expression in 1126 genes, while period 4 had higher baseline expression of 1917 genes (Table 3.3). These differences were most pronounced in genes related to cell wall modification, signaling, transcription and transport, all of which showed at least 1.6 times the number of genes with higher expression later in the season. The only groups with more genes expressed at a higher level in period 1 were photosynthesis and secondary metabolism. Two *TPS* genes showed greater expression in period 1, and none were expressed more highly in period 4, eliminating the possibility that the lack of *TPS* expression in period 4 was due to higher constitutive expression. Phytohormone biosynthesis genes show some differences, although no clear biases are apparent. Six SA biosynthesis and signaling genes were expressed at greater levels later in the season, while only one was higher in period 1. JA biosynthesis and signaling genes were split evenly between the two periods. Auxin- and ethylene signaling genes showed the greatest number of differences, perhaps due to their changing physiological roles.

To further test the hypothesis that lower constitutive expression of controls potentiated elevated expression of induced genes, genes that were induced or suppressed in periods 1 and 4 were compared against the list of genes that showed the opposite expression pattern for that sampling period in the control comparison. Very few genes met these criteria. Of the 2,517 genes that were induced in period 1, only 60 had lower expression of controls compared to period 4, and only 45 out of 767 suppressed genes in period 1 had higher expression of control genes compared to period 4. Conversely, 44 out of the 75 induced genes in period 4 had lower levels of expression among controls, representing a relatively large proportion of the total induced genes for that period, and

two out of 19 suppressed genes showed higher expression in controls. In summary, although higher constitutive expression in period 4 may have masked the higher expression of a small number of genes, this phenomenon is insufficient to account for the lack of gene expression in that sample overall, including terpene synthase expression. On the contrary, changes in controls may have led to an increase in the number of induced genes in period 4.

Sampling periods 4 and 8 occurred late in the season in the first and second years of the study, respectively. These two periods showed extreme differences in the number of induced genes, with period 8 differentially expressing 2,689 genes compared to only 94 in period 4, however the comparison of expression in undamaged samples shows this is not due to differences in baseline expression levels in either sample (Table 3.4). Overall, 1,363 genes were expressed at higher levels among control plants in period 4, and 1,569 in period 8. Most notably, more photosynthesis and cell wall modification genes were expressed at greater levels in period 4, while period 8 had greater expression of more genes related to protein metabolism, stress responses, transcription, and signaling. Among signaling genes, however, there was no clear bias in expression of hormone biosynthesis genes, with most pathway showing higher expression of a small number of genes split equally between both periods. Secondary metabolism showed twice as many genes expressed highly in period 8, but only one gene was a putative terpene synthase gene; changes in constitutive expression are therefore not sufficient to explain the differences in expression found among this class of gene.

Induced and suppressed genes from periods 4 and 8 were also compared to control genes that differed in baseline expression. Only three genes that were induced in period 4 had lower expression of controls compared to period 8, and no suppressed genes had higher expression in controls. For period 8, 206 induced genes had lower expression in controls, and 255 suppressed genes had higher expression in controls. Overall, these groups represent a small portion of the set of induced genes, and changes in baseline expression do not appear to have driven the large differences in expression between periods 4 and 8.

qPCR

Overall, qPCR techniques confirm the expression levels of genes as measured by RNA-Seq, recording mean expression levels that were, with few exceptions, quantitatively very similar to the RNA-Seq results (Fig. 3.7). *HPL1* expression was elevated in periods 1, 2, 4, and 8 as measured by RNA-Seq. qPCR results were nearly identical in the first three periods measured, and registered a slightly lower expression level in period 8. Expression of *HPL1* decreased slightly in period 5 according to qPCR, and RNA-Seq reported a value below the expression threshold. Expression of *LOX2* was similarly elevated in the first two periods, regardless of method. No amplification was recorded for period 4 qPCR samples, suggesting that, although RNA-Seq recorded an increase in expression, baseline expression may have been very low. For period 8, qPCR disagreed with RNA-Seq, although the qPCR result was based on only one replicate due to lack of amplification. RNA-Seq did not record any expression changes for *PAL* or

RUB above the threshold for differential expression. For all sampling periods, qPCR showed expression that was at or below this threshold for both genes. *TPS* expression matched closely, with no expression changes recorded in periods 1 and 5, and increased expression in periods 2 and 8, although the results for period 8 had a large error term. For period 4, no amplification was detected in the qPCR analysis, and RNA-Seq similarly registered no expression in that sample.

Synthesis

Among the various PCAs tested, PC 1 of the model that includes all phytohormone-related genes has more power to explain mean total induced VOC emissions (R^2=0.865, *p*=0.0221; Fig. 3.8) than components from analyses that included only genes related to the main resistance hormones (JA, SA ETH, and ABA) or terpene synthase genes. PC1 of this full hormone model includes strong factor loadings from genes related to each of the ethylene, brassinosteriod, jasmonic acid, auxin, abscisic acid, salicylic acid, cytokinin, and gibberellin pathways, and represents 38.7% of the variation in hormone-related gene expression (Table 3.5). PC1 of the JA-only model was the next best predictor (R^2=0.709, p=0.0736) followed by PC1 of the terpene synthase model (R^2=0.634, p=0.1071; see Table 3.6 for factor loadings).

The individual compounds that make up the VOC blend were also subjected to a PCA to test the ability of the various gene expression models to predict correlated subsets of the emitted blend. PC1 of the VOC PCA comprises 39.5% of the variation in the data set; *cis*-3-hexen-1-ol, hexenyl acetate, limonene, linalool, methyl salicylate, nerolidol and

α-farnesene all loaded positively on this axis, while *trans*-2-hexenal, (*E*)-β-caryophyllene, geranyl acetone, and β-selenine loaded negatively (Table 3.7). PC2 accounts for 28% of the variation and differentiates samples rich in *trans*-2-hexenal, hexenyl acetate, linalool and DMNT from those with high quantities in limonene, (*E*)-β-caryophyllene, and α-farnesene. The complete hormone model is again the most capable of explaining the variation in VOC emissions; PC1 of the hormone model showed a strong positive correlation with VOC PC1, although the analysis was only marginally significant (R^2=0.756, p=0.0556; Fig. 3.9) and PC3 of the hormone model correlated negatively but non-significantly with VOC PC2 (R^2=0.672, p=0.0892; Fig. 3.10). *TPS* gene expression is a relatively poor predictor of VOC emission: *TPS* PC1 explained less of the variation in VOC PC1 (R^2=0.4, p=0.25), while *TPS* PC3 accounted for a similar proportion as PC3 of the hormone model (R^2=0.678, p=0.0868).

Bioassay

On average, 24.7% of *L. daturaphila* larvae reared on uninduced plants survived to eclosion, while 28.5% survived on induced plants (Fig. 3.11). The effect of sampling period was significant ($F_{6,124}$=6.23, p=0.0388), although neither the main effect of treatment ($F_{1,124}$=0.44, p=0.5098) or the treatment by period interactions were significant (F_{6124}=0.52, p=0.7914). The bioassay results from period 4 were excluded from this analysis because fewer than 5% of the larvae survived, with most of the mortality occurring within the first 24 hours after transfer. Larval mass at eclosion showed a similar pattern: sampling period had a large effect ($F_{6,72}$=9.81, p<0.0001; Fig. 3.12), but

treatment ($F_{1,75}$=1.71, p=0.1944) and the treatment by period interaction ($F_{6,72}$=1.86, p=0.1) were both non-significant. Both models retained the random effect of plant nested within sampling period.

Discussion

The observed response to herbivory confirms a clear pattern of ontogenetic decline in inducibility for VOCs, but a highly variable transcriptomic response. Although the first two sampling periods showed similar inducibility in terms of the quantity of VOCs emitted and number of genes induced and suppressed, the identity of the compounds and genes involved in the response showed major differences. Further, the three sampling periods showing no induced VOC emissions each showed markedly different transcriptional responses. Despite the lack of obvious transcriptional patterns, an exploratory PCA suggests that a composite index of genes putatively involved in phytohormone synthesis and signaling could serve as a reliable indicator of VOC emission quantity.

Herbivore performance was not affected by prior herbivory

Regardless of sampling date and VOC inducibility, survival and mass at eclosion of *L. daturaphila* larvae remained independent of host plant induction status. This result corroborates results from prior greenhouse studies that showed no effect of *D. wrightii* induction status on survival of *L. daturaphila* in the absence of natural enemies (Hare and Sun 2011a). Despite evidence that the chronic, profuse damage inflicted by these

herbivores reduces *D. wrightii* fitness through removal of leaf canopy (Elle *et al* 1999; Elle and Hare 2000), selection has apparently not resulted in an efficacious, contemporary induced resistance response to *L. daturaphila*.

Several foundational studies suggest that qualitative changes in chemical resistance traits drive speciation of plants through enemy release (Ehrlich and Raven 1964; Berenbaum 1983; Berenbaum 2001); effective resistance against adapted herbivores, however, may be determined by other types of traits. Although indirect resistance through natural enemies, mediated by VOCs, increases mortality of eggs and larvae (Kessler and Baldwin 2001; Hare and Sun 2011a), evidence of a corresponding increase in plant fitness is lacking (Hare 2011; Kessler and Heil 2011). Many plants may instead rely on physical traits associated with mature leaves, such as leaf toughness and poor nutritional content, to reduce the palatability of leaves for herbivores (e.g. Feeny 1970). Incidence data suggests that *L. daturaphila* are present on *D. wrightii* throughout the season (Elle and Hare 2000; Hare 2010), however, suggesting that either physical resistance is not enough to inhibit the herbivore population or sufficient numbers of vulnerable leaves are available throughout the season to support the population.

Tolerance represents an effective trait for coping with pervasive damage, especially in cases involving specialist herbivores that circumvent resistance traits (Boege *et al*. 2007). A study involving *D. stramonium* showed that selection by *L. daturaphila* favored tolerance in host plants rather than highly resistant phenotypes, although intermediate resistance was favorable when another herbivore was present (Carmona and Fornoni 2013). Phylogenetic patterns indicate that overall resistance in milkweeds has declined

with speciation, while tolerance has increased (Agrawal and Fishbein 2008). Although the tolerance of *D. wrightii* has not been assessed in the context of herbivory, the large underground storage capacity of its root system allows plants to regrow after being cut back to the root crown (Hare 2010), suggesting this species is capable of extensive compensatory regrowth.

The significant effect of sampling period on larval survival might be interpreted as a change in constitutive resistance on the part of the plant, however it is more likely attributable to heat exposure that occurred during transfer from a chilled cooler to the plants that varied by sampling date. Many of the larvae that were not immediately transferred died, and so it is expected that even those that were transferred quickly may have suffered effects. Such an effect was clearly seen in period 4, when the majority of larvae failed to survive the first 24 hours. Heat-related mortality should have affected induction treatment groups equally, with the caveat that the large environmental effect may have obscured detection of a signal from the induction treatment. On the other hand, a strong effect of sampling period was also evident in the larval mass bioassay. These larvae necessarily survived the transfer, although lingering or ongoing effects of environmental conditions cannot be excluded from explanations of the strong sampling period effect. Regardless, no clear trend of plant ontogeny emerges that might explain differences between periods.

Herbivore-induced VOC emissions decline with plant ontogeny

The periods of inducibility in this study were marked by emissions roughly one-tenth the rate of previous field studies (Hare 2010, Hare and Sun 2011a). Those studies collected VOCs after 48 and 72 hours of treatment, as opposed to the 24-hour induction treatment used here. Accordingly, the present study showed low emission of terpenes, including (*E*)-β-caryophyllene, (*E*)-β-ocimene, and α-humulene, which typically appear later in the time course of induction (Turlings *et al*. 1998), but was richer in *cis*-3-hexen-1-ol, methyl salicylate and nerolidol.

The differences between blends collected in the first year of the study mirror results seen previously: VOCs are highly inducible in juvenile plants, but mature, flowering plants show no response (Hare 2010; Hare and Sun 2011a). Unlike previous studies, however, a return of inducibility was not seen in the spring of the second year. This anomaly is likely the result of study plants' delayed progression to maturity in the first year as they recovered from transplantation to the field. In the second year, despite the first sampling period occurring at an earlier date, the plants were advanced in maturity because they already possessed an established root system and began producing leaves at an earlier date. Therefore, the lack of inducible VOCs in the second year of this study should not be taken as a contradiction of previous work, but rather as an indication of the importance of accounting for plant ontogeny independent of calendar date when measuring resistance traits.

The induced transcriptional response is highly variable

In the present study, gene expression analyses suggest that the response to herbivory at the transcriptional level is more nuanced than VOC emissions would suggest. Despite similar levels of induced VOC emissions, as well as a similar number of DEGs overall, only 29.2% of DEGs in sampling periods 1 and 2 were expressed in common. Conversely, plants that emitted no VOCs in response to herbivory each showed remarkably different expression profiles. Interestingly, regardless of the number and identities of DEGs measured in each sample, the similarity of gene distribution across functional categories was striking. This pattern suggests that herbivory elicits a response from each pathway fairly consistently, yet some aspect of the elicitation modulates the number and identity of the genes that are affected within each pathway. This could indicate a functional redundancy if the affected genes share similar roles, but perhaps more likely indicates the importance of coordination across each aspect of the variable plant phenotype, regardless of the specific outcomes of induction.

The difference in transcriptomic activity responsible for this ontogenetic shift in VOC response appears to be complex, although other factors may have obfuscated the identification of clear patterns. The first year of the study suggests that flowering *D. wrightii* stops responding almost entirely, as indicated by the major reduction in DEGs detected in damaged plants. However, widespread transcriptomic response in periods 5 and 8, as well as the major differences in the genes responding among inducible plants in periods 1 and 2, indicate that herbivore-elicited response is interacting with other regulatory mechanisms to govern the response.

Although the mono- and sesquiterpenes found in the VOC blend are synthesized by terpene synthase enzymes, the results of the present study suggest that the expression of genes producing these enzymes may not function to regulate VOC production, and may be an unreliable marker of induction. The accumulation of *TPS* genes induced in period 8, for instance, appeared similar to that of the highly-induced plants in periods 1 and 2, yet did not result in increased emissions in damaged plants. Alternatively, there may be a delay between the expression of biosynthetic genes and the appearance of their eventual products (Tzin *et al*. 2015). Since this study sampled gene expression and VOC emissions more or less simultaneously, it cannot be ruled out that the *TPS* gene expression detected in this study would better predict emissions at a later time. Considering that induced VOCs have not been detected in *D. wrightii* late in the season (Hare 2010; Hare and Sun 2011a), however, this explanation is not likely to account for the unexpected *TPS* expression in sampling period 8.

A better marker of induction was found to include the many genes involved in synthesis and signaling of phytohormones. The roles of the primary defense-related phytohormones such as JA, SA, and ET are well established in the context of plant resistance (Howe 2004; von Dahl and Baldwin 2007; Wasternack 2007; Thaler *et al*. 2010). Due to the overwhelmingly complex biochemistry involved, studies of the cross-talk between phytohormones has been largely restricted to these three hormones (e.g. Zarate *et al*. 2007; Leon-Reyes *et al*. 2009; Leon-Reyes *et al*. 2010; Onkokesung *et al*. 2010). The mutual antagonism of SA and JA pathways has received extensive attention due to their central role in generating resistance responses following attack (Robert-

Seilenantz *et al*. 2011; Thaler *et al*. 2012). The relationship between these two hormone pathways is thought to have evolved in response to the need to coordinate effective responses; JA-centric responses are generally expected to be more effective against most insect herbivores and necrotrophic pathogens, while SA-based responses are generated in response to biotrophic pathogens and phloem-feeding insects (Thaler *et al*. 2012). Coordination between the two resistance pathways is critical to mounting an effective resistance response because inducing inappropriate responses would likely increase susceptibility to attackers, and inducing both responses simultaneously might decrease the effectiveness of each or at least incur unnecessary metabolic costs. In addition to these two main hormones, ET modulates the response of JA; although ET is not independently sufficient to produce a resistance response, the full resistance response cannot be generated without the influence of ET (Onkokesung *et al*. 2010).

Dampening of induced resistance in response to ontogenetic changes in the plant may involve mechanisms that allow hormones ostensibly regulating growth and reproduction to prevent a response to herbivory. In *N. attenuata*, the levels of ET, JA, and JA-isoleucine, a JA derivative also involved in response elicitation, declined as plants began elongating and flowering, but the high levels seen in young plants could be restored by removing the inflorescences (Diezel *et al*. 2011). The authors of that study hypothesized that auxin, and possibly SA, are responsible for suppression of the damage response due to their involvement in reproduction and antagonistic relationship with JA and ET. The results of the present study are less clear in this regard. Although we did not measure phytohormone levels directly, genes putatively involved in signaling of all

phytohormones loaded both positively and negatively on PC1 of the full phytohormone analysis, and showed a stronger correlation with VOC emissions than analyses including any other combination of hormones. Whether differences in hormone levels existed, as well as the influence of measured genes on those hormone levels, are both unknown. Currently, there is insufficient information about the function of the genes included in the analysis to explain why some are associated with higher VOC quantities and others are not. The methodology used in this study also ignores any hormones or signaling molecules that are transported into the focal leaves, which is suggested as a proximate cause of the response suppression seen in *N. attenuata* (Diezel *et al*. 2011). It's possible that such influences alter the efficacy of particular hormones, such that damage-response signals are still produced but ultimately do not precipitate a phenotypic change.

The increase in predictive power gained by the inclusion of phytohormone genes ostensibly unrelated to resistance should perhaps not be surprising since an increasing number of studies have linked abscisic acid, gibberellin, auxin, cytokinin, and brassinosteroids to the induced response (Pieterse *et al*. 2009; Robert-Seilaniantz *et al*. 2011). Although the specific influence of other phytohormones on the induced response is not well understood at this time, most appear to have inhibitory effects on JA, SA, or ET. For instance, ABA-deficient tomato plants had increased levels of SA-associated resistance, and decreased JA-mediated resistance. Additionally, drought-triggered ABA induction in intact tomato plants reduced the antagonistic effect of SA on JA, thereby facilitating the JA response when plants were subsequently attacked by herbivores (Thaler and Bostock 2004).

The apparent ability of plants to use a range of hormone pathways to regulate the induced response suggests that subsets of genes are independently controlled, at least to some degree. Of the five sampling periods surveyed, four produced widespread changes in gene expression, yet only two released elevated levels of VOCs. This suggests that plants are able to respond to external cues, but that this response is not "all-or-nothing", which suggests that evolution may be able to act on individual components of the response. Some have taken differences in the induced response at the trait level to mean that plants are tailoring their response to the specific attacker (e.g. Turlings *et al*. 1995; de Moraes *et al*. 1998; Dicke 1999; Kessler *et al*. 2010). Several studies explicitly testing this hypothesis in direct resistance traits have failed to find support for it (Agrawal 2000; Chung and Felton 2011; Bode, *et al*. 2013). Because VOC-mediated interactions between plants and natural enemies are information-based, relatively low-cost to the plant, and may not provide a fitness benefit, ostensible specificity in the induced VOC blend may be better explained by associative learning by predators and parasitoids (Allison and Hare 2009; Gols *et al*. 2012)

The involvement of other hormones in the induced response suggests that abiotic factors have a major influence on the transcriptome. The plant response to abiotic conditions, including ultraviolet solar radiation, drought, heat, wind, and nutrient availability, often involves traits that increase herbivore resistance, and the presence of these stressors can impact the induced response to herbivory (Conconi *et al*. 1996; Cipollini 1997; Izaguirre *et al*. 2003; Izaguirre *et al*. 2007; Kuhlmann and Müller 2009; Srinivasan *et al*. 2009; Kruidhof *et al*. 2012; Rasmussen *et al*. 2013). The importance of

environmental context is exemplified by the differences between studies conducted in the laboratory and field (Schmidt and Baldwin 2006; Kant and Baldwin 2007). The results of the current study reinforce previous findings that cross-talk between phytohormones can have a strong influence on the induced response (Robert-Seilaniantz *et al*. 2011; Balmer and Mauch-Mani 2012; Thaler *et al*. 2012). Although this study is not designed to separate the effects of the abiotic environment and plant ontogeny, it is clear that plant ontogeny consistently affects induced VOC emissions while the effects on induced gene expression are highly dependent on the context of attack.

The evolutionary context of ontogenetic variation in resistance

Broadly, the changing response observed over the course of the two-year study can be viewed through the framework of the ODT. The ODT predicts that plants should have evolved to allocate defenses to tissues only when the loss of those tissues would cause a greater decrease in fitness than the cost of the defenses, and that such an investment is further dependent on the probability of attack. This suggests that leaves should only be defended so long as they are required to support reproduction, presumably through resource acquisition. Some species, like *Datura*, have distinct vegetative and reproductive stages. Early in the season, these plants focus on vegetative growth, building up a significant reserve of stored sugars in an extensive root system. Later in the season, vegetative growth slows while plants invest in hundreds of large fruits. At this point, the ODT predicts that plants will invest less in protecting leaves, since the loss of photosynthetic tissue may not impede production of reproductive tissue. Additionally,

late stage investment in defensive compounds might have allocation costs as well as ecological costs if such compounds affect pollination, dispersal, or act as feeding stimulants to florivores or frugivores.

This study supports previous work showing that VOC inducibility in *Datura* declines as the growing season progresses and plants begin flowering, but that this inducibility returns the following spring when plants regrow vegetative tissue (Hare, 2010). Inducibility can also be partially restored if plants are cut back to the root crown late in the season. These results are perhaps consistent with predictions of the ODT, as *D. wrightii* appears to be less actively resisting leaf herbivory during reproductive stages. The preponderance of DEGs even in plants that did not increase VOC emissions, however, suggests there may be more to the story. There may have been changes to other metabolites that function as direct resistance traits, or changes in resource dynamics that affect herbivore performance. *L. daturaphila* larvae were not affected by induction status or ontogenetic period, but such an adapted specialist herbivore might be a poor indicator of resistance; differences might be seen in generalist members of the herbivore community.

A previous study showed that predation of *L. daturaphila* eggs on *D. wrightii* was positively correlated with VOC emissions when they were present, but that predation was not restricted to periods in which VOCs were inducible (Hare and Sun 2011a). Evidence of a fitness benefit from the attraction of natural enemies is lacking. The benefits of VOCs as within-plant signals appear self-evident, but also remain to be rigorously tested. On the other hand, the potential ecological costs of VOC emissions include herbivore and

florivore attraction (e.g. Ballhorn *et al*. 2013; Kessler *et al*. 2013). The presence of this ontogenetic decline in inducible resistance traits in other herbaceous species from several families (Barton and Koricheva 2010) suggests this response may be synapomorphic, rather than independently evolved in *D. wrightii* (Hare and Sun 2011a).

Although the framework of the ODT makes clear predictions for annual plants, the life history of perennial plants is more complex. Because perennials may experience vegetative growth stages during multiple seasons, and resources accumulated during one season may contribute to fitness in subsequent years, the "optimal" allocation configuration is less clear. Mobilizing resources to defend photosynthetic tissue late in the season may mitigate losses to potential resources useful for regrowth in the second year, but presumably would do so at a cost to fitness in the first year.

Such a fitness cost in the first year could be overcome by potentiation of increased reproduction in subsequent years, particularly if reproductive output is correlated with biomass. In such a scenario, the finite rate of increase, an interval-based measure of reproductive output, would increase with plant age (Hare *et al*. 2003). Whereas net reproductive rate only considers lifetime reproduction of a cohort of individuals based on reproductive output and survival in each age class, the finite rate of increase also involves the compounding effects of early reproduction as offspring begin reproduction contemporary with the parental reproduction. Therefore, withholding reproductive effort in the first year would only increase fitness if reproductive output of the parent plant in later years outpaced offspring reproduction.

Unique aspects of the *D. wrightii* system have allowed for testing of this hypothesis previously. Plants with the sticky trichome phenotype tend to invest in larger canopies at the expense of reproduction. Over multiple growing seasons, the greater investment in vegetative growth in the sticky phenotype overcomes the lower reproductive rate in the first two years, such that by the third year both phenotypes have a similar net reproductive output. Incorporating the compounding fitness benefit of offspring reproduction, however, shows that the sticky phenotype's lower investment in reproduction in the first year results in a 40-43% lower finite rate of increase (Hare *et al*. 2003). Therefore, reproduction in the subsequent growing seasons contributes proportionally less fitness than reproduction in the first year and carries a risk if fitness is reduced in the first year and the plant dies before it can capitalize on the investment. Natural selection may therefore act against plants that allocate resources to the defense of photosynthetic tissue instead of reproduction, suggesting that some perennial plants may experience a fitness landscape not unlike that of annuals.

The difference in transcriptional responses seen in the second year of the study compared to the first may represent non-adaptive responses that occur because natural selection acts weakly on traits expressed after the first season of reproduction. Maladaptive traits that compromise the optimal defense of the plant in the first year, such as allocating resources to resistance traits instead of reproduction, would presumably be under relatively strong selection if such a trait reduced the fitness of the plant. The effect of any selection on traits after the first period of reproductive output is necessarily more diffuse. The extent of this reduced efficacy of natural selection likely depends on the

relative lifespan of the plant and the proportion of lifetime fitness that the first year represents. Many long-lived tree species reproduce repeatedly over many years, often increasing reproductive potential as they grow. For these species, selection pressure is likely to remain largely undiminished throughout much of their lifetime, assuming consistent herbivore loads, since the relative contribution of the first year represents a vanishingly small proportion of their potential lifetime reproduction. In the case of *D. wrightii*, plants seldom survive to their third year, and the first year's reproduction represents the highest finite rate of increase, and may accounting for 33-100% of total seed set (Hare *et al*. 2003).

For shorter-lived perennial species, the mechanisms ensuring consistent responses over the course of each season may not be as robust as those in longer-lived species. For instance, if leaf resistance traits are under the influence of inhibitory hormones emitted by apical inflorescences, then regulation of those traits may decrease as plants increase in size and those signals become diluted. This same scenario is involved in apical dominance: lateral bud growth is inhibited by apical auxin until the distance between the apex and the axillary bud increases to the point that the auxin is too dilute to inhibit growth (Cline 1991). If auxin similarly modulates resistance trait suppression, as hypothesized by Diezel *et al*. (2011), then the extent and efficacy of suppression may depend on the distance from the leaf to the flower. Nevertheless, the widespread gene expression seen in the second year of this study was not accompanied by induced VOC emissions. Separate, more robust mechanisms may be in place to regulate VOC

emissions by competing for precursor availability, suppressing expression of critical biosynthetic genes, or sequestering potentially volatile compounds within the leaf.

Conclusion

Ultimately, this study has demonstrated that the ontogenetic decline of herbivore-induced VOC emission is not necessarily associated with a wholesale inhibition of the transcriptional response. Even in sampling periods where VOCs were not inducible, terpene synthases were upregulated and therefore do not appear to control the rate of induced emission. The induced response may instead depend on the hormonal context in which damage occurs, as it appears that a plethora of hormone-signaling genes may be responsible for collectively influencing the emission of VOCs. Taken together, these results indicate that the inducibility of VOCs may be separately regulated from other responses, and not necessarily tied to other induced responses. Therefore, natural selection may act on different aspects of the induced response independently.

Table 3.1. List of genes and their associated primers analyzed by qPCR to confirm RNA-Seq results.

Gene	Abbreviation	Gene ID	Forward (5'→3')	Reverse (3'→5')
Hydroperoxide lyase 1	*HPL1*	Solyc07g049690	AAATGTGGTGGCGGTTCTG	GGCACAAACACGCATATCTC
Phenylalanine ammonia lyase	*PAL*	Solyc09g007900	GCTCCACCTACCCTTTGATG	TCGTCCTCGAAAGCTACAATC
Terpene synthase	*TPS*	Solyc03g006550	GGGATGCAAAGAACCTTGAT	TGGCAACATCACACACAAGA
Lipoxygenase 2	*LOX2*	Solyc01g006540	GGTGATGGAGTTGGAGAAAGA	GGACGGAGTAAACGGTGTTG
RuBPCase small chain 2B	*RUB*	Solyc02g063150	TCCCGTTACCAAGAAGAACAAC	AGCAAGGAACCCATCCACTT
Actin	*ACT*	Solyc04g071260	TCTTCCAGCCATCCTTGATT	TGACCCACCACTAAGCACAA
Ubiquitin	*UBI*	Solyc12g099030	TCCACCTTGTCCTTCGTCTC	CCTCTGAACCTTGCCAGAA

Figure 3.1. Mean (±SE) total VOC emissions by treatment and sampling period. Black bars correspond to plants that were damaged by two *Lema daturaphila* caged on an individual leaf for 24 hours, while grey bars indicate control plants that received empty cages. VOCs were collected from the same individual leaves immediately following cessation of treatment. Asterisk denotes $p \leq 0.05$.

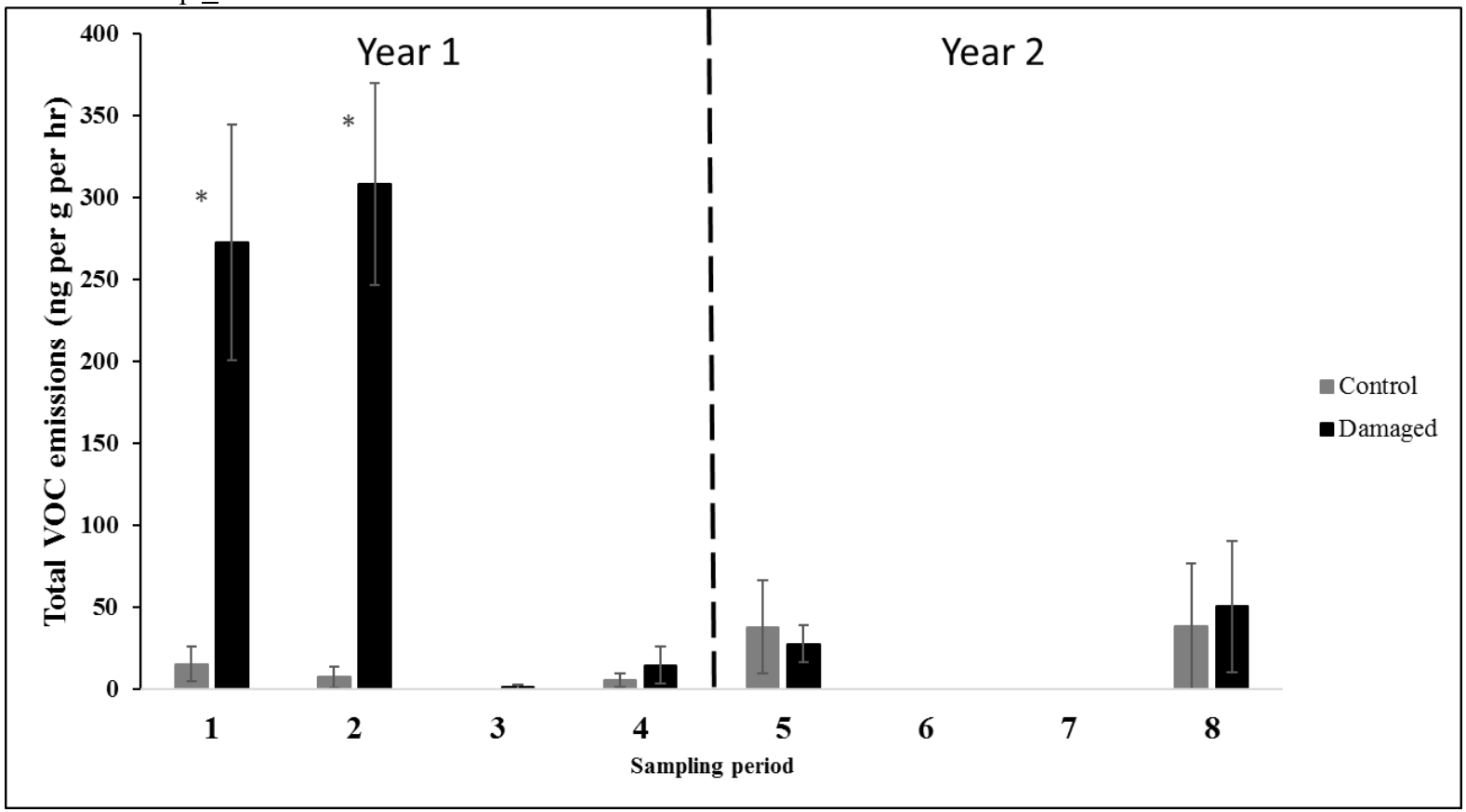


Figure 3.2. Number of genes differentially expressed by continuously damaged plants at several time points. Black bars above the horizontal line indicate the number of genes that with increased expression; grey bars below the line indicate suppressed genes.

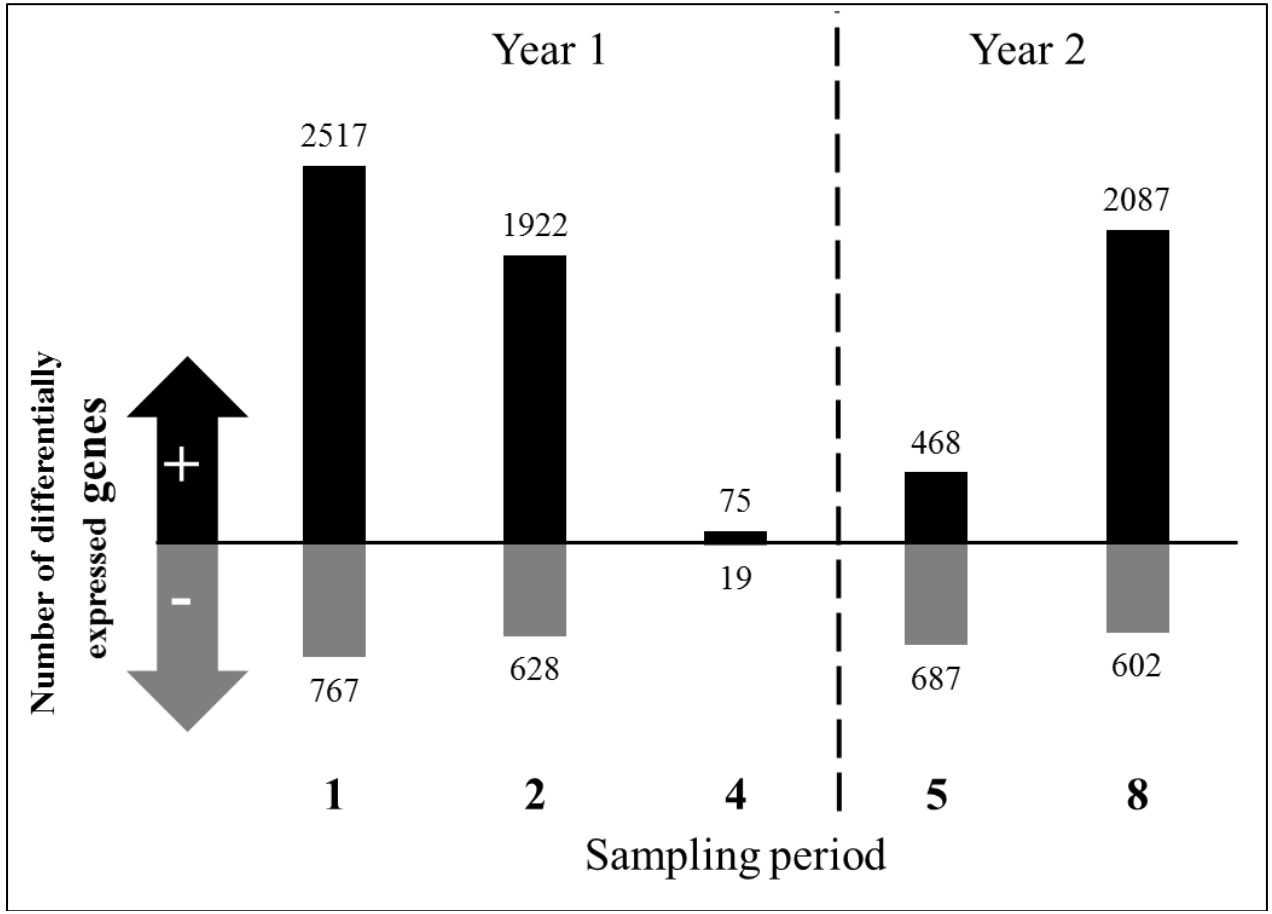


Figure 3.3. Heatmap of 6,016 genes induced by 24 hours of feeding by *Lema daturaphila* across sampling periods, shown in columns, with hierarchical clusters showing similarity of expression across rows. Red indicates increased expression relative to controls, and green indicates decreased expression.

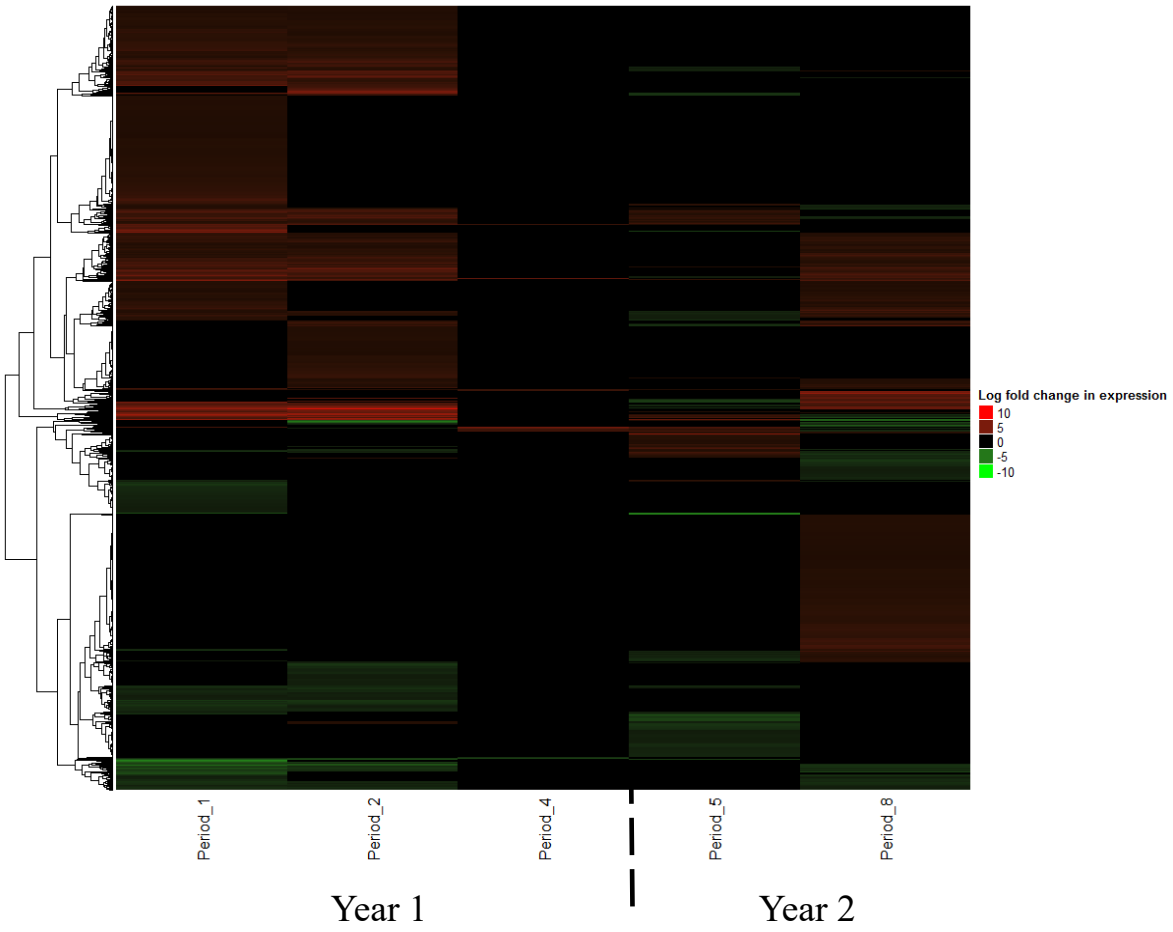


Figure 3.4. Number of genes differentially expressed in each sampling period grouped by functional category assigned by MAPMAN. Top, genes with increased expression relative to controls. Bottom, genes that were downregulated compared to controls. Sampling periods from year 2 of the study denoted with stripes.

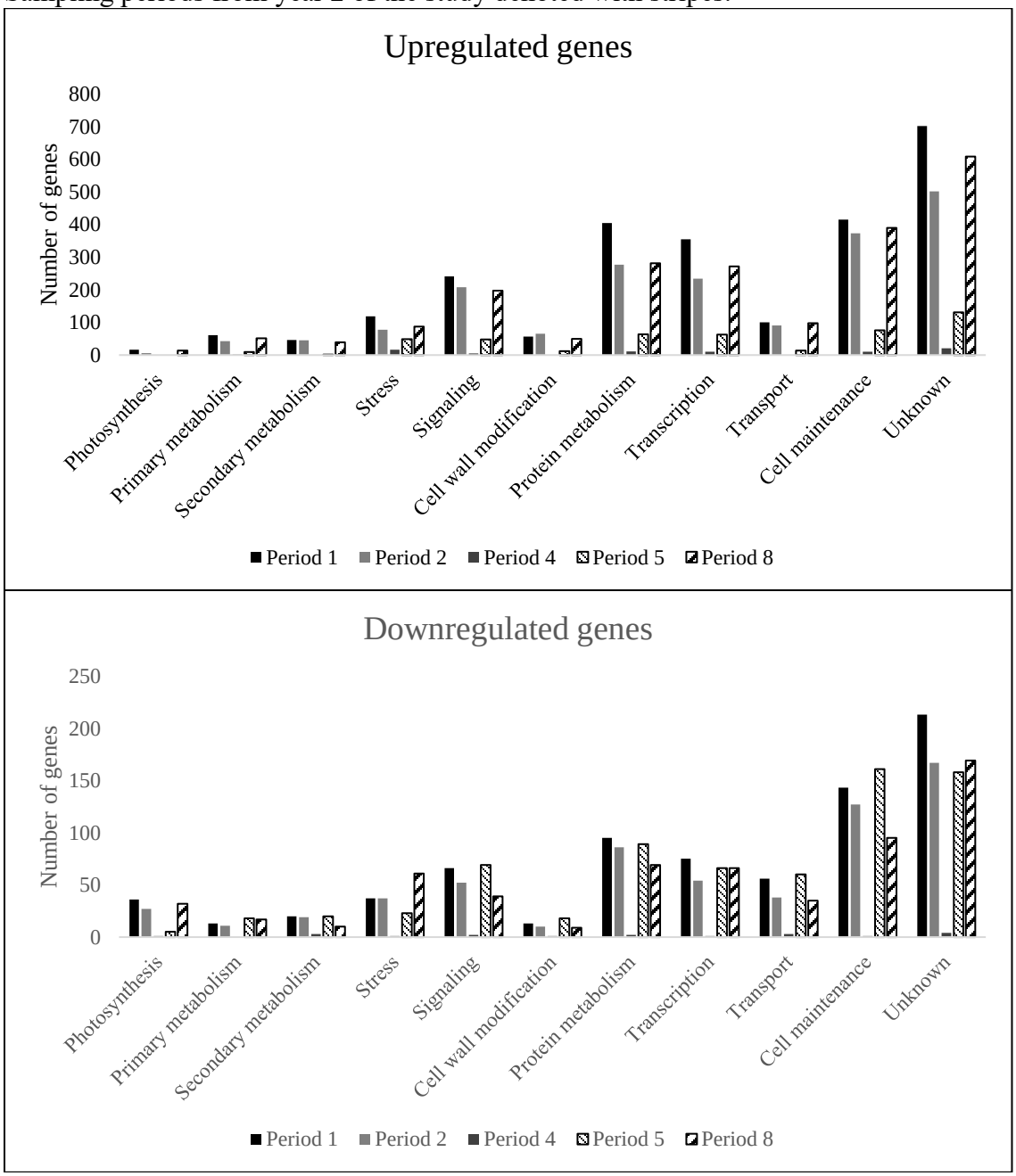


Figure 3.5. Heatmap of 17 putative terpene synthase genes induced by 24 hours of feeding by *Lema daturaphila* across sampling periods, shown in columns, with hierarchical clusters showing similarity of expression across rows. Red indicates increased expression relative to controls, and green indicates decreased expression.

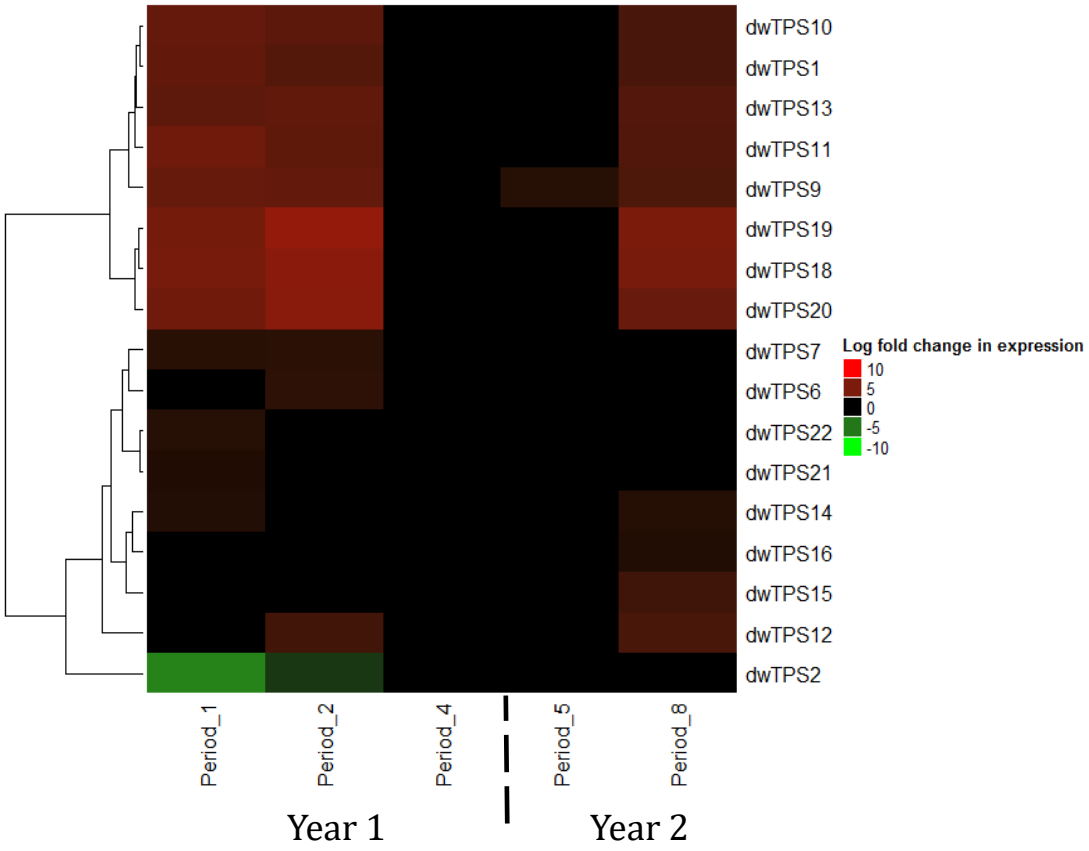


Table 3.2. A list of terpene synthase genes identified as being differentially expressed over both seasons in *Datura wrightii*. Nucleotide sequences from the *D. wrightii* transcriptome were used to predict amino acid sequences and open reading frames, and the resulting sequences were compared to the tomato proteome. Predicted products for each gene are from the reference listed.

Datura wrightii gene	Tomato ortholog gene ID	Tomato homolog	Predicted amino acid length	Non-similar amino acid substitutions from tomato	Expect (E) value	Main predicted product	Reference
DwTPS1	solyc00g154480		521	7	0	Unknown terpenoid	Fernandez-Pozo et al. 2015
DwTPS2	solyc01g101180	Truncated slTPS32-like	249	6	e-154	alpha-humulene / (-)-(e)-β-caryhophyllene	Falara et al., 2012
DwTPS6	solyc01g105890	slTPS5-like	609	2	0	Linalool	Falara et al., 2012
DwTPS7	solyc01g105910		131	0	2E-90	D-limonene	Fernandez-Pozo et al. 2015
DwTPS9	solyc02g079890	slTPS25-like	122	5	5E-67	β-ocimene	Falara et al., 2012
DwTPS10	solyc02g079900		56	2	4E-32	(E E)-α-farnesene	Fernandez-Pozo et al. 2015
DwTPS11	solyc02g079910	slTPS27-like	160	2	4E-67	Limonene	Falara et al., 2012
DwTPS12	solyc03g006550		621	8	0	(-)-*ent*-kaurene	Fernandez-Pozo et al. 2015
DwTPS13	solyc03g007730		562	3	0	β-ocimene	Fernandez-Pozo et al. 2015
DwTPS14	solyc03g025560		165	3	e-113	Undecaprenyl pyrophosphate	Fernandez-Pozo et al. 2015
DwTPS15	solyc04g070980		760	9	e-104	cycloartenol	Fernandez-Pozo et al. 2015
DwTPS16	solyc06g060010		555	0	0	α-humulene / (-)-(*E*)-β-caryhophyllene	Fernandez-Pozo et al. 2015
DwTPS18	solyc10g005390	Nearly identical to slTPS39	563	0	0	Nerolidol / linalool	Falara et al., 2012
DwTPS19	solyc10g005410	Truncated slTPS37-like	137	11	7E-26	Nerolidol / β-ocimene	Falara et al., 2012
DwTPS20	solyc10g005420	Truncated	38	0	5E-21	Limonene	Fernandez-Pozo et al. 2015
DwTPS21	solyc10g085150	Truncated	137	0	7E-97	Undecaprenyl pyrophosphate	Fernandez-Pozo et al. 2015
DwTPS22	solyc12g006530		761	0	0	Cycloartenol	Fernandez-Pozo et al. 2015

Figure 3.6. Heatmap of 208 genes putatively involved with hormone biosynthesis and signaling. Genes were induced by 24 hours of feeding by *Lema daturaphila* across sampling periods, shown in columns, with hierarchical clusters showing similarity of expression across rows. Red indicates increased expression relative to controls, and green indicates decreased expression. Colored labels, right, indicate each gene's pathway assignment.

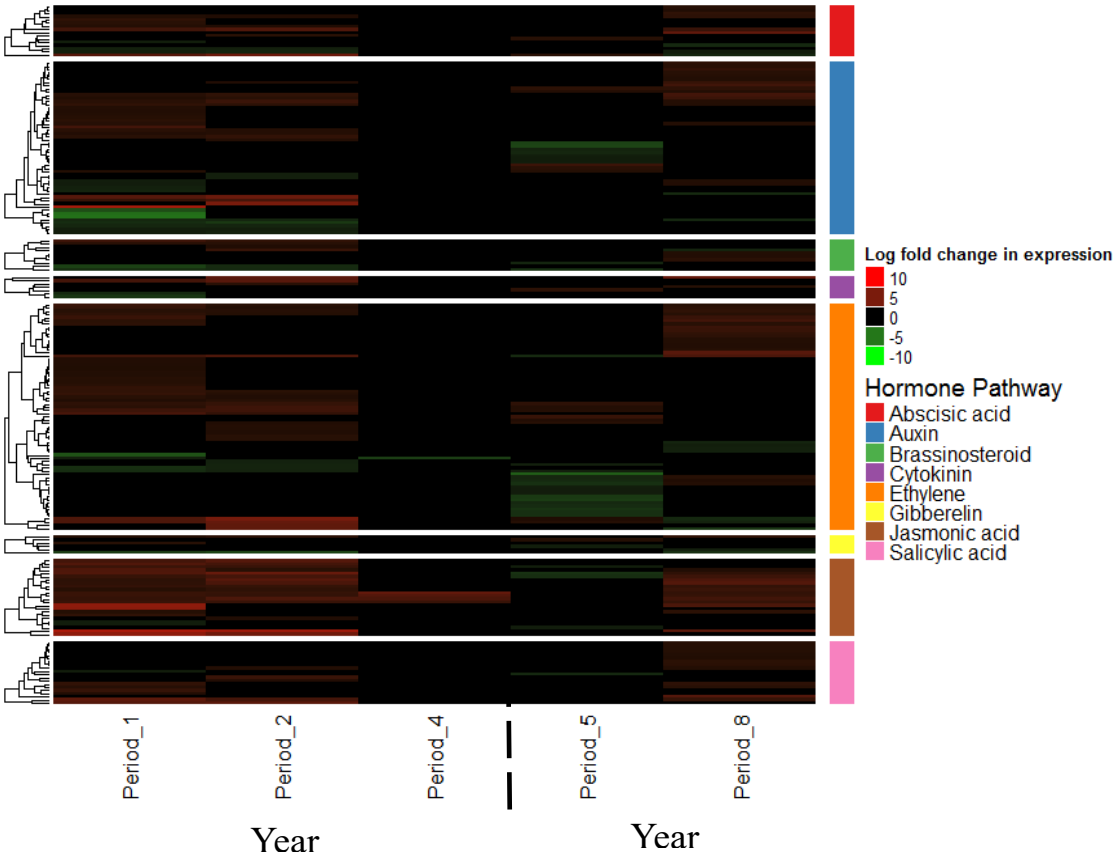


Table 3.3. Comparison of gene expression in undamaged plants sampled in periods 1 and 4, corresponding to the first and last sampling periods of the first year of the study. Genes were assigned to functional groups based on MapMan gene ontology designations. Note that some genes are assigned to multiple categories.

		Greater expression in period	
Functional group	Sub-group	**1**	**4**
Overall		**1126**	**1917**
Photosynthesis		**51**	**29**
Primary metabolism		**44**	**48**
Cell wall modification		**27**	**48**
Cell maintenance		**216**	**331**
Protein metabolism		**202**	**285**
Secondary metabolism		**23**	**14**
	Terpene synthases	2	0
Signaling		**88**	**145**
	Abscisic acid	1	3
	Auxin	12	9
	Brassinosteroid	0	1
	Cytokinin	2	0
	Ethylene	10	14
	Gibberelin	3	4
	Jasmonic acid	4	5
	Salicylic acid	1	6
Biotic and abiotic stress		**70**	**105**
Transcription		**118**	**222**
Transport		**48**	**109**
Unknown		**269**	**630**

Table 3.4. Comparison of gene expression in uninduced plants sampled in periods 4 and 8, corresponding to the first and last sampling periods of the first year of the study. Genes were assigned to functional groups based on MapMan gene ontology designations. Note that some genes are assigned to multiple categories.

		Greater expression in period	
Gene group	Sub-group	**4**	**8**
Overall		**1363**	**1569**
Photosynthesis		**41**	**23**
Primary metabolism		**37**	**43**
Cell wall modification		**46**	**27**
Cell maintenance		**245**	**284**
Protein metabolism		**157**	**278**
Secondary metabolism		**12**	**24**
	Terpene synthases	0	1
Signaling		**112**	**128**
	Abscisic acid	3	2
	Auxin	7	6
	Brassinosteroid	0	0
	Cytokinin	0	2
	Ethylene	15	12
	Gibberelin	1	1
	Jasmonic acid	3	5
	Salicylic acid	4	2
Biotic and abiotic stress		**62**	**110**
Transcription		**135**	**221**
Transport		**83**	**71**
Unknown		**462**	**402**

Figure 3.7. Comparison of gene expression patterns across sampling periods using quantitative PCR (qPCR) and RNA-Seq for plants continuously damaged by *Lema daturaphila*. Dark bars represent mean (±SE) qPCR analyses. Light bars show RNA-Seq results. Some qPCR values reflect fewer than three biological replicates (see text for details).

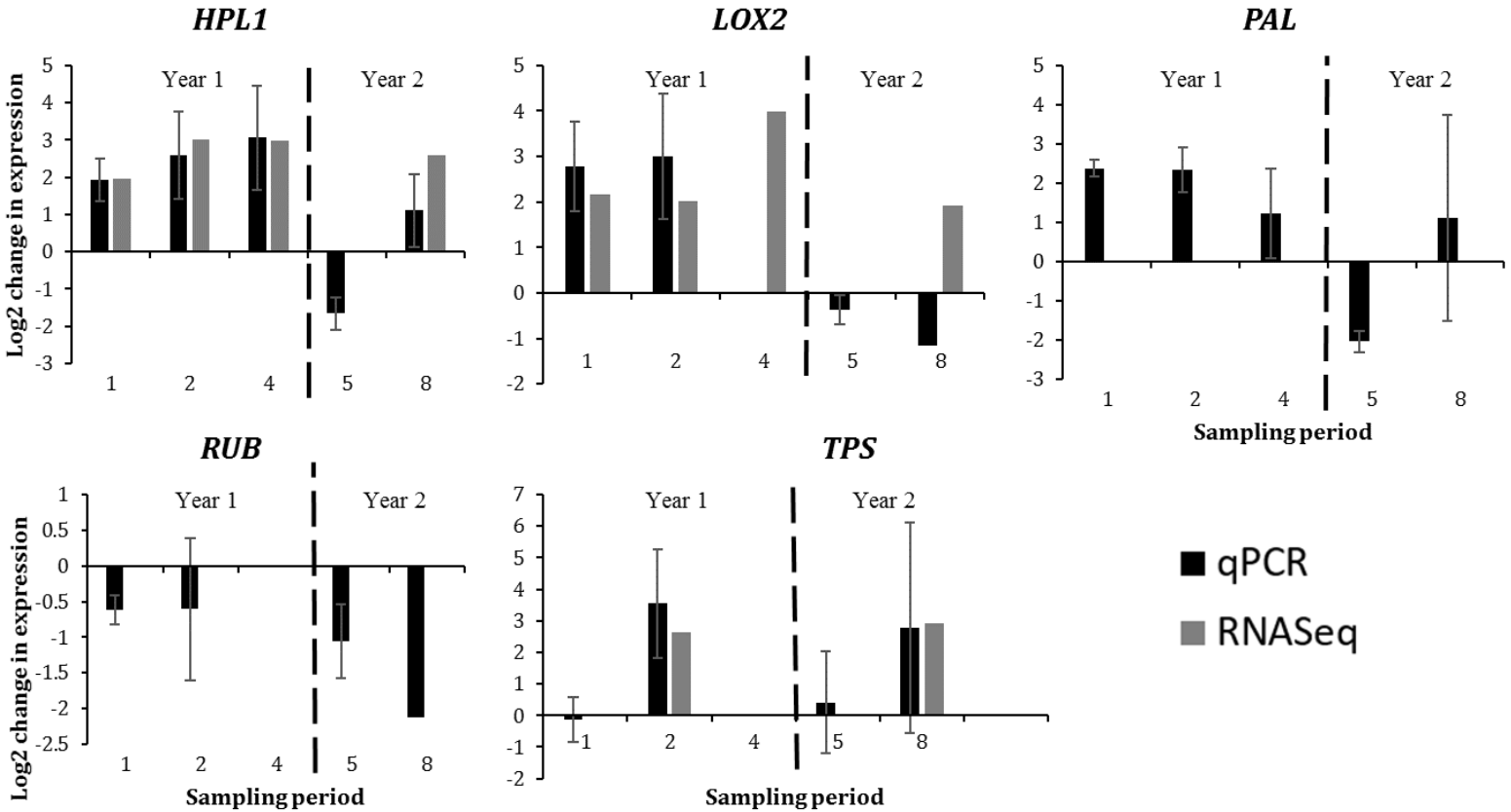


Figure 3.8. Regression of mean induced VOC emissions against principal component 1 of the PCA that included all genes putatively involved in phytohormone biosynthesis. Mean induced VOCs is calculated based on the mean emissions of induced plants from each sampling period minus mean emissions of control plants from that same period. R^2=0.865, p=0.0221.

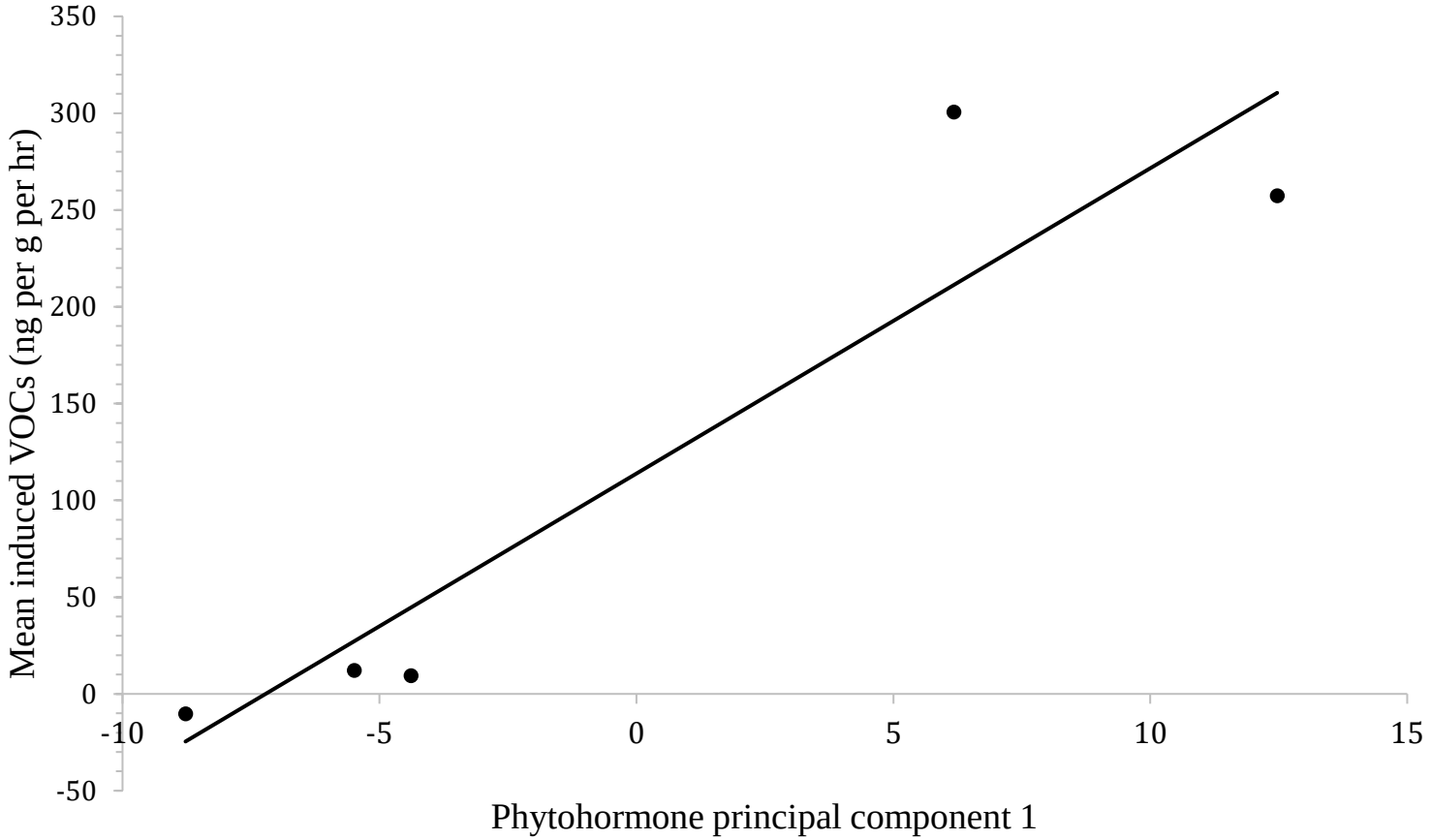


Table 3.5. List of all genes putatively assigned to phytohormone biosynthesis and signaling. Included are gene IDs, factor loading scores for each gene showing their impact on the principal components analysis that included all genes shown here, and putative functions as assigned by MapMan.

	PC1	PC2	PC3	
Percent variation	38.7	32.5	19.1	
Gene ID				**Function**
Solyc12g014290.1	0.110	-0.012	-0.005	ethylene.induced-regulated-responsive-activated
Solyc08g008110.2	0.110	-0.012	-0.006	ethylene.synthesis-degradation.1-aminocyclopropane-1-carboxylate synthase
Solyc03g119910.2	0.110	-0.013	0.006	ethylene.synthesis-degradation
Solyc02g071990.2	0.110	-0.013	0.007	brassinosteroid.signal transduction.BZR
Solyc12g009220.1	0.109	-0.013	0.013	jasmonate.signal transduction
Solyc04g079730.1	0.109	-0.013	0.017	jasmonate.synthesis-degradation.allene oxidase synthase
Solyc10g080880.1	0.109	-0.013	0.017	auxin.signal transduction
Solyc01g009760.2	0.109	-0.013	0.020	abscisic acid.induced-regulated-responsive-activated
Solyc09g089610.2	0.109	-0.013	0.021	ethylene.signal transduction
Solyc07g007870.2	0.108	0.014	0.037	jasmonate.synthesis-degradation.12-Oxo-PDA-reductase
Solyc03g005990.2	0.107	-0.013	0.032	brassinosteroid.signal transduction.BZR
Solyc10g049500.1	0.105	-0.014	0.042	salicylic acid.synthesis-degradation
Solyc02g092820.2	0.104	-0.014	0.046	auxin.induced-regulated-responsive-activated
Solyc08g079140.1	0.102	-0.014	0.057	auxin.induced-regulated-responsive-activated
Solyc01g099210.2	0.101	0.030	-0.044	jasmonate.synthesis-degradation.lipoxygenase
Solyc07g042170.2	0.101	-0.014	0.060	jasmonate.signal transduction
Solyc03g082510.1	0.100	-0.014	0.061	auxin.induced-regulated-responsive-activated
Solyc07g048070.2	0.100	-0.014	0.062	auxin.induced-regulated-responsive-activated
Solyc01g088160.2	0.100	-0.014	0.062	cytokinin.synthesis-degradation
Solyc09g011680.1	0.099	-0.014	0.064	ethylene.signal transduction
Solyc12g049400.1	0.098	0.041	0.027	jasmonate.signal transduction
Solyc09g091530.1	0.098	0.047	-0.002	salicylic acid.synthesis-degradation
Solyc03g114730.2	0.094	0.053	0.019	auxin.induced-regulated-responsive-activated
Solyc02g085730.2	0.094	0.050	0.058	jasmonate.synthesis-degradation.allene oxidase cyclase
Solyc08g008100.2	0.092	0.058	-0.021	ethylene.synthesis-degradation.1-aminocyclopropane-1-carboxylate synthase
Solyc10g006130.1	0.088	-0.049	0.029	ethylene.signal transduction
Solyc09g082780.2	0.087	-0.005	-0.099	auxin.induced-regulated-responsive-activated
Solyc09g089920.1	0.087	-0.005	-0.099	ethylene.signal transduction
Solyc08g014120.2	0.087	-0.005	-0.099	ethylene.induced-regulated-responsive-activated
Solyc08g075490.2	0.087	-0.005	-0.099	abscisic acid.synthesis-degradation.synthesis.9-*cis*-epoxycarotenoid dioxygenase
Solyc01g109140.2	0.087	-0.005	-0.099	jasmonate.synthesis-degradation.allene oxidase synthase
Solyc03g033590.1	0.087	-0.005	-0.099	auxin.induced-regulated-responsive-activated
Solyc02g093020.2	0.087	-0.005	-0.099	ethylene.signal transduction
Solyc04g009850.2	0.087	-0.005	-0.099	ethylene.synthesis-degradation
Solyc06g082860.2	0.087	-0.005	-0.099	auxin.induced-regulated-responsive-activated
Solyc08g078220.2	0.087	-0.005	-0.099	ethylene.signal transduction
Solyc10g007350.2	0.087	-0.005	-0.099	ethylene.induced-regulated-responsive-activated
Solyc06g069790.2	0.087	-0.005	-0.099	gibberelin.induced-regulated-responsive-activated
Solyc04g014250.2	0.087	-0.005	-0.099	abscisic acid.induced-regulated-responsive-activated
Solyc05g053720.2	0.087	-0.005	-0.099	auxin.induced-regulated-responsive-activated
Solyc03g117570.2	0.087	-0.005	-0.099	salicylic acid.synthesis-degradation
Solyc07g049200.2	0.087	-0.005	-0.099	auxin.induced-regulated-responsive-activated
Solyc04g077410.2	0.087	-0.005	-0.099	ethylene.synthesis-degradation.1-aminocyclopropane-1-carboxylate synthase
Solyc01g103160.2	0.087	-0.005	-0.099	salicylic acid.synthesis-degradation
Solyc01g111010.2	0.087	-0.005	-0.099	auxin.induced-regulated-responsive-activated

Solyc02g064830.2	0.087	-0.005	-0.099	auxin.induced-regulated-responsive-activated
Solyc02g064950.2	0.087	-0.005	-0.099	ethylene.synthesis-degradation
Solyc04g080030.2	0.087	-0.005	-0.099	ethylene.signal transduction
Solyc01g107490.1	0.087	-0.005	-0.099	ethylene.induced-regulated-responsive-activated
Solyc08g029000.2	0.087	-0.005	-0.099	jasmonate.synthesis-degradation.lipoxygenase
Solyc07g056570.1	0.085	-0.063	0.047	abscisic acid.synthesis-degradation.synthesis.9-*cis*-epoxycarotenoid dioxygenase
Solyc07g053740.1	0.085	-0.061	0.051	ethylene.signal transduction
Solyc03g006320.1	0.085	-0.057	0.065	ethylene.signal transduction
Solyc11g065930.1	0.083	0.058	0.054	abscisic acid.synthesis-degradation
Solyc05g006220.2	0.082	0.045	0.074	auxin.synthesis-degradation
Solyc09g089780.2	0.081	0.074	-0.002	ethylene.synthesis-degradation
Solyc09g089810.1	0.077	0.077	-0.027	ethylene.synthesis-degradation
Solyc01g009680.2	0.076	0.075	0.029	jasmonate.synthesis-degradation.lipoxygenase
Solyc10g007960.1	0.074	0.083	0.049	jasmonate.synthesis-degradation.allene oxidase synthase
Solyc10g076660.1	0.072	0.086	0.044	ethylene.synthesis-degradation
Solyc05g052030.1	0.070	-0.054	0.069	ethylene.signal transduction
Solyc01g109150.2	0.067	0.053	-0.105	jasmonate.synthesis-degradation.allene oxidase synthase
Solyc12g011040.1	0.065	0.070	0.032	jasmonate.synthesis-degradation.lipoxygenase
Solyc03g118740.2	0.061	0.076	0.032	auxin.signal transduction
Solyc10g011660.2	0.061	0.076	0.032	auxin.induced-regulated-responsive-activated
Solyc07g009340.2	0.061	0.076	0.032	auxin.induced-regulated-responsive-activated
Solyc07g056670.2	0.061	0.076	0.032	ethylene.synthesis-degradation
Solyc03g122340.2	0.061	0.076	0.032	jasmonate.synthesis-degradation.lipoxygenase
Solyc03g117260.1	0.061	0.076	0.032	ethylene.signal transduction
Solyc02g063240.2	0.061	0.076	0.032	brassinosteroid.synthesis-degradation.sterols.DWF7
Solyc11g013110.1	0.061	0.076	0.032	ethylene.synthesis-degradation
Solyc01g097390.2	0.061	0.076	0.032	auxin.induced-regulated-responsive-activated
Solyc02g093140.2	0.061	0.076	0.032	ethylene.synthesis-degradation
Solyc02g084950.2	0.061	0.076	0.032	salicylic acid.synthesis-degradation
Solyc09g097960.2	0.061	0.076	0.032	auxin.induced-regulated-responsive-activated
Solyc10g005360.2	0.061	0.076	0.032	ethylene.synthesis-degradation
Solyc01g079200.2	0.061	0.076	0.032	ethylene.synthesis-degradation
Solyc01g111080.2	0.061	0.076	0.032	gibberelin.induced-regulated-responsive-activated
Solyc02g070430.2	0.061	0.076	0.032	ethylene.synthesis-degradation
Solyc02g083860.2	0.061	0.076	0.032	ethylene.synthesis-degradation
Solyc10g007570.2	0.061	0.076	0.032	ethylene.synthesis-degradation
Solyc04g008670.1	0.061	0.076	0.032	ethylene.synthesis-degradation
Solyc01g112070.2	0.061	0.076	0.032	auxin.induced-regulated-responsive-activated
Solyc01g095080.2	0.061	0.076	0.032	ethylene.synthesis-degradation.1-aminocyclopropane-1-carboxylate synthase
Solyc07g008290.2	0.061	0.076	0.032	auxin.induced-regulated-responsive-activated
Solyc05g052740.1	0.059	-0.071	0.030	ethylene.synthesis-degradation
Solyc09g089790.2	0.059	0.093	0.017	ethylene.synthesis-degradation
Solyc06g007910.2	0.057	0.089	0.042	gibberelin.induced-regulated-responsive-activated
Solyc04g077440.2	0.052	-0.064	0.114	brassinosteroid.synthesis-degradation.sterols.other
Solyc08g078180.1	0.051	-0.059	0.118	ethylene.signal transduction
Solyc07g066480.2	0.050	0.100	0.010	abscisic acid.synthesis-degradation
Solyc09g075860.2	0.048	0.078	0.084	jasmonate.synthesis-degradation.lipoxygenase
Solyc09g089770.2	0.046	0.080	-0.095	ethylene.synthesis-degradation
Solyc07g066560.1	0.046	0.080	-0.095	auxin.induced-regulated-responsive-activated
Solyc11g069800.1	0.046	0.097	0.039	jasmonate.synthesis-degradation.allene oxidase synthase
Solyc05g010780.1	0.045	0.082	-0.094	salicylic acid.synthesis-degradation
Solyc10g049540.1	0.043	-0.011	0.143	salicylic acid.synthesis-degradation
Solyc04g081270.1	0.043	-0.011	0.143	auxin.induced-regulated-responsive-activated
Solyc01g109060.2	0.043	-0.011	0.143	salicylic acid.synthesis-degradation

Solyc08g079130.1	0.043	-0.011	0.143	auxin.induced-regulated-responsive-activated
Solyc02g091990.2	0.043	-0.011	0.143	ethylene.synthesis-degradation.1-aminocyclopropane-1-carboxylate synthase
Solyc12g005940.1	0.043	-0.011	0.143	ethylene.synthesis-degradation
Solyc12g056180.1	0.043	-0.011	0.143	ethylene.synthesis-degradation.1-aminocyclopropane-1-carboxylate synthase
Solyc08g016720.1	0.043	-0.011	0.143	abscisic acid.synthesis-degradation.synthesis.9-*cis*-epoxycarotenoid dioxygenase
Solyc12g089040.1	0.043	-0.011	0.143	brassinosteroid.signal transduction.BZR
Solyc03g093550.1	0.043	-0.011	0.143	ethylene.signal transduction
Solyc03g031450.2	0.043	-0.011	0.143	ethylene.induced-regulated-responsive-activated
Solyc04g016430.2	0.043	-0.011	0.143	cytokinin.synthesis-degradation
Solyc01g099160.2	0.043	-0.011	0.143	jasmonate.synthesis-degradation.lipoxygenase
Solyc10g009110.1	0.043	-0.011	0.143	ethylene.signal transduction
Solyc08g014000.2	0.042	0.093	0.060	jasmonate.synthesis-degradation.lipoxygenase
Solyc09g089760.1	0.039	0.087	-0.091	ethylene.synthesis-degradation
Solyc07g049690.2	0.039	0.067	0.075	jasmonate.synthesis-degradation.allene oxidase synthase
Solyc10g006640.2	0.038	-0.111	0.012	ethylene.induced-regulated-responsive-activated
Solyc01g108870.2	0.038	-0.111	0.012	gibberelin.synthesis-degradation.GA20 oxidase
Solyc03g098230.2	0.038	-0.111	0.012	ethylene.synthesis-degradation
Solyc08g079150.1	0.038	-0.111	0.012	auxin.induced-regulated-responsive-activated
Solyc11g044560.1	0.038	-0.111	0.012	abscisic acid.signal transduction
Solyc01g108860.2	0.038	-0.111	0.012	ethylene.synthesis-degradation
Solyc11g072130.1	0.038	-0.111	0.012	ethylene.synthesis-degradation
Solyc02g079830.1	0.038	0.088	-0.089	salicylic acid.synthesis-degradation
Solyc10g079640.1	0.036	0.103	0.035	auxin.synthesis-degradation
Solyc01g006560.2	0.034	0.048	0.016	jasmonate.synthesis-degradation.lipoxygenase
Solyc07g054580.2	0.030	0.105	0.036	auxin.induced-regulated-responsive-activated
Solyc07g061720.2	0.030	0.110	0.020	ethylene.synthesis-degradation
Solyc02g080120.1	0.029	0.111	0.019	ethylene.synthesis-degradation
Solyc01g109160.2	0.028	0.096	-0.082	jasmonate.synthesis-degradation.allene oxidase synthase
Solyc01g006540.2	0.025	0.032	0.017	jasmonate.synthesis-degradation.lipoxygenase
Solyc10g045420.1	0.021	-0.066	-0.108	auxin.induced-regulated-responsive-activated
Solyc07g061730.2	0.017	0.117	0.014	ethylene.synthesis-degradation
Solyc09g089800.1	0.012	0.105	-0.069	ethylene.synthesis-degradation
Solyc11g072110.1	0.010	0.068	-0.098	ethylene.synthesis-degradation
Solyc01g095110.2	0.003	0.083	0.106	salicylic acid.synthesis-degradation
Solyc10g079870.1	0.000	0.087	0.100	cytokinin.synthesis-degradation
Solyc02g036350.2	-0.008	0.021	0.044	ethylene.synthesis-degradation
Solyc10g008520.2	-0.016	0.104	0.063	auxin.induced-regulated-responsive-activated
Solyc02g077370.1	-0.025	-0.077	0.071	ethylene.signal transduction
Solyc02g021280.1	-0.038	0.111	-0.012	salicylic acid.synthesis-degradation
Solyc02g030170.2	-0.038	0.111	-0.012	brassinosteroid.synthesis-degradation.sterols.DWF1
Solyc11g011410.1	-0.038	0.111	-0.012	salicylic acid.synthesis-degradation
Solyc08g081540.2	-0.038	0.111	-0.012	ethylene.synthesis-degradation.1-aminocyclopropane-1-carboxylate synthase
Solyc04g055260.2	-0.038	0.111	-0.012	salicylic acid.synthesis-degradation
Solyc09g089820.1	-0.038	0.111	-0.012	ethylene.synthesis-degradation
Solyc12g014000.1	-0.038	0.111	-0.012	salicylic acid.synthesis-degradation
Solyc11g006270.1	-0.038	0.111	-0.012	brassinosteroid.synthesis-degradation.BRs.DET2
Solyc04g014530.1	-0.038	0.111	-0.012	ethylene.signal transduction
Solyc10g076840.1	-0.038	0.111	-0.012	ethylene.synthesis-degradation
Solyc11g072310.1	-0.038	0.111	-0.012	ethylene.synthesis-degradation
Solyc08g068480.1	-0.038	0.111	-0.012	auxin.induced-regulated-responsive-activated
Solyc00g085070.2	-0.038	0.111	-0.012	brassinosteroid.synthesis-degradation.sterols.other
Solyc06g010270.1	-0.038	0.111	-0.012	salicylic acid.synthesis-degradation
Solyc12g013780.1	-0.038	0.111	-0.012	ethylene.synthesis-degradation
Solyc04g009220.1	-0.038	0.111	-0.012	salicylic acid.synthesis-degradation

Solyc08g069010.2	-0.038	0.111	-0.012	salicylic acid.synthesis-degradation
Solyc07g025250.1	-0.038	0.111	-0.012	salicylic acid.synthesis-degradation
Solyc11g013270.1	-0.038	0.111	-0.012	auxin.induced-regulated-responsive-activated
Solyc11g071620.1	-0.038	0.111	-0.012	abscisic acid.synthesis-degradation
Solyc05g013730.2	-0.038	0.111	-0.012	auxin.induced-regulated-responsive-activated
Solyc09g010010.1	-0.038	0.111	-0.012	ethylene.synthesis-degradation
Solyc06g083140.2	-0.038	0.111	-0.012	auxin.induced-regulated-responsive-activated
Solyc06g073080.2	-0.038	0.111	-0.012	ethylene.synthesis-degradation
Solyc03g117100.2	-0.038	0.111	-0.012	ethylene.signal transduction
Solyc11g071590.1	-0.038	0.111	-0.012	abscisic acid.synthesis-degradation
Solyc02g072490.2	-0.038	0.111	-0.012	auxin.induced-regulated-responsive-activated
Solyc12g038670.1	-0.038	0.111	-0.012	auxin.induced-regulated-responsive-activated
Solyc07g005060.2	-0.038	0.111	-0.012	auxin.signal transduction
Solyc08g029150.1	-0.038	0.111	-0.012	salicylic acid.synthesis-degradation
Solyc09g074910.1	-0.038	0.111	-0.012	abscisic acid.induced-regulated-responsive-activated
Solyc11g071600.1	-0.038	0.111	-0.012	abscisic acid.synthesis-degradation
Solyc09g064910.1	-0.038	0.111	-0.012	cytokinin.synthesis-degradation
Solyc02g078380.2	-0.043	0.011	-0.143	auxin.induced-regulated-responsive-activated
Solyc02g071380.2	-0.043	0.011	-0.143	ethylene.synthesis-degradation
Solyc06g060750.2	-0.043	0.011	-0.143	auxin.induced-regulated-responsive-activated
Solyc11g006300.1	-0.050	0.079	0.005	brassinosteroid.synthesis-degradation.BRs.DET2
Solyc11g011210.1	-0.061	-0.076	-0.032	gibberelin.induced-regulated-responsive-activated
Solyc11g006180.1	-0.061	-0.076	-0.032	ethylene.signal transduction
Solyc03g123550.1	-0.061	-0.076	-0.032	auxin.induced-regulated-responsive-activated
Solyc07g043420.2	-0.061	-0.076	-0.032	ethylene.synthesis-degradation
Solyc11g070150.1	-0.061	-0.076	-0.032	cytokinin.signal transduction
Solyc03g006490.2	-0.061	-0.076	-0.032	auxin.induced-regulated-responsive-activated
Solyc09g097890.2	-0.073	-0.080	-0.021	auxin.induced-regulated-responsive-activated
Solyc11g072120.1	-0.078	0.062	0.012	ethylene.synthesis-degradation
Solyc09g098000.2	-0.078	0.076	0.053	auxin.induced-regulated-responsive-activated
Solyc11g013310.1	-0.078	0.076	0.053	auxin.signal transduction
Solyc12g013700.1	-0.081	0.032	-0.036	auxin.induced-regulated-responsive-activated
Solyc01g087260.2	-0.082	-0.062	-0.047	abscisic acid.synthesis-degradation.synthesis.9-*cis*-epoxycarotenoid dioxygenase
Solyc06g073060.2	-0.082	0.025	-0.037	auxin.synthesis-degradation
Solyc05g051200.1	-0.087	0.005	0.099	ethylene.signal transduction
Solyc01g068410.2	-0.087	0.005	0.099	auxin.signal transduction
Solyc11g032130.1	-0.087	0.005	0.099	jasmonate.synthesis-degradation.12-Oxo-PDA-reductase
Solyc04g052980.1	-0.087	0.005	0.099	auxin.induced-regulated-responsive-activated
Solyc01g110920.2	-0.087	0.005	0.099	auxin.induced-regulated-responsive-activated
Solyc08g075480.2	-0.087	0.005	0.099	abscisic acid.synthesis-degradation.synthesis.9-*cis*-epoxycarotenoid dioxygenase
Solyc05g008060.2	-0.087	0.005	0.099	auxin.signal transduction
Solyc08g061930.2	-0.087	0.005	0.099	cytokinin.synthesis-degradation
Solyc08g061920.1	-0.087	0.005	0.099	cytokinin.synthesis-degradation
Solyc08g078280.1	-0.087	0.005	0.099	salicylic acid.synthesis-degradation
Solyc11g011680.1	-0.087	0.005	0.099	auxin.induced-regulated-responsive-activated
Solyc11g032230.1	-0.087	0.005	0.099	jasmonate.synthesis-degradation.12-Oxo-PDA-reductase
Solyc04g017720.2	-0.088	-0.054	-0.044	gibberelin.induced-regulated-responsive-activated
Solyc01g110940.2	-0.100	0.014	-0.061	auxin.induced-regulated-responsive-activated
Solyc03g112750.2	-0.103	0.014	-0.051	auxin.signal transduction
Solyc06g072680.2	-0.107	0.014	-0.034	abscisic acid.induced-regulated-responsive-activated
Solyc09g097990.1	-0.107	0.013	-0.032	auxin.induced-regulated-responsive-activated
Solyc10g085050.1	-0.108	0.013	-0.028	auxin.induced-regulated-responsive-activated
Solyc03g006880.2	-0.110	0.012	-0.003	ethylene.synthesis-degradation
Solyc11g006290.1	-0.110	0.012	0.008	brassinosteroid.synthesis-degradation.BRs.DET2

Table 3.6. List of all genes putatively identified as coding for terpene synthases. Included are gene IDs, factor loading scores for each gene showing their impact on the principal components analysis that included all genes shown here, and predicted product based on similarity to predicted or observed products from similar genes found in tomato.

	PC1	PC2	PC3		
Percent variation	61.5	23.1	15.1		
Gene ID				**Name**	**Predicted product**
solyc00g154480	0.31	0.00	0.02	DwTPS1	Unknown terpenoid
solyc01g101180	-0.23	0.34	-0.07	DwTPS2	α-humulene / (-)-(*E*)-β-caryophyllene
solyc01g105890	0.13	0.03	-0.56	DwTPS6	Linalool
solyc01g105910	0.24	-0.25	-0.23	DwTPS7	D-limonene
solyc02g079890	0.30	-0.01	-0.04	DwTPS9	β-ocimene
solyc02g079900	0.31	-0.01	-0.01	DwTPS10	(*E*,*E*)-alpha-farnesene
solyc02g079910	0.31	0.00	0.03	DwTPS11	Limonene
solyc03g006550	0.16	0.40	-0.20	DwTPS12	(-)-*ent*-kaurene
solyc03g007730	0.31	0.06	-0.04	DwTPS13	β-ocimene
solyc03g025560	0.19	0.11	0.47	DwTPS14	Undecaprenyl pyrophosphate
solyc04g070980	0.07	0.44	0.28	DwTPS15	Cycloartenol / Lanosterol / Squalene
solyc06g060010	0.07	0.44	0.28	DwTPS16	α-humulene / (-)-(*E*)-β-caryophyllene
solyc10g005390	0.30	0.10	-0.05	DwTPS18	Nerolidol / linalool
solyc10g005410	0.30	0.11	-0.09	DwTPS19	Nerolidol / β-ocimene
solyc10g005420	0.30	0.08	-0.10	DwTPS20	Limonene
solyc10g085150	0.17	-0.34	0.30	DwTPS21	Undecaprenyl pyrophosphate
solyc12g006530	0.17	-0.34	0.30	DwTPS22	Cycloartenol

Table 3.7. List of VOCs included in a principal components analysis, as well as their factor loadings toward each individual component. The percent of the variation explained by each PC is listed at the top.

	PC1	PC2	PC3	PC4
Percent variation	39.53	27.98	21.62	10.87
Compound				
trans-2-hexenal	-0.33	0.31	-0.18	0.01
cis-3-hexen-1-ol	0.43	0.00	0.11	0.08
Hexenyl acetate	0.23	0.42	-0.06	-0.23
Limonene	0.33	-0.31	0.18	-0.01
(*E*)-β-ocimene	0.06	0.10	0.28	0.72
Linalool	0.24	0.43	-0.05	0.08
DMNT	-0.04	0.28	0.50	0.04
Methyl salicylate	0.43	0.05	0.07	0.14
(*E*)-β-caryophyllene	-0.18	-0.46	0.12	-0.09
Geranyl acetone	-0.17	-0.17	-0.28	0.61
β-selenine	-0.26	0.01	0.48	-0.06
Nerolidol	0.24	-0.07	-0.49	0.04
α-farnesene	0.33	-0.32	0.15	-0.10

Figure 3.9. Regression of principal component 1 of the VOC PCA against principal component 1 of the PCA that included all genes putatively involved in phytohormone biosynthesis. R^2=0.756, p=0.0556.

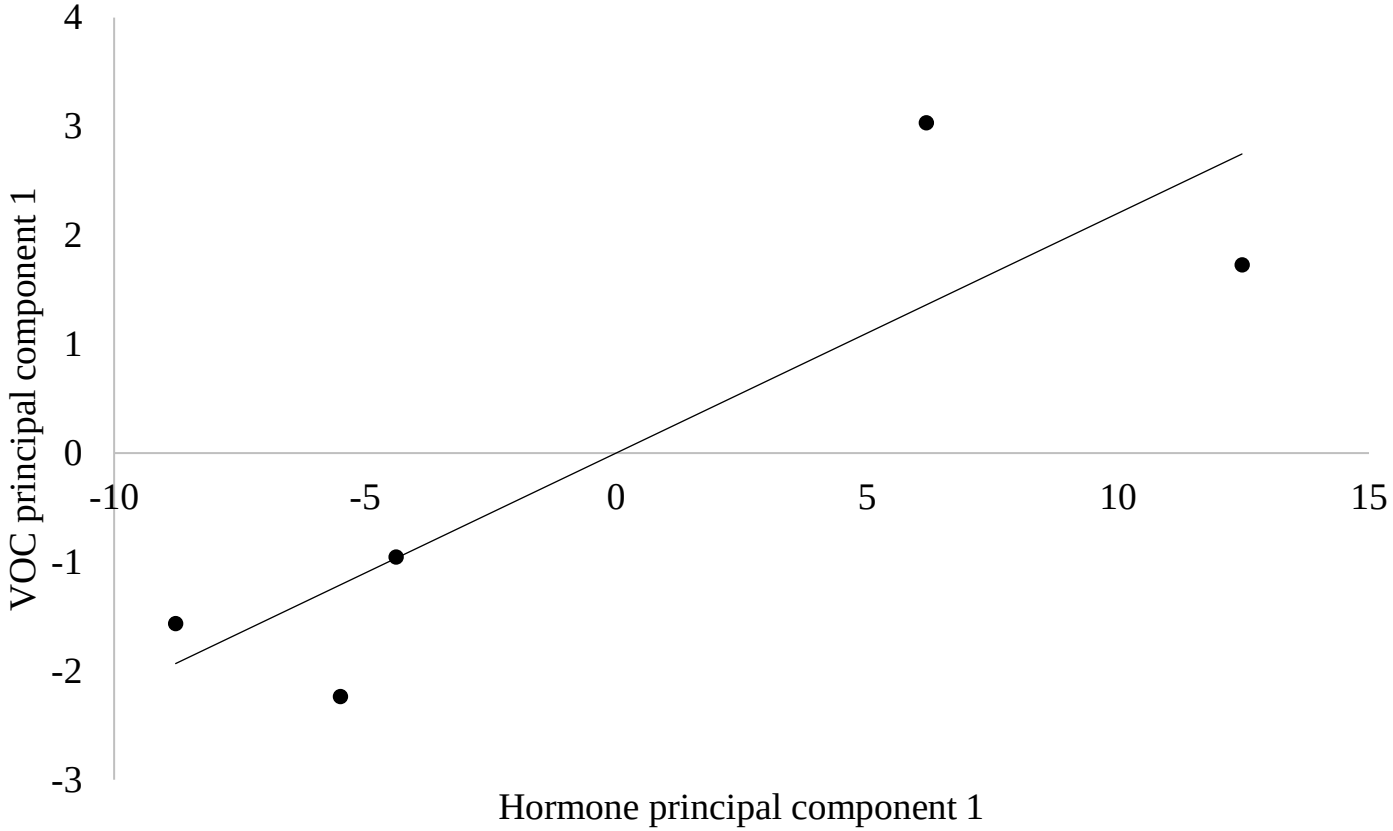


Figure 3.10. Regression of principal component 2 of the VOC PCA against principal component 3 of the PCA that included all genes putatively involved in phytohormone biosynthesis. R^2=0.672, p=0.0892.

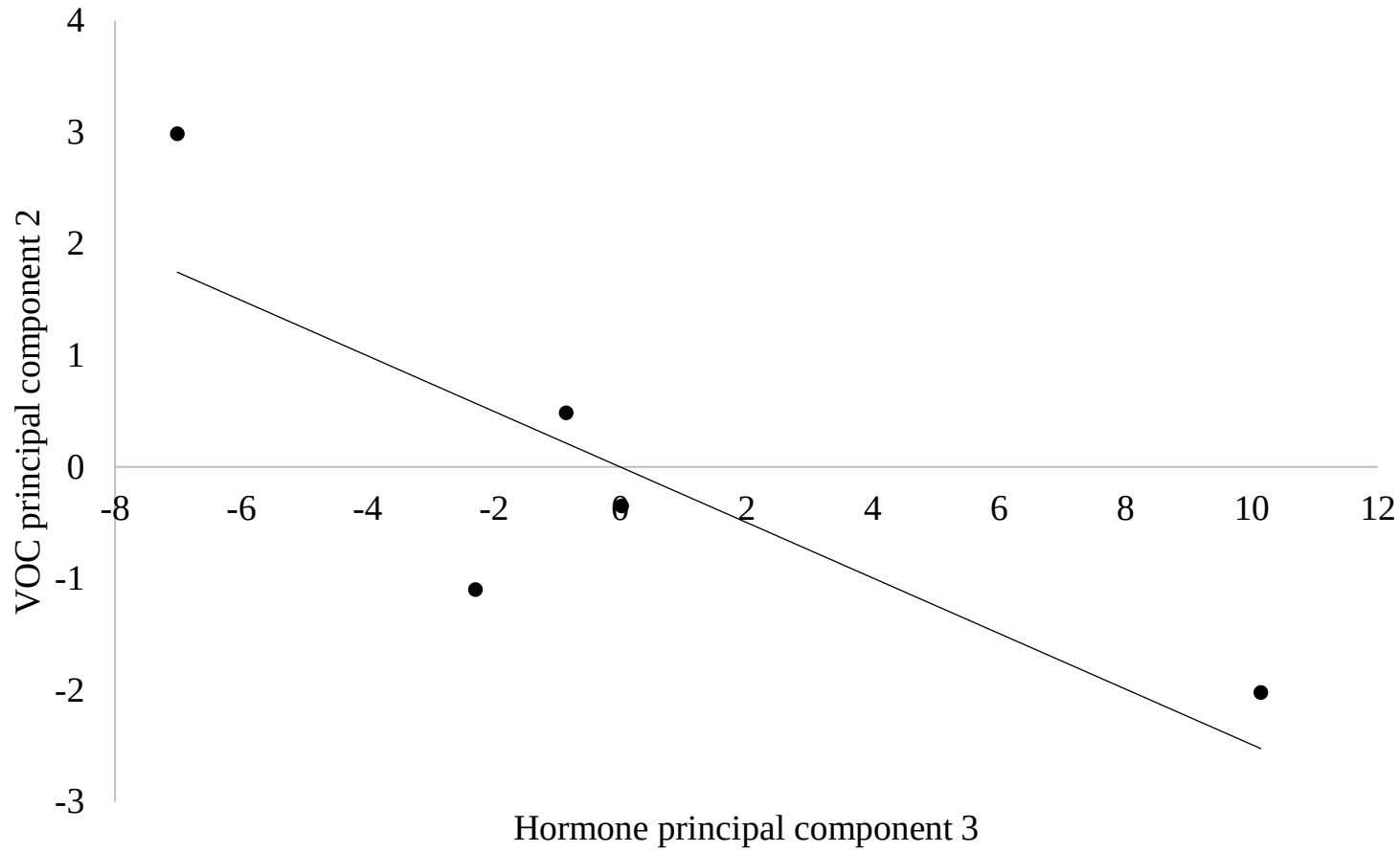


Figure 3.11. Mean (±SE) proportion of *L. daturaphila* larvae that survived to eclosion across sampling periods. Black bars indicate plants that were damaged by two *L. daturaphila* adults for 24 hours prior to the start of the bioassay, and grey bars indicate plants were not previously damaged. Larvae started the bioassay as freshly hatched first instar larvae. Data from period 4 were excluded due to excessive mortality within 24 hours of starting the bioassay. The effect of sampling period was significant ($F_{6,124}$=6.23, p=0.0388), although neither the main effect of treatment ($F_{1,124}$=0.44, p=0.5098) or the treatment by period interactions were significant ($F_{6,124}$=0.52, p=0.7914).

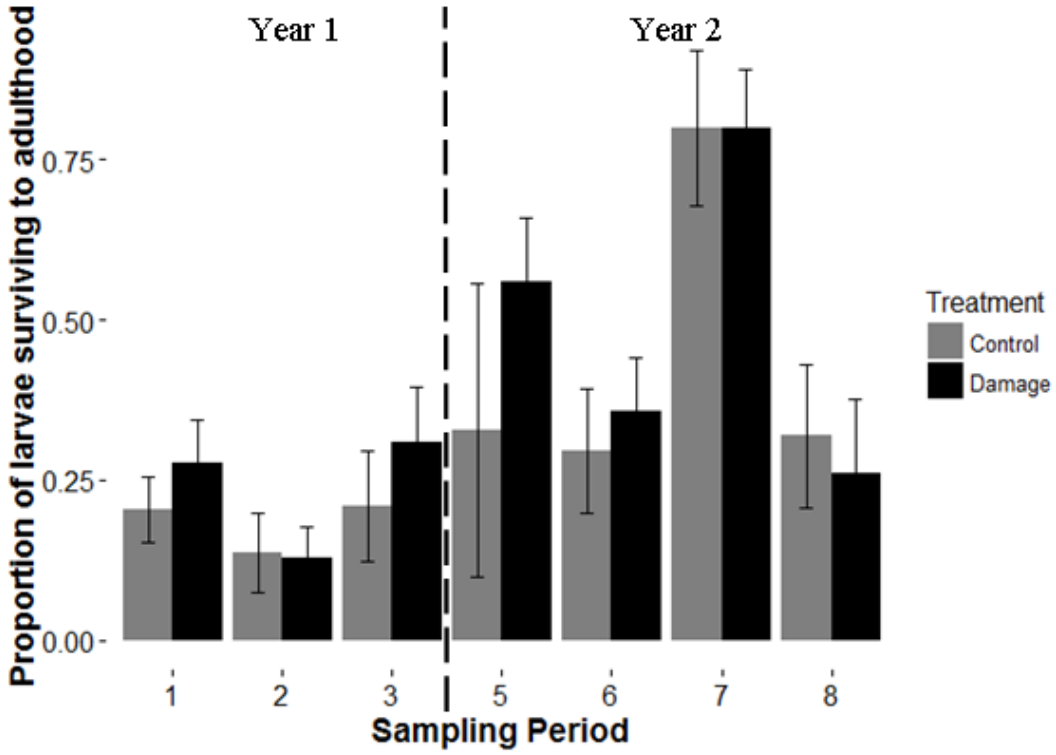
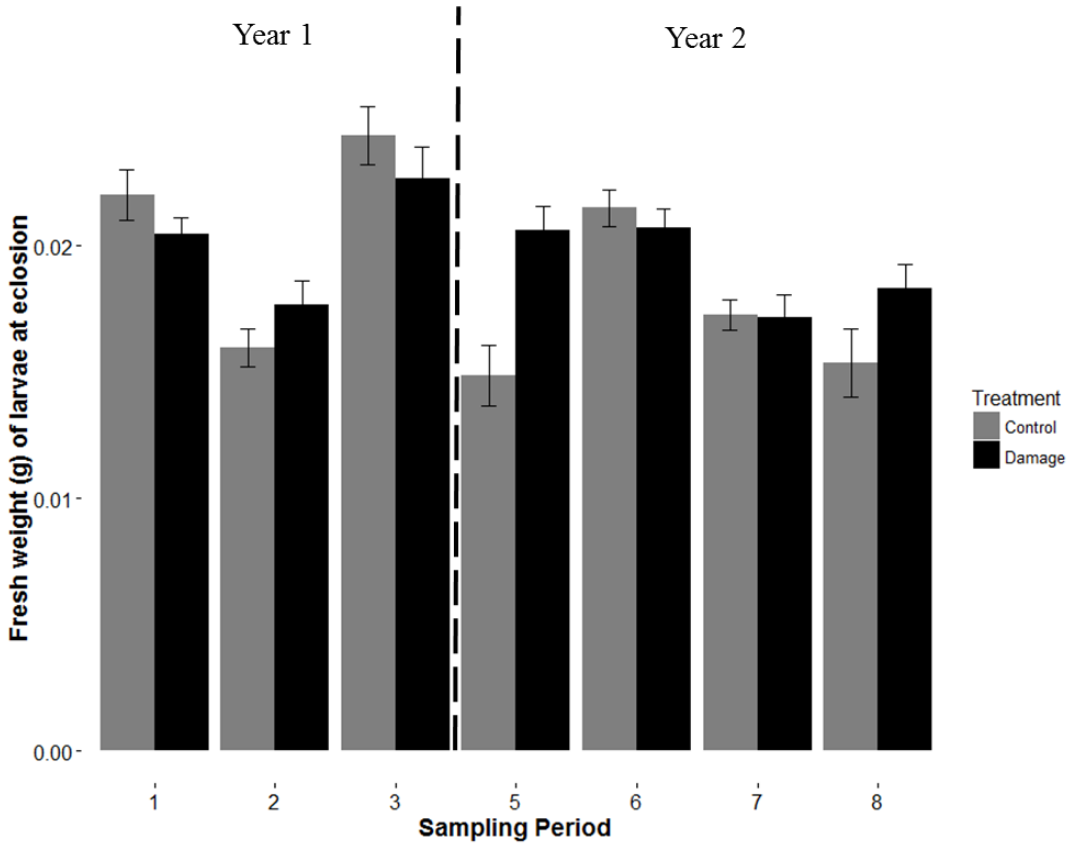


Figure 3.12. Mean (±SE) weight (g) of *L. daturaphila* at eclosion across sampling periods. Black bars indicate plants that were damaged by two *L. daturaphila* adults for 24 hours prior to the start of the bioassay, and grey bars indicate plants were not previously damaged. Data from period 4 were excluded due to excessive mortality within 24 hours of starting the bioassay. Sampling period had a large effect ($F_{6,72}$=9.81, p<0.0001), but treatment ($F_{1,75}$=1.71, p=0.1944) and the treatment by period interaction ($F_{6,72}$=1.86, p=0.1) were both nonsignificant.



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Chapter Four: Ontogeny and Genotype Have Interactive Effects on Induced Responses to Herbivory

Introduction

Variation of traits within a population is a fundamental characteristic of biological systems and a prerequisite for evolution by natural selection. Volatile organic compounds (VOCs) emitted by plants vary tremendously due to both genetic and environmental factors (Hare, 2011). While it may be unsurprising that crop species under artificial selection show variation in herbivore-induced leaf VOCs between cultivars (McCall *et al*.1994; Takabayashi *et al*. 1994; Degen *et* al. 2004; Lou *et al*. 2006; Gouinguené *et al*. 2001; Gols *et al*. 2012), variation in emitted blends is also persistent among genotypes found in undomesticated populations. Horsenettle (*Solanum carolinense*) showed marked variation in herbivore-induced VOCs between 12 genotypes (Delphia *et al*. 2009). A study of *Nicotiana attenuata* showed that HIPV blends differed by orders of magnitude across five accessions, with some accessions emitting twice as many compounds as others (Schuman *et al*. 2009). Blends differed both quantitatively and qualitatively among eight distinct genetic lines from four populations of *Datura wrightii*. In this case, the total volatile blend induced by insect attack was 4-16 times greater than in uninduced plants, depending on the genotype. (*E*)-β-caryophyllene was the most prominent compound released and was shown to comprise roughly 18% up to 60% in insect-damaged plants (Hare, 2007).

VOC emissions that increase after herbivore attack appear to benefit the plant by acting as a within-plant signal (Karban *et al*. 2006; Heil and Silva Bueno 2007; Frost *et al*. 2008). Leaves that perceive such a signal show increased preparedness for herbivore

attack, either by inducing resistance or by priming their resistance mechanisms to produce a faster, stronger response following attack. Although vascular signals are well described for several species, airborne signals provide a benefit when leaves of the same plant are near each other spatially, but vascularly disconnected or distant, as with different branches of a large tree (Orians 2005).

HIPVs also inform carnivorous arthropods (henceforth "natural enemies") of the location of potential prey and allow them to focus searching behavior at the source of emission (Dicke *et al*. 1990). The efficacy of information-based indirect resistance has been demonstrated under field conditions, where the experimental release of authentic compounds led to a 4.9-7.5-fold increase in predation of hornworm (*Manduca sexta*; Sphingidae) eggs by the generalist hemipteran *Geocoris pallens* on *N. attenuata* (Kessler and Baldwin, 2001). Evidence for an adaptive benefit of attracting natural enemies through HIPVs remains circumstantial at best, however, and true tests of Darwinian fitness are lacking (Hare 2011; Kessler and Heil, 2011). Because natural enemies are capable of associative learning, release of any specific blend from plants may be less important than prior experience with successful oviposition opportunities (Allison and Hare, 2009). Furthermore, VOCs broadcast information about the plant, including the location and possibly the identity of the species (Pearse *et al*. 2013). This can lead to negative consequences for the plant if herbivores are attracted to damaged plants (Ballhorn *et al*. 2013). Therefore, although HIPV emission is undoubtedly an important trait mediating tritrophic interaction, it is unclear what evolutionary forces may have led to contemporary variation in VOC emission.

The impact of intraspecific variation in HIPVs on the foraging behavior of natural enemies may depend on the species involved. Several studies suggest that the quantity of blend released is unimportant. When presented with a choice between HIPVs from cowpea (*Vigna unguiculata*) or maize, *Cotesia marginiventris* preferred the cowpea blend, despite its quantitative inferiority (Hoballah *et al*. 2002). Attraction of parasitoids to maize cultivars could not be explained by quantitative variations in the HIPV blend under field or laboratory conditions (Hoballah *et al*. 2002; Degen *et al*. 2012). In *D. wrightii*, however, predation by *G. pallens* was positively correlated with the quantity of volatiles released under field conditions (Hare and Sun, 2011a). Variation in HIPVs may also affect the ability of natural enemies to discriminate between blends. Inexperienced *Diadegma semiclausum* and *D. fenestrale*, Hymenopteran parasitoids, preferred *Brassica oleracea* plants induced by suitable hosts over those induced by non-host herbivores, but could not distinguish host from non-host on *Sinapis alba* or feral *Brassica*. Given an opportunity to learn through repeated successful oviposition experiences, however, *D. semiclausum* was able to learn to distinguish between host and non-host induced feral *Brassica* plants (Gols *et al*. 2012).

The question of whether qualitative or quantitative differences are more important as cues for natural enemies may be obfuscated by the release of compounds that are not perceived. Although *N. attenuata* releases dozens of compounds following herbivory, *cis*-3-hexen-1-ol, linalool, and *cis*-α-bergamotene were each individually capable of increasing attraction of *G. pallens* (Kessler and Baldwin, 2001). Furthermore, the relative ratio of blend components can also have a strong influence on attraction.

Geocoris pallens is attracted to (*Z*) and (*E*) isomers of green leaf volatiles (GLVs), however predator attraction was highest when (*Z*) and (*E*) isomers were released in equal amounts (Allmann and Baldwin, 2010). *D. wrightii* releases at least 60 unique floral VOCs, but attraction of the dominant pollinator in Arizona populations is dependent on only nine of those compounds. Furthermore, although these nine compounds could only be perceived as a mixture, they elicited foraging behavior over a 1000-fold range of dilution (Riffell *et al*. 2009). Disentangling the full function of each blend component is therefore onerous, requiring extensive, factorial testing at various doses.

Given that variation in plant traits exists within native populations and may affect the ecology of at least three trophic levels, it is important to ask how the induction pathways differ between individuals and what traits may covary. Phenotypic plasticity of plants is governed by a small number of phytohormones (Pieterse *et al*. 2009). The majority of the response to chewing insects is mediated by the jasmonic acid (JA) pathway, although this response is sometimes modulated by the activity of other hormones, such as abscisic acid (ABA), ethylene (ET), auxin (IAA), and salicylic acid (SA; Mewis *et al*. 2006; von Dahl and Baldwin, 2007; Wasternack, 2007; Leon-Reyes *et al*. 2010; Robert-Seilaniantz *et al*. 2011; Zhang *et al*. 2013). The SA pathway tends to dominate the response to phloem-feeding insects, although this may be manipulative on the part of the insect, since SA antagonizes JA in some species and prevents the plant from mounting an effective defense (Zarate *et al*. 2007; Ali and Agrawal, 2012). Ultimately, the induced response to herbivores involves a widespread restructuring of the plant transcriptome that may

involve differential regulation of roughly 10% of the entire plant genome (Reymond *et al*. 2004).

In order to coordinate such an extensive phenotypic shift, plants rely on a plethora of signaling molecules. The earliest events leading to an induced response in the plant involve perception of the attack and signaling to downstream structural genes that generate the phenotypic changes (Wu and Baldwin 2009). Plants respond to insect attack after perceiving stimuli that reliably indicate an attack is underway. As a consequence, the plant response to herbivory differs from the response to general mechanical damage (Baldwin 1990; Alborn *et al*. 1997; Halitschke *et al*. 2001; Wu and Baldwin 2009). Responses characteristic of herbivory can be induced by at least two types of elicitors: herbivore-associated molecular patterns (HAMPs) and mechanical damage that resembles herbivore feeding patterns.

HAMPs are chemical signatures that reliably indicate the presence of an herbivore. The first such compound, volicitin, was identified in the oral secretions produced by beet armyworms as they fed on corn seedlings (Alborn *et al*. 1997). Several other such elicitors have since been characterized from the oral secretions of herbivores (Musser et al 2002; Schäfer et al 2011; Tian et al 2012; Louis et al 2013), as well as from oviposition fluids (Hilker and Meiners 2006; Kim *et al*. 2012; Hilfiker *et al*. 2014) and frass (Ray *et al*. 2015). Such chemical elicitors can be applied to mechanical wounds inflicted on plants to induce a response that mimics real herbivory of those plants. In lieu of chemical elicitors, mechanical damage that mimics the feeding patterns of particular herbivores can achieve the same effect (Mithöfer *et al*. 2005).

The early components of the signaling pathway involve rapid changes proximal to the site of attack that disseminate the signal to local tissue. Responses include ion fluxes causing depolarization of cell membranes in the surrounding tissues, production of reactive oxygen species (ROS), and mitogen-activated protein kinase (MAPK) signaling cascades (Wu and Baldwin 2009; Zebelo and Maffei 2015). These signals occur within minutes of wounding and trigger phytohormone biosynthetic pathways (Wu *et al*. 2007), although the specific relationships between these two signaling components are not yet well understood (Zebelo and Maffei 2015).

Many defense responses are dependent on WRKY transcription factors (TFs), a family of proteins unique to plants. The function of the more than 70 WRKY TFs found in some species has yet to be fully detangled, in part because they regulate themselves and other WRKY TFs (Ulker and Somssich, 2004; Eulgem, 2006). In *Nicotiana,* WRKY3 modulates the downstream traits induced by WRKY6, including direct resistance traits such as proteinase inhibitors (PIs) and diterpene glycosides, as well as some VOCs (*cis*-α-bergamotene) but not others (*cis*-3-hexenol; Skibbe *et al*. 2008). In this system, both TFs are required for the full defense response, and the lack of either leads to substantial increases in herbivory under field conditions. Although some WRKY TFs appear to be upstream of JA signaling in *N. attenuata* (Skibbe *et al*. 2008), recent work in *Arabidopsis* suggests that at least several WRKY TFs are downstream of ABA signaling and may represent a mechanism by which plant hormone cross-regulation occurs (Rushton *et al*. 2012).

A comparison of *N. attentuata* and *Solanum nigrum* transcription profiles showed that most induced transcripts were species-specific and very few were differentially expressed in both species (Schmidt *et al*. 2005). Furthermore, major differences between these two species were seen in the signaling pathways involved, with far fewer signaling transcripts differentially regulated in *S. nigrum*. In *N. attenuata*, relative expression of genes from the octadecanoid pathway, which synthesizes JA, was positively correlated with JA. JA, along with SA, were correlated with the quantitative emission of three components of the blend emitted following herbivory. The qualitative and quantitative differences among the remaining 17 compounds comprising the blend could not be explained by these upstream regulatory processes and were attributed to downstream regulatory differences (Schuman *et al*. 2009). Differences in the inducibility of traits were found to be associated with an expression level polymorphism in the accumulation of two mitogen-activated protein kinase transcripts in *N. attenuata* accessions. Reduced expression of these two upstream regulators was associated with reduced JA biosynthesis pathway elements and a dramatic reduction in emission of the volatile *cis*-α-bergamotene, but surprisingly an increase in phenolic compounds and nicotine (Wu *et al*. 2008).

Researchers have gone to great lengths to map biosynthetic pathways that produce secondary metabolites relevant to plant defense. Although specific compounds may differ, plants from many diverse taxa produce many traits with comparable functions (Duffey and Stout, 1996). In some cases, however, traits that are constitutively expressed in one taxon are inducible in another. Leaf trichomes, for instance, are found in many plants, including *Datura*, and yet are inducible in only a few, including cruciferous

species, some tomatoes, *Mimulus* and others (Hare and Walling, 2006; Holeski *et al*. 2010). Similarly, alkaloids are inducible in *Nicotiana* species but not in *Datura* (Baldwin, 1996; Hare and Walling, 2006).

Elucidating the proximate mechanisms underlying such variation can assist in developing hypotheses to explain why variation occurs and how selection on phenotypes operates. The attention given to intraspecific variation has been eclipsed with the advent of "-omics" techniques that emphasize the use of carefully controlled laboratory conditions and isogenic lines in order to tease apart the workings of specific genetic elements. While this effort has led to myriad breakthroughs in the understanding of the relationships between molecular and biosynthetic pathways, these types of studies may underestimate the variation present in natural populations. Ecogenomic approaches seek to integrate molecular techniques into evolved ecological systems in order to study gene function in an evolutionary context (Kant and Baldwin, 2007).

Datura wrightii Regel (Solanaceae) is a perennial species native to the southwestern United States and Mexico. Within southern California populations, *D. wrightii* exhibits two leaf trichome phenotypes, and aspects of the ecology and genetics of the trichome dimorphism have been described elsewhere (van Dam et al. 1999). The "velvety" phenotype is densely covered by short, non-glandular trichomes, whereas the "sticky" phenotype is less densely covered with glandular trichomes that secrete esters of glucose and aliphatic acids. The trichome phenotype is governed by a single locus and is inherited in a Mendelian fashion; the allele for the sticky phenotype is dominant over that of the velvety phenotype (van Dam et al. 1999).

Previous studies have shown herbivore-specific differences in resistance imparted by the two trichome types. The sticky morph confers resistance to *Manduca sexta* larvae, flea beetles, leafhoppers, and whitefly species, but is highly susceptible to the mirid *Tupiocoris notatus*; *L. daturaphila*, used in this study to damage plants, performs equally well on both morphs (van Dam and Hare 1998a; van Dan and Hare 1998b; Hare and Elle 2002). The natural enemy *Geocoris pallens* fares poorly on sticky plants, exhibiting lower predation rates due to difficulties navigating the sticky trichomes on the leaf surface (Gassman and Hare 2005).

The herbivore community that attacks *D. wrightii* consists of approximately 5-10 species depending on location and season (Elle and Hare 2000). *Lema daturaphila*, the most damaging herbivore, is a multivoltine chrysomelid that feeds on both sticky and velvety morphs of *D. wrightii* throughout the growing season as both larva and adult. Damage by *L. daturaphila* increases emission of up to 20 compounds. The blend is composed of GLVs, mono- and sesquiterpenes, with (*E*)-β-caryophyllene, (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, (E,E)-4,8,12-trimethyl-1,3,7,11- tridecatetraene (hereafter TMTT), (*E*)-4,8-dimethyl-1,3,7- nonatriene (hereafter DMNT), and β-selinene typically comprising the bulk of the emitted compounds (Hare 2010; Hare and Sun 2011a; Hare and Sun 2011b). Among several genetic lines of *D. wrightii* assayed, VOC production increases 3.9 to 16.2 times after attack, and the relative abundance of individual compounds varies, with the most abundant compound, (*E*)-β-caryophyllene, comprising 17 to 59% of the blend. This variation has been shown to be heritable. The trichome phenotype has no effect on volatile production (Hare 2007).

When grown in the field, *D. wrightii* plants are highly inducible during the vegetative growth stages early in the growing season, but plants that have reached the reproductive stage cease to emit volatiles in response to herbivory (Hare 2010). When present, the quantity of VOC emission is positively correlated with predation by *Geocoris pallens*, the dominant predator in this system and a generalist that feeds on eggs and young larvae of many insects (Hare and Sun 2011a). The efficacy of VOCs in attracting *G. pallens* is independent of genotypic differences in the composition of blends, although predation is higher on velvety plants (Gassmann and Hare 2005).

Using this system, we asked the following questions: 1) How do intraspecific differences in inducibility manifest at the transcriptional level, and can these differences explain variation in VOC emissions? 2) Does ontogenetic decline in inducibility affect each genotype similarly? 3) Finally, given the ecological differences associated with the two trichome morphs, how do sticky and velvety plants differ in inducibility at the transcriptional level?

Methods

Overview

To characterize interactive effects of genotypic and ontogenetic changes in expression, the plants used in this study were germinated and planted simultaneously, but sampled at two different times throughout the growing season. Three genetic lines of *D. wrightii* were used to compare their response to herbivory. In each sampling period, VOCs were collected from individual plants immediately after 24 hours of herbivore

damage or control treatment. Following the VOC collection, the focal leaves were excised for gene expression analyses. A separate set of plants from a single line were used were used to assess differences in inducibility between trichome morphs. These plants were only assayed for gene expression; no VOC emissions were collected.

Plants

Plants used in this study were from three lines originally collected from natural populations in southern California, as described elsewhere (Hare and Elle 2004). The three lines used here were selected for their quantitatively varied total VOC emissions in a previous laboratory study (Hare 2007). The MVV6 line greatly increases volatile emissions following herbivory, the BCV7 line has relatively low induced emissions, and the UCV9 line has intermediate induced emissions to the other lines (Hare 2007). These lines were developed by backcrossing heterozygous sticky progeny to their original velvety pollen parent for five generations. Because the trichome phenotype is controlled by a single gene, crossing heterozygotes with the velvety recessive homozygotes results in an expected 1:1 ratio of sticky to velvety offspring. The method of backcrossing within the line results in sibs that are expected to be 98.4% similar after five generations, except at the trichome locus (Hare and Sun 2011a).

Seedlings were allowed to germinate and grow under greenhouse conditions until they had 8-10 leaves and could be transferred to the field. The greenhouse was equipped with high-pressure sodium lamps providing supplemental illumination for 14 hr/d so that

midday light intensities averaged 1250±39 μmol m−2 s−1 PAR illumination at plant height. Greenhouse temperatures ranged between 15° and 35°C (Hare and Sun 2011a).

Plants were transplanted to the field on 8 May, 2015. Prior to planting, the field was treated with a pre-emergence herbicide (Trifluralin at the rate of 668 ml/ha) to suppress natural weed growth. Furrows were irrigated for 24 hours directly before and after transplanting to allow plants to establish after normal winter rains had ceased. Plants were irrigated one final time on 17 June, 2015, one month after planting. There was no rainfall during the 2015 growing season, typical of sage scrub communities.

Plants were arranged in blocks of four individuals. Half the individuals within each block had the sticky phenotype, and half were velvety. Plants within blocks were 0.76 m from their nearest neighbor. Blocks were a minimum of 1.5 m from any other block. At the University of California experimental farm where this study took place, prevailing winds consistently travel from west to east, and control plants were planted at least 3 m west of treatment plants in order to prevent any possible induction or priming due to airborne damage signals (Kruidhof *et al*. 2012). Each plant was only sampled once per season.

A separate set of plants was used for the trichome phenotype comparison. These plants consisted of 36 sibling plants from the MVV6 line. Eighteen displayed the sticky phenotype, while the remainder were velvety. Plants were arranged in blocks of three individuals that alternated trichome type.

Treatment

Sampling was conducted during two time periods, during July and September of 2015. Prior to sampling, plants were treated with biweekly applications of acephate (Orthene 97, Valent Chemical Co., Walnut Creek, CA, USA 1.2 g/L) in water to prevent natural herbivory. Treatment with this insecticide has no effect on gene expression or induced resistance (described in Chapter 3). Acephate treatment was withheld on plants for two weeks prior to sampling to allow herbivores to feed and resumed afterwards.

Young, fully-expanded leaves were chosen on each of 54 plants at the start of each sampling period. Within each block of four plants, three plants were used for sampling based on overall health and lack of herbivore damage. Due to limits on number of possible concurrent volatile collections, as well as the need to sample early in the day to minimize heat stress associated with the sampling technique, samples within each collection period were collected over two days. Four or five plants within each genotype by treatment combination were sampled on each date, to avoid confounding the effect of sampling date with any particular treatment level. Two adult *L. daturaphila* were caged on focal leaves of the treatment group using fine mesh bags. Control plants received empty mesh bags. Insects were caged on leaves for 22.5 hours and then cages and insects were removed. *L. daturaphila* used in this study were reared on greenhouse-grown *D. wrightii* in an insectary; this stock population was periodically supplemented with field-collected individuals to minimize effects of inbreeding.

For the trichome study, plants from half of the blocks in each trichome group received the 24-hour damage treatment on a single leaf, while the other half received empty mesh bags. All of the plants were sampled in a single day.

Volatile collection and analysis

VOCs were collected non-destructively from all focal leaves one day after treatment was terminated, using a push-pull collection system described previously (Hare 2010). Briefly, aeration chambers were constructed from polyester cooking bags (unprinted 45 x 55 cm, Terinex, Bedford, England), placed over individual leaves, and secured using a twist tie. Intake and exhaust ports were created in the chambers using gastight fittings (Swagelok, San Diego, CA, USA). Two 12-V portable air pumps (Model # MOA- P125-JH, Gast Manufacturing, Benton Harbor, MI, USA) powered by a 12-V deep-cycle marine battery, created a "push-pull" airflow scheme regulated by flow meters (Aalborg, Orangeburg, NY, USA) to ensure a constant rate of 1.0 L/min for each leaf. Air was filtered through activated charcoal and flowed to the chamber through PTFE tubing (4.6-mm O.D.). Air flowing out of the chamber passed through a glass trap containing Super-Q (25 mg, Alltech, State College, PA, USA), an adsorbent used to collect volatiles, and subsequently through PVC tubing. VOCs were collected from 16 plants concurrently, with two 90-minute collections per day occurring from 9-10:30 and 11:30-13:00. Plants included in each collection were divided equally between control and treatment groups. At the end of each collection, traps were wrapped in aluminum foil, returned to the laboratory, and frozen at -20° C for extraction later that day.

Volatiles were eluted from the traps with 150 µl of CH_2Cl_2 containing 4 ng/µl of n-bromoheptane (Sigma-Aldrich) as an internal standard into autosampler vials with 250-µl glass inserts, and vials were sealed with crimp caps and PTFE-lined rubber septa. Samples were analyzed by gas-liquid chromatography as described in Hare and Sun (2011b). Peaks were quantified in units of ng g^{-1} leaf (dry wt.) hr^{-1} using Agilent ChemStation® software based on comparison of the peak height of each VOC component with that of the internal standard.

Gene expression

Gene expression was measured in the leaves 24 hours after the initiation of damage. Immediately following collection of the VOC sample, focal leaves were excised and flash frozen in liquid nitrogen. To accommodate the requirements for robust expression analyses, leaf samples from three plants in each block were pooled to form one biological replicate, leaving three replicates per genotype by treatment level.

RNA was extracted from leaf tissue of pooled replicates using Qiagen RNEasy Plant Mini Kit following manufacturer instructions. RNA concentrations were confirmed by NanoDrop spectrophotometer prior to final concentration and integrity check using Agilent 2100 Bioanalyzer courtesy of University of California, Riverside's Institute for Integrative Genome Biology. RNA-Seq libraries were prepared using NEBNext Ultra RNA Library Prep Kit for Illumina according to manufacturer recommendations. Six individual samples were multiplexed per lane using compatible index primers and

sequenced using an Illumina HiSeq 2000 sequencing system using single-end 50-bp reads.

After trimming the index primers from each read, sequencing data was aligned against the *D. wrightii* reference transcriptome, developed as described in Chapter 2, with Mosaik (Lee *et al* 2014), using a maximum mismatch threshold of four. The edgeR package in R was used to compare gene expression in the treatment and control groups using the exact test for the negative binomial distribution (Robinson and Smyth 2007; Robinson and Smyth, 2008; Robinson *et al*. 2010; McCarthy *et al*. 2012; Zhou *et al* 2014). Gene expression greater than ± 2 log-fold changes compared to controls were considered differentially expressed using a false discovery rate of ≤ 0.05 (Reymond *et al* 2004). Similar pair-wise comparisons were also made using control samples from each genotype, to compare baseline expression levels, as well as with samples from damaged leaves to determine differences among induced plants. In order to simultaneously compare all samples, a hierarchical cluster of scaled count data was also created using the clustering function in JMP Pro 12.

RNA-Seq data was validated using quantitative PCR (qPCR) for the following genes, identified by their sequence similarity to annotated tomato genes: *hydroperoxide lyase 1* (*HPL1*), *phenylalanine ammonia lyase* (*PAL*), *terpene synthase 12* (*DwTPS12*), *lipoxygenase 2* (*LOX2*), and *RuBPCase small chain 2B* (*RUB*), with *actin* (*ACT*) and *ubiquitin* (*UBI*) chosen as reference genes. Primers were designed using Primer3 (Koressaar *et al.*, 2007; Untergasser *et al.*, 2012) and assayed for efficiency in triplicate

with cDNA templates diluted over five orders of magnitude (Table 4.1). Melting curves for each primer used in this study produced a single, sharp peak.

To create cDNA, total RNA extracted during the procedure described above was first treated with RQ1 DNase (Promega, Chicago, IL, USA) to remove any remaining DNA. DNase was inactivated and cDNA was synthesized using oligo(dT) primer (Promega), SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), dNTPs (Sigma Aldrich, St. Louis, MO, USA), and RNase inhibitor (Promega). qPCR was performed in triplicate using SYBR Green master mix (Bio-Rad, Los Angeles, CA, USA) and 96-well optical plates (Bio-Rad) in a Bio-Rad iQ5 Real-Time Detection System, with PCR conditions as follows: denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 60 s, and a melting curve that showed each experimental sample yielded a single, sharp peak at the amplicon's melting point. Expression values were calculated using $2^{-\Delta\Delta Ct}$ to find the log fold change in expression in the treatment group (Pfaffl 2001).

Statistics

VOC emissions rates were calculated based on the weight of the leaves after they were flash frozen. Dry leaf weight was determined by dividing frozen tissue weight by 3.079, a figure derived by comparing the proportional difference in weight loss when leaves are oven-dried vs. flash frozen in liquid nitrogen. VOC emissions were $\log_{10}(x+1)$ transformed to ensure normality of errors and accommodate samples for which no volatiles were detected. Total VOC emissions were analyzed using a mixed model

analysis of variance (ANOVA) in JMP Pro 12, with treatment, sampling period, genotype and their interactions as fixed effects; block and plant nested within block, and sampling date were random effects. One-way ANOVAs were used to test each genotype for significant increases in emission within sampling periods.

Principal components analyses (PCAs) were used to explore the relationship between gene expression and VOC emission. Gene expression and VOC emission data have important structural differences that make comparative analyses difficult. Although the samples originated from the same set of plants, the number of effective biological replicates differed. Rigorous analyses of gene expression require pooling of tissue from multiple plants to form a single biological replicate; therefore, although VOC emissions were collected from each plant individually for a maximum of 18 replicates in each genotype by sampling period group, gene expression produced a single value for each gene at each sampling period. Furthermore, unlike VOC emissions data, gene expression is a relative value based on the difference between the treatment and control sample, and all control samples are effectively assigned a value of zero. To accommodate these differences, VOC emission data were transformed into a relative value: for each individual compound, the average emission of control plants in each sampling period was subtracted from the average of the treatment group, producing a single value.

Both relative VOC emissions data and specific gene groups were separately subjected to PCAs, creating fewer, uncorrelated variables based on the covariance and correlation matrices of individual compounds and genes, respectively. The factor loadings show the contribution of each original variable to the new component, and factor

scores for the new components are calculated for the original observations. In order to assess the contribution of subsets of genes with presumed roles in induced plant resistance, PCAs were conducted on terpene synthase genes and various combinations of hormone biosynthesis genes, including JA; JA and ET; JA and ABA; JA, ET, and ABA; JA, ABA, and SA; JA, ET, ABA, and SA; and, finally, JA, ET, ABA, SA, IAA, and CK. Principal components derived from these analyses were then regressed against both the total induced emissions, as well as principal components of the VOC emissions.

Results

Volatile emissions

Thirteen unique compounds were detected in at least one sample across all treatments, genotypes, and sampling periods of the study. The monoterpene linalool and the green leaf volatile *trans*-2-hexenal comprised the bulk of the VOCs detected. (*E*)-β-caryophyllene, (*E*)-β-ocimene, DMNT, methyl salicylate, *cis*-3-hexen-1-ol, hexenyl acetate, and decanal were detected in intermediate quantities. Geranyl acetone and limonene were emitted in trace quantities.

These collections show significant effects of treatment ($F_{1,92}$=7.58, p=0.0071; Fig. 4.1) but not sampling period or genotype (both p>0.5). The genotype by treatment interaction was highly nonsignificant ($F_{2,87}$=0.79 p=0.46). The treatment by sampling period and genotype by sampling period interactions were both slightly nonsignificant ($F_{1,89}$=3.13, p=0.081 and $F_{2,89}$=2.53, p=0.085, respectively). Decomposing the treatment by sampling period interaction shows that the treatment effect was significant in the early

sampling period ($F_{1,89}$=10.34, p=0.0018) but not in the later period ($F_{1,89}$=0.48, p=0.49). Random effects did not significantly improve any of the models and were not used. One-way ANOVAs shows significant increases in VOC emissions from BCV7 and UCV9 in the early sampling period (both $p<0.012$), while all others were nonsignificant ($p>0.05$).

Gene expression

Overall, 931 unique genes were differentially regulated over the course of this study (see supplemental table 3). Across both sampling periods, the UCV9 genotype altered the expression of more genes in response to herbivore damage than either of the other genotypes, with 330 and 221 genes induced and 92 and 43 genes suppressed in the early- and late- season samples, respectively. The MVV6 line showed similar expression in the early season sample, with 313 genes induced and 35 suppressed. This line differed from the former in the late-season sample, when only 38 genes were induced and 8 were suppressed. Finally, a minimal response was recorded for the BCV7 line. Early in the season, 47 genes were induced and 18 were suppressed, and late in the season 35 genes were induced and only one was suppressed.

There was very little overlap between DEGs collected from different samples (Fig. 4.2). Samples clustered primarily based on a lack of altered expression, showing that late-season samples from the MVV6 and BCV7 lines were most similar simply because both had very few DEGs. To circumvent this shortcoming, Venn diagrams of induced and suppressed genes were used to better examine the similarity of samples based on expression (Fig. 4.3). Among early-season samples, only 30 were induced

across all three genotypes. MVV6 and UCV9 commonly increased expression of 62 additional genes, but the vast majority of induced genes in those samples were unique to samples from those lines. BCV7 and MVV6 shared 14 induced genes. Only three genes were uniquely induced in the BCV7 line, and none were shared only by BCV7 and UCV9.

Similarly, only four genes were suppressed in all three early-season samples. The MVV6 line uniquely suppressed 19 genes, shared 11 with UCV9, and only shared one with BCV7. Twelve genes were only suppressed in BCV7, which shared only one with UCV9. UCV9 had the largest share of uniquely suppressed genes, with 76 in total.

Among late-season samples, only five genes were induced in all three lines, 20 were commonly regulated in MVV6 and UCV9 and 19 were induced in both BCV7 and UCV9. These figures represent more genes than were uniquely induced in either BCV7 or MVV6. Only one gene was shared by MVV6 and BCV7 that was not induced in UCV9. For suppressed genes, all genes detected were unique to the sample in which they were detected, except for one gene that was common to BCV7 and UCV9.

Within lines, there was little overlap between genes induced early in the season and those induced later in the season (Fig. 4.4). Of the 551 DEGs detected in the UCV9 line, only 66 were differentially expressed both early and late in the season. For the MVV6 line, 24 of the 38 genes induced later in the season were also induced in the early sample. The BCV7 samples had very little overlap: only five of the 77 DEGs were expressed in both samples. For suppressed genes, both the UCV9 and MVV6 lines had

only one gene commonly expressed in early and late samples, while none were commonly suppressed in the BCV7 line (Fig. 4.5).

Although disparate pathways were represented among the 30 genes induced in all three lines early in the season, a few redundancies were noted (See Table XKCD1). Three genes putatively code for enzymes involved in rate-limiting steps of the ET pathway (discussed below), an important modulator of JA-mediated resistance traits (Adie *et al*. 2007). In addition, two protease inhibitor precursor genes and one polyphenol oxidase (*PPO*) genes were also consistently induced. Protease inhibitors are common resistance traits that may decrease herbivore digestion, and PPO enzymes can activate phenolic compounds that reduce the palatability of leaves for attacking herbivores (Duffey and Stout 1996); further work will be required to determine whether these particular genes contribute to such resistance traits. Four genes detected in all early samples are *TPS* genes (*DwTPS1*, *DwTPS11*, *DwTPS13*; discussed below), which are commonly induced following herbivore attack (e.g. Falara *et al*. 2011). Three genes putatively code for the beta subunit of tryptophan synthase and are involved in amino acid synthesis, and three other genes code for UDP-glucosyl/UDP-glucoronosyl transferases that are involved in carbohydrate biosynthesis (Barber *et al*. 2006). It is unclear whether the redundant differential expression of these genes holds significance for plant resistance or occurs for incidental reasons.

DEGs were separated into functional categorizations based on assigned pathways identified using MAPMAN (Fig. 4.6; Thimm et al. 2004). Excluding genes of unknown function, the pathways showing the greatest number of induced changes include cell

maintenance, protein metabolism, signaling, and transcription. Despite pervasive differences in the identity of the induced genes among samples, the relative proportion of genes assigned to each pathway remained relatively consistent across the UCV9-early, UCV9-late, and MVV6-early samples. For the remaining samples, the pattern was less consistent across pathways, likely due to the low overall number of DEGs.

There was less consistency of samples across pathways for suppressed genes (Fig. 4.7). UCV9-early had the most suppressed genes overall, and showed correspondingly greater numbers of suppressed genes across most categories. Notably, UCV9-early showed fewer suppressed genes than at least one other sample in several categories, including primary metabolism, cell wall modification, and transport. For the signaling categories, the number of suppressed genes was roughly equal to those of the UCV9-late sample. Notably, the UCV9-late sample showed no suppression of genes in the photosynthesis pathway, but a greater number of genes related to transport relative to other samples. No samples showed suppression of genes related to secondary metabolism. Overall, the distribution of DEGs across functional categories suggests a general consistency across genotypes, but also a small number of notable exceptions.

Nine *TPS* genes were induced over the course of the study (See Fig. 4.8 and Table 4.2 for descriptions). None were suppressed. Early samples from MVV6 and UCV9 showed induction of all nine genes, while BCV7 increased expression of only four. The UCV9-late sample showed expression of five *TPS* genes, while the MVV6-late sample failed to induce any. The BCV7 line showed only two induced *TPS* genes late in the

season. Within each line, more *TPS* genes were expressed early in the season, but each line showed different patterns of expression.

Phytohormone expression also showed little consistency across samples (Fig. 4.9). The MVV6 line showed widespread induction of the JA pathway in the early sample, with 14 genes upregulated, including four putative *LOX* genes, four *allene oxide synthase* (*AOS*) genes, one *HPL* gene, one *allene oxide cyclase* (*AOC*) gene, one *12-oxophytodienoate reductase* (*OPR*) gene, and three jasmonate-ZIM-domain protein (JAZ) coding genes. This differential expression was entirely absent later in the season. The UCV9-early sample showed a surprising pattern, inducing only three JA-related genes, including a *LOX*, an *AOS*, and a *JAZ* gene also upregulated in MVV6-early. UCV9-late expression included four genes, an *AOC*, *AOS*, and two *LOX* genes, none of which were induced in the early-season sample from that line. The BCV7-early sample upregulated only one JA-related gene, a *LOX* gene, in the early sample, but no genes at the later timepoint.

The UCV9-early sampled showed increased expression of ten ET-related genes, out of a total of 16 detected in this study. Four were detected in that line later in the season. The MVV6 line only induced six in response to damage early in the season, and this number decreased to a single gene at the later timepoint. BCV7-early showed increased expression of five ET genes as well, although only one overlapped with those expressed by MVV6. BCV7 failed to express these genes later in the season.

Of the thirty genes commonly induced by all three genotypes in the early sampling period, the only three that were related to hormone biosynthesis all belonged to

the ET pathway. These include two putative 1-aminocyclopropane-1-carboxylate (ACC) synthase (*ACS*) genes and a gene moderately similar to 1-aminocyclopropane-1-carboxylate oxidase (*ACO*), according to designations assigned by MapMan. This latter gene was also induced in the UCV9-late sample. The remainder of the ET pathway showed little consistency between samples: out of the 16 ET-related DEGs detected in the study, 11 were unique to a single sample. Additionally, six of these uniquely expressed genes were detected in the UCV9-early sample.

Four abscisic acid genes were induced in the study: two xanthine dehydrogenase (*XDH*) genes, one abscisic aldehyde oxidase (*AAO*) gene, and one ABA-responsive GRAM domain-containing protein coding gene. The UCV9-early sample induced both *XDH* genes and the *AAO* gene. The UCV9-late sample induced one *XDH* gene, the *AAO* gene, and the GRAM domain-containing protein coding gene. Only one other sample induced any abscisic acid-related genes: the MVV6-late sample induced one of the *XDH* genes. Only one gene from the SA pathway was detected in this study; it was induced in both UCV9 samples, but no others. The only gene related to cytokinin (CK) biosynthesis was a cytokinin oxidase/dehydrogenase gene induced in the UCV9-early sample. Two genes putatively coding for gibberellin-responsive genes were also detected: one of which was suppressed in both UCV9 samples, and BCV7-early samples, but induced in the MVV6-early sample; the other was found only in the UCV9-early sample. All five auxin-related genes detected in this study were differentially regulated in the UCV9-late sample. Two of these genes were suppressed rather than induced; one of these was also suppressed in UCV9-early. One of the induced genes was also induced in MVV6-early.

Taken together, the phytohormone data suggest very different responses of each phytohormone pathway among the three genetic lines early in the season, and a high level of expression in the UCV9 line late in the season that differed almost entirely from the pattern observed in that line earlier in the season.

Gene expression represents a relative value; to assess the possibility that differences in induced expression between genotypes is due to differences in constitutive expression, comparisons were drawn across control and induced samples from all genotypes using both pairwise comparisons and hierarchical clustering. Among early season samples, striking differences were apparent comparing the MVV6 line to the other two (Table 4.3). The MVV6-early control vs BCV7-early control comparison revealed that the BCV7 line had higher constitutive expression of 542 genes, while the MVV6 line had higher expression of 171 genes. The MVV6-UCV9-early control comparison was less pronounced, with UCV9-early control samples showing 66 genes with higher baseline expression, compared to 19 in MVV6. Little difference was seen between early control samples from BCV7 and UCV9. Among damaged samples from the early sampling period, the least difference was seen between MVV6 and BCV7, with 102 and 94 genes showing higher expression in those lines, respectively. Intermediate differences were seen between BCV7 and UCV9, with BCV7 having higher expression of 127 genes and UCV9 having higher expression of 182. UCV9 showed higher expression of 839 genes compared to the MVV6 line, which in turn showed higher expression of 410 genes. Taking into account the number of DEGs that were detected for each of these lines, these comparisons indicate that the difference in DEGs between the BCV7-early and MVV6-

early samples is likely due in part to a higher constitutive expression of genes in the BCV7 line. The UCV9 line may have a level of constitutive expression intermediate to the other two lines, since the control samples do not greatly differ from either of the other two lines. Among induced genes, however, the widespread differences between the induced phenotypes of MVV6 and UCV9 are corroborated by the pairwise comparison of treatment samples.

All late-season pair-wise comparisons between control samples showed greater differences between lines than the corresponding early-season comparisons. Additionally, the differences between control samples were greater than the differences between treatment samples. Despite low numbers of DEGs and only six commonly expressed genes in the MVV6 and BCV7 late-season samples, these lines showed extensive differences in baseline expression without corresponding differences between damaged samples. More than 400 genes were expressed at higher levels in one line or the other in control samples, yet only 86 and 56 were expressed at greater levels in MVV6 or BCV7 treatment samples, respectively. This pattern may indicate that the lack of differential expression was due to greater variation in treatment samples that prevented differences from reaching statistical significance. BCV7 and UCV9 showed greater expression of 132 and 139 genes in control samples, and a similar 176 and 171 genes in treatment samples, respectively, despite fairly large differences in induced gene expression patterns between the lines. The largest differences were between MVV6 and UCV9. Control samples showed 465 and 642 genes expressed at higher levels in MVV6 and UCV9, respectively, but only 197 and 226 genes expressed at greater levels in

treatment samples. These data suggest that differences in the induced transcriptional patterns can at least partially be explained by differences in baseline levels of expression.

Hierarchical clusters of scaled transcript count data are used to simultaneously examine the overall similarity of samples from each treatment by genotype group (Fig. 4.10). Notably, the first bifurcation separates two of the MVV6-early control samples and all three UCV9-late control samples from all other samples. Among these remaining samples, two clades are apparent: one which contains early-season samples, and one which contains late-season samples. BCV7-early control and induced samples are spread among clusters of the other genotype samples, suggesting that the low expression values detected from this line may be due to a relatively greater variability. UCV9-early control samples are distributed similarly, although extensive induction was detected in this case. The only early season samples that cluster relatively close to each other are the MVV6-early induced samples and the UCV9-early induced samples.

Among late-season samples, clusters of samples from genotype-by-treatment groups are much tighter. MVV6 treatment and control samples are all grouped together, corroborating the lack of expression in this line. Induced UCV9 samples also cluster together, and this cluster occurs near the BCV7 control samples. Interestingly, although the location of the BCV7 control samples near the induced UCV9 samples suggests that the lack of expression in these samples may be due to high baseline expression, two of the induced BCV7 samples clustered with the MVV6 samples, which showed little induction. Ultimately, the lack of induction detected in BCV7 samples from both early-

and late-season samples appears at least partially attributable to variation in samples from damaged plants.

Comparison of gene expression between MVV6 trichome phenotypes

The velvety phenotype induced roughly three times more genes in response to herbivory compared to the sticky phenotype (Fig. 4.11). Specifically, the velvety phenotype induced 1,349 genes in response to herbivory while suppressing 113, while the sticky phenotype induced 343 and suppressed 24. Pairwise comparisons of control and induced samples across phenotypes, however, suggested that the differences may not be as substantial as they seem. Between control samples, sticky plants had higher expression of 11 genes, compared to nine in velvety plants (Table 4.4). Among induced samples, only five genes were expressed at higher levels in sticky plants, and 12 showed higher levels in velvety plants (Table 4.5). In both comparisons, the genes belonged to a variety of pathways, including cell maintenance, photosynthesis, primary metabolism, protein metabolism, signaling, stress, transcription, transport, and several with unknown functions (Fig. 4.12). Insect damage increased expression of 11 *TPS* genes in the velvety phenotype; three of these genes were also induced in the sticky phenotype, and one additional gene was suppressed in sticky plants (Fig. 4.13; see table 4.6 for descriptions). None of these genes, however, were differentially expressed in the treatment and control comparisons. Taken together, these results indicate that the differences in inducible gene expression between the two phenotypes are primarily quantitative differences. Genes that were differentially induced in the velvety plants but not the sticky plants were also likely

near the threshold in sticky plants, or more genes would have been identified as differentially expressed in the comparison of treatment samples. This is not to say that the difference in gene expression between the trichome phenotypes is negligible, but rather that the difference is not qualitative. Studies combining the two phenotypes are likely to underestimate the extent of differential expression because of the increase in variation.

qPCR

Overall, qPCR techniques confirm the expression levels of genes as measured by RNA-Seq, recording mean expression levels that were, with few exceptions, quantitatively very similar to the RNA-Seq results (Figure 4.14). *HPL1* expression was elevated only in MVV6-early according to RNA-Seq, although several elevated, but non-significant results were recorded for other samples. BCV7-early and MVV6-late samples recorded no data for this gene. qPCR results were quantitatively very similar in the remaining samples, although RNA-Seq detected a lower, nonsignificant result for *HPL1* in UCV9-late compared to qPCR analysis. RNA-Seq expression values for *LOX2* matched qPCR values very closely for the *LOX2* gene, although no data was recorded for BCV7-early. No significant RNA-Seq values were detected for the *PAL* gene in this study, although not all samples reported results. Similarly, all of the qPCR results showed low or highly variable expression. The *RUB-SSU* gene showed minimal expression changes in this study. RNA-Seq showed significant, negative expression in the UCV9-early sample, which is confirmed by qPCR. All other samples showed very

slight, negative expression with qPCR, except for MVV6 late, which showed a mean positive expression value with very high variance. For the *DwTPS12* gene, the MVV6-early sample showed a strong, positive expression from both methods. For the UCV9-early and MVV6-late samples, there was some disagreement, with qPCR showing relatively lower and higher expressions, respectively. No RNA-Seq values were recorded for the remaining samples, but qPCR showed low expression values for these samples.

Explaining variation in VOC emissions

Variation in total induced volatiles is best explained by the PCA that included JA, ET, and ABA. Within that PCA, PC 4 accounted for only 8.8% of the variation (Table 4.7). A regression of total induced volatile emissions from all six samples against PC4 showed a large explanatory power, though the relationship was not statistically significant (R^2=0.594, p=0.0728; Fig. 4.15). Other PCs from this analysis were poor predictors of VOC emissions (all R^2<0.32, p>0.24). Six of the eight genes that load most heavily on PC4 are from the ET pathway. Their putative identities are *ERF DOMAIN PROTEIN 9* (*ERF9*), *ACO*, three *ACS* genes, as well as one gene weakly resembling an ethylene responsive transcription factor that loaded negatively on the PC. In addition, a *LOX* gene from the JA pathway loaded positively on this PC, and a *XDH* gene from the ABA pathway loaded negatively.

The individual compounds that make up the VOC blend were also subjected to a PCA to test the ability of the various gene expression models to predict correlated subsets of the emitted blend. PC1 of the VOC PCA comprised 42.8% of the variation in the

volatile emissions (Table 4.8). *Trans*-2-hexanal and (*E*)-β-caryophyllene loaded positively on this PC, while geranyl acetone and, to a lesser extent, hexenyl acetate loaded negatively. Variation in PC1 of the VOC model was best explained by the third PC of a PCA of that included phytohormone-related genes from the JA, ET, ABA, SA, IAA, and CK pathways (R^2=0.743, p=0.0273; Fig. 4.16). PC3 of this model accounts for 17.9% of the variation in expression of these genes (Table 4.9). Genes from the JA, ET, and ABA pathway had strong positive loadings on the model, while two auxin genes loaded negatively along with several ET-related genes.

Discussion

Herbivory induced transcriptional profiles that varied considerably across genotypes, and ontogenetic changes associated with waning VOC emission did not eliminate these differences. Induced volatile emissions were detected from the UCV9 and BCV7 lines, but not the MVV6 line, in the earlier sampling period. In the later sampling period, none of the lines increased volatile emissions in response to herbivory. In contrast, the number of induced genes detected varied considerably between the genotypes. The UCV9 line induced a large number of genes in both sampling periods, while the BCV7 line showed very little differential expression. The MVV6 line, however, differed from the others in that gene expression was extensive in the first sampling period but not later in the season. Within lines, few genes were differentially regulated in both sampling periods, even in the UCV7 line, which showed similar overall numbers of DEGs in both periods.

Volatiles

The overall quantities of volatile emissions detected in this study were considerably lower than previous studies (Hare 2010; Hare and Sun 2011a), primarily due to the shorter interval between the initiation of herbivory and volatile sampling. Differences between the two sampling periods confirm the previously observed trend that inducibility seen in juvenile *D. wrightii* plants declines as they begin to flower and produce fruit. The lack of inducible volatiles in the early-season sample from the MVV6 line, which is known to emit increase VOC emissions after herbivory, suggests that these plants had already progressed into the later ontogenetic stage.

Trichome phenotype affects inducibility

Herbivore attack induced roughly three times as many DEGs in the velvety plants compared to sticky plants, suggesting that the trichome phenotype may impart a greater sensitivity in plants with primarily non-glandular trichomes. The comparisons between control samples and treatment samples, however, show that this difference is primarily quantitative: only 20 genes showed differential expression between undamaged plants expressing the two trichome types, and 17 were expressed differently between damaged plants. This analysis suggests that the difference between plants with the trichome phenotype is primarily quantitative rather than qualitative.

Through gene linkage or pleiotropic effects, the gene coding for glandular trichomes affects the life history of *D. wrightii*, with sticky plants tending to favor vegetative growth and investing less into reproduction than velvety plants (Elle *et al*.

1999). It was hypothesized that the differences in the ecology associated with this morph might lead to a suite of differences representing an alternative strategy. For instance, the decrease in natural enemy performance on sticky plants might be accompanied by lower VOC emissions, since the attraction of natural enemies would provide less benefit on those plants.

This study fails to show strong support for the alternative strategy hypothesis. Previous work showed no differences in VOC emission between siblings from the same backcross line but differing in trichome morph (Hare 2007). Here, the difference between trichome types suggests no major qualitative differences in their induced phenotypes, but more work will be needed to identify the functions and ecological effects, if any, of the few genes that showed consistent differential expression between the two morphs. The quantitatively greater expression of many genes in the velvety phenotype, however, adds to the list of traits affected by the trichome locus through pleiotropy or gene linkage.

Induced transcriptional profiles differ across genotypes

Intraspecific variation in phenotypic traits in general, and plant resistance traits in particular, is well-documented and is a basic premise of evolution by natural selection (Darwin 1880; Gouinguené *et al*. 2001; Zangerl and Berenbaum 2003; Hare 2007; Wu *et al*. 2008; Schuman *et al*. 2009). The mechanisms generating such variation, however, have received less attention. This study sought to determine whether differences in the

induced transcriptional profile might suggest proximal mechanisms responsible for phenotypic variation.

Previous work showed large, quantitative differences in VOC emission between genetic lines of *D. wrightii* under laboratory conditions (Hare 2007). The first aim of this study was to determine whether there were intraspecific differences in the induced response at the transcriptional level among young plants that were capable of inducing VOC emissions, and, if so, to characterize those differences under field conditions. A reasonable hypothesis would predict that genes involved in signaling (e.g. the JA pathway) and core functions typically affected by herbivore attack (e.g. photosynthesis) should be commonly regulated in all three genotypes, with a smaller set of downstream genes showing qualitative or quantitative differences indicating differential regulation of volatile production. The results of this study indicate that, in addition to quantitative differences in the number of genes activated following herbivory, major qualitative differences exist between the responses of the three genotypes tested, as well.

Of the 835 genes detected in the early sampling period of this study, only 30 were commonly regulated by all three genotypes. Furthermore, genes from major pathways associated with the induced response, including photosynthesis or JA signaling (Karban and Baldwin 1997), are not found among these 30. Among those consistently expressed in the early samples, three are putatively responsible for critical steps in the ET pathway, an important modulator of JA-mediated resistance traits (Adie *et al*. 2007). Four additional genes are terpene synthases, predicted to produce β-ocimene (two genes), (E-E)-α-farnesene, and an unknown terpene. The induction of these genes in each genotype

following damage indicates their expression is relatively robust across these genotypes and may be under selection for consistent expression, but also disqualifies these genes as potential sources of intraspecific variation detected in this study.

Comparisons across early-season control and treatment samples suggest that these differences are due, at least in part, to differences in constitutive expression levels of many genes. The BCV7 line showed greater baseline expression levels of more than 500 genes compared to the MVV6 line. This difference was far less between treatment samples, suggesting that induced gene expression in the MVV6 line reduced the differences in abundance of many gene transcripts. Pairwise comparisons of BCV7 and UCV9 lines showed few differences in baseline samples but slightly more among induced samples. Induced UCV9 samples also showed major differences from induced MVV6 samples. Taken together, these findings suggest that the three lines examined in this study differ in their baseline level of expression of hundreds of genes and the degree to which expression is inducible. Perhaps most importantly, however, they appear to differ in their trajectory of induction: herbivore attack exacerbated the differences in gene expression between the samples of induced plants from each line, beyond those genes that were classified as being differentially expressed.

The induced response relies on perception and signaling

The extent of the variation seen across samples from the three genotypes suggests that "upstream" regulatory elements, prior to phytohormone biosynthesis, are driving variation in the induced phenotype. The most likely scenario is that the differences in

perception and early signaling lead to the variation seen in this study, and the pervasive differences in phytohormone biosynthesis pathways are merely the result. Since herbivores were randomly drawn from a laboratory colony for use in the damage treatment, we can exclude the role of differences in elicitors and damage patterns in causing the variation. Because several hormone pathways were affected, it is more parsimonious to attribute differences to a small number of early-signaling mechanisms than to conclude that each pathway differed in its perception or response to elicitation independently. Such a hypothesis is supported by studies of intraspecific variation in induced resistance in *N. attenuata* (Wu *et al*. 2008). In that species, an expression level polymorphism in the negative regulation of a salicylic acid-induced protein kinase and associated transcripts explained a plethora of downstream differences in signaling and secondary metabolism profiles.

The main hormone pathway expected to regulate the induced response to herbivory is the JA pathway (Wasternack and Song 2017). In the present study, the transcriptional response of *D. wrightii* genes attributed to this pathway showed no clear pattern of response. The BCV7-early, BCV7-late, and MVV6-late samples showed little to no induction in this pathway, consistent with their overall lack of transcriptional response. Yet the three samples that induced significant transcriptional responses showed markedly different patterns of JA-related transcription. This was especially surprising in the UCV9-early sample that only showed activity from three JA-related genes, yet still showed a robust induced response in terms of both gene expression and VOC emission. Four JA-related genes, none of which were induced in the UCV9-early sample, were

induced in the UCV9-late sample and were accompanied by a transcriptional response that included fewer genes overall, most of which were not induced in the earlier sample. Despite the active response of the UCV9 line late in the season, however, volatile emissions did not increase after herbivory compared to control samples. The MVV6 line showed the most active response from the JA-pathway early in the season, increasing expression of 14 JA-related genes, but showed no increase in VOC emissions.

Genes belonging to the ET pathway were nearly as well-represented as those in the JA pathway. ET is known to modulate the induced response generated through the JA pathway, although ET does not generate a resistance response when expressed alone (Adie *et al*. 2007). The consistent expression of two *ACS* and one *ACO* genes among induced early-season plants, but not late-season plants, suggests that the ET pathway may partially differentiate early-season plants that are inducible from those that are no longer inducible late in the season.

The enzyme produced by *ACS* genes converts *S*-adenosyl-methionine precursors to ACC. This reaction represents the first committed step to ET production, and is the primary rate-limiting step in ET biosynthesis (Adie *et al*. 2007). *ACO* gene products oxidize ACC to convert it to ET, cyanide, and carbon dioxide, and thus represents the final step in the pathway (Broekaert *et al*. 2006, Adie *et al*. 2007). The consistency of expression of these genes across the early-season samples suggests that the role of the ET pathway and its involvement in the resistance response may shift consistently across genotypes as *D. wrightii* matures. Although posttranscriptional regulation of the ET pathway further modifies the level of hormone production (Adie *et al*. 2007), the activity

of these genes suggests that the late-season response may lack involvement of the ET pathway. It is interesting to note that while the MVV6-early sample showed expression of far more JA-related genes than the other pathways, the UCV9-early sample showed held this distinction for ET-related genes. Although these hormones coregulate aspects of the induced resistance phenotype (Broekaert *et al*. 2006; Adie *et al*. 2007; Onkokesung *et al*. 2010), there appears to be little covariation among the genes that comprise their biosynthetic pathways.

Interestingly, genes related to the ABA pathway were detected in all of the UCV9 and MVV6 samples, but were absent from both BCV7 samples. A gene that MapMan identified as nearly identical to *Arabidopsis XANTHINE DEHYDROGENASE 1* (*XDH1)* was induced in UCV9 and MVV6 samples from both sampling periods. *XDH1* is stress-inducible in *A. thaliana* and appears to function downstream of ABA signaling (Hesberg *et al*. 2004). A putative abscisic aldehyde oxidase (*AAO*) gene that, according to MapMan, is nearly identical to the *A. thaliana AAO3* gene was only expressed in the early-season samples of the UCV9 and MVV6 lines. The *AAO* gene produces an enzyme that catalyzes the final step in ABA production (Seo *et al*. 2000a; Seo *et al*. 2000b). Although *XDH1* is ABA-responsive and suggests activation of that pathway late in the season, the restriction of increased transcription of *AAO* genes to the early portion of the season indicate there may have been greater ABA activity at that time.

In addition to its major role in regulating plant responses to drought, salinity, and temperature stressors, the ABA pathway activity is a prerequisite for induction of herbivore resistance traits in both *A. thaliana* and *N. attenuata* through coregulation of

the MYC2 transcription factor pathway (Nguyen *et al*. 2016). In addition to increased local susceptibility at the site of induction, plants with ABA-signaling deficiencies have impaired systemic signaling capabilities (Vos *et al*. 2013). Distal leaves of *A. thaliana* show evidence of priming, but subsequent damage to those leaves will not elicit a response consistent with expectations of priming. Exogenous application of ABA restores this response, however, demonstrating its role in triggering induced resistance (Vos *et al*. 2013).

ABA has a complex relationship with other phytohormones. Although ABA is required for some components of the JA-regulated response that are regulated by the MYC2 transcription factor, ABA suppresses the ET pathway and therefore also the suite of traits that are coregulated by JA and ET (Adie *et al*. 2007; Nguyen *et al*. 2016). Such suppression has been shown to increase susceptibility to pathogens through suppression of effectual JA- and ET-responsive pathways (Ton *et al*. 2009). In fact, ABA-driven suppression of ET appears to be a basic regulator of plant growth in *A. thaliana* (LeNoble *et al*. 2004). Additionally, drought-triggered ABA induction in intact tomato plants reduced the antagonistic effect of SA on JA, thereby facilitating the JA response when plants were subsequently attacked by herbivores (Thaler and Bostock 2004).

Ontogenetic decline in inducibility affects each line uniquely

Ontogenetic decline in inducibility followed no clear pattern across the three genotypes assayed. Although all three lines perceived the herbivore attack later in the season, they each differentially expressed fewer genes in response. The magnitude of

decline, however, differed substantially. The UCV9 line showed a moderate decline in the number of DEGs induced across the two sampling periods. BCV7 showed the least change, although that pattern is perhaps confounded by the minimal induced change seen early in the season. On the other hand, the MVV6 line was highly inducible early in the season but showed a major decrease in inducibility late in the season.

Apart from differences in the number of DEGs, the identity of the genes involved in the induced response were almost entirely different from the response seen earlier in the season. Both the BCV7 and UCV9 lines showed very little overlap with those induced earlier, but the MVV6 showed relatively fewer genes unique to the late-season sample. Interestingly, in the UCV9-late sample, the distribution of DEGs across functional categories showed similar proportions to those induced in that line earlier in the season, as well as in the MVV6-early sample. Although it is unclear what function those induced DEGs serve, this pattern suggests that the widespread transcriptional changes associated with herbivory require coordination of genes representing each functional pathway.

Dampening of induced resistance in response to ontogenetic changes in the plant may involve mechanisms that allow hormones ostensibly regulating growth and reproduction to alter the response to herbivory. In *N. attenuata*, the levels of ET, JA, and JA-isoleucine, a JA derivative also involved in response elicitation, declined as plants began elongating and flowering, but the high levels seen in young plants could be restored by removing the inflorescences (Diezel *et al*. 2011). The authors of that study hypothesized that auxin, and possibly SA, are responsible for suppression of the damage

response due to their involvement in reproduction and antagonistic relationship with JA and ET.

The mechanism responsible for ontogenetic changes in inducibility are unknown in *D. wrightii*. The changes brought about by maturation are not ostensibly consistent across genotypes, but such a pattern may be obfuscated by differences in the baseline level of inducibility characteristic of each line. Even a systemic hormone signal emanating consistently from the inflorescence of each genotype could have drastically different effects on the inducible response of each line through crosstalk with the seemingly unique hormone profile of each line of plants. Regardless of the consistency of the mechanism, it can conclusively be stated that ontogeny affects each of the genotypes. However, the relative difference in induced transcriptional profile between early- and late-sampling periods, as well as the genes that were induced both early and late in the season, were overwhelmingly unique to each genotype.

Phytohormone gene expression explains VOC emissions

The mono- and sesquiterpenes found in the VOC blend are synthesized by terpene synthase enzymes, but the results of the present study suggest that expression of the genes producing these enzymes may not function to regulate VOC production, and may be an unreliable marker of VOC induction. Of the nine *TPS* genes differentially regulated in this study, all were induced in the MVV6-early sample, yet VOC emissions from damaged plants were not elevated. Only four *TPS* genes were induced in the BCV7-early sample, which showed increased emissions. Late-season samples from the

BCV7 and UCV9 lines both increased expression of multiple *TPS* genes, yet this did not lead to increased emissions.

Instead, the changes in VOC emissions observed in this study are better explained by expression of phytohormone biosynthesis and signaling genes. Although the small sample size in this study makes it difficult to detect relationships between genes and metabolites, the variation in VOC PC1, representing the main axis of variation in emissions, was explained by variation in genes from the JA, ET, ABA, CK and IAA pathways.

It is notable that the third PC from the phytohormone model explained the variation in VOC emissions. PCAs create variables based on covariance matrices of the original variables. Each additional PC represents variation in the matrix that is orthogonal to the covariance previously accounted for. Therefore, VOC emissions are explained by a small, relatively obscure aspect of the variation in the data.

This raises the question of what effects the first two PCs of the phytohormone analysis have. Such variation could be linked to other aspects of the inducible phenotype, such as direct resistance traits, tolerance, primary metabolism, or photosynthesis. On the other hand, variation in expression does not necessarily indicate biochemical or physiological changes. Instead, a small subset of genes that are rate-limiting, or have outsized effects, may be more important regulators than a larger number of covarying genes that are less tightly regulated.

For instance, it worth noting that the best model for predicting VOC emissions included genes from the IAA pathway. Two genes from this pathway loaded strongly

and negatively on the third phytohormone PCA, potentially accounting for the increased coefficient of determination associated with that gene expression model. One gene is an unknown auxin-responsive gene, while the other is a putative *PIN8* gene involved in auxin transport. Auxin is a hormone involved in growth and reproduction and has previously been hypothesized as a mechanism responsible for ontogenetic suppression of induced resistance, due in part to its negative relationship with the JA and ET pathways. (Onkokesung *et al*. 2010). Although this study only shows an associative relationship between phytohormone genes and VOC expression, it provides an important starting point by identifying genes of potential interest.

One of the basic assumptions of chemical ecology is that inducible secondary metabolites are under selection based on their ability to defend plants from herbivory, and that the production of these compounds is therefore carefully regulated following detection of herbivores (Wu and Baldwin 2010; Raguso *et al*. 2015). Synthesis of VOCs involves complex biochemical pathways that branch from primary metabolism to form the LOX, MVA, MEP, and shikimate pathways (Dudareva *et al*. 2013). None of these pathways are entirely independent of one another, and necessarily compete for precursor molecules. It is therefore not surprising that regulation of the final products of these pathways are regulated by signaling molecules rather than downstream components. Phytohormones presumably coordinate the activity of numerous rate-limiting steps along the metabolic pathways in order to preferentially synthesize particular compounds. It is notable, however, that in this study, the three genetic lines responded very differently to the same stimulus. The divergent transcriptional responses indicate that the response to

herbivory may not be as finely tuned as expected (e.g. Pieterse and Dicke 2007), although the even distribution of genes across functional categories in each genotype imply the responses are not random. Further research will be needed to determine the specific contributions of each phytohormone to the various resistance traits.

Evolutionary implications

The large degree of intraspecific variation in the induced transcriptional profile may allow for relatively rapid adaptive changes in resistance phenotype. Campbell and Kessler (2013) noted the association between inducibility and mating system. Their results suggest that Solanaceous species reliant on outcrossing tend to exhibit a greater degree of constitutive resistance, while self-pollinating species are, on average, more inducible, and that these phenotypic combinations evolved through numerous independent events. The variation in response observed in this study indicates that natural populations of *D. wrightii* in southern California are likely to have a high degree of standing variation in the genetic components influencing inducibility, a prerequisite for adaptive evolution.

That lack of consistency in transcriptional profiles suggests that selection pressures on this character may be diffuse or inconsistent. The BCV7 line showed higher baseline expression levels for hundreds of genes compared to the MVV6 line, while the MVV6 and UCV9 lines were far more inducible, although their induced states were very different. Although these ostensibly alternative resistance phenotypes may represent adaptation to local herbivore communities, none of the source populations differed in

obvious ways when surveyed when the original genotypes were collected (Elle and Hare 2000). Since overall regulation of VOCs, at least, appears to be governed by early signaling molecules, resistance phenotypes may vary due to pleiotropy as plants adjust their hormone signaling responses to other external stimuli, such as abiotic conditions or growth-related pressures. On the other hand, much of the variation seen between these genotypes may be of little importance: critical, rate limiting processes may be tightly regulated while expression of remaining genes is superfluous so long as it produces products in excess of the rate-limiting step. To test these ideas, either large-scale, longitudinal studies or methods utilizing subtractive molecular methods, such as silencing genes, will be needed to tease apart the specific contributions of each factor.

Conclusion

The results of this chapter indicate that intraspecific variation in the induced response of *D. wrightii* is pervasive, and varies more than induced VOC emissions might otherwise suggest. Ontogenetic changes in this species affect the induced transcriptomic profile, but these changes manifest uniquely in each genotype studied here, leading to unique late-season profiles as well. The extent to which these variations contribute to differences in resistance traits other than VOC emission is unclear. These results refute the notion that induced responses characteristic of plant species can adequately be deduced from a single clonal genotype. Indeed, intraspecific variation in inducible resistance may be better described as a spectrum of response.

Table 4.1. List of genes and their associated primers analyzed by qPCR to confirm RNA-Seq results.

Gene	Abbreviation	Gene ID	Forward (5'→3')	Reverse (3'→5')
Hydroperoxide lyase 1	*HPL1*	Solyc07g049690	AAATGTGGTGGCGGTTCTG	GGCACAAACACGCATATCTC
Phenylalanine ammonia lyase	*PAL*	Solyc09g007900	GCTCCACCTACCCTTTGATG	TCGTCCTCGAAAGCTACAATC
Terpene synthase	*TPS*	Solyc03g006550	GGGATGCAAAGAACCTTGAT	TGGCAACATCACACACAAGA
Lipoxygenase 2	*LOX2*	Solyc01g006540	GGTGATGGAGTTGGAGAAAGA	GGACGGAGTAAACGGTGTTG
RuBPCase small chain 2B	*RUB*	Solyc02g063150	TCCCGTTACCAAGAAGAACAAC	AGCAAGGAACCCATCCACTT
Actin	*ACT*	Solyc04g071260	TCTTCCAGCCATCCTTGATT	TGACCCACCACTAAGCACAA
Ubiquitin	*UBI*	Solyc12g099030	TCCACCTTGTCCTTCGTCTC	CCTCTGAACCTTGCCAGAA

Figure 4.1. Mean (±SE) total VOC emissions by treatment, genotype, and sampling period. Grey bars correspond to plants that were damaged by two *Lema daturaphila* caged on an individual leaf for 24 hours, while black bars indicate control plants that received empty cages. VOCs were collected from the same individual leaves immediately following cessation of treatment. Asterisk denotes $p \leq 0.05$.

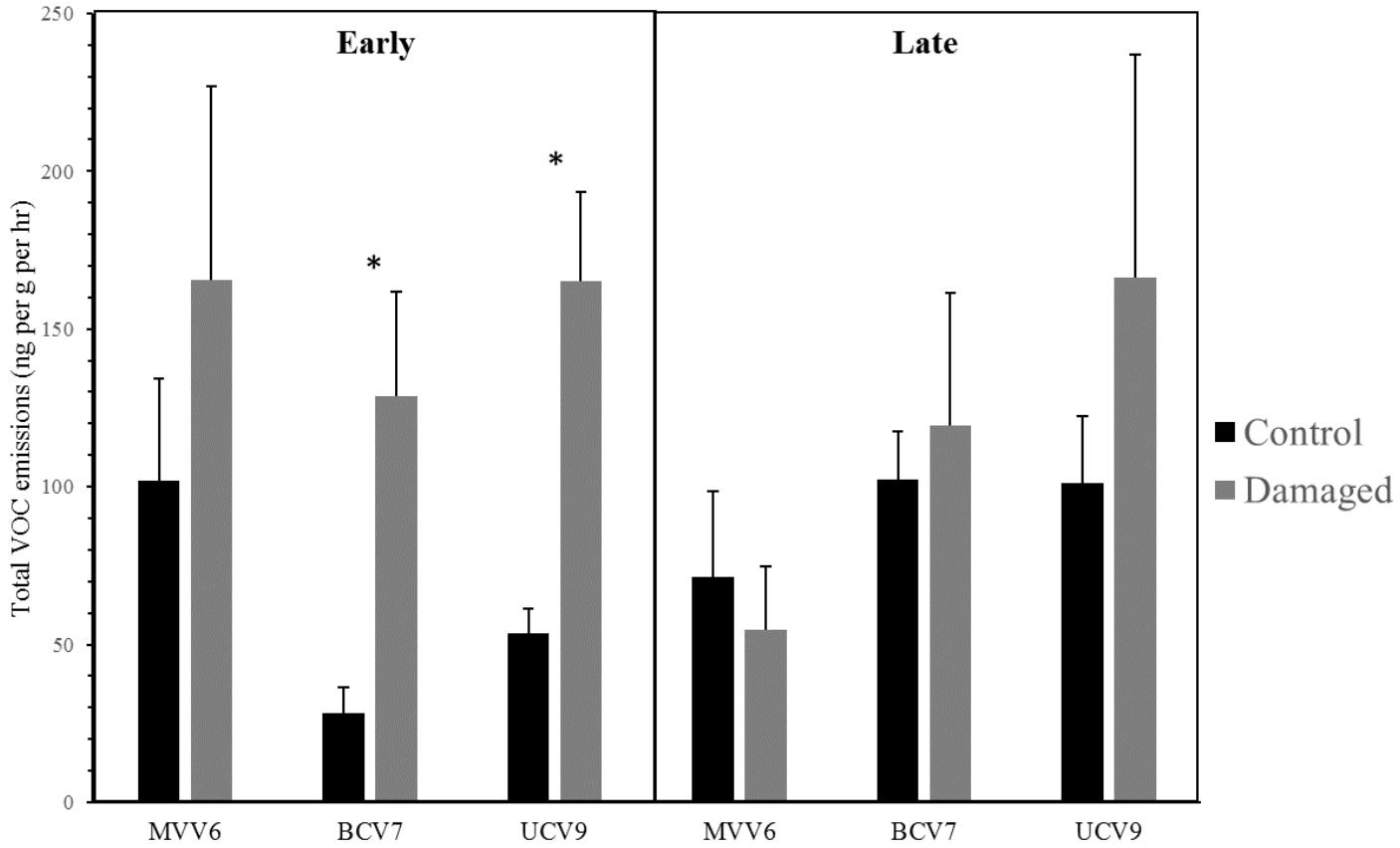

Figure 4.2. Heatmap of 932 genes induced by 24 hours of feeding by *Lema daturaphila* across genotypes and sampling periods, shown in columns, with hierarchical clusters showing similarity of expression across rows. Red indicates increased expression relative to controls, and green indicates decreased expression.

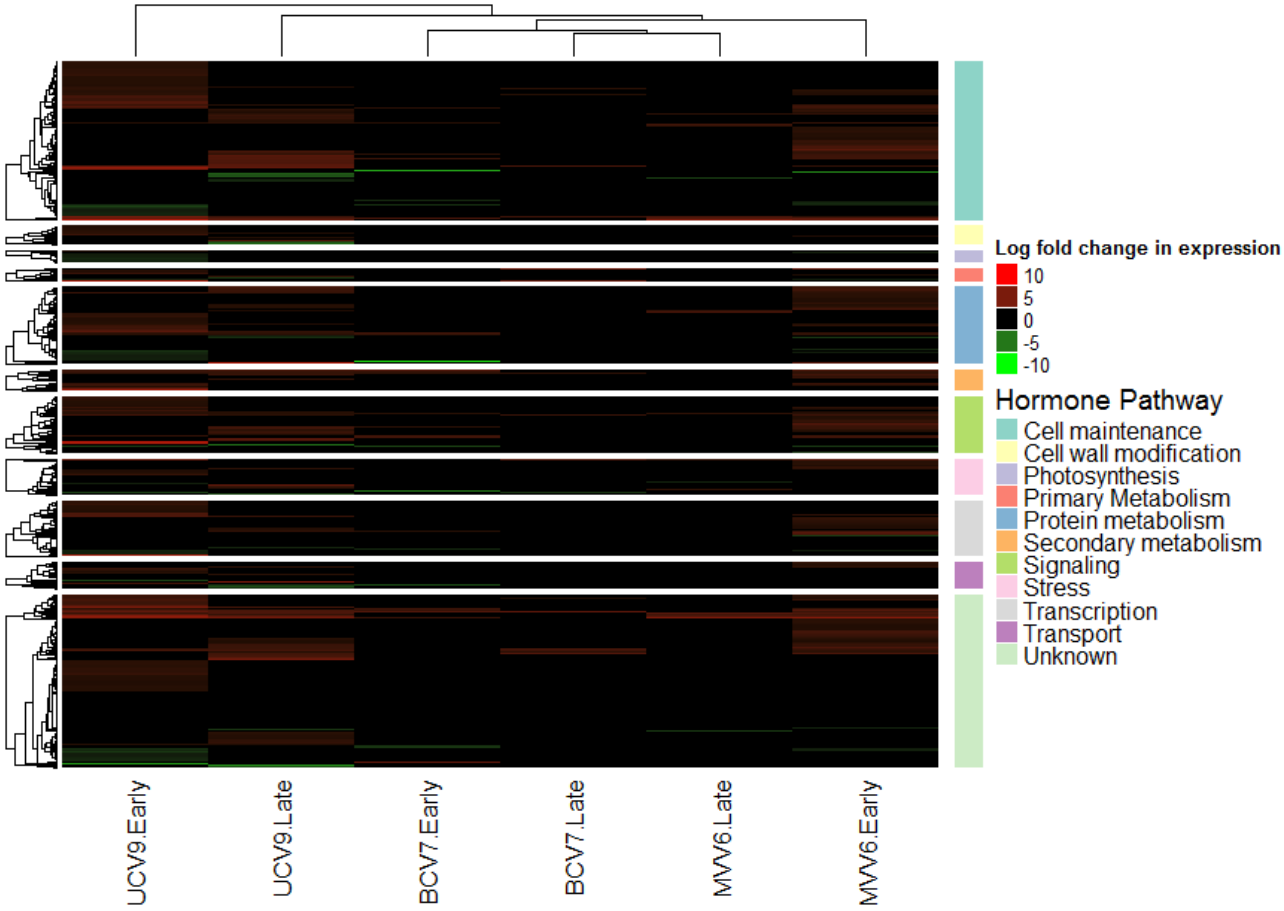

Figure 4.3. Venn diagrams showing overlapping expression of upregulated or downregulated genes by each genotype, separated by sampling period

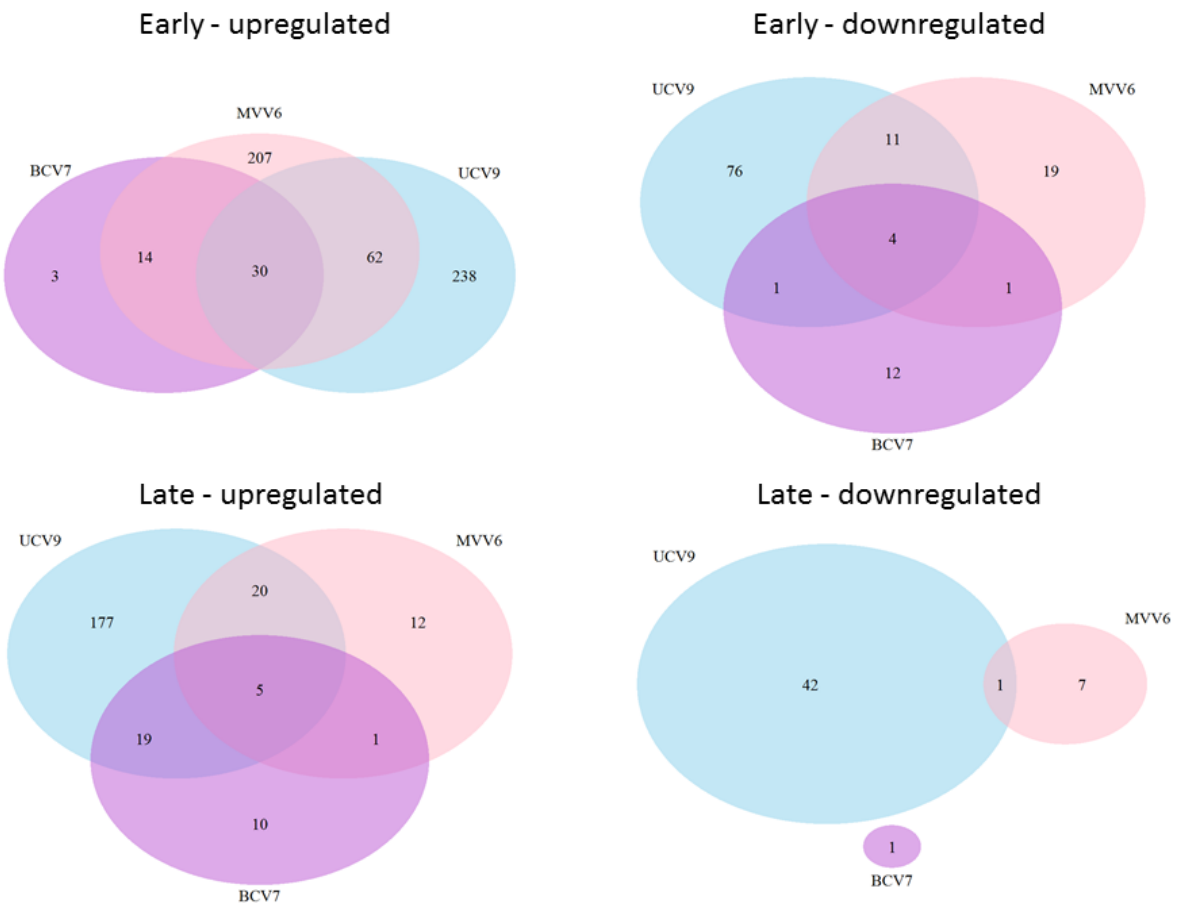

Figure 4.4. Venn diagrams showing genes that were upregulated in both early and late sampling periods by each genotype.

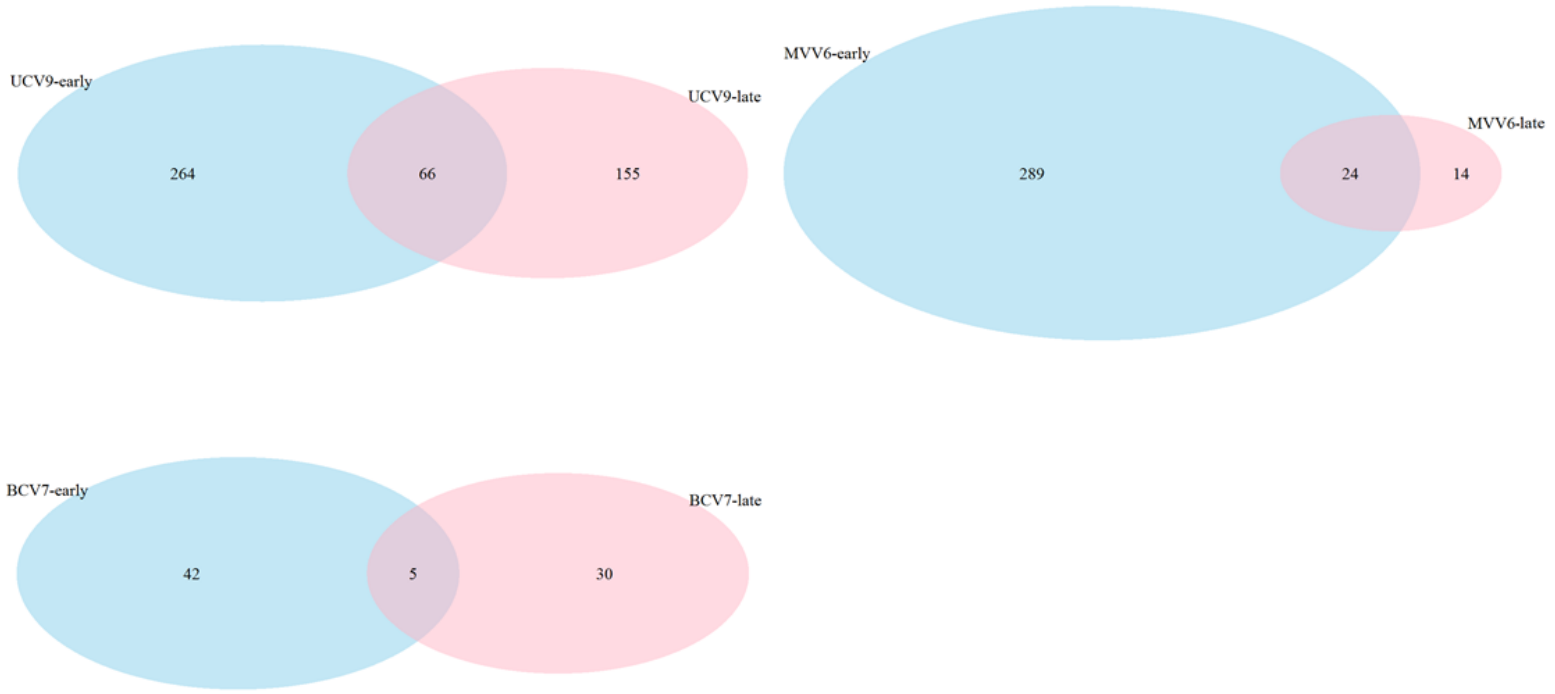

Figure 4.5. Venn diagrams showing genes that were downregulated in both early and late sampling periods by each genotype.

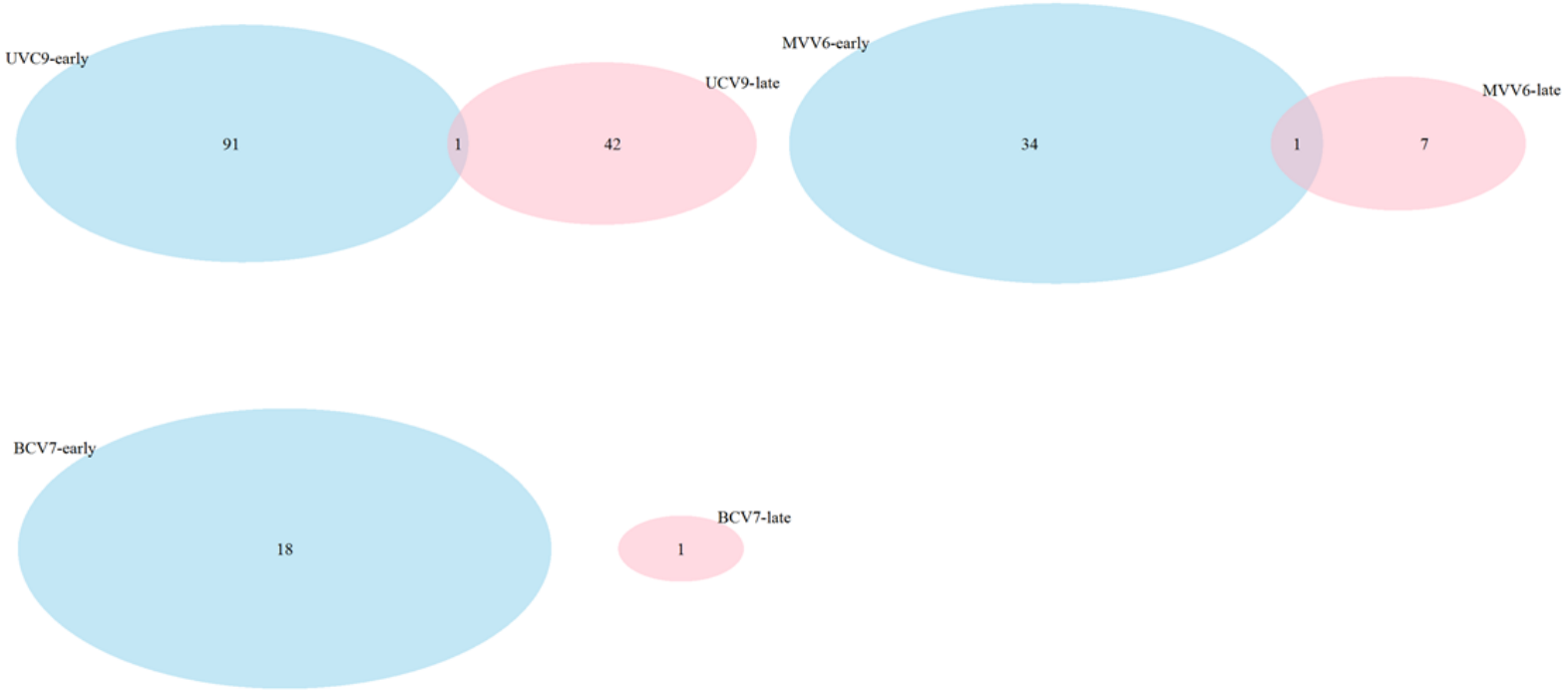

Table 4.2. List of genes that showed common regulation in all early-season samples. Descriptions are based on MapMan gene ontology (GO) terms.

Pathway	Function	Tomato ortholog	UCV9-Early	MVV6-Early	BCV7-Early	UCV9-Late	MVV6-Late	BCV7-Late
Cell wall modification	MUR4-like	Solyc07g043550.2	1.52	2.61	1.63	1.49	2.07	0.00
Cell maintenance	Fatty acyl-CoA reductase (alcohol-forming)	Solyc06g074390.2	2.60	2.40	1.60	0.00	0.00	0.00
Protein metabolism	Tryptophan synthase, beta subunit, putative	Solyc10g018390.1	1.66	2.07	2.72	1.62	0.00	0.00
Protein metabolism	Tryptophan synthase, beta subunit, putative	Solyc10g005320.2	1.62	2.09	2.74	1.78	0.00	0.00
Protein metabolism	Tryptophan synthase, beta subunit, putative	Solyc10g018380.1	1.31	1.74	1.82	1.36	0.00	0.00
Secondary metabolism	β-ocimene synthase	Solyc03g007730.1	2.30	1.74	1.84	2.03	0.00	0.00
Secondary metabolism	β-ocimene synthase (*TPS25*)	Solyc02g079890.1	1.65	1.77	1.74	1.98	0.00	1.70
Secondary metabolism	Terpene synthase	Solyc00g154480.1	2.17	3.03	2.60	2.40	0.00	0.00
Secondary metabolism	(E E)-alpha-farnesene	Solyc02g079910.1	2.22	3.07	3.35	2.86	0.00	0.00
Signaling	1-aminocyclopropane-1-carboxylate oxidase	Solyc12g005940.1	1.35	1.83	1.62	1.25	0.00	0.00
Signaling	1-aminocyclopropane-1-carboxylate (ACC) synthase (*ACS6*)	Solyc08g008110.2	2.34	2.34	2.34	0.00	0.00	0.00
Signaling	1-aminocyclopropane-1-carboxylate (ACC) synthase (*ACS1*)	Solyc08g008100.2	2.09	2.47	2.61	0.00	0.00	0.00
Stress	lactoylglutathione lyase family protein / glyoxalase I family protein	Solyc01g103590.2	7.02	4.56	4.20	6.25	0.00	0.00
Cell maintenance	UDP-glucoronosyl/UDP-glucosyl transferase family protein	Solyc12g006430.1	3.80	3.60	3.01	3.14	3.16	0.00
Cell maintenance	UDP-glucoronosyl/UDP-glucosyl transferase family protein	Solyc02g067690.2	1.80	1.66	1.86	1.89	0.00	0.00
Cell maintenance	UDP-glucoronosyl/UDP-glucosyl transferase family protein	Solyc12g088690.1	1.41	2.12	1.48	1.68	0.00	0.00
Cell maintenance	Weakly similar to SAP domain-containing protein	Solyc06g063020.2	1.11	1.14	1.89	0.00	0.00	0.00
Protein metabolism	Protein kinase family protein	Solyc09g011320.2	2.58	2.83	3.00	2.75	0.00	0.00
Signaling	GLR5 (GLUTAMATE RECEPTOR 5)	Solyc07g052390.2	1.42	1.47	1.38	1.29	1.65	0.00
Unknown	Catechol oxidase B (PPO)	Solyc08g074630.1	4.42	4.15	3.55	3.70	5.85	3.17
Unknown	Unknown	Solyc01g081620.2	5.27	5.42	2.17	4.60	5.04	0.00
Unknown	Aspartic protease inhibitor 1 precursor	Solyc03g098780.1	4.02	2.24	3.40	3.12	0.00	3.96
Unknown	*NYE1 (NON-YELLOWING 1)*	Solyc12g056480.1	1.80	1.86	1.78	0.00	0.00	0.00
Unknown	FLOT1-like	Solyc03g117250.2	3.31	4.93	6.14	4.11	4.96	0.00
Unknown	Agenet domain-containing protein	Solyc06g072200.1	2.12	1.37	1.85	1.82	0.00	2.06
Unknown	Ulp1 protease family C-terminal catalytic domain containing protein	Solyc01g105640.2	3.05	3.55	2.03	2.74	0.00	0.00
Unknown	Chymotrypsin inhibitor I, D subunit	Solyc09g084470.2	4.79	2.82	3.85	0.00	0.00	0.00
Unknown	Serine protease inhibitor 6 precursor	Solyc03g019690.1	3.93	2.29	3.29	3.13	0.00	4.07
Unknown	Unknown	Solyc01g105440.1	1.49	2.06	2.57	0.00	0.00	0.00
Primary metabolism	FOLDED PETAL 1-like	Solyc01g095960.2	1.61	1.76	2.40	0.00	0.00	0.00
Unknown	haloacid dehalogenase-like hydrolase family protein	Solyc11g009020.1	-1.81	-1.05	-1.56	0.00	0.00	0.00
Signaling	Gibberellin-responsive protein, putative	Solyc04g017720.2	-2.52	-2.48	-2.89	0.00	0.00	0.00
Cell maintenance	Phosphatidic acid phosphatase-related	Solyc10g086690.1	-1.80	-1.34	-2.12	0.00	0.00	0.00
Primary metabolism	Aldose 1-epimerase family protein	Solyc02g085100.2	-1.43	-1.40	-1.67	0.00	0.00	0.00

Figure 4.6. Number of genes upregulated in each genotype by sampling period combination grouped by functional categories assigned by MAPMAN.

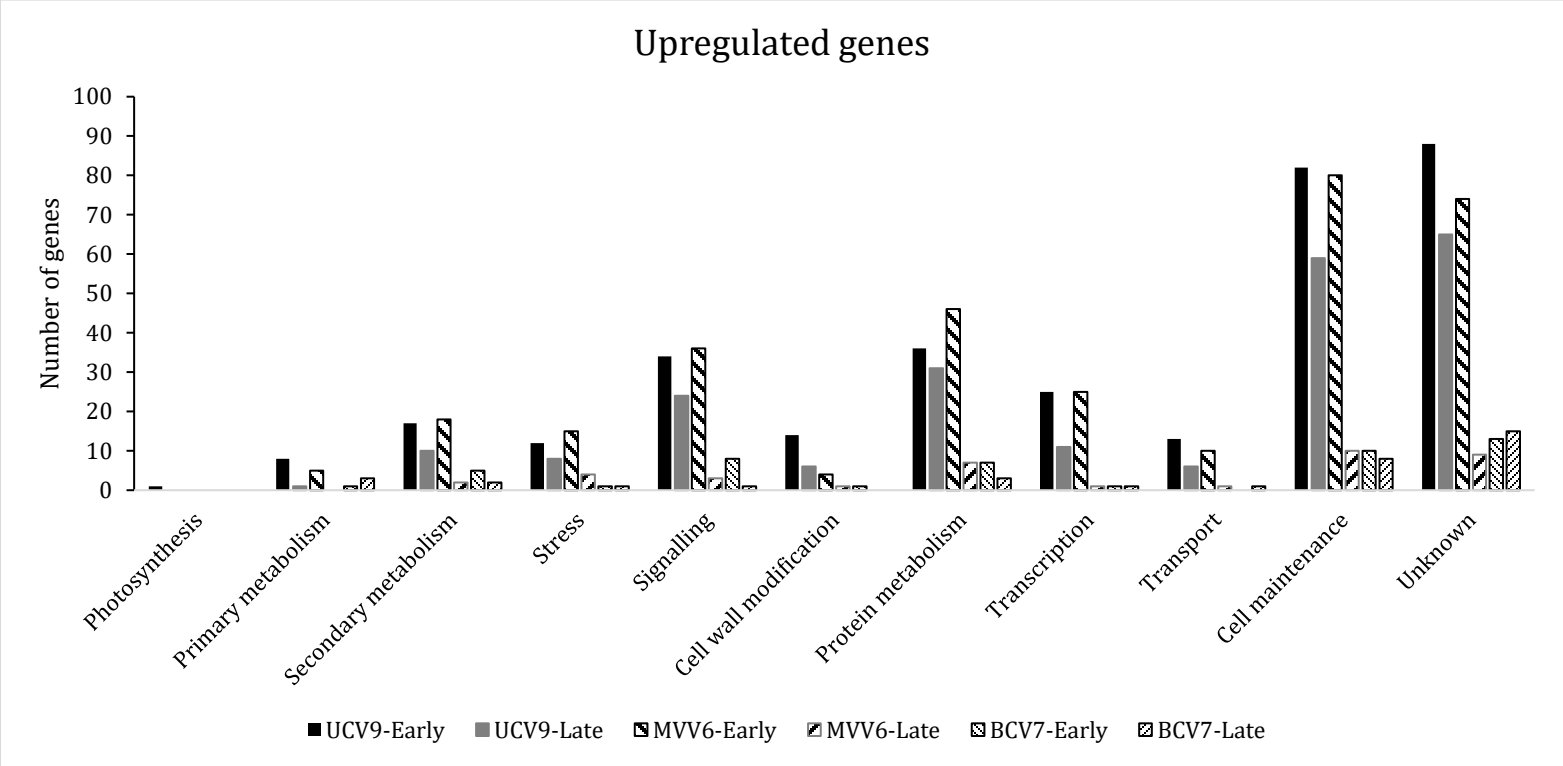

Figure 4.7. Number of genes downregulated in each genotype by sampling period combination grouped by functional categories assigned by MAPMAN. Note the difference in scale compared to Figure 4.6.

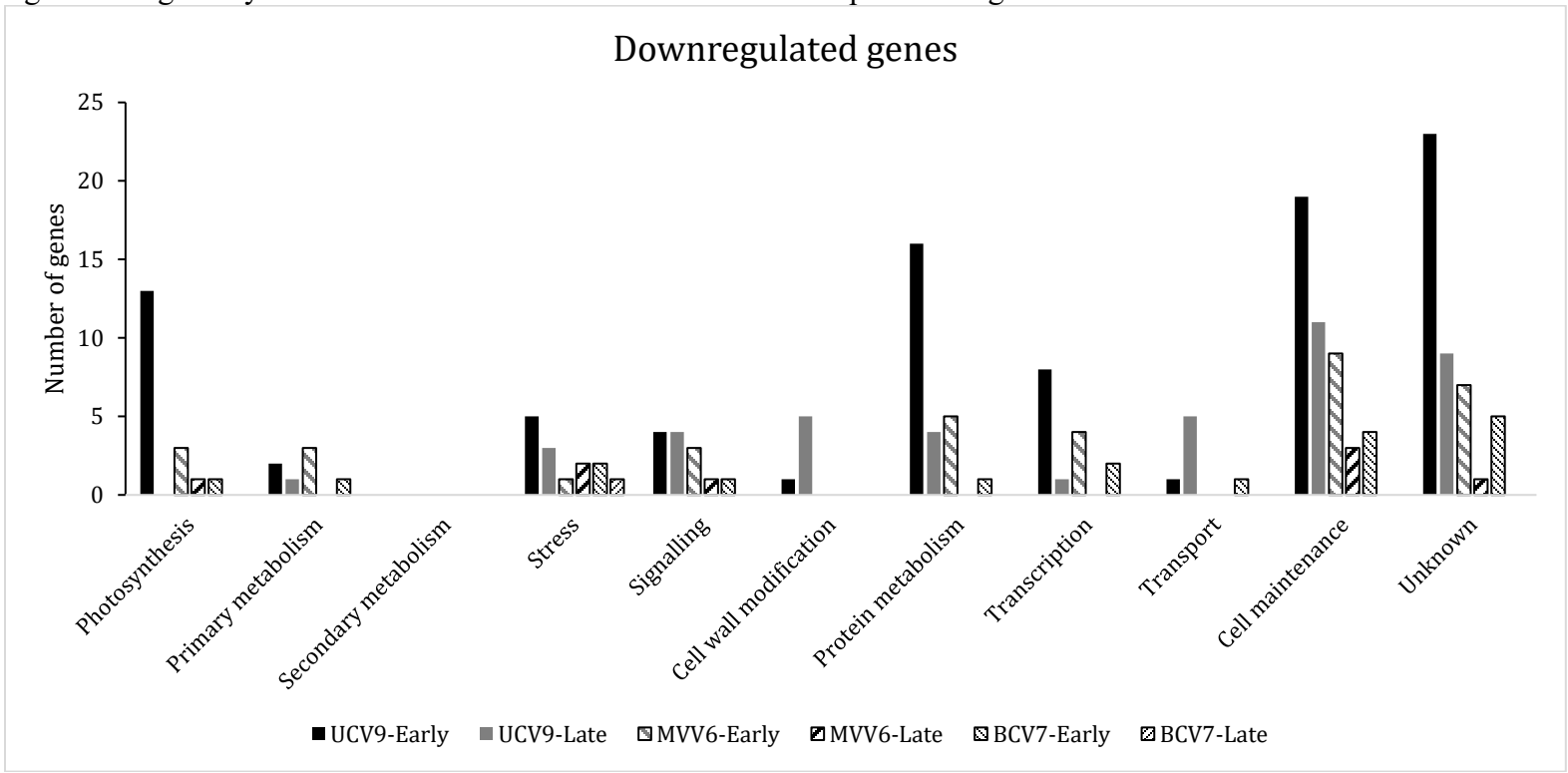

Figure 4.8. Heatmap of nine putative terpene synthase genes induced by 24 hours of feeding by *Lema daturaphila* across genotypes and sampling periods, shown in columns, with hierarchical clusters showing similarity of expression across rows. Red indicates increased expression relative to controls, and green indicates decreased expression.

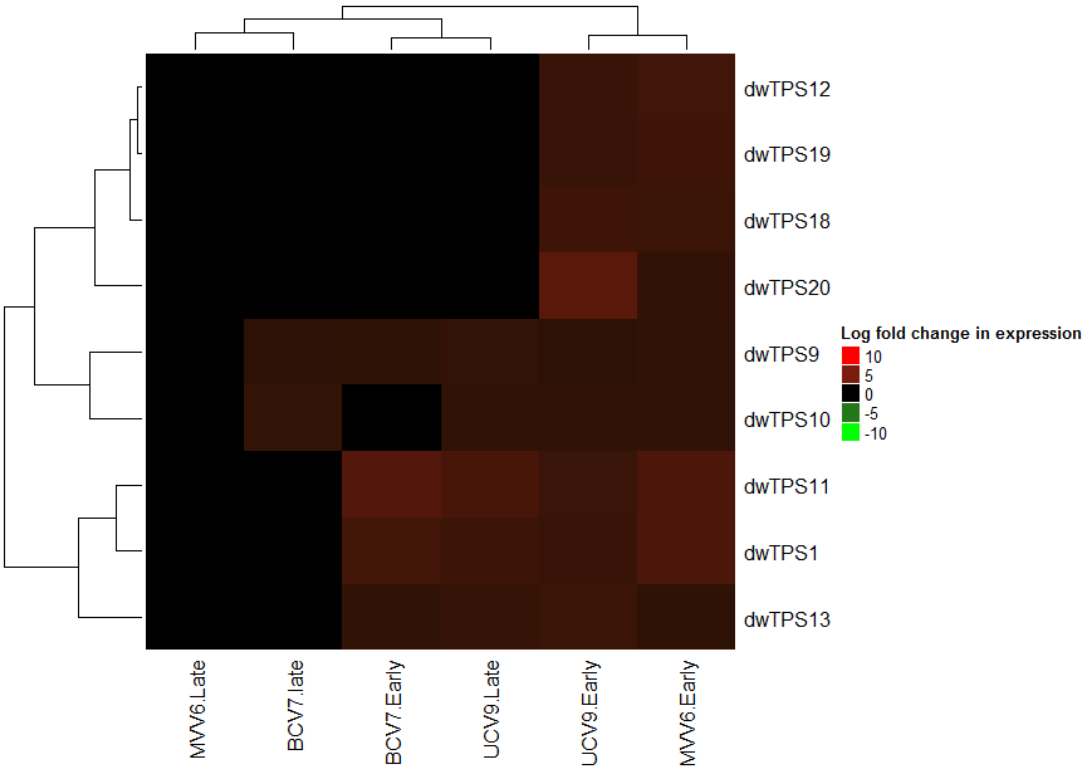

Table 4.3. A list of terpene synthase genes identified as being differentially expressed following herbivory in *Datura wrightii*. Nucleotide sequences from the *D. wrightii* transcriptome were used to predict amino acid sequences and open reading frames, and the resulting sequences were compared to the Tomato proteome. Predicted products for each gene are from the reference listed.

Datura wrightii gene	Tomato ortholog gene ID	Tomato homolog	Predicted amino acid length	Non-similar amino acid substitutions from tomato	Expect (E) value	Main predicted product	Reference
dwTPS1	solyc00g154480		521	7	0	Unknown terpenoid	Fernandez-Pozo et al. 2015
dwTPS9	solyc02g079890	slTPS25-like	122	5	5E-67	β-ocimene	Falara et al., 2012
dwTPS10	solyc02g079900		56	2	4E-32	(E E)-α-farnesene	Fernandez-Pozo et al. 2015
dwTPS11	solyc02g079910	slTPS27-like	160	2	4E-67	Limonene	Falara et al., 2012
dwTPS12	solyc03g006550		621	8	0	(-)-*ent*-kaurene	Fernandez-Pozo et al. 2015
dwTPS13	solyc03g007730		562	3	0	β-ocimene	Fernandez-Pozo et al. 2015
dwTPS18	solyc10g005390	Nearly identical to slTPS39	563	0	0	Nerolidol / linalool	Falara et al., 2012
dwTPS19	solyc10g005410	Truncated slTPS37-like	137	11	7E-26	Nerolidol / β-ocimene	Falara et al., 2012
dwTPS20	solyc10g005420	Truncated	38	0	5E-21	Limonene	Fernandez-Pozo et al. 2015

Figure 4.9. Heatmap of 46 genes putatively involved with hormone biosynthesis and signaling. Genes were induced by 24 hours of feeding by *Lema daturaphila* across sampling periods, shown in columns, with hierarchical clusters showing similarity of expression across rows. Red indicates increased expression relative to controls, and green indicates decreased expression. Colored labels, right, indicate each gene's pathway assignment.

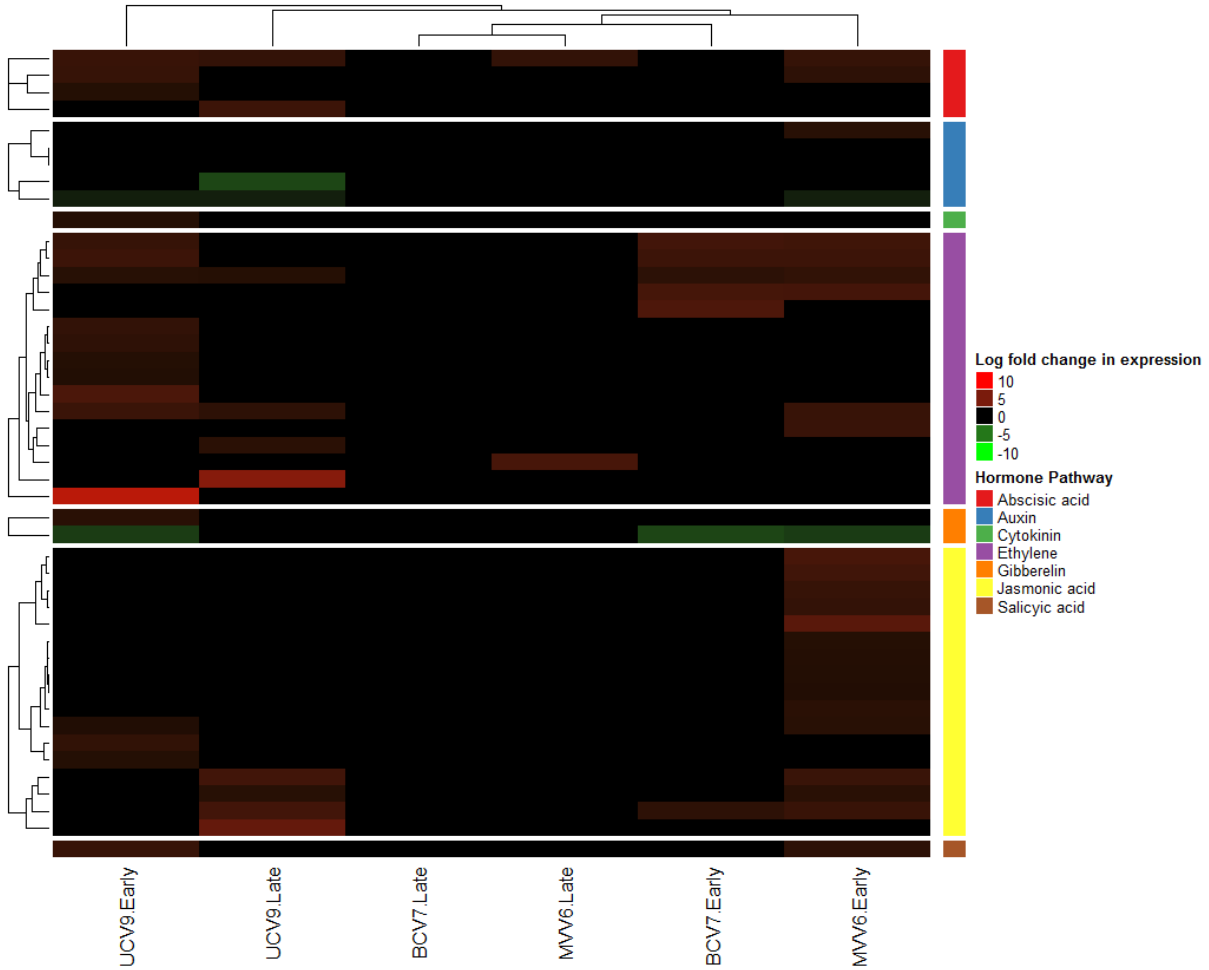

Table 4.4. Pair-wise comparisons of gene expression in undamaged control plants (top), and damaged plants (bottom) from genotypes sampled in early and late sampling periods. Genes were assigned to functional groups based on MapMan gene ontology designations.

Control

	Early						**Late**					
	BCV7 vs. UCV9		MVV6 vs. BCV7		MVV6 vs. UCV9		BCV7 vs. UCV9		MVV6 vs. BCV7		MVV6 vs. UCV9	
	BCV7	UCV9	MVV6	BCV7	MVV6	UCV9	BCV7	UCV9	MVV6	BCV7	MVV6	UCV9
Cell maintenance	1	0	43	115	2	3	33	27	113	78	103	110
Cell wall modification	0	0	3	26	0	3	2	4	9	10	10	20
Photosynthesis	0	0	1	6	0	2	0	0	10	2	9	3
Primary Metabolism	0	0	1	12	0	0	4	2	6	9	11	11
Protein metabolism	5	0	24	63	5	7	16	18	63	64	60	91
Secondary metabolism	0	2	3	15	0	5	5	5	12	7	10	7
Signaling	3	1	16	54	3	11	19	16	35	34	59	56
Stress	0	0	10	27	1	4	5	11	25	48	18	54
Transcription	1	2	16	58	4	14	8	15	36	60	44	87
Transport	1	0	12	34	1	2	7	8	19	18	29	27
Unknown	5	6	42	132	3	15	33	33	99	139	112	176
Total	**16**	**11**	**171**	**542**	**19**	**66**	**132**	**139**	**427**	**469**	**465**	**642**

Damaged

	Early						**Late**					
	BCV7 vs. UCV9		MVV6 vs. BCV7		MVV6 vs. UCV9		BCV7 vs. UCV9		MVV6 vs. BCV7		MVV6 vs. UCV9	
	BCV7	UCV9	MVV6	BCV7	MVV6	UCV9	BCV7	UCV9	MVV6	BCV7	MVV6	UCV9
Cell maintenance	19	31	27	26	58	155	46	34	18	11	42	30
Cell wall modification	1	2	0	6	3	27	3	5	2	3	4	13
Photosynthesis	14	1	0	2	32	5	1	1	5	2	4	2
Primary Metabolism	4	4	2	1	16	17	3	8	3	0	4	4
Protein metabolism	23	23	16	14	65	104	22	20	15	5	37	39
Secondary metabolism	1	5	3	3	4	19	3	5	4	2	9	3
Signaling	10	26	6	3	27	92	16	21	7	9	24	21
Stress	3	11	6	2	16	56	7	9	3	2	6	10
Transcription	6	23	12	9	40	108	17	18	7	3	15	25
Transport	7	11	9	5	25	39	9	9	5	1	14	8
Unknown	39	45	21	23	124	217	49	41	17	18	38	71
Total	**127**	**182**	**102**	**94**	**410**	**839**	**176**	**171**	**86**	**56**	**197**	**226**

Figure 4.10. Hierarchical clustering of scaled transcript count data obtained from RNA-Seq analysis. Samples are color coded according to treatment by genotype by sampling period combinations. CON = control; DAM = damage

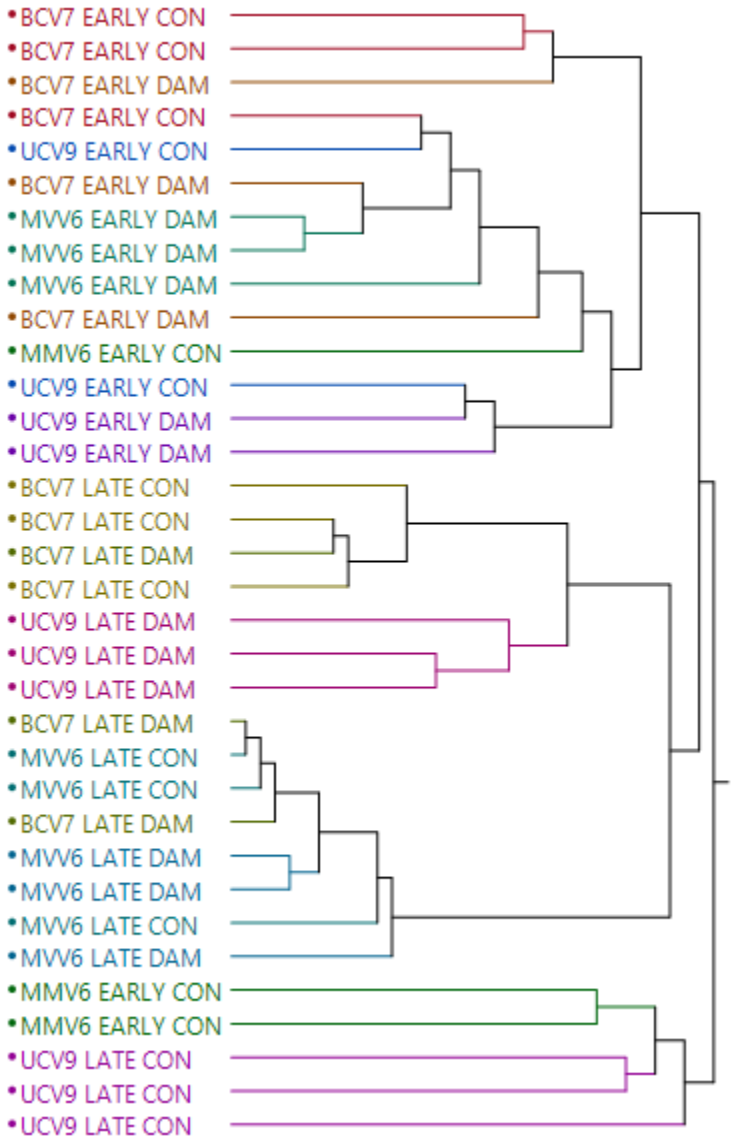

Figure 4.11. Diagram showing comparing expression of sibling plants from the MVV6 line that differed with respect to their trichome phenotype. Horizontal comparisons at the top (sticky) and bottom (velvety) represent differential expression of damaged plants relative to undamaged plants within trichome type. Vertical comparisons represent comparisons between control samples from each trichome group (left) and treatment samples from each trichome group (right).

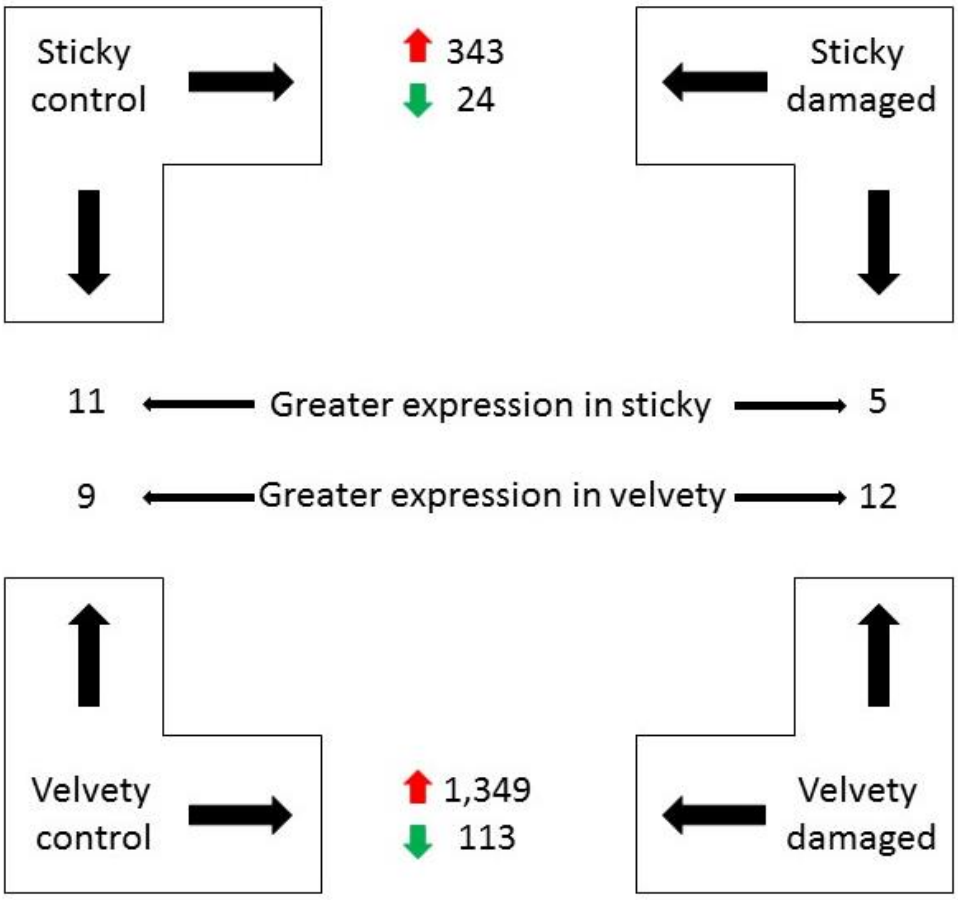

Table 4.5. List of genes that showed differential expression in the pair-wise comparison between samples of undamaged plants belonging to the two trichome types. Expression values of each gene listed on the left represent relative expression compared to the expression level in the other sample. Descriptions are based on MapMan gene ontology (GO) terms.

Undamaged comparison	Fold change higher in				
	Sticky	Velvety	Gene ID	Pathway	Description
		4.24	Solyc06g074680.2	Cell maintenance	Highly similar to lecithin:cholesterol acyltransferase family protein / LACT family protein
		2.13	Solyc07g049440.2	Cell maintenance	moderately similar to GDSL-motif lipase/hydrolase family protein
		1.84	Solyc08g081170.2	Cell maintenance	Highly similar to MURE
	7.60		Solyc07g017950.2	Photosynthesis	Weakly similar to RBCS1A (RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN 1A)
	1.93		Solyc12g008510.1	Primary Metabolism	Highly similar to HXK2 (HEXOKINASE 2); ATP binding / fructokinase/ glucokinase/ hexokinase
	6.47		Solyc11g012990.1	Protein metabolism	Moderately similar to peroxisomal membrane protein
		3.31	Solyc01g091040.2	Signaling	Moderately similar to PDLP8 (PLASMODESMATA-LOCATED PROTEIN 8)
		3.26	Solyc09g007010.1	Stress	Weakly similar to pathogenesis-related protein
	1.95		Solyc06g048410.2	Stress	Moderately similar to FSD2 (FE SUPEROXIDE DISMUTASE 2)
	6.80		Solyc12g014240.1	Stress	Weakly similar to pollen Ole e 1 allergen and extensin family protein
		4.83	Solyc12g009490.1	Transcription	Weakly similar to SHN1 (SHINE 1); DNA binding / sequence-specific DNA binding / transcription factor
		2.82	Solyc09g090960.2	Transcription	Moderately similar to ribonucleoprotein, chloroplast
		2.64	Solyc09g090970.2	Transcription	Moderately similar to pathogenesis-related protein STH-2
	5.57		Solyc06g072200.1	Unknown	Unknown
	5.18		Solyc06g072190.1	Unknown	Unknown
	8.61		Solyc05g053070.2	Unknown	Unknown
	8.61		Solyc05g053060.1	Unknown	Unknown
	2.82		Solyc07g007240.2	Unknown	Unknown
		8.21	Solyc03g020050.2	Unknown	Moderately similar to proteinase inhibitor type-2 precursor
	2.55		Solyc03g082610.1	Unknown	Unknown

Table 4.6. List of genes that showed differential expression in the pair-wise comparison between samples of damaged plants belonging to the two trichome types. Expression values of each gene listed on the left represent relative expression compared to the expression level in the other sample. Descriptions are based on MapMan gene ontology (GO) terms.

	Fold change higher in				
	Sticky	Velvety	Gene ID	Pathway	Description
Damaged comparison	7.93		Solyc07g043660.2	Cell maintenance	Highly similar to AMP-dependent synthetase and ligase family protein
		3.50	Solyc06g074680.2	Cell maintenance	Highly similar to lecithin:cholesterol acyltransferase family protein / LACT family protein
		2.64	Solyc04g082000.2	Cell maintenance	Nearly identical to ATPLDDELTA; phospholipase D
	8.53		Solyc07g017950.2	Photosynthesis	Weakly similar to RBCS1A (RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN 1A)
	8.12		Solyc11g012990.1	Protein metabolism	Moderately similar to peroxisomal membrane protein
	7.59		Solyc07g043680.2	Protein metabolism	Moderately similar to enoyl-CoA hydratase/isomerase family protein
		2.73	Solyc09g007010.1	Stress	Weakly similar to pathogenesis-related protein
		2.37	Solyc09g006010.2	Stress	Weakly similar to PR1 (PATHOGENESIS-RELATED GENE 1)
		2.58	Solyc09g090960.2	Transcription	Moderately similar to ribonucleoprotein, chloroplast, putative / RNA-binding protein cp29
		3.22	Solyc04g071050.2	Transcription	Weakly similar to nucleic acid binding / nucleotide binding
		3.49	Solyc03g025200.2	Transport	Highly similar to MATE efflux family protein
		3.87	Solyc03g025220.2	Transport	Highly similar to MATE efflux family protein
	1.98		Solyc08g081970.2	Unknown	Unknown
		3.27	Solyc04g071080.1	Unknown	Unknown
		3.75	Solyc08g074620.1	Unknown	Nearly identical to catechol oxidase B, chloroplast precursor
		2.96	Solyc04g071070.2	Unknown	Unknown
		3.60	Solyc04g071060.1	Unknown	Unknown

Figure 4.12. Number of genes induced in each trichome type grouped by functional categories assigned by MAPMAN. Note the difference in scale for the number of upregulated compared to downregulated genes

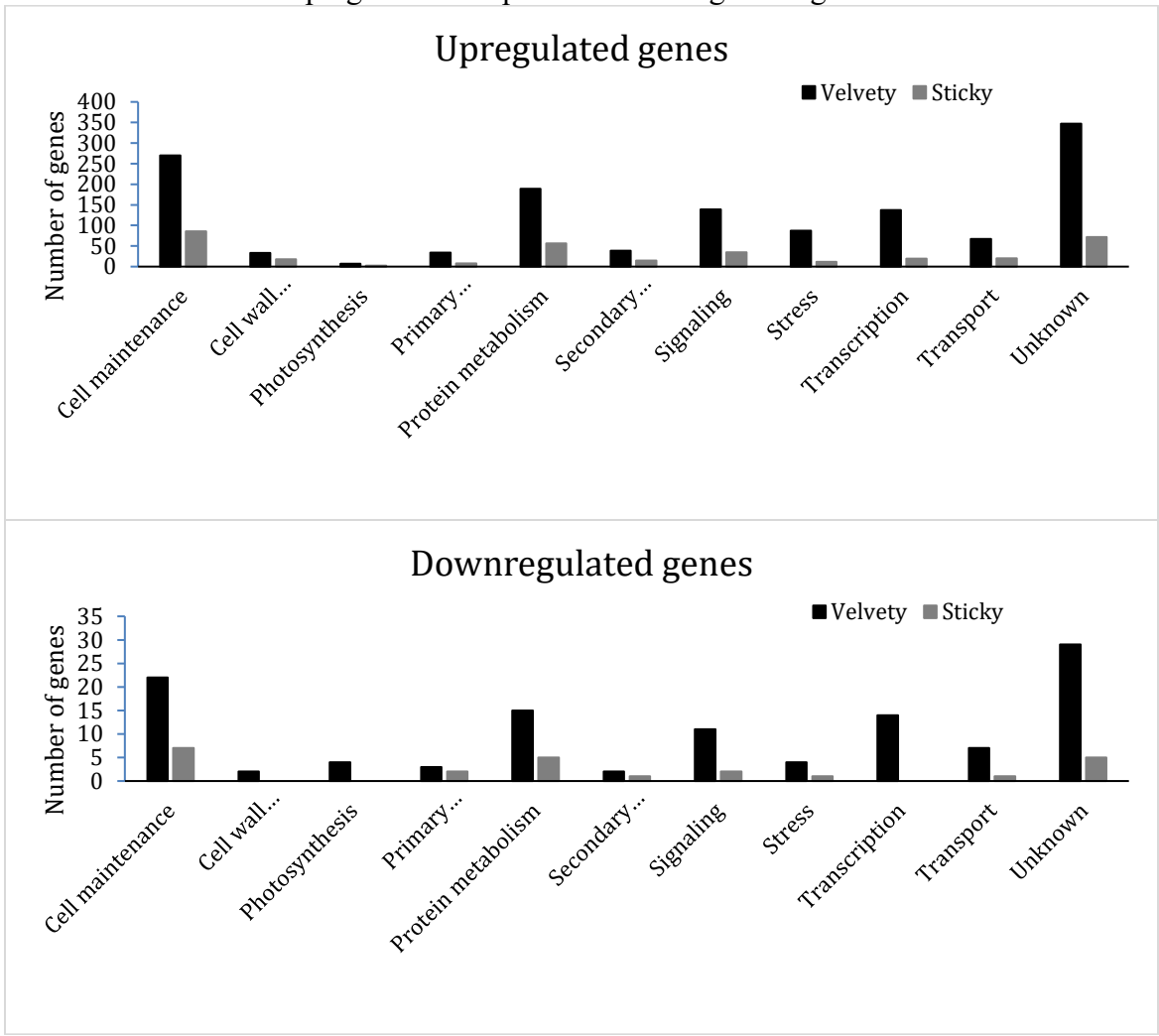

Figure 4.13. Heatmap of 12 putative terpene synthase genes induced by 24 hours of feeding by *Lema daturaphila* in sticky and velvety plants from the MVV6 line of *Datura wrightii*, shown in columns, with hierarchical clusters showing similarity of expression across rows. Red indicates increased expression relative to controls, and green indicates decreased expression.

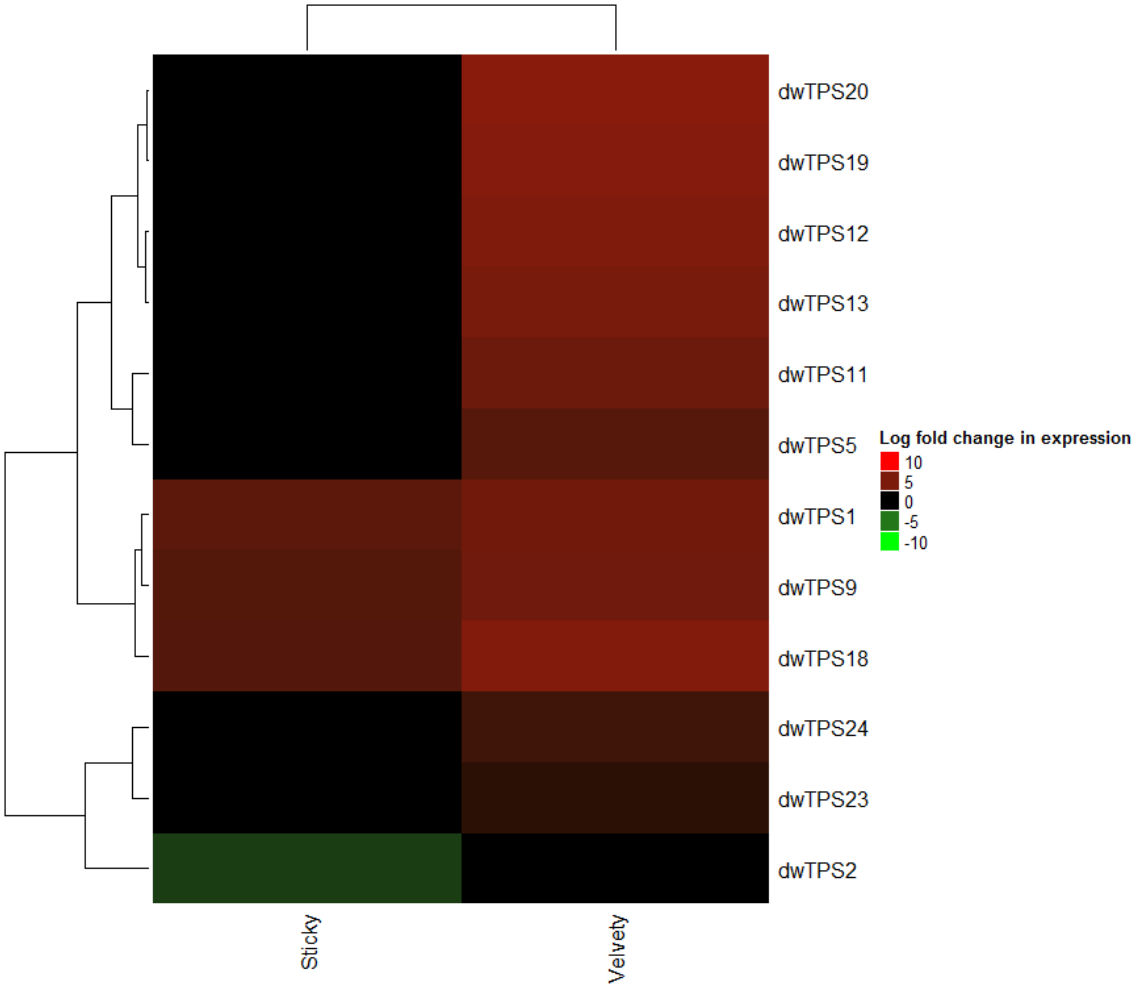

Table 4.7. A list of terpene synthase genes identified as being differentially expressed following herbivory in *Datura wrightii* plants from the MVV6 line that differed in trichome morph. Nucleotide sequences from the *D. wrightii* transcriptome were used to predict amino acid sequences and open reading frames, and the resulting sequences were compared to the Tomato proteome. Predicted products for each gene are from the reference listed.

Datura wrightii gene	Tomato ortholog gene ID	Tomato homolog	Predicted amino acid length	Non-similar amino acid substitutions from tomato	Expect (E) value	Main predicted product	Reference
DwTPS1	solyc00g154480		521	7	0	Unknown terpenoid	Fernandez-Pozo et al. 2015
DwTPS2	solyc01g101180	Truncated slTPS32-like	249	6	e-154	α-humulene / (-)-(*E*)-β-caryhophyllene	Falara et al., 2012
DwTPS5	solyc01g105880	Nearly identical to slTPS4	590	0	0	β-phellandrene	Van Schie et al. 2007
DwTPS6	solyc01g105890	slTPS5-like	609	2	0	Linalool	Falara et al., 2012
DwTPS7	solyc01g105910		131	0	2E-90	D-limonene	Fernandez-Pozo et al. 2015
DwTPS8	solyc02g079840	slTPS38-like	538	4	0	α-bergamotene	Falara et al., 2012
DwTPS9	solyc02g079890	slTPS25-like	122	5	5E-67	β-ocimene	Falara et al., 2012
DwTPS11	solyc02g079910	slTPS27-like	160	2	4E-67	Limonene	Falara et al., 2012
DwTPS12	solyc03g006550		621	8	0	(-)-*ent*-kaurene	Fernandez-Pozo et al. 2015
DwTPS13	solyc03g007730		562	3	0	β-ocimene	Fernandez-Pozo et al. 2015
DwTPS18	solyc10g005390	Nearly identical to slTPS39	563	0	0	Nerolidol / linalool	Falara et al., 2012
DwTPS19	solyc10g005410	Truncated slTPS37-like	137	11	7E-26	Nerolidol / β-ocimene	Falara et al., 2012
DwTPS20	solyc10g005420	Truncated	38	0	5E-21	Limonene	Fernandez-Pozo et al. 2015
DwTPS23	solyc01g105940		105	0	6.00E-73	Limonene	Fernandez-Pozo et al. 2015
DwTPS24	solyc06g059930	Truncated	174	0	e-124	Germacrene synthase	Fernandez-Pozo et al. 2015

Figure 4.14. Comparison of gene expression patterns across samples using quantitative PCR (qPCR) and RNA-Seq for plants continuously damaged by *Lema daturaphila*. Dark bars represent mean (±SE) qPCR analyses. Light bars show RNA-Seq results.

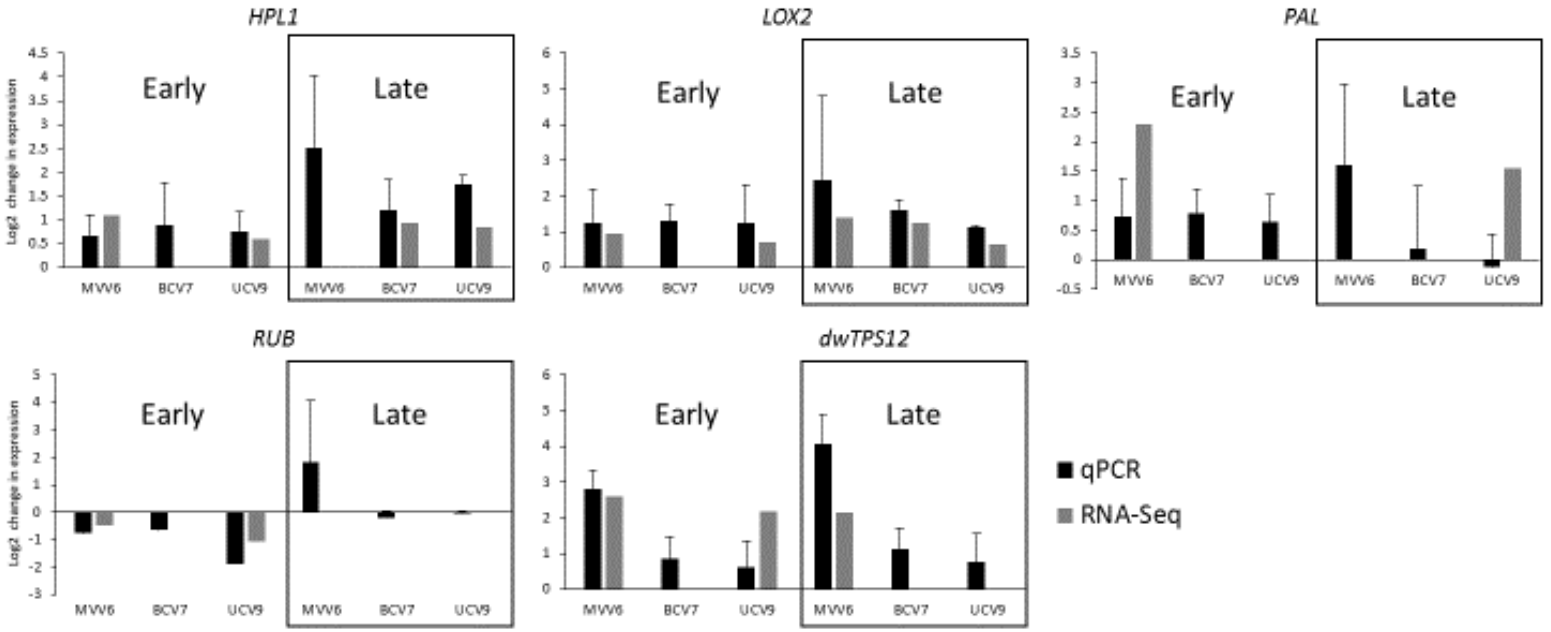

Table 4.8. List of all genes putatively assigned to biosynthesis and signaling of jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA). Included are putative functions as assigned by MapMan, pathway designations, factor loading scores for each gene showing their impact on the principal components analysis that included all genes shown here, and tomato orthologs. Percent variation accounted for by each PC is listed at the top.

		PC1	PC2	PC3	PC4	
Percent variation		40.7	31.8	16.5	8.8	
Function	**Pathway**					**Tomato ortholog**
ERF domain protein	ET	-0.03	-0.05	-0.18	0.47	Solyc10g009110.1
1-aminocyclopropane-1-carboxylate oxidase	ET	0.16	0.09	0.11	0.37	Solyc12g005940.1
1-aminocyclopropane-1-carboxylate oxidase	Et	0.14	0.15	-0.12	0.33	Solyc08g008100.2
1-aminocyclopropane-1-carboxylate synthase	ET	0.18	-0.04	-0.16	0.32	Solyc12g056180.1
1-aminocyclopropane-1-carboxylate synthase	ET	0.13	0.18	-0.10	0.30	Solyc08g008110.2
Lipoxygenase	JA	0.14	-0.12	0.20	0.28	Solyc08g014000.2
Allene oxide synthase	JA	-0.03	-0.11	0.37	0.08	Solyc10g007960.1
Gibberellin 20-oxidase	ET	-0.03	-0.11	0.37	0.08	Solyc11g072310.1
Gibberellin 20-oxidase	ET	-0.03	-0.11	0.37	0.08	Solyc12g013780.1
GRAM domain-containing protein	ABA	-0.03	-0.11	0.37	0.08	Solyc03g098660.2
Lipoxygenase	JA	0.16	-0.09	0.29	0.01	Solyc09g075860.2
Allene oxide synthase	JA	-0.05	0.28	0.06	0.00	Solyc01g109160.2
Lipoxygenase	JA	-0.05	0.28	0.06	0.00	Solyc01g099160.2
Gibberellin 2-beta-dioxygenase	ET	-0.05	0.28	0.06	0.00	Solyc04g008670.1
Leucocyanidin oxygenase	ET	-0.05	0.28	0.06	0.00	Solyc08g080040.2
Gibberellin 2-beta-dioxygenase	ET	-0.05	0.28	0.06	0.00	Solyc02g070430.2
2-oxoglutarate-dependent dioxygenase	ET	-0.05	0.28	0.06	0.00	Solyc09g089770.2
2-oxoglutarate-dependent dioxygenase	ET	-0.05	0.28	0.06	0.00	Solyc12g006380.1
2-oxoglutarate-dependent dioxygenase	ET	-0.05	0.28	0.06	0.00	Solyc09g089810.1
Xanthine dehydrogenase	ABA	-0.05	0.28	0.06	0.00	Solyc11g065920.1
Allene-oxide cyclase	JA	0.19	-0.08	0.25	0.00	Solyc02g085730.2
Leucoanthocyanidin dioxygenase	ET	0.14	0.17	0.25	-0.01	Solyc10g076660.1
Abscisic aldehyde oxidase	ABA	0.13	0.25	0.04	-0.05	Solyc01g088170.2
Jasmonate-ZIM-domain protein	JA	0.19	0.19	0.02	-0.06	Solyc07g042170.2
Jasmonate-ZIM-domain protein	JA	0.25	0.00	-0.03	-0.07	Solyc12g049400.1
Jasmonate-ZIM-domain protein	JA	0.25	0.00	-0.03	-0.07	Solyc12g009220.1
12-oxophytodienoate reductase	JA	0.25	0.00	-0.03	-0.07	Solyc07g007870.2
Allene oxide synthase	JA	0.25	0.00	-0.03	-0.07	Solyc01g109150.2
Allene oxide synthase	JA	0.25	0.00	-0.03	-0.07	Solyc01g109140.2
Allene oxide synthase	JA	0.25	0.00	-0.03	-0.07	Solyc11g069800.1
Allene oxide synthase	JA	0.25	0.00	-0.03	-0.07	Solyc04g079730.1
Hydroperoxide lyase	JA	0.25	0.00	-0.03	-0.07	Solyc07g049690.2
Lipoxygenase	JA	0.25	0.00	-0.03	-0.07	Solyc01g009680.2
Lipoxygenase	JA	0.25	0.00	-0.03	-0.07	Solyc12g011040.1
2-oxoglutarate-dependent dioxygenase	ET	0.25	0.00	-0.03	-0.07	Solyc09g089800.1
Xanthine dehydrogenase	ABA	0.08	0.11	0.23	-0.25	Solyc11g065930.1
Ethylene responsive transcription factor	ET	-0.07	-0.06	-0.11	-0.33	Solyc09g089910.1

Figure 4.15. Regression of mean induced VOC emissions against the first four principal components of the PCA that included all genes putatively involved in jasmonic acid-, ethylene-, and abscisic acid-signaling pathways. Mean induced VOCs is calculated based on the mean emissions of induced plants from each genotype by sampling period group minus mean emissions of control plants from that same period. All relationships $p>0.05$.

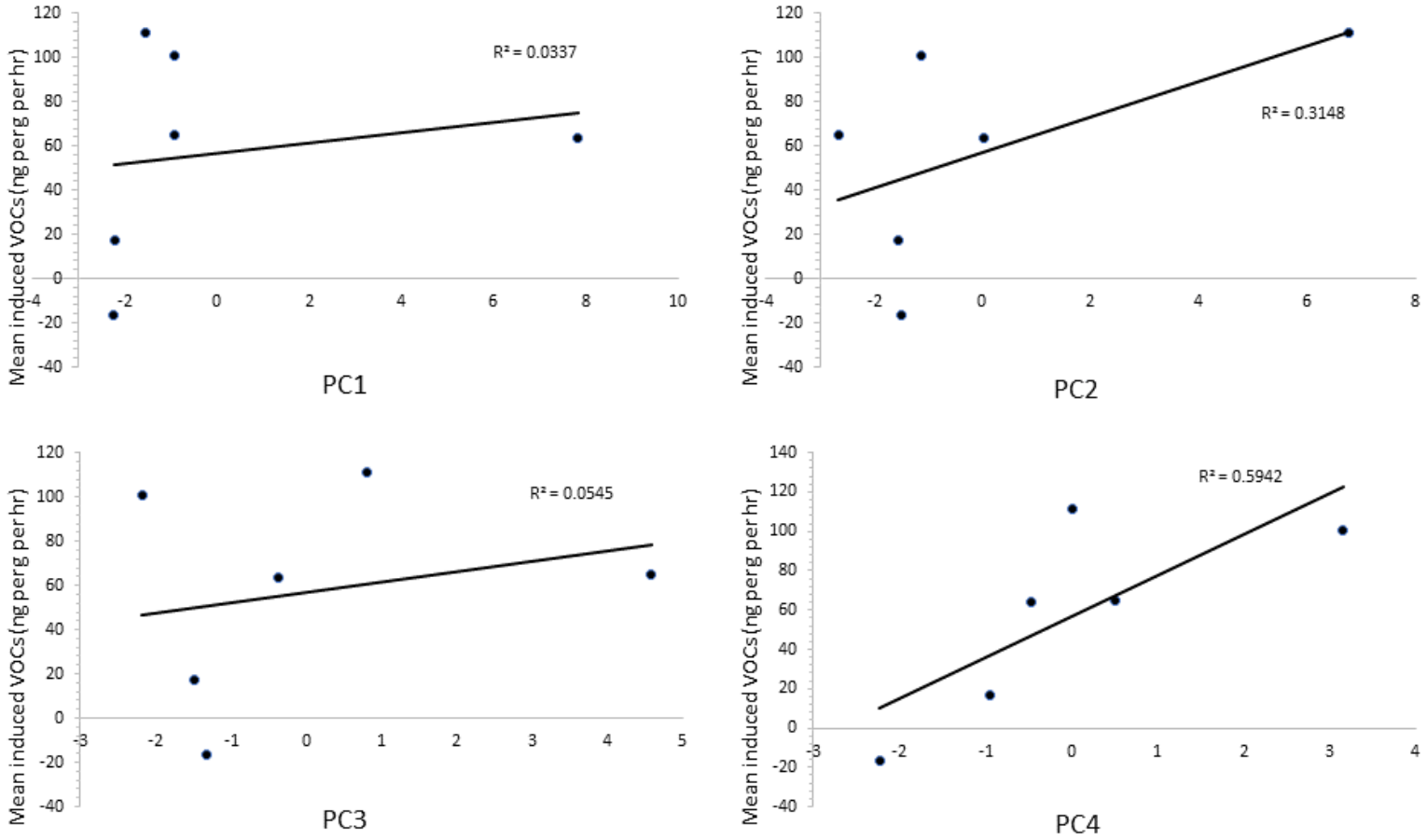

Table 4.9. List of individual compounds included in a principal components analysis of VOC emissions, as well as their factor loadings toward each individual component. The percent of the variation explained by each PC is listed at the top.

	PC1	**PC2**	**PC3**
Percent variation	42.8	30.1	20.1
Compound			
trans-2-hexenal	0.46	0.25	0.72
cis-3-hexen-1-ol	-0.08	0.27	0.08
trans-2-hexen-1-ol	-0.01	-0.02	-0.07
Hexenyl acetate	-0.24	0.13	0.17
Limonene	0.00	-0.01	0.00
(*E*)-β-ocimene	0.00	-0.19	-0.07
Linalool	0.07	0.74	-0.21
DMNT	0.10	0.46	-0.17
Methyl salicylate	0.18	0.07	-0.20
(*E*)-β-caryophyllene	0.49	-0.20	0.25
Geranyl acetone	-0.66	0.11	0.52
α-farnesene	0.02	-0.01	0.01

Figure 4.16. Regression of principal component 1 against the first three principal components of the PCA that included all genes putatively involved in jasmonic acid, ethylene, abscisic acid, salicylic acid, auxin, and cytokinin signaling pathways. Phytohormone PCs 1 and 2 had nonsignificant relationships with VOC PC1 ($p>0.05$), but phytohormone PC3 showed a significant relationship ($p=0.0273$)

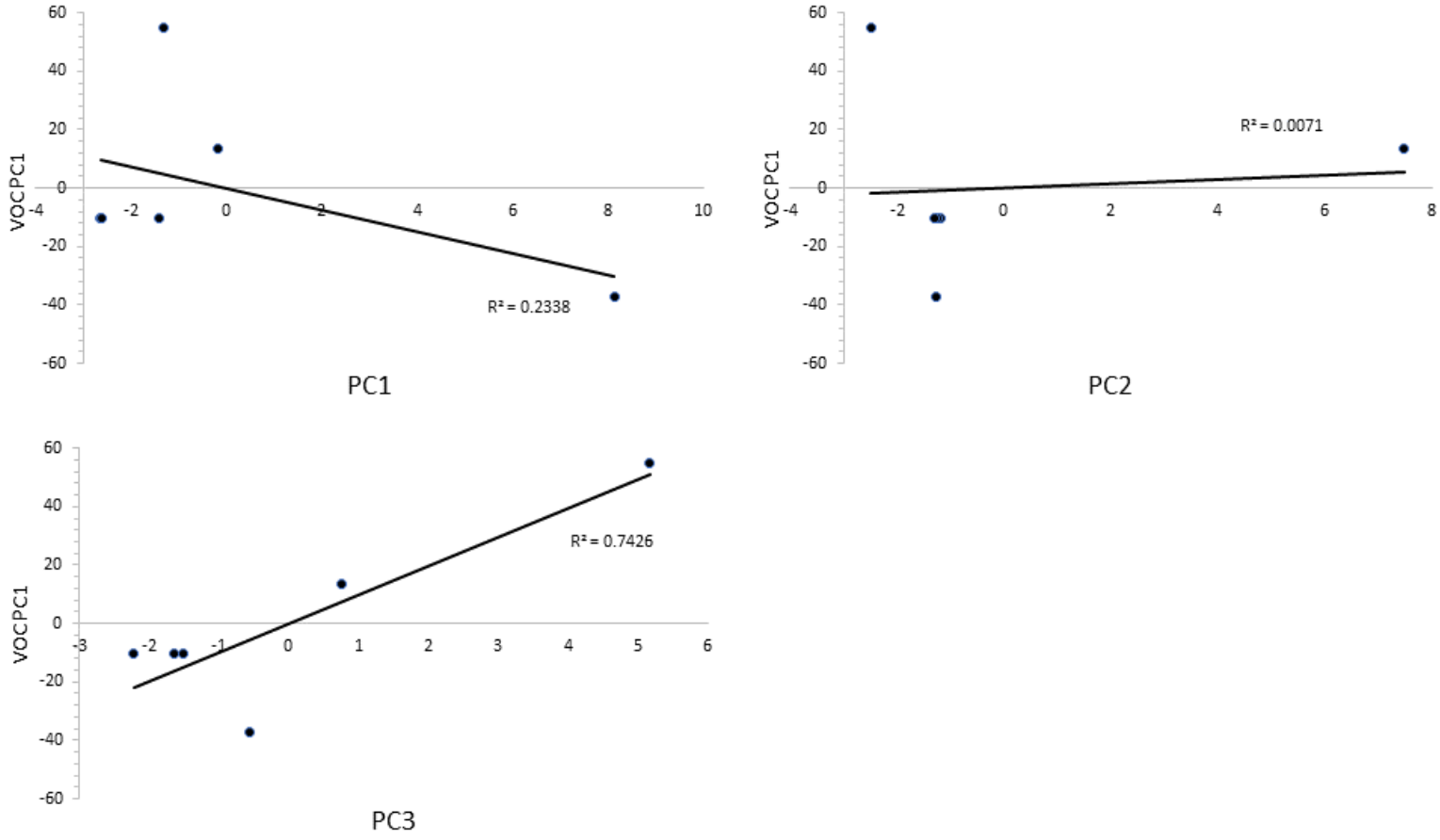

Table 4.10. List of all genes putatively assigned to biosynthesis and signaling of jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), auxin (IAA), cytokinin (CK), and salicylic acid (SA). Included are putative functions as assigned by MapMan, pathway designations, factor loading scores for each gene showing their impact on the principal components analysis that included all genes shown here, and tomato orthologs. Percent variation accounted for by each PC is listed at the top.

		PC1	PC2	PC3	
	Percent variation	39.8	32.5	17.9	
Function	**Pathway**				**Tomato ortholog**
Allene oxide synthase	JA	-0.04	-0.09	0.34	Solyc10g007960.1
Gibberellin 20-oxidase	ET	-0.04	-0.09	0.34	Solyc11g072310.1
Gibberellin 20-oxidase	ET	-0.04	-0.09	0.34	Solyc12g013780.1
GRAM domain-containing protein	ABA	-0.04	-0.09	0.34	Solyc03g098660.2
Lipoxygenase	JA	0.14	-0.11	0.26	Solyc09g075860.2
Allene-oxide cyclase	JA	0.17	-0.11	0.23	Solyc02g085730.2
Leucoanthocyanidin dioxygenase	ET	0.16	0.13	0.21	Solyc10g076660.1
Xanthine dehydrogenase	ABA	0.10	0.09	0.20	Solyc11g065930.1
Lipoxygenase	JA	0.12	-0.14	0.19	Solyc08g014000.2
1-aminocyclopropane-1-carboxylate oxidase	ET	0.16	0.05	0.10	Solyc12g005940.1
Allene oxide synthase	JA	0.00	0.27	0.05	Solyc01g109160.2
Lipoxygenase	JA	0.00	0.27	0.05	Solyc01g099160.2
Gibberellin 2-beta-dioxygenase	ET	0.00	0.27	0.05	Solyc04g008670.1
Leucocyanidin oxygenase	ET	0.00	0.27	0.05	Solyc08g080040.2
Gibberellin 2-beta-dioxygenase	ET	0.00	0.27	0.05	Solyc02g070430.2
2-oxoglutarate-dependent dioxygenase	ET	0.00	0.27	0.05	Solyc09g089770.2
2-oxoglutarate-dependent dioxygenase	ET	0.00	0.27	0.05	Solyc12g006380.1
2-oxoglutarate-dependent dioxygenase	ET	0.00	0.27	0.05	Solyc09g089810.1
Xanthine dehydrogenase	ABA	0.00	0.27	0.05	Solyc11g065920.1
cytokinin dehydrogenase	CK	0.00	0.27	0.05	Solyc01g088160.2
S-adenosyl-L-methionine:carboxyl methyltransferase	SA	0.16	0.20	0.02	Solyc09g091530.1
Abscisic aldehyde oxidase	ABA	0.16	0.20	0.02	Solyc01g088170.2
Jasmonate-ZIM-domain protein	JA	0.21	0.14	0.00	Solyc07g042170.2
Jasmonate-ZIM-domain protein	JA	0.24	-0.05	-0.04	Solyc12g049400.1
Jasmonate-ZIM-domain protein	JA	0.24	-0.05	-0.04	Solyc12g009220.1
12-oxophytodienoate reductase	JA	0.24	-0.05	-0.04	Solyc07g007870.2
Allene oxide synthase	JA	0.24	-0.05	-0.04	Solyc01g109150.2
Allene oxide synthase	JA	0.24	-0.05	-0.04	Solyc01g109140.2
Allene oxide synthase	JA	0.24	-0.05	-0.04	Solyc11g069800.1
Allene oxide synthase	JA	0.24	-0.05	-0.04	Solyc04g079730.1
Hydroperoxide lyase	JA	0.24	-0.05	-0.04	Solyc07g049690.2
Lipoxygenase	JA	0.24	-0.05	-0.04	Solyc01g009680.2
Lipoxygenase	JA	0.24	-0.05	-0.04	Solyc12g011040.1
2-oxoglutarate-dependent dioxygenase	ET	0.24	-0.05	-0.04	Solyc09g089800.1
IAA-Ala conjugate hydrolase/ metallopeptidase	IAA	0.24	-0.05	-0.04	Solyc05g006220.2
1-aminocyclopropane-1-carboxylate synthase	ET	0.14	0.13	-0.10	Solyc08g008110.2
Ethylene responsive transcription factor	ET	-0.08	-0.04	-0.10	Solyc09g089910.1
1-aminocyclopropane-1-carboxylate oxidase	Et	0.15	0.10	-0.11	Solyc08g008100.2
1-aminocyclopropane-1-carboxylate synthase	ET	0.15	-0.07	-0.14	Solyc12g056180.1
ERF domain protein	ET	-0.04	-0.04	-0.15	Solyc10g009110.1
auxin-responsive protein, putative	IAA	-0.15	-0.10	-0.26	Solyc01g110940.2
PIN-FORMED 8; auxin transporter	IAA	0.04	0.09	-0.34	Solyc02g087660.2

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Chapter Five: Concluding Remarks on the Variation of Induced Transcriptomic Responses to Herbivory in *Datura wrightii*

A central goal of biology is to understand how the genotype of an organism influences its phenotype. Heritable variation in traits is a basic requirement of evolution by natural selection, and elucidating the molecular basis for variation in traits is crucial for understanding how natural selection can act on traits within a population. The studies within this dissertation characterized the variation in transcriptional activity that occurred due to several ecological and genetic factors, and related these differences to the emission of volatile organic compounds (VOCs).

The second chapter examined the response dynamics of the plant over the course of a single, continuous herbivore attack. This approach allowed for a relatively fine-scale analysis of gene expression over time, and the relationship between transcript accumulation and the deployment of induced VOCs was explored. The results of this study show that the quantity of induced emissions is not proportional to the amount of damage inflicted by herbivores over the course of a single attack. Despite continuing damage, VOC emissions peaked 48 hours after the start of damage. The number of differentially expressed genes (DEGs) increased in a step-wise fashion, reaching a zenith at 48 hours before substantially decreasing in number by 96 hours. Furthermore, most DEGs in this study were unique to the 48-hour time point. Overall, the damage response was highly complex and suggests a regulatory mechanism preventing additional herbivore damage from eliciting further response from the plant.

Chapter three built upon prior studies showing an ontogenetic decline in herbivore-induced VOC emissions as *D. wrightii* entered the reproductive stage. This

study characterized ontogenetic changes to pathways associated with the induced response over the course of two growing seasons under field conditions, with the aim of identifying potential mechanisms responsible for changes in inducible VOC emissions. The results of this study show that the variation in DEGs is far greater than VOC emissions would suggest. During early time points when plants were inducible, the quantity of herbivore-induced DEGs was similar, despite very little overlap in the identity of the genes. Furthermore, the lack of inducible gene expression was not associated with a particular transcriptional profile. Among the three timepoints absent induced volatiles, each showed a remarkably different gene expression response ranging from 94 DEGs at the end of the first year to 2,689 at the end of the second year. Although terpene synthase gene expression was a poor predictor of volatile emissions, multivariate analyses showed that an aggregation of phytohormone gene expression could predict 86.5% of the variation in total volatile emissions.

Finally, the fourth chapter looked at differences in induced responses across fifth generation inbred lines of *D. wrightii*, to examine the influence of genetic variation on transcriptional pathways involved in resistance. Given the patterns identified in the second chapter, this facet of the study asked how induced responses and ontogenetic decline differed between plant lines originating in natural populations located in southern California, as well as across trichome phenotypes. The results of this chapter suggest that although all three lines used in the study experienced ontogenetic decline in response in terms of gene expression, volatile emissions, or both, the specific transcriptional profiles differed tremendously. Within lines, very few genes were differentially expressed at both

early and late time points. Between lines, very few DEGs were commonly regulated at either early or late time points. Differences in trichome phenotype are associated with herbivore-specific resistance and vulnerability in *D. wrightii*, leading to the hypothesis that this trait might be associated with a suite of compensatory resistance traits. Siblings from a single genetic line differing in trichome phenotype showed modest differences in gene expression. Although the velvety phenotype showed roughly three times as many DEGs as the sticky trichome phenotype, comparisons between undamaged or damaged samples showed that the differences between phenotypes are primarily quantitative, with a small number genes showing consistently different expression levels between phenotypes.

Taken together, these studies demonstrate that induced responses to herbivory are far from immutable. Each chapter showed that differences in gene expression between samples were far greater than phenotypic traits would suggest. Indeed, samples within each chapter showed remarkable variation in the numbers of genes induced in response to herbivory, and there was no clear association between the number of DEGs and the quantities of volatiles emitted. Furthermore, there was little overlap in the specific genes expressed, regardless of the total number of DEGs or VOC emission quantities. These findings suggest that the concept of a "typical" induced response for a given species may not exist. Although few genes were differentially expressed in multiple samples, the notable consistency of gene distributions across functional categories suggests that overall patterns of gene expression are non-random. The physiological and ecological significance of this activity remains an open question.

The ability of multivariate aggregations of phytohormones to explain variation in VOC emissions is a crucial finding of these studies. Although the involvement of hormones in the elicitation of resistance traits is not a new idea, it confirms that phenotypic traits are ultimately regulated by early signaling molecules. In contrast, downstream terpene synthase genes were consistently upregulated following herbivory, but this was not necessarily accompanied by increased VOC emissions. Additionally, the improvement of explanatory models by including more hormones, especially ones not typically expected to factor in induced resistance, suggests that resistance phenotypes are the product of the entire phytohormonal landscape. This phenomenon also suggests that the ontogenetic decline in VOC inducibility occurs due to—or is associated with changes to—hormones involved with reproduction that negatively regulate VOC emissions.

Ultimately, this dissertation has important implications for the study of plant-insect interactions. First, it suggests that many studies of the transcriptional response of plants to herbivore attack may be underestimating the variability of induced responses. For this reason, it is imperative to explicitly account for plant age to minimize experimental error. On the other hand, future studies will need to expand in scope to account for the differences in responses across ontogenetic stages and genotypes. Arbitrarily isolated genetic lines of model plants are not likely representative of variation among natural populations.

The inconsistencies of response observed in these studies suggest that selection on induced responses is diffuse. Phenotypic plasticity has evolved in plants as a response to variations in environmental conditions, and herbivores represent only one set of selective

pressures on plant populations. Given that plants have a limited number of signaling pathways through which to coordinate responses to multiply external stimuli, plants are very likely constrained in their ability to respond to each external stressor. As such, induced resistance is unlikely to represent an ideal response to herbivory, but is instead a constrained, yet presumably somewhat effective, response to herbivores that is the result of selection by herbivores attacking across a wide breadth of environmental contexts. Moving forward, the proliferation of studies involving undomesticated, non-model organisms will be critical to furthering our understanding of breadth of responses and their ecological and evolutionary implications.