LEAF SALICYLIC ACID CONCENTRATION AND INSECT HERBIVORY

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Due to their stationary existence, plants are exposed to an array of attackers including pathogens and insect herbivores. In defense, plants employ sophisticated responses mediated by the hormones salicylic acid (SA) and jasmonic acid (JA). These two hormones act antagonistically to fine-tune expression of defense compounds to a particular attacker. This is an important component of optimal defense theory (ODT), which states that expression of defenses is costly due to limited metabolic resources. Several studies have shown that increased expression, or exogenous application, of SA inhibits expression of JA-mediated defenses, and vice versa. However, no studies have investigated how constitutive SA levels affect JA-mediated defenses. After reviewing the literature on ODT, I present a review of the mustard family (Brassicaceae) and their specialist insect herbivores *Pieris* butterflies, which is the model system used in my first study. In this study, I compared constitutive SA levels in five spring-flowering and five summer-flowering mustard species and found that the spring-flowering species received significantly less herbivory and supported lower caterpillar relative growth rate (RGR) than the summer-flowering species. I then asked whether the differences that I observed could be explained by the underlying leaf SA concentrations. I found that the species with the lowest constitutive SA concentrations were the most resistant to herbivory and supported the lowest larval RGR. The highest herbivory rate and RGR occurred in species with intermediate concentrations of constitutive SA. In a second study, I investigated the inhibition of JA-mediated defenses by induced SA levels. I applied a commonly-used SA analog to five mustard species then measured RGR of the generalist caterpillar, *Trichoplusia ni*. I found that exogenous SA increased plant susceptibility in most, but not all plants. The exception being an agricultural variety of the perennial plant, *Lesquerella fendleri*.

I conclude that low constitutive SA concentrations may benefit plants by reducing herbivory and larval growth rates of insect herbivores. The finding that constitutive SA levels affect herbivore

performance is unprecedented and has implications for our understanding of the evolution of plant defenses and for the cultivation of more insect-resistant crops.

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1. INTRODUCTION

Plants are under constant attack from many types of organisms and must simultaneously defend themselves against pathogens, herbivores and parasites. Since plants are immobile, they are especially vulnerable to herbivorous animals, which can move and search for food. Of the herbivores that threaten plants, insects are the most numerous and cause the most damage. Among insects there exist drastically different modes of attack. For example some insects chew plant tissue, while others use a pointed proboscis to extract phloem from plants and still others, called leaf miners, tunnel through the inner layers of leaves. In response to these threats, plants have evolved various ways of defending themselves against the many different attackers they face. Plant defenses include structural modifications that make feeding on plant tissue difficult. Trichomes, for example, are tiny outgrowths of the epidermis that interfere with insect feeding on plant tissue (Traw & Dawson, 2002a; Traw & Feeny, 2008). Other plants have waxes that make their tissues difficult to chew or digest (Stoner, 1990). Plants also produce many different chemical compounds that reduce the digestibility of plant tissue (Jongsma & Beekwilder, 2011; Franco et al., 2002), act as direct toxins to ingesting insects (Agrawal et al., 2012; Kos et al., 2012) or attract enemies of herbivores, such as predators or parasitoids (Geervliet et al., 1996; van Poecke et al., 2003; Halitschke et al., 2008; Hopkins et al., 2009; Dicke & Baldwin, 2010). Despite great variation among the thousands of chemical defenses produced by various plant species, evolutionarily-conserved metabolic pathways that regulate defense signaling have been found in all angiosperms studied. Gene products in these pathways interact with each other to create a complex metabolic network that is only partially understood.

In this thesis I first review our current understanding of plant defense theory and plant signaling pathways most important for defense against insects and pathogens (Chapter 2). Next I summarize the research that has been conducted on mustards and their specialist herbivores (Chapter 3). I then describe two studies that I conducted that investigate whether varying constitutive defense hormone levels among plant species correlate with the performance of insect herbivores. In the first study, I assessed how variation in endogenous SA production correlates with the performance of the cabbage white butterfly, *Pieris rapae* (Chapter4). In the second study I investigate whether exogenous application of SA increases the susceptibility of plants to the cabbage looper, *Trichplusia ni* (Chapter 5). Finally, I briefly summarize the findings from the two studies and suggest the direction of future experiments (Chapter 6).

2. A REVIEW OF PLANT DEFENSE THEORIES

In the following chapter, I first describe plant chemical defenses and answer the question of why some plant defenses are produced constitutively while others must be induced. Next, I discuss insect counteradaptations, and why insects become specialists on particular plant groups. Finally, I present the two major biochemical pathways by which plants induce defense responses.

WHAT CHEMICAL DEFENSES DO PLANTS USE

Across the plant kingdom thousands of chemicals are produced in response to constant herbivory pressure from insects and other animals. For example, cardenolides are toxic steroid-derived compounds that block animal enzyme Na/K-ATPase (Dobler et al., 2012; Petschenka et al., 2013). Alkaloids are structurally diverse, typically toxic compounds consisting of more than 20 classes and grouped together because they contain nitrogen and have a basic pH (Wink et al., 1998). Glucosinolates (GS) are derived from glucose and contain sulfur, nitrogen and a variable side group (Halkier & Gershenzon, 2006). Many more defensive secondary metabolites exist across the plant kingdom (for a review see Mithöfer & Boland, 2012). These compounds are typically not directly involved in the primary metabolic processes necessary for growth, development and reproduction of plants and are therefore referred to as secondary metabolites. Many secondary compounds target insect biochemistry and either deter attack or reduce the damage caused by an attack.

Defensive chemicals may be produced constitutively, as an induced response after an attack has been initiated, or both. They may also be classified as quantitative or qualitative defenses. Constitutive

defenses are frequently quantitative in nature. That is, they are continuously made and stored in the plant in order to prevent herbivory, but their effect is dose dependent (Feeny, 1968). For example, many plants, especially woody species, produce tannins. These are large compounds that are difficult for herbivores to digest and are typically greater than 5% of the dry weight plant tissue, but may be as high as 40% (Feeny & Bostock, 1968). In contrast, induced defenses are frequently qualitative in nature. That is, they are toxic compounds that function at much lower concentrations in plant tissue, typically less than 2% of dry weight. Glucosinolates are an example of a qualitative defense and have been measured to comprise only 0.0148-0.0276% of leaf dry weight in *Brassica oleracea* (van Emden & Bashford, 1969) and up to 1% of leaf dry weight in *Arabidopsis thaliana* (Brown et al., 2003). This lower concentration is enough to deter herbivory by non-adapted specialist and most generalist insect species (Erickson and Feeny, 1974; Lichtenstein, et al., 1964).

Plant defenses against insects may also act directly or indirectly, depending on the trophic level of the target organism. Direct defense compounds either reduce the digestibility of plant tissue or are toxic to organisms that ingest them. In contrast, indirect defenses attract natural enemies of herbivores, such as predators and parasitoids. A well-studied example is the attraction of parasitoids of *Pieris* caterpillars to insect-damaged Brassicaceae plants (see Gols & Harvey, 2009). These plants produce and store biologically inactive glucosinolates (GS) and their activating enzymes, myrosinases, in separate cellular compartments. Upon tissue damage, e.g. by a *Pieris* caterpillar, the GS and myrosinase mix, facilitating the hydrolysis of the GS to an aromatic isothiocyanate that attracts the parasitoid wasp *Cotesia glomerata* (Gols et al., 2008c). A gravid female *C. glomerata* wasp then deposits her eggs in the caterpillar and they hatch a few days later and consume the caterpillar, killing it.

WHY ARE SOME PLANT DEFENSES PRODUCED CONSTITUTIVELY WHILE OTHERS MUST BE INDUCED?

Optimal Defense Theory

The optimal defense theory (ODT) was described in the mid-1970s to explain the evolution of plant secondary chemical defenses and how plants allocate metabolic resources to defense and the necessary physiological functions of growth, development and reproduction, in a way that maximizes individual fitness (Feeny, 1975; Rhoades & Cates, 1976; Stamp, 2003). The primary assumption of this theory is that defenses are costly. Accordingly, numerous studies have gone on to demonstrate fitness costs associated with the production of physical or chemical defenses (Ågren & Schemske, 1993; Traw, 2002; Agrawal et al., 2002). Despite the costs, plants express defensive traits due to the fitness benefit conferred by reducing the damage done by herbivores. In the absence of enemies, however, plants that allocate fewer resources to defense will have more resources to allocate to growth and reproduction and therefore should experience higher fitness compared to plants that allocate more resources to unnecessary defenses. One mechanism many plants have evolved to reduce expression of defense in the absence of enemies is plasticity in production of defense structures and compounds. Plasticity in plant defense refers to the inducible expression of defensive traits in response to particular stimuli. This allows an organism to allocate resources to traits that will maximize fitness in a particular situation. For example, in the absence of herbivores, available carbon and nitrogen resources can be allocated toward growth and reproduction. This enables a plant to be more competitive and produce more seeds when expression of inducible defenses is not necessary. When attacked by an herbivore, a plant can divert some of its resources toward the production of structural defenses such as trichomes or chemical defenses such as GS (Karban & Baldwin, 1997; Traw, 2002; Traw & Dawson, 2002a; Björkmann et al., 2008).

Other assumptions of ODT postulate that there is genetic variation in the production of secondary metabolites which allows for differential selection of appropriately defended individuals. It also assumes that the primary selective force for expression of defensive traits is herbivory and that induced defenses reduce herbivory. These assumptions have been supported by research data. Intraspecific variation in expression of defensive secondary metabolites has been demonstrated in numerous studies (Dirzo and Harper 1982; Zangerl and Berenbaum 1990; van Dam and Vrieling 1994; Agrawal et al., 2002; Gols et al., 2008b; Agerbirk et al., 2010). Other studies have concluded that herbivory can negatively affect the growth and reproduction of plants (e.g., Marquis 1984; Strauss 1991; Smallegange et al., 2008) and is indeed a major selective force acting on the expression of secondary metabolites (Simms & Rausher, 1989; Mauricio & Rausher, 1997; Agrawal, 2005).

Tradeoffs Between Primary and Secondary Physiological Processes

The biosynthesis of secondary metabolites has metabolic costs that depend on the substrate and cofactors produced as well as on costs associated with transport and storage (Mooney et al., 1983; Baas, 1989; Gershenzon 1994). The reallocation of resources from growth and reproduction to defense against enemies is usually described as a tradeoff between primary and secondary physiological processes (Herms & Mattson, 1992; Mole, 1994). Accordingly, plants that express defensive traits should theoretically produce fewer seeds than plants that do not express these traits. This hypothesis has been supported by several studies (Mitchell-Olds et al., 1996; Traw, 2002; Strauss et al., 2002). However, the expressed defense reduces herbivory and increases the fitness of a plant when compared to less defended individuals. In the presence of herbivory pressure, effectively-defended plants have higher flower and seed production. Based on these results, natural selection should act on intraspecific variation in costs associated with the expression of defenses and select against individuals with a high

cost of defense, or with costs associated with the expression of defenses at inappropriate times, which reduce relative fitness in the absence of enemies (Simms 1992).

Inducible defenses and phenotypic plasticity

Constitutive defenses are preformed physical or chemical barriers that interfere with herbivory and are generally quantitative in nature. They tend to be large, metabolically-expensive compounds that are effective against all herbivores and are typically expressed by larger, perennial plants that are relatively easy to find (apparent) and face higher numbers of enemies. Inducible defenses, on the other hand, are smaller molecules that are toxic in low concentrations and therefore incur lower metabolic costs. However, they are also relatively easier for insects to overcome through the evolution of counterdefenses. Inducible defenses may also be either direct or indirect, depending on the organism they target and the mechanism of the defense. Direct defenses affect the ability of herbivores to consume plant material (Kessler & Baldwin, 2002). They can be physical in nature, such as trichomes that make plant tissue difficult to chew, or they may be chemicals, such as proteinase inhibitors which decrease the ability of the insect to digest plant material or toxins that injure the insect by interfering with physiological processes. Indirect defenses, on the other hand, do not directly affect the herbivore itself. They are typically volatile compounds, termed herbivore-induced plant volatiles (HIPV), which attract natural enemies of the herbivores to the plant upon tissue damage. Herbivore predators and parasitoids attracted to a plant emitting a HIPV 'distress signal' reduce herbivore population size and the damage caused to the plant. Some compounds such as GS, however, are both present constitutively (phytoanticipins; Rask et al., 2000; Fahey et al., 2001; Halkier & Gershenzon, 2006) and are inducible upon insect attack (phytoalexins; Traw, 2002; Traw & Dawson, 2002b; Agrawal et al., 2002; Agrawal & Kurashige, 2003; Mewis et al., 2005; Gols et al., 2008a).

Glucosinolates are small molecules and are relatively inexpensive when compared to large indigestible molecules like tannins. Therefore plants that allocate smaller amounts of metabolic resources to defense can afford to produce a limited amount of these compounds constitutively in anticipation of herbivore attack. However, these plants also demonstrate phenotypic plasticity as a cost-saving measure in the expression of GS. Phenotypic plasticity is the ability of a single genotype to express multiple phenotypes in response to variable environmental conditions (Sultan, 2000; van Buskirk & Steiner, 2009; Auld et al., 2010). A plastic trait, therefore, may be better adapted to several different environments (Bradshaw, 1965; Pigliucci, 2001; Auld et al., 2010). Plasticity is an important mechanism by which plants maximize fitness when confronted with environmental heterogeneity. The fact that plasticity is not maximized by all organisms for all traits demonstrates that there are ecological constraints on the evolution of phenotypic plasticity (Agrawal et al., 2002; Valladares et al., 2007; Auld et al., 2010).

Plants cannot, however, rely entirely on induced defenses. Allocating a portion of resources to constitutive defenses is necessary due to the constant presence of insect herbivores and the great threat they pose to plants. These constitutive defenses are, in fact, effective at deterring most generalists and all non-adapted specialists. Insect herbivores, therefore, indirectly limit plant growth and reproduction, even in the absence of actual herbivory damage.

Plant Apparency

An important consideration in determining optimal defense of plants is the susceptibility of an individual plant to attack by its enemies. Feeny (1976) associated risk of attack with plant apparency, which is the likelihood that a given plant will be found by its enemies. Factors that influence apparency include plant size, growth form, persistence and the abundance of conspecifics in the community

(Feeny, 1976). An insect herbivore that is a family specialist may also find a plant more easily if that plant is associated with a community of confamilial plants producing similar chemical defenses (Root, 1973). This scenario exists when the similar chemistry of related is used by herbivores as cues for locating hosts. These last two ideas are an adaptation of Root's (1973) resource concentration hypothesis. In this seminal work, Root found that specialist herbivores tended to be more abundant on *Brassica oleracea* that existed in monospecific stands when compared to individuals growing in communities of nonrelated plants. The abundance of plants in monocultures makes each individual easier to find and more susceptible to discovery by its herbivores.

Feeny (1976) argued that the proportion of metabolic resources allocated to defense is directly related to a plant's apparency. More apparent plants are at a greater risk of herbivory and thus must be better defended with higher concentrations of defensive compounds (Cates & Orians, 1975; Futuyma, 1976). Whereas, unapparent plants are harder to find, have a decreased risk of herbivory and therefore can allocate proportionally more metabolic resources toward growth and reproduction. Apparent plants tend to be larger, more persistent, longer-lived members of climax communities. Feeny began his career working with oaks (dominant climax species) in England and noted that tannins in the leaves of *Quercus robur* constitute approximately 5% of leaf dry weight (Feeny & Bostock, 1968). Tannins are what Feeny described as quantitative defenses; they typically form large molecules that are difficult for animals to digest and were termed "digestibility-reducing" defenses by Rhoades & Cates (1976). Tannins bind to plant proteins and form large, indigestible complexes, thus reducing the nutritional status of plant tissues. Since these compounds have no toxic effects on herbivores, they are only as effective as the amount of nutritional content they make unavailable to herbivores. Therefore, large quantities of these compounds are necessary to make the plants that produce them unattractive host choices for insects. Being large molecules required in large quantities makes these compounds metabolically expensive to produce. Defense theory predicts that only long-lived plants that can delay

reproduction and can store resources are likely to depend on this type of defense mechanism. Furthermore, qualitative defenses may be less effective for apparent plants due to differences in generation time with the insects that consume their tissues. Larger, more apparent, slower-growing and later-reproducing plants, such as many woody species, have longer generation times than the insects that feed upon them, which may have several generations per year. Thus, due to their shorter generation time, insects may quickly evolve adaptations that allow them to overcome the toxic effects of qualitative, but not quantitative, secondary metabolites (Fox, 1981).

In contrast to quantitative defenses, qualitative defenses target insect metabolic pathways and are therefore toxic, frequently causing paralysis or death. These compounds, however, are easier for insects to overcome. Specialist herbivores feeding on plants with toxic qualitative defenses have repeatedly evolved counterdefenses to the toxins produced by their particular host species (Ehrlich & Raven, 1964; Janz 2011). Furthermore, specialists may use toxic compounds against a plant by evolving sensory mechanisms that enable them to use the secondary metabolites as cues for locating food plants (Feeny et al., 1983; Chew, 1988; Renwick & Chew, 1994; Städler et al., 1995; Hopkins et al., 2009).

Unapparent plants, however, are generally more difficult for herbivores to find. These plants are typically early successional herbaceous species, such as many members of the family Brassicaceae. The life history of these plants is concentrated on rapid growth, early reproduction, high fecundity and greater dispersal and colonization abilities (Feeny, 1976; Grime, 1977, 2001). Because of their rapid growth and early and high reproductive output, these plants allocate fewer metabolic resources toward defense against herbivores and pathogens. Instead they rely on two different methods of defense when compared to the expensive, quantitative defenses produced by larger, longer-lived plants. First, they rely on their unapparent nature to escape detection by enemies (Feeny, 1976; Fox, 1981). With fewer herbivores finding them, they are at lower risk of herbivory than more apparent species. Second, they

produce smaller amounts of qualitative defensive compounds. These chemical defenses are typically toxic to animals in small amounts, reducing their metabolic burden and costs of production. Erickson & Feeny (1974) found that GS concentrations as low as 0.1% fresh weight was enough to cause 100% mortality in swallowtail butterfly (*Papilio polyxenes*) larvae. GS are the primary defensive compounds of the Brassicaceae (Feeny, 1977; Rodman et al., 1996; Halkier & Gershenzon, 2006) and are effective at preventing nonadapted specialists and many generalist insect species from feeding on them (Lichtenstein et al., 1964; Erickson & Feeny, 1974; Lazzeri et al., 2004).

INSECT COUNTERADAPTATIONS

Several Brassicaceae specialists have been shown to sequester GS in their tissue, hijacking these defensive compounds for their own use. The harlequin bug, *Murgantia histrionica* (Pentatomidae; Aliabadi et al., 2002), the aphids *Brevicoryne brassicae* and *Lipaphis erysimi* (Aphididae; Weber et al., 1986; Bridges et al., 2002) and turnip sawfly, *Athalia rosae*, larvae (Tenthredinidae; Müller et al., 2001) all sequester GS in their tissues. However, not all Brassicaceae specialists sequester GS in their tissues (Müller et al., 2003). *Pieris rapae* larvae divert degradation of ingested GS away from toxic isothiocyanates and toward less toxic nitriles using nitrile specifier protein (NSP), an enzyme present in their gut (Wittstock et al., 2004). Feeny (1976) recognized that secondary metabolites were easily overcome by specialist herbivores and noted that variation in concentration had little, if any, negative effect on specialist herbivores. He was, however, cautious enough to note that the experimental evidence for this claim was lacking.

Glucosinolates have, however, been shown to be toxic to specialist insect herbivores as well as generalists. Berenbaum et al. (1989) showed that growth of the parsnip webworm, *Depressaria*

patinacella (Lepidoptera: Oecophoridae) and the digestibility of their diet was decreased by the furanocoumarin bergapten. And Agrawal (2000) showed that performance of *P. rapae* and *Plutella xylostella* on *Brassica nigra* was reduced after induction of GS by prior herbivore feeding. The effect of GS on specialist herbivores, however, is inconclusive. Traw & Dawson (2002b) likewise showed that performance of *P. rapae* and the mustard flea beetle, *Phyllotreta cruciferae*, feeding on *B. nigra* was reduced after induction of defenses by prior herbivore feeding, but they attributed the reduced performance to induction of trichomes. Finally, Kliebenstein et al. (2002) used QTL mapping to find loci controlling resistance to insect herbivores. They concluded that performance of the generalist *Trichoplusia ni* was reduced by higher GS levels, but the higher levels had no effect on the performance of the specialist *Plutella xylostella*. Results such as these have the greatest implications for herbivore species that go through multiple generations per season since ensuing generations after defense induction are adversely affected.

WHY DO INSECTS SPECIALIZE?

Coevolution of Plant and Insects

Current theory posits that plants and the herbivorous insects that feed upon them coevolved through adaptation and counteradaptation (Ehrlich & Raven, 1964; Berenbaum, 1983; Braby & Tueman, 2006; Janz, 2011). Ehrlich & Raven (1964) recognized that there are frequent correlations between families of butterflies and clades of their food plants. The critical mechanism contributing to the arms race, in their view, was the development of plant secondary metabolites that act as deterrents, digestibility reducers or toxins. This observation led them to attribute the coevolution of plants and their insect herbivores to an evolutionary arms race. That is, plants evolved chemical defenses in response to herbivory from

insects and insects, in turn, evolved mechanisms to counter the toxins or digestibility reducers of the plants. Some individual plants within a species in turn evolved new chemical defenses which allowed them to escape from the herbivores, but their split from the parent lineage was shadowed by insects that split from their own parent lineage, coevolving with the plants. A basic tenet of this view is that insects that are adapted to the chemistry of one clade of plants will have a relatively easy time evolving to one of these plants that develop a new chemical defense since the plants are closely related and their overall chemistry is similar. Highly specific defensive compounds that affect a narrow range of herbivores promotes pairwise evolution of plant species and insect herbivores (Futuyma & Agrawal, 2009) and favors the evolution of insect specialization on a narrow range of host plants with similar chemistry. This theory is supported by phylogenetic comparisons of butterflies and plants that show that host shifts have been more common between closely related plants than between more distantly related plants over evolutionary time (Janz and Nylin, 1998, Braby and Trueman, 2006). However, the evolution of two interacting species does not occur in isolation. More complex, less evident interactions also occur, which affect selective pressure on both species. This has been called diffuse evolution and occurs when species evolve in response to the selective pressure from multiple species, which, in turn are evolving in response to multiple other species (Hougen-Eitzman & Rausher, 1994; Becerra & Benable, 1999; Strauss et al., 2005).

Opposing theory

Predators and parasitoids may also influence the coevolution of insects and their food plants. This top-down approach, as presented by Bernays & Graham (1988; see also Hairston et al., 1960), posits that predators and parasitoids select for a change in the host species their insect herbivore prey. They argue that host shifts occur more often and more rapidly than can be explained by mutation and natural selection. They describe the driving force behind host shifts as a move to enemy-free space. This

hypothesis assumes that the predator or parasitoid species will not find the prey on the new host. That is, the prey species becomes less apparent. They also argue that the shift to a new plant species may confer chemical defenses in the insect through sequestration of toxins. However, this mechanism requires that the prey species sequester the toxin in question and sequestration of toxic defenses is used by only a portion of insect species that feed on toxin-producing plants. Therefore this mechanism would be able to explain only a fraction of the predator-driven host shifts. Furthermore, their argument assumes that insects do not reach high enough densities to exert selective pressure on plants. However, induction of defenses in response to herbivores and the tradeoffs observed when plants express defenses in the absence of predators argue that certain evolved defenses are specific for reducing or preventing herbivory. Indeed, these defenses have been shown to reduce the damage caused by herbivores (Mauricio & Rausher, 1997; Traw & Dawson, 2002b; Agrawal & Kurashige, 2003; Gols et al., 2008a, b; Agrawal et al., 2012).

HORMONAL REGULATION OF PLANT DEFENSES

Despite such great variation in plant defense chemistry, there is great similarity in regulation of defense signaling in all angiosperms studied. The plant hormone salicylic acid is known to regulate expression of defenses against biotrophic pathogens, which require living plant tissue for an infection to progress. Plants frequently recognize these pathogens by common molecular markers on the cell surface of most microorganisms called pathogen-associated molecular patterns (PAMP). Recognition of a particular PAMP triggers a low level of resistance mediated by SA termed PAMP-triggered immunity (PTI; Chisholm et al., 2006, Jones & Dangl, 2006; Vlot et al., 2009). Virulent pathogens have evolved mechanisms to counter PTI by injecting effector proteins into plant cells by means of a type III secretion system. Effector proteins have been shown to inhibit plant PTI. To counter pathogen effectors, plants have

evolved mechanisms to recognize and inhibit these proteins in what has been termed a gene-for-gene response. This response is systemic, termed systemic acquired resistance (SAR), and provides enhanced resistance to subsequent infections from the triggering pathogen. SAR is also mediated by SA and involves direct or indirect interaction between the protein product of a plant resistance (R) gene and its associated pathogen effector protein (Chisholm et al, 2006; Jones & Dangl, 2006; Halim et al, 2006; Vlot et al., 2009).

3. MUSTARDS AND THEIR HERBIVORES AS A MODEL SYSTEM

In the following chapter, I first describe the importance and distribution of the plant family Brassicaceae. I then describe the major chemical defenses that are found in the mustards and how they function. Next, I present the pierid butterflies that specialize on these mustards and how the females use glucosinolate compounds as oviposition cues in their search for host plants.

Importance and Distribution of the Plant Family Brassicaceae

The Brassicaceae are an economically and ecologically important plant family consisting of over 3700 species in approximately 330 genera. Various Brassicaceae species are grown worldwide as vegetable crops and for their seed oil (e.g. *Brassica oleracea, B. napus, B. rapa, B. carinata, B. juncea, B. nigra, Sinapis alba, Armoracia rusticana, Raphanus sativus, Wasabia japonica* ; Ahuja et al., 2011), species from several genera are grown as ornamentals (e.g. *Aubrieta, Erysimum, Iberis, Matthiola, Lobularia, Lunaria, Arabis*), and a few crucifers have been used for medicinal, bioindustrial, biocontrol and phytoremediation purposes (Anjum et al., 2012). The Brassicaceae family also includes scientifically important species such as *Arabidopsis thaliana*, which has become a model plant species studied around the world, primarily due to its short generation time, small genome (five chromosomes), small size, availability of numerous genetic mutants and being the first plant species to have its entire genome sequenced (The Arabidopsis Genome Initiative, 2000). Other species are emerging as model research species including *Capsella bursa-pastoris* and several species of the genera *Brassica* (e.g. *B. oleracea* and *B. nigra*) and *Boeachera*.

A disproportionate number of wild Brassicaceae species are found in temperate regions of the Northern Hemisphere. However, numerous genera, such as *Draba, Lepidium* and *Cardamine*, can also be found in the Southern Hemisphere with a few genera found exclusively in southern Africa (e.g. *Heliophila, Silicularia, Brachycarpa, Chamira, Schlechteria*; Koch & Kiefer, 2006). Distribution of the Brassicaceae in tropical regions is very limited, with the few taxa present being limited to mountainous and alpine regions (Koch & Kiefer, 2006).

Many Brassicaceae species possess characteristics that make them successful colonizers of ruderal habitats and also successful at establishing in new locations. These characteristics include an annual and rapid growth form, which make them exceptional early-successional species (Grime, 1977, 2001). Such ruderals species do not survive well in agricultural systems (although *Brassica kaber* (syn. *Sinapis arvensis*) and other Brassicaceae species are common weeds in among agricultural crops (Buchanan et al., 2009). Rather, they grow best with low competition where they can complete their rapid life cycle before other plant species overgrow them, blocking sunlight. Establishment of a species in a new region where it did not previously exist is a colonization process. Successful colonizing plant species have been found to share a handful of common traits that help them become established in new locations. In addition to a rapid growth form, these traits include polyploidy, predominant self-fertilization or clonal reproduction, substantial local differentiation due to founder effects and restricted gene flow and adaptation to a wide range of habitats which can be achieved either through phenotypic plasticity or ecotypic differentiation (Baker and Stebbins, 1965; Brown and Marshall, 1981; Barrett and Richardson, 1986; Brown and Burdon, 1987). However, despite these shared characteristics, each successful invasion event appears to be a unique case (Brown and Marshall, 1981).

Although these mustards may be considered early-successional species, they will be found at many sites year-after-year due to recurring disturbances caused by humans. These sites include

roadsides, land adjacent to railroad tracks and mowed fields, among others. Cessation of yearly disturbance at these sites would inevitably lead to successional changes that would allow mustards to be outperformed and replaced by competitor species (Grime, 2001).

Certain species of Brassicaceae are successful invaders, have achieved a nearly global distribution and possess several of the traits of successful colonizers. *Capsella bursa-pastoris* is tetraploid (2n = 4x = 32), predominantly inbreeding and is an exceptional colonizer, being one of the most widespread plants on the planet (Neuffer and Albers, 1996). This species is native to Eurasia and has spread to North and South America, Australia and New Zealand (Hurka et al 2003). Early studies using isozyme data indicated that *C. bursa-pastoris* arrived in the New World via multiple independent introduction events (Neuffer and Hurka, 1999), another indication that this species is a capable invader. Two of the approximately 25 species of *Diplotaxis* are successful colonizers. *Diplotaxis muralis*, like *C. bursa-pastoris*, is tetraploid and predominantly selfing. *D. tenuifolia*, on the other hand, is diploid and is an obligate outbreeder. Both species are native to Europe and the Mediterranean region and have successfully invaded North and South America, Australia, New Zealand and South Africa. In addition, the spread of both *C. bursa-pastoris* and *D. muralis* has been highly influenced by humans (Kowarik and Sukopp, 2000).

Glucosinolate Production by Mustards

Glucosinolate Structure. Across the plant kingdom thousands of defensive secondary compounds are produced. However, individual species are limited to a narrow repertoire of chemicals at their disposal. A characteristic shared by all Brassicaceae species is the production of GS, also known as mustard oil glucosides, as secondary metabolites employed as defensive compounds against herbivores, parasites and pathogens. The basic structure of GS was first accurately described by Ettlinger and Lundeen

(1956). These water-soluble thioglucosides are derived from glucose and contain sulfur and nitrogen moieties along with a variable side group (R) that is derived from one of eight amino acids (Figure 1.1; Ettlinger & Lundeen, 1956; Fahey et al., 2001; Halkier & Gershenzon, 2006; Hopkins et al., 2009). Over 120 different GS have been isolated and identified (Halkier & Gershenzon, 2006) and they are classified based on the amino acid that the R group is derived from and the modifications made to that group.

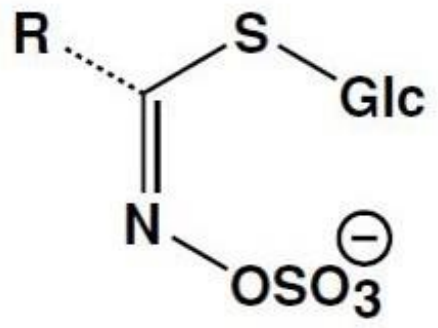

Figure 3.1. Basic chemical structure of glucosinolates. Variation in the side chain (R) determines specificity. Adapted from Halkier & Gershenzon (2006).

Aliphatic GS are derived from Ala, Ile, Leu, Met, or Val, aromatic GS are derived from Phe or Tyr and indole GS are derived from Trp (Halkier & Gershenzon, 2006). The precursor amino acids of most GS side chains are extensively modified (Fahey et al., 2001). R groups can be modified by elongation with one or more methylene groups as well as by hydroxylation, *O*-methylation, desaturation, glycosylation and acylation (Halkier & Gershenzon, 2006). Anders Kjaer in Denmark isolated and characterized more GS than anyone else and has also contributed greatly to our knowledge of the chemistry, biosynthesis, metabolism and biology of GS (see Fahey et al., 2001 for many Kjaer references.)

Glucosinolate Distribution. Glucosinolates are synthesized almost exclusively by plants in the order Brassicales, especially plants in the families Brassicaceae, Capparaceae, Caricaceae and Resedaceae (Feeny, 1977; Rodman, 1991; Rodman et al., 1996; Halkier & Gershenzon, 2006). The lone exception being members of the genus *Drypetes* in the family Euphorbiaceae (order Euphorbiales) which are phylogenetically distant from Brassicales but also produce GS (Rodman et al., 1996). GS synthesis and function has been most extensively studied in the Brassicaceae, with hundreds of species having been investigated (Kjaer, 1976; Fahey et al., 2001). Despite the large number of GS identified across species, each individual plant is capable of producing only a handful of GS in significant concentrations, while producing a few other types in trace amounts (Rask et al., 2000). A few species, however, have been found to produce larger numbers of GS. For example, the Col-0 genotype of the model species *Arabidopsis thaliana* was found to make as many as twenty three different GS (Hogge et al., 1988).

Glucosinolate Storage and Activation. Glucosinolates are produced and stored by plants prior to encounters with enemies. From a plant physiological standpoint these compounds are phytoanticipins. However, after an attack has been initiated, production of increased concentrations of GS are triggered by induction of plant defense hormones, such as jasmonic acid. GS themselves, however, are biologically inactive. All plants that produce and store GS also make and store one of a group of activation enzymes known as myrosinases (Rask et al., 2000). Myrosinases are a group of thioglucosidase enzymes that hydrolyze GS, cleaving the glucose and sulfate moieties and forming one of several potentially toxic products including thiocyanates, isothiocyanates, nitriles or epithionitriles among other less common products (Fahey et al., 2001; Halkier & Gershenzon, 2006). It is the hydrolysis products that are biologically active and toxic to many organisms.

In healthy, undamaged plant tissue GS and myrosinases are stored separately, (Björkman, 1976; Kjær, 1976; Rask et al., 2000). Physical separation prevents unnecessary hydrolysis and production of GS

degradation products, which not only defend the plant, but are also strongly phytotoxic and therefore may have damaging autotoxic effects on the plant (Hooker et al., 1945; Bell and Muller, 1973; Feeny, 1977). Upon tissue damage, by a foraging insect for example, the precursor GS and the activating myrosinase mix together causing the rapid conversion of GS to the biologically active isothiocyanate or other compound. This spatial separation and subsequent rapid mixing upon tissue damage has been termed the "mustard oil bomb" (Matile, 1980; Luthy & Matile, 1984). The mustard oils that are produced are highly toxic to most insect herbivores that are not specifically adapted to these chemicals, making them effective defense compounds (Feeny, 1977; Blau et al., 1978; Chew, 1988; Rask et al., 2000; Wittstock et al., 2004). These compounds have also been shown to be effective toxins against bacteria, fungi, nematodes, slugs, snails, amphipods and even birds and mammals (Glen et al., 1990; Newman et al., 1992; Mithen, 1992; Giamoustaris & Mithen, 1995; Fahey et al., 2001) and to also be allelopathic toward other plants species allowing crucifers to successfully compete with other vegetation (Brown & Morra, 1997; Vaughn & Berhow, 1999).

Evidence shows that myrosinases are stored in vacuoles separate from GS. But the actual location of these vacuoles may vary for different plant species. It has long been thought that these two interactors were stored in separate cells within each particular plant tissue, with myrosinase being stored in special "myrosin cells" (Björkman, 1976; Kjær, 1976; Bones & Iversen, 1985). This has been supported by recent work using GUS fusion constructs (Husebye et al., 2002; Thangstad et al., 2004). However, other work suggests that storage of myrosinases may vary between plant species (Kelly et al., 1998; Andreasson et al., 2001). Kelly et al. (1998) showed that myrosinase storage vacuoles in *Brassica* species may occur within the same cells that store their target GS precursors. Regardless of which cells the two compounds are stored in, physical separation prevents activation of GS until tissue damage occurs.

The Specialist Insect Herbivore *Pieris rapae*

Pieris rapae larvae feed almost exclusively on GS-producing plants in the order Brassicales (Verschaffelt, 1910; Rodman et al., 1996; Rask et al., 2000). Verschaffelt (1910), and many investigators since (Gautier & Riel, 1919; Dethier, 1947; Thorsteinson, 1953; Johansson, 1951; David & Gardiner, 1966), showed that by mixing mustard oils with synthetic diets or with the leaves of other plants, the larvae of *Pieris* butterflies are stimulated to feed. Johansson (1951) found that fifth instar *Pieris brassicae* larvae were conditioned to whichever food plant on which they were initially reared. Thorsteinson (1953) repeated Verschaffelt's (1910) work with more species. He found that seven of twenty one species not containing mustard oil glycosides were partially accepted by *Pieris brassicae* larvae when smeared with 3% sinigrin or 3% sinalbin.

Pieris rapae larvae have limited mobility and therefore rely on the mother to find and oviposit on appropriate food plants. Female butterflies typically lay their eggs on or very near the food plant on which the larvae feed. She may find an appropriate plant using a variety of senses, including smell, sight, touch and taste. After a review of the literature, Renwick & Radke (1988) concluded that location of host plants initially is guided primarily by color vision. Acceptance or rejection of the host plant then depends on chemical cues interpreted by contact chemoreceptors after alighting onto the leaf surface. It has been well established that butterflies (and insects in general) use chemoreceptors on their tarsi, antennae, proboscises and ovipositors to help locate appropriate plants for oviposition (reviewed by Renwick & Chew, 1994). A great amount of research has been performed particularly on the tarsi chemoreceptors of Lepidopterans (Ma & Schoonhoven, 1973; Du et al., 1995; Städler et al., 1995; for a review see Feeny et al., 1983). They may lay their eggs in a variety of places on the plant depending on butterfly species, such as on leaves, flowers or within crevices in the bark of a tree. In the case of the genus *Pieris*, the females lay their eggs on the underside of the leaves of their host. Once an

appropriate plant is found, a female will alight on the top of a leaf, curl her abdomen around the edge of the leaf and lay her eggs on the underside of the leaf. Eggs may be laid singly (e.g. *Pieris rapae*; peronal observation) or they may be laid in groups of 100 or more (e.g. *Pieris brassicae*; Opler, 2011). Placing the eggs on the underside of the leaf offers them some protection from predators and from the sun. After hatching, the egg casing may provide nutrition to the larvae prior to them feeding on the leaf.

Specialist insect herbivores adapted to the Brassicaceae have been shown to use GS and their hydrolysis products as cues for finding plants and as oviposition and feeding stimulants. Since the occurrence of GS is limited primarily to plants in the order Brassicales with the Brassicaceae being very abundant and prolific producers, these compounds can provide reliable cues for food plant location for crucifer specialists. More than 25 species of specialist insects in the Coleoptera, Lepidoptera and Diptera have been shown to use GS as feeding and oviposition stimulants (Chew, 1988; Louda & Mole, 1991; Hopkins et al., 2009). Several reports have demonstrated the role of GS as effective oviposition stimulants by applying them to nonhost plants that do not produce GS or even by application to nonplant substances, such as paper. Reed et al. (1989), used ion-exchange liquid chromatography to show that eight different GS fractions from three different Brassicaceae species individually stimulated oviposition by the diamondback moth, *Plutella xylostella*. Furthermore, they showed that the stimulatory effects were equally strong with all eight GS. However, the GS hydrolysis products produced by adding myrosinase to the compounds eliminated the stimulatory effects. Renwick et al. (1992) isolated three different GS from cabbage (*Brassica oleracea*) also using chromatography. However, they showed that glucobrassicin (3-indolylmethyl glucosinolate) was a stronger oviposition stimulant than sinigrin. The third GS, glucoiberin, did not show any activity as an oviposition stimulant for *P. rapae* when applied to the nonhost plant Sieva bean (*Phaseolus vulgaris*, var. Sieva). This work improved upon some of their earlier work in which they applied sinigrin and cabbage extracts to green index cards in order to stimulate oviposition by *P. rapae* females (Renwick & Radke, 1983). Van Loon et al. (1992), also

using green cards and chromatography-isolated GS, showed that the oviposition stimulant in *B. oleracea* was the GS glucobrassicin. Chemorecoptors in the tarsi of *Pieris* butterflies also sense deterrents produced by nonhost plants as has been demonstrated by several investigators (Renwick & Radke, 1985; Sachdey-Gupta et al. 1990; Städler et al., 1995).

Renwick et al. (2006) went on to show that the volatile GS degradation products, isothiocyanates, can also act as oviposition stimulants for the diamondback moth, *Plutella xylostella*. They applied the isolated isothiocyanates iberin and sulforaphane in a chloroform solution to filter paper. Both isothiocyanates were highly active as oviposition stimulants. GS and isothiocyanates however, may not be the only stimulants in Brassicaceae plants for specialist insect herbivores. An important finding by Roessingh et al. (1997) was that, in addition to GS, an unidentified, non-GS compound isolated from *B. oleracea* served as a strong oviposition stimulant for the cabbage root fly (*Delia radicum*). They later identified the compound as 1,2-dihydro-3-thia-4,10,10b-triaza-cyclopenta[.a.]fluorene-lcarboxylic acid, which they called “cabbage identification factor” (Hurter et al., 1999).

Non-GS compounds have long been known to also stimulate feeding in specialist insects. Nielsen et al. (1979) showed that flavonol glycosides isolated from horseradish (*Armoracia rusticana*) stimulated feeding in the flea beetle *Phyllotreta armoraciae*. In fact, the combination of flavonol glycosides plus GS were more stimulatory than any of the compounds alone. This additive effect of multiple stimulatory compounds may be more ecologically relevant, since GS and other secondary metabolites are presented to insects as mixtures of several compounds. And cumulative, or even synergistic, effects of combinations of GS, or GS with other compounds, has been found by Spencer (1996), Spencer et al. (1999) and van Loon et al. (2002).

There are a few different ways insects overcome the harmful effects of plant chemical defenses. Some insects sequester toxic compounds, using them for their own advantage to deter predation (Nishida, 2002; Opitz & Müller, 2009). Other insects excrete the toxins largely unchanged from their original form (Schramm et al., 2012). However, Müller et al. (2003), showed that *Pieris* butterflies do not sequester GS and Wittstock et al. (2004) demonstrated that the larvae of these butterflies instead produce an enzyme present in their guts called nitrile specifier protein (NSP) that hydrolyzes the GS into nontoxic nitriles that are then excreted, instead of allowing myrosinase to degrade the GS to a toxic isothiocyanate. Evolution of the detoxifying compounds and adaptation to GS-producing plants are hypothesized to have facilitated exploitation, specialization and radiation of these butterflies on species in the order Brassicales (Ehrlich & Raven, 1964; Feeny P, 1975; Chew, 1975, 1977; Thompson & Pellmyr, 1991; Janz & Nylin, 1998; Braby and Trueman, 2006; Wheat et al., 2007; Janz, 2011). An indication of diversion to nontoxic products instead of sequestration of toxins by *Pieris* butterflies is the nonaposematic coloration of these butterflies. They are white or yellow with a couple simple black spots. In line with these results, studies a have shown that crucifer-feeding *Pieris* species are palatable to predators and do not form mimetic associations with other butterfly species (Kingsolver, 1987; Ley & Watt, 1989; Lyytinen et al., 2001).

Slansky and Feeny (1977) compared *P. rapae* larval growth on a wide range of wild and cultivated mustards. They found no relationship between larval growth and type or quantity of GS in the plants. They did, however, find that larval growth was strongly influenced by the availability of nitrogen in the food plant. Similarly Blau et al. (1978) looked at the effects of allylglucosinolates present in the leaves of *Brassica oleracea* on *P. rapa*e caterpillars. They reported that artificially increasing the levels of allylglucosinolates by twenty fold had no effect on growth of *P. rapae* larvae. However, in the same study growth of the southern armyworm (*Spodoptera eridania*) was unaffected at low, natural concentrations, but significantly reduced with artificially elevated concentrations of GS. This is in

contrast to results reported by Stowe (1998) and Agrawal & Kurashige (2003) who found that elevated GS levels slowed the growth rate of larval *P. rapae* caterpillars. Agrawal & Kurashige's results may be more ecologically relevant since higher GS levels were induced by previous feeding by *P. rapae* caterpillars, a situation that is likely to occur in nature, whereas the GS levels of the plants in the study by Stowe were artificially controlled. Van Leur et al. (2008) showed that the primary GS expressed in a plant can differentially affect generalist and specialist insect herbivores. Using two different genotypes of *Barbarea vulgaris* that differ in expression of their dominant GS, either glucobarbarin or gluconasturtiin, they showed that larvae of the generalist *Mamestra brassicae* preferred and performed better on the genotypes dominated by the GS gluconasturtiin, while larvae of the specialist *P. rapae* neither preferred nor performed better on either genotype. Interestingly, Poelman et al. (2008) showed that herbivory by *P. rapae* caterplillars alter the community of herbivores and parasitoids found on damaged plants when compated to the community found on nondamaged plants.

CONCLUSION

An understanding of plant-insect interactions will help ecologists develop a general theory of the evolution of defensive traits in plants. Furthermore, knowledge of the mechanisms of defense can assist agricultural researchers in developing varieties of crop plants that are more resistant to insects. In the work presented next I address several questions regarding the distribution and evolutionary conservation of plant defense signaling pathways active against insect herbivores. In both studies I investigate negative crosstalk between the signaling pathway responsible for triggering defenses against chewing insects and the pathway responsible for regulating defenses against biotrophic pathogens. I first look at life history and constitutive defenses of mustards against the specialist insect herbivore *P. rapae* and how these defenses are affected by the major signaling pathway responsible for triggering

defenses against pathogens. I then present work that investigates the conservation of induced-defense-pathway negative crosstalk in a group of related mustards.

4. HERBIVORY AND RELATIVE GROWTH RATE OF *PIERIS RAPAE* IS CORRELATED WITH HOST CONSTITUTIVE SALICYLIC ACID CONCENTRATION AND FLOWERING TIME

In the following chapter, I describe a study I conducted that investigates how plant life history influences constitutive leaf salicylic acid (SA) concentrations and how these two factors correlate with insect herbivore performance.

INTRODUCTION

Insect herbivores and pathogens cause significant reductions in the performance of plants in nature (Schoonhoven et al., 2005) and in agriculture (Oerke, 2006). Plants, in turn, have evolved a sophisticated set of defensive responses that are mediated in large part by the hormones salicylic acid (SA) and jasmonic acid (Karban and Baldwin 1997). Much of what we know about these hormones has come from the study of a few major model systems (Vlot et al., 2009). Very little is known about how concentrations of these hormones vary in natural populations and the extent to which this variation may structure plant – enemy interactions in nature (but see Todesco et al 2010, Zhang et al. 2014).

Application of exogenous SA decreases symptoms of bacterial disease and pathogen abundance in agricultural crops (Vallad & Goodman 2004). However, an interesting indirect effect of the application of exogenous SA to plants has been that plants treated with SA or bacterial pathogens experience increased susceptibility to insect herbivores (Thaler et al., 1999, 2002a; Cipollini et al., 2004). This is an example of what are collectively referred to as ecological costs of defense, where the act of defending against one particular enemy makes a plant more susceptible to other enemies (Thaler et al.,

2002a, b; Karban, 2011, Johnson et al 2014). Indeed, such ecological costs appear widespread in plants. In a meta-analysis of the literature, Strauss et al. (2002) found that 62% of studies that investigated plant defense against insect herbivores found an ecological cost of resistance.

While exogenous treatment of plants with SA has been shown to increase the susceptibility to insect herbivores, the effects of constitutive variation in SA on plant resistance to insect herbivores has not been reported previously. Tissue constitutive concentrations of SA do differ among genotypes within species (Zhang et al., 2014) and among species (Raskin, 1992). Constitutive differences in wild plant allocation to SA have also been shown recently to structure resistance to bacterial and fungal pathogens (Todesco et al., 2010, Zhang et al. 2014).

Constitutive salicylic acid concentrations in plants have been linked previously to plant phenology and the transition to flowering specifically (Martinez et al., 2004; Jin et al., 2008, Wada et al., 2010). Plants that have their major growth and flowering stages during the spring, such as spring ephemeral mustards, are likely to experience different herbivore and pathogen pressures relative to plants that emerge and flower in the summer (Feeny 1976, 1977). Some evidence has suggested that spring ephemeral mustards experience lower levels of herbivory from insect herbivores (Vail et al, 1991; Gaines & Kok, 1995) and have lower levels of constitutive resistance against damage than do summer mustards (Feeny and Rosenberry 1982). The effects of plant life history on resistance to generalist herbivores have been studied previously (Silvertown and Dodd 1996, Van Zandt 2007), but the effects of plant life history on specialist herbivores, those that are limited to hosts within one or a few host families, has received less attention.

Because neonate butterfly larvae, particularly of specialists, cannot typically switch hosts and therefore die if placed on an incorrect host, it is critical that adult females oviposit on acceptable hosts. Female butterflies are known to use leaf chemistry in selecting host plants (Dethier, 1982; Renwick &

Chew, 1994) and have been shown to prefer host plants that promote larval growth and development (Chew, 1975; Mayhew, 1997). If constitutively high SA concentration suppresses production of constitutive JA-mediated defenses, then it is possible that female butterflies may prefer plants with constitutively high SA concentrations, as these plants may provide the best food source for their progeny. There have been no studies investigating whether constitutive SA concentration influences female butterfly oviposition choice.

I focus here on a group of ten mustard species that co-occur in marginal environments across the mid-Atlantic region of the United States, and all interact with pierid butterflies (Chew, 1975). This group is notable first because it includes the genetic model plant, *Arabidopsis thaliana*, and several other species (e.g. *Capsella bursa-pastoris*) for which full genome sequences are currently available. Likewise, *Pieris rapae* (hereafter "*Pieris*") is one of the best studied butterflies and co-occurs with these mustards across much of the US and Europe (Capinera, 2001). This system is also notable because these mustards, while all occurring in ruderal habitats (e.g. agricultural edges, railroad beds, stream washes, and trailsides) possess substantial variation in life history strategies. In the Northeastern US, five of the species (*A. thaliana*, *C. bursa-pastoris*, *Draba verna*, *Cardamine impatiens*, and *Barbarea vulgaris*) typically flower in March, April and May whereas the other five species (*Arabis canadensis*, *Brassica nigra*, *Lepidium campestre*, *Sinapis arvensis*, and *Sisymbrium altissimum*) flower in June, July, and August (Uva et al., 1997). In New York and Pennsylvania, this difference in flowering phenology has been very consistent across years (B. Traw, pers. observation). Each of the ten species in this focal group co-occurs in close proximity with at least three other species in the group. As such, emerging *P. rapae* females typically have several mustard species to choose from within very close proximity in these habitats.

In this study, I asked first whether leaf herbivory rates, larval relative growth rates, or adult *Pieris rapae* female oviposition rates differed between the spring- and summer-flowering species when measured under common garden conditions. I then asked whether the differences that I observed corresponded to the underlying leaf salicylic acid concentrations and also to what extent they could be explained by the underlying phylogenetic relationships among the species. Finally I asked whether host plant selection by the maternal butterflies correlated positively with the performance of larvae on these same hosts.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of all ten mustard species (*Arabis canadensis, Arabidopsis thaliana, Barbarea vulgaris, Brassica nigra, Capsella bursa-pastoris, Cardamine impatiens*, *Draba verna, Lepidium campestre*, *Sinapis arvensis*, *Sisymbrium altissimum*) were all collected in Tompkins County, NY in the vicinity of Cornell University. Seeds were sown in Pro-Mix BX soil (Premier Tech, Quakertown, PA) in 36-well flats and placed in a 4°C cold-room for 3 d of cold stratification. Flats were then transferred to an environmentally-controlled growth chamber at the University of Pittsburgh with constant conditions of 22°C, 12h day-night cycle and 350µmol $m^{-2}sec^{-1}$ light provided by a 1:1 mixture of sodium and metal halide lamps. All plants were grown simultaneously, watered as needed, fertilized every 10 d and moved at least once per week within the growth chamber to minimize positional effects.

Pieris rapae Material and Performance Assay

Pieris rapae were obtained from the Morehouse lab at the University of Pittsburgh, (Pittsburgh, PA, USA). Caterpillars were reared on kale in a growth chamber at 24°C with 60% relative humidity and 18:6-hour light:dark cycle. Six weeks after germination of the plants, leaf samples of each species were obtained by hole punch (8 mm diameter). Two discs were placed in each well of a 12-well plate so that each well contained two discs of only one species. Filter paper moistened with distilled water was placed on the bottom of each well to prevent the leaf discs from drying out. Four or five replicates were performed and positions of each plant species in the wells were haphazardly varied with each replicate. First instar *P. rapae* caterpillars were individually weighed to the microgram on a MX5 Microbalance (Mettler Toledo) and placed in the plate, one per well. Lids were placed on the plates and the plates maintained in a growth chamber with conditions as stated above. After 24 hr, caterpillars were reweighed. The RGR of the caterpillars was calculated as follows:

RGR = (ln (final weight) – ln (initial weight))/day

Leaf area consumed was visually estimated for each caterpillar and the results of four or five replicates averaged.

Pieris rapae Adult Female Oviposition Choice Test

One healthy, four-week-old plant of each of the ten species was placed in a clear chamber (0.5, 0.5, and 1m for width, length, and height) exposed to natural light. Gravid female *P. rapae* butterflies, mated within the previous 24 hr, were placed in the chamber with the plants. The availability of females differed for each trial and so the numbers of females used were 30, 15, 23, and 15 females used in Trial 1, 2, 3, and 4, respectively. The number of eggs deposited on each plant was counted at the end of the trial. Four separate trials were conducted, each consisting of new plants and naïve females. Each trial

was terminated when 5% of the females had died, which occurred at 36, 60, 48, and 120 h in Trial 1, 2, 3, and 4, respectively.

Measurement of Leaf SA Concentration

After four weeks of growth, whole plants were harvested by cutting at the base with a razor blade, placing in a coin envelope and immediately flash-frozen in liquid nitrogen. Plant tissue was stored in a -80°C freezer prior to the assessment for leaf salicylic acid concentrations. Samples were removed from the freezer onto dry ice and then immediately placed in a lyophilizer for 3 d to freeze-dry the tissue. SA extraction followed the methods of Dewdney et al. (2000). Approximately 20-25 mg of dry leaf tissue was weighed, pulverized and suspended in 3 ml of 90% methanol. An internal control of 1µg of O-anisic acid (Sigma # 169978) was added to each sample (100µl of a 10µg/ml solution in 100% methanol) and the tubes were placed in a shaker at 300 rpm at room temperature for 24hr. The liquid was transferred to a new tube and the pellet was resuspended in 3ml of 100% methanol and rotated at 300 rpm for 24hr for a second time. The supernatant fractions were combined and vortexed to mix.

Because SA exists in plants with two forms, I then split each sample. The first aliquot was used to measure free SA, and the second was used to measure total SA, which includes the large portion of SA that is conjugated to sugar (SA *O-β* –glucoside). I split each sample in equal volumes into two screwcap tubes and placed the tubes in a fume hood until dry (roughly 24hr later). The aliquot for measurement of total SA received 40U of β-glucosidase (Sigma # 0395) in 400µl of 100mM sodium acetate buffer (pH 5.5) which cleaves the sugar from SA glucoside, thus providing an estimate of total SA present in the sample (free plus glucoside-conjugated). The other aliquot received the 400µl of buffer but no enzyme. All samples were incubated overnight at 37^{o}C and then received 400µl of 10% trichloroacetic acid. All samples were then partitioned twice with 1ml of an organic extraction solvent

(100:99:1 of ethyl acetate: cyclopentane: 2-propanol), vortexing each time before collecting the two organic phase fractions in a centrifuge tube. Tubes were then placed in a fume hood until dry (24 to 48hr). Samples were resuspended in 600µl of 55% methanol, vortexed, and placed in a rocker overnight. Samples were centrifuged at 5000g for 15min, the supernatant was then transferred to a 0.2µm nylon spin-prep membrane filters (Fisher #07-200-389), and centrifuged at 14,000g for 5min. Concentrations of SA were then measured by high performance liquid chromatography (HPLC) on an HP1100 (Agilent # G1380-90000) system with a 4.6 x 150mm Eclipse XDB C-18 column (Agilent # 993967-902) and fluorescence detector (excitation at 301nm and emission at 412nm for SA and excitation at 301nm and emission at 365nm for O-anisic acid). Solvent flow was 1ml/min, beginning with 30% of 100% methanol and 70% of 0.5% acetic acid for five minutes, increasing to 40% methanol at 7.5min and 60% methanol at 15min, returning to 30% methanol at 18min. Concentrations (µg/g leaf dry mass) of free and total SA were calculated as the peak area of each compound divided by the product of the peak area of the O-anisic acid internal standard and sample mass.

Statistical Analyses

Data were natural log transformed prior to analysis. To assess the difference between spring-flowering and summer-flowering species, I performed a nested ANOVA with the mustard species nested with flower time group for the following three variables: oviposition rate, free salicylic acid, and total salicylic acid, which all had balanced numbers of replicates per species. Two of the variables, percent herbivory and relative growth rate, had unbalanced numbers of replicates measured for each species. For those two variables, I calculated species averages from the available replicates and then assessed the difference between the five spring and five summer-flowering species by one-way ANOVA. Linear regression was performed with all independent and response variable. Polynomial regression was performed when this fit the data better and made biological sense, e.g. very high and very low levels of

SA affecting caterpillar performance. All calculations were performed using Minitab v. 17.1 (Minitab Inc., State College, Pennsylvania).

RESULTS

On average, the five spring-flowering mustards received significantly less herbivory ($F_{1, 8}$ = 9.43, P=0.015, Figure 4.1A) and supported lower relative growth rates ($F_{1, 8}$=13.24, P = 0.007, Figure 4.1B) by first instar larvae of *Pieris rapae* in the feeding assay relative to the five summer-flowering mustards. The average leaf disk from the spring-flowering mustards lost 47.8% of its area, whereas the average leaf disk from the summer-flowering mustards lost 89.9% of its area, an amount nearly two-fold greater. The average larva feeding on a spring-flowering mustard disk gained 0.19 mg per mg of initial mass, whereas the average larva feeding on a summer-flowering mustard gained 0.53 mg per mg of initial mass, an amount nearly three-fold greater. Larvae were unable to consume *Capsella bursa-pastoris* and lost weight on those leaf disks. While this mustard was conspicuously resistant, it was not the only spring-flowering mustard that had high resistance to the larvae. *Draba verna*, *Cardamine impatiens* and *Barbarea vulgaris* all exhibited substantially less damage and lower larval growth rates than the three most acceptable summer-flowering species. Of the spring-flowering species, only *Arabidopsis thaliana* did not differ in quality relative to the summer-flowering species.

When gravid *P. rapae* females were offered an array including all ten species, the average summer-flowering mustard received an average of 68 eggs per female per day per square meter of foliage, whereas the average spring-flowering mustard received less than 15 eggs, which amounted to a four-fold difference, but this was not statistically significant ($F_{1, 8}$=2.89, P = 0.127, Figure 4.1C), owing to strong differences among species. Two summer-flowering (*Arabis canadensis* and *Lepidium campestre*)

and two spring-flowering mustards (*Capsella bursa-pastoris* and *Cardamine impatiens*) received essentially no eggs. If those four non-accepted species were removed, the remaining three summer-flowering mustards (*Brassica nigra, Sinapis arvense*, and *Sisymbrium altissimum*) had an average of 111 eggs per female per day per square meter of foliage, which was significantly greater than the 24 eggs per female per day per square meter received by the three spring-flowering mustards ($F_{1, 4}$=10.1, P = 0.034).

Leaf constitutive salicylic acid concentrations did not differ significantly between the spring-flowering and summer-flowering species for either free SA ($F_{1, 8}$= 1.59, P=0.242, Figure 4.2A) or total SA ($F_{1, 8}$= 0.07, P=0.793, Figure 4.2B), when all species were included. However, one spring-flowering mustard (*Barbarea vulgaris*) and one summer-flowering mustard (*Arabis canadensis*) had unusually high concentrations of salicylic acid. When these two high concentration species were removed, the average of the four remaining summer-flowering mustards was 0.35 ug/g free SA, whereas the average of the four remaining spring-flowering mustards was 0.07 ug/g free SA, a nearly five-fold difference, which was significant ($F_{1, 6}$= 14.27, P=0.009). For total SA, the difference between the average of the reduced set of four summer-flowering mustards and four spring-flowering mustards was 3.5-fold, which was also significant ($F_{1, 6}$= 12.80, P=0.012).

Leaf herbivory by *Pieris rapae* neonates was not correlated with leaf constitutive free SA concentration (R^2=46.5%, P=0.112, Figure 4.3A), but exhibited a strong polynomial relationship with total constitutive SA concentration (R^2=75.3%, P=0.007, Figure 4.3B). Relative growth rate of these neonate larvae was correlated positively with the amount that they consumed of the leaf disks (R^2=80.3%, P<0.001, Figure 4.4A). Relative growth rate of the larvae was not correlated with leaf constitutive free SA concentration (R^2=34.5%, P=0.227, Figure 4.4B), but exhibited a polynomial relationship with total constitutive SA concentration (R^2=59.4%, P=0.043, Figure 4.4C). Female adult

butterflies laid significantly more eggs on hosts that resulted in higher larval relative growth rates, as shown by the positive correlation between these two variables at the species level (R^2=48.1%, P=0.039, Figure 4.5A). Oviposition rates were not correlated with either the leaf constitutive concentrations of either free SA (R^2=25.1%, P=0.363, Figure 4.5B) or total SA (R^2=9.0%, P=0.720, Figure 4.5C).

Phylogenetic grouping of the ten species based on maturase K (matK) gene sequences (Koch et al. 2001) resulted in the identification of four fully resolved clades, each containing two or three species (Figure 4.6A). These clades did explain significant variation in the oviposition rate by *Pieris rapae*, with the species in Clade 1 receiving seven-fold more eggs on average than species in the other three clades (R^2=79.7%, P=0.017, Figure 4.6D). These three species also all share the summer-flowering habit (Figure 4.6G). Phylogenetic groupings did not explain significant variation in either herbivory rate (R^2=41.0%, P=0.33, Figure 4.6B), larval growth rate (R^2=42.2%, P=0.31, Figure 4.6C), leaf constitutive free SA concentration (R^2=27.2%, P=0.56, Figure 4.6E), or leaf constitutive total SA concentration (R^2=22.7%, P=0.64, Figure 4.6F).

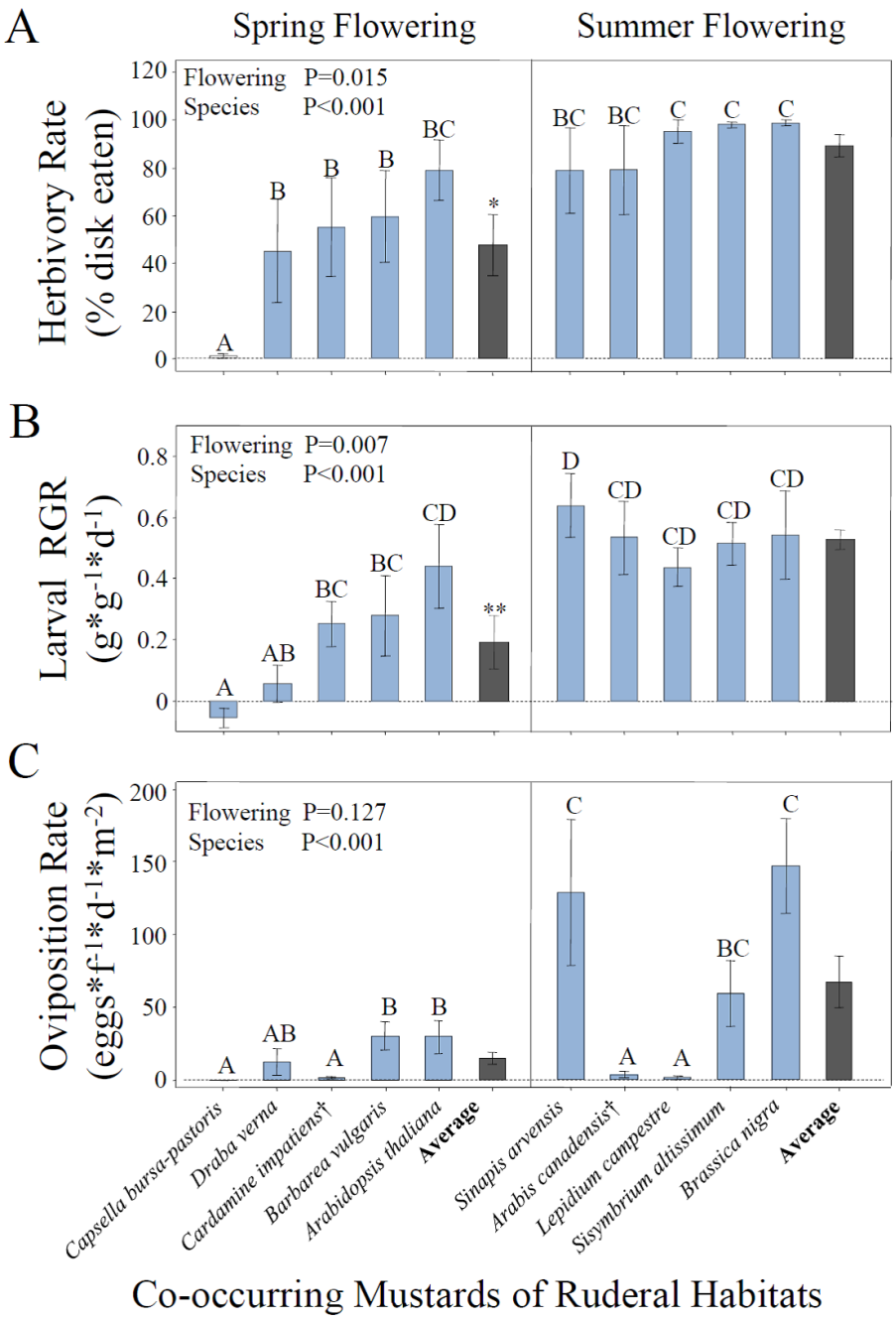

Figure 4.1. Comparison of **A**) herbivory (%) and **B**) relative growth rate ($g*g^{-1}*d^{-1}$) of *Pieris rapae* larvae in simultaneous disc feeding assays, and **C**) oviposition rates of females in choice arenas that included one individual plant of each of the ten species. Shown are means (+/- SE) for larval tests (N = 4 or 5) and adult female choice assays (N = 4). Significant differences at P = 0.05 between species are indicated by the absence of shared letters. Overall means (+/-SE) of the spring and summer groups are included

(gray bars). P values are shown for flowering group and species nested within flowering group.*P<0.05, **P<0.01.

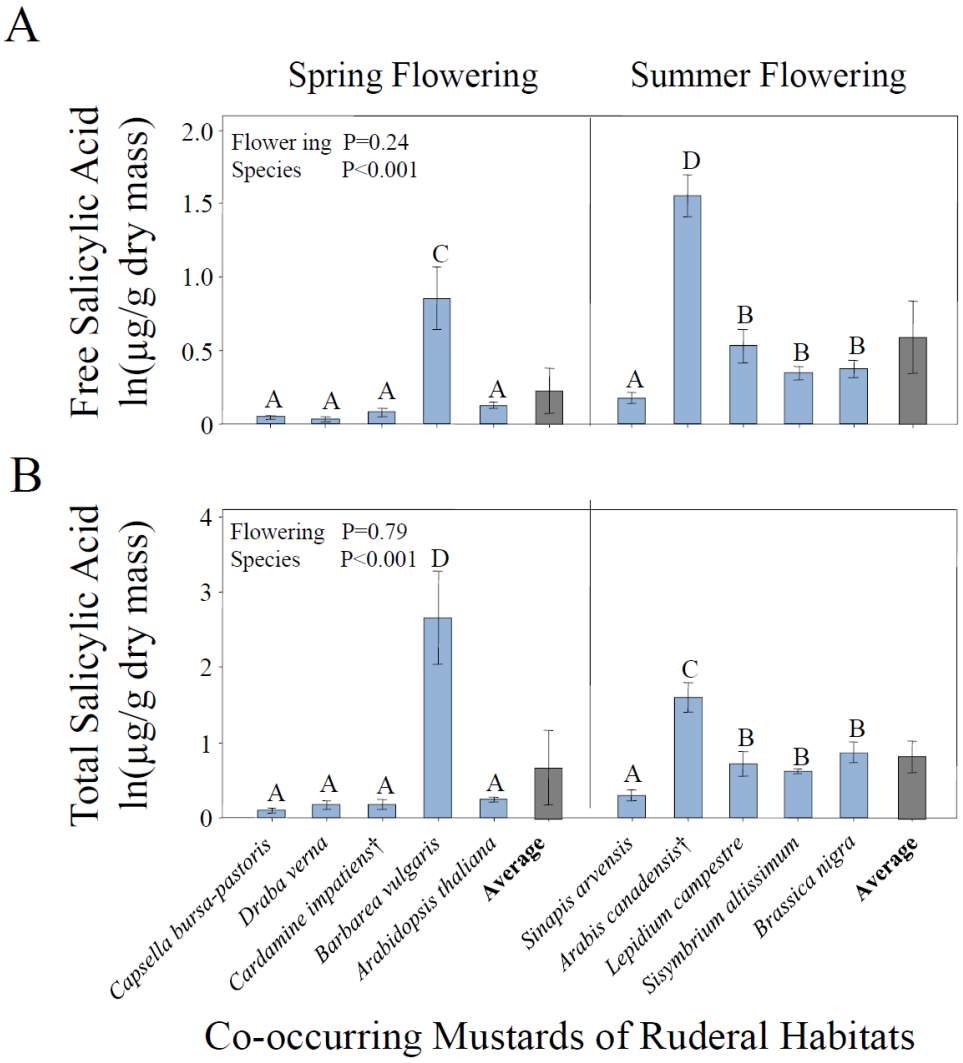

Figure 4.2. Comparison of natural log transformed values of constitutive **A**) free salicylic acid (µg/g dry mass) and **B**) total salicylic acid (µg/g dry mass) of ten Brassicaceae species. Shown are means (+/- SE) for leaf samples from four replicate plants. Significant differences at P = 0.05 between species are indicated by the absence of shared letters. Overall means (+/-SE) of the spring and summer groups are included (gray bars). P values are shown for flowering group and species nested within flowering group.

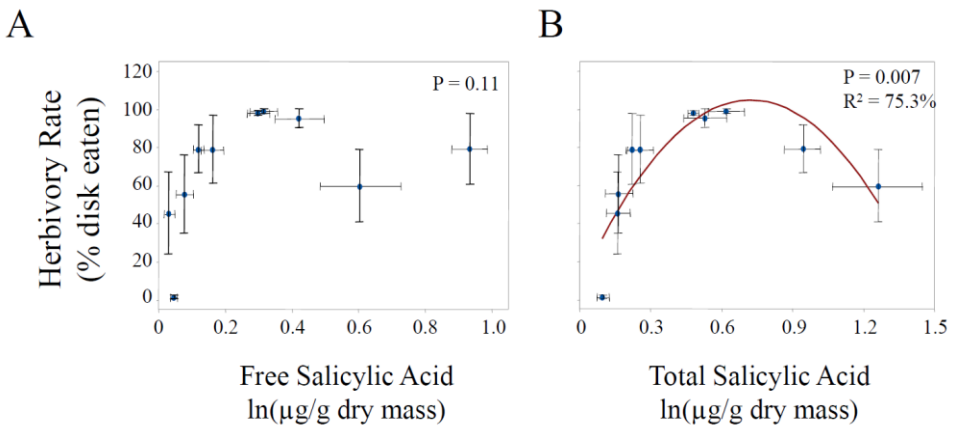

Figure 4.3. Scatterplots showing relationship between herbivory rate (% disk eaten) and leaf **A**) free salicylic acid ln(µg/g dry mass) and B) total salicylic acid ln(µg/g dry mass) measured from plants reared in a separate experiment in the absence of herbivores. P values from polynomial regression are shown. R^2 value indicates percent variance in herbivory rate that is explained by the fitted polynomial regression line.

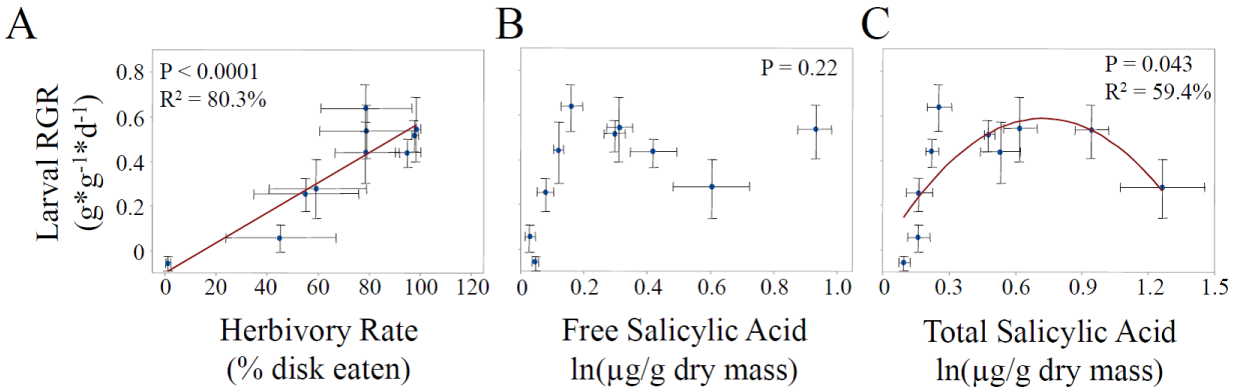

Figure 4.4. Scatterplots showing relationship between larval relative growth rate (RGR, $g*g^{-1}*d^{-1}$) and **A**) larval herbivory rate (% disk eaten), **B**) free salicylic acid ln(µg/g dry mass) and **C**) total salicylic acid ln(µg/g dry mass) measured from plants reared in a separate experiment in the absence of herbivores. P values from linear or polynomial regression are shown. R^2 value indicates percent variance in larval relative growth rate that is explained by the fitted regression line.

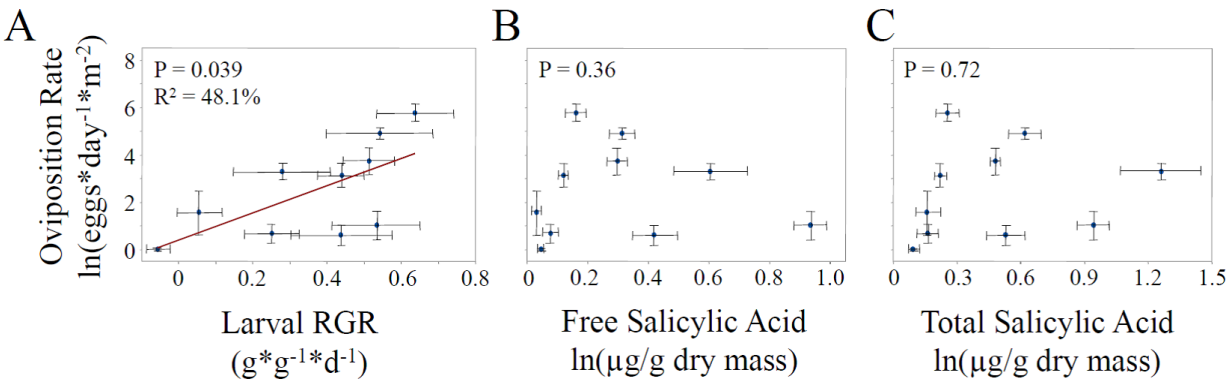

Figure 4.5. Scatterplots showing relationship between adult female oviposition rate (eggs*d^{-1}*m^{-2}) and **A**) larval relative growth rate (RGR, g*g^{-1}*d^{-1}), **B**) free salicylic acid ln(µg/g dry mass) and **C**) total salicylic acid ln(µg/g dry mass) measured from plants reared in a separate experiment in the absence of herbivores. P values from linear or polynomial regression are shown. R^2 value indicates percent variance in larval relative growth rate that is explained by the fitted regression line.

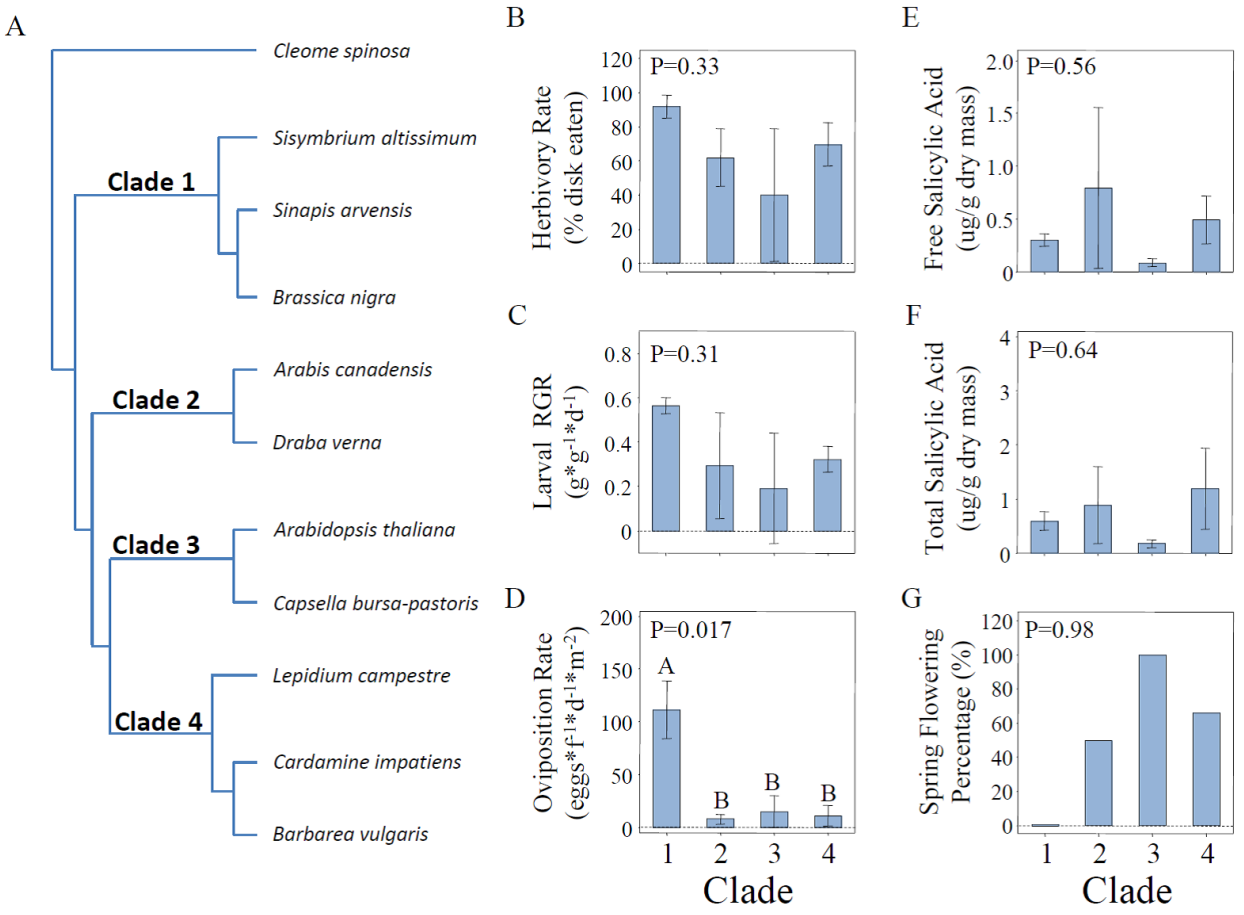

Figure 4.6. Assessment of the phylogenetic relationships among the mustards on their correlations with the performance of *Pieris rapae* and plant phenological behavior of spring- versus summer-flowering. **A**) Identification of four clades based on analysis of maturase K (*matK*) gene sequences (Koch et al., 2001). Relationship between the four clades and average **B**) herbivory rates (% disk eaten), **C**) larval relative growth rate (g*g-1*d-1), **D**) oviposition rate (eggs*f-1*d-1*m-2), **E**) leaf free salicylic acid concentration (µg/g dry mass), **F**) leaf total salicylic acid concentration (µg/g dry mass), and **G**) percentage of spring-flowering species in each group.

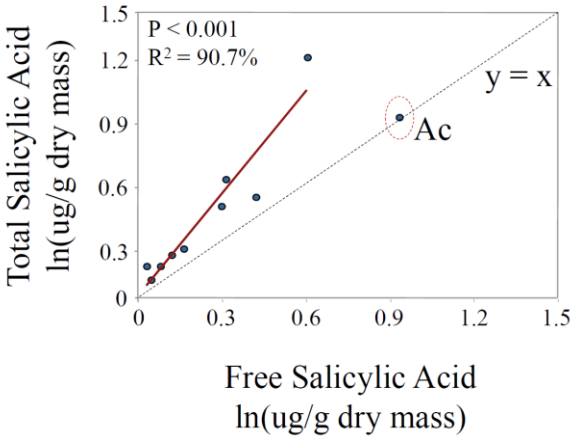

Figure 4.7. Relationship between total and free leaf constitutive salicylic acid concentrations for all ten species of mustard studied. *Arabis canadensis* (Ac) is an exception in that it falls on the y = x line, suggesting that none of its salicylic acid was conjugated to sugar.

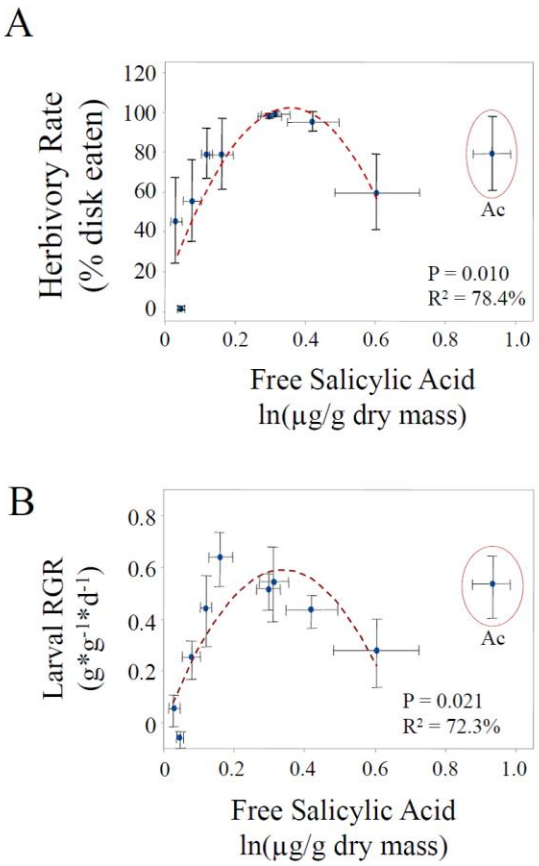

Figure 4.8. Scatterplots showing relationship between **A**) herbivory rate (% disk eaten) and **B**) larval relative growth rate as a function of leaf constitutive free salicylic acid ln(µg/g dry mass) measured from plants reared in a separate experiment in the absence of herbivores. P values from polynomial regression are shown. R^2 value indicates percent variance in herbivory rate that is explained by the fitted polynomial regression line.

Table 4.1. Comparison of means (+/- SE) from common garden assessment of five spring and five summer flowering mustards from the ruderal community in upstate New York for larval percent herbivory, larval relative growth rate, and adult oviposition rate by *Pieris rapae*, as well as constitutive free and total salicylic acid concentrations (µg/g dry mass) in leaf tissue.

Flowering	Name	N	Percent Herbivory (% disk eaten)	Relative Growth Rate ($g * g^{-1} * d^{-1}$)	N	Oviposition Rate ($eggs * f^{-1} * d^{-1} * m^{-2}$)	Free Salicylic Acid ($\mu g * g^{-1}$ dry mass)	Total Salicylic Acid ($\mu g * g^{-1}$ dry mass)
Spring	*Capsella bursa-pastoris*	5	1.0 +/- 1.0	-0.05 +/- 0.03	4	0.0 +/- 0.0	0.04 +/- 0.01	0.09 +/- 0.02
	Draba verna	4	45.0 +/- 21.6	0.05 +/- 0.06	4	12.7 +/- 9.3	0.03 +/- 0.01	0.17 +/- 0.05
	Cardamine impatiens	4	55.0 +/- 20.6	0.25 +/- 0.07	4	1.5 +/- 0.9	0.08 +/- 0.02	0.18 +/- 0.06
	Barbarea vulgaris †	5	59.4 +/- 19.1	0.27 +/- 0.13	4	30.4 +/- 9.7	0.85 +/- 0.21	2.65 +/- 0.61
	Arabidopsis thaliana	4	78.7 +/- 12.6	0.44 +/- 0.13	4	29.8 +/- 11.4	0.12 +/- 0.01	0.25 +/- 0.03
Summer	*Sinapis arvensis*	4	78.7 +/- 18.0	0.63 +/- 0.10	4	128.8 +/- 50.0	0.17 +/- 0.03	0.29 +/- 0.07
	Arabis canadensis	5	79.0 +/- 18.6	0.53 +/- 0.11	4	3.4 +/- 2.1	1.55 +/- 0.13	1.59 +/- 0.19
	Lepidium campestre	5	95.0 +/- 5.0	0.43 +/- 0.06	4	1.5 +/- 1.2	0.53 +/- 0.11	0.72 +/- 0.16
	Sisymbrium altissimum	5	98.0 +/- 1.2	0.51 +/- 0.07	4	59.0 +/- 22.5	0.34 +/- 0.04	0.61 +/- 0.03
	Brassica nigra	4	98.7 +/- 1.2	0.54 +/- 0.14	4	147.1 +/- 32.4	0.37 +/- 0.05	0.87 +/- 0.13

† *Barbarea vulgaris* had three replicates for free and total SA

Table 4.2. Regression analyses for percent herbivory and larval relative growth rate as a function of natural log-transformed constitutive free salicylic acid concentration including the ten species of mustards from ruderal habitats in the Northeastern United States.

Response	Predictor	Source	DF	SS	F	P	R-Sq
% Herbivory	Ln(Free SA)	Full Model	2	3800.72	3.0	0.112	46.5%
		Linear	1	1257.55	1.5	0.262	
		Quadratic	1	2543.17	4.1	0.084	
		Error	7	4379.45			
		Total	9	8180.17			
	Ln(Total SA)	Full Model	2	6160.76	10.7	**0.007**	75.3%
		Linear	1	993.24	1.1	0.324	
		Quadratic	1	5167.52	17.9	**0.004**	
		Error	7	2019.41			
		Total	9	8180.17			
Larval RGR	Ln(Free SA)	Full Model	2	0.15	1.8	0.227	34.5%
		Linear	1	0.08	1.8	0.207	
		Quadratic	1	0.07	1.6	0.240	
		Error	7	0.30			
		Total	9	0.46			
	Ln(Total SA)	Full Model	2	0.27	5.1	**0.043**	59.4%
		Linear	1	0.05	1.0	0.353	
		Quadratic	1	0.22	8.4	**0.023**	
		Error	7	0.19			
		Total	9	0.46			

Table 4.3. Polynomial regressions for percent herbivory and larval relative growth rate as a function of natural log transformed constitutive free salicylic acid concentration excluding *Arabis canadensis*.

Response	Predictor	Source	DF	SS	F	P	R-Sq
% Herbivory	Ln(Free SA)	Full Model	2	6320.93	10.9	**0.010**	78.4%
		Linear	1	1834.20	2.1	0.194	
		Quadratic	1	4486.74	15.4	**0.008**	
		Error	6	1745.10			
		Total	8	8066.03			
Larval RGR	Ln(Free SA)	Full Model	2	0.31	7.8	**0.021**	72.3%
		Linear	1	0.06	1.18	0.314	
		Quadratic	1	0.24	12.6	**0.012**	
		Error	6	0.11			
		Total	8	0.42			

DISCUSSION

In this study I asked if differences in constitutive SA concentration among species of closely-related plants are correlated with larval performance of a specialist insect herbivore. To address this question, I measured SA in the leaves of ten mustard species and also determined RGR and tissue damage by first-instar *P. rapae* caterpillars. I found that total constitutive SA concentration was indeed correlated with both *P. rapae* larval RGR and the susceptibility of mustard plants to herbivory by first instar caterpillars. These results indicate that low SA concentration in this group of plants is correlated with high resistance to herbivory. Previous studies have shown that exogenous application of SA or analogs of SA directly increase plant susceptibility to insect herbivores (Cipollini et al., 2004; Thaler et al., 2002a, b, 2012), but this is the first study to show that constitutive levels of SA themselves are correlated with insect performance. My data suggest that the relationship between constitutive SA concentration and resistance to *P. rapae* feeding is non-linear, with the highest resistance occurring at the lowest and highest levels of SA.

There are three possible mechanisms by which low constitutive levels of SA may correlate with high plant resistance to insect herbivory. First, this pattern could result from reduced negative pathway crosstalk between SA- and JA-mediated defenses. Here, low constitutive SA levels in leaves would have lower disruptive effects on constitutive or induced expression of defenses aimed at deterring herbivory, which are mediated by the hormone JA (Traw et al., 2003; Cipollini et al., 2004; Thaler et al., 2002a, b, 2012). Second, it is also possible that SA itself directly stimulates insect feeding and performance (but see Raju et al., 2009; Akbar et al., 2012). Third, both SA levels and resistance to insect feeding may not have a causal relationship, but instead be both correlated with a third factor, such as flowering time. SA has been shown to promote flowering in *Arabidopsis* (Martinez et al., 2004; Jin et al., 2008) and other species (Wada et al., 2010).

Total SA concentrations include the large fraction of SA that is conjugated to sugar (Vlot et al. 2009). I found that total constitutive SA had a stronger correlation with insect herbivory and performance than did free SA alone. Surprisingly, one of the mustard species, *Arabis canadensis*, produced only free SA and did not have any sugar conjugated SA (Figure 4.7). In the absence of this outlier, both larval herbivory and relative growth rates were highly significantly correlated with leaf constitutive free SA concentration (Figure 4.8, Table 2.3). Collectively, my data suggest that constitutive levels of total and free SA are generally tightly correlated with each other. Therefore it is not possible to distinguish between the relative ecological importance of the free and sugar-conjugated forms at present.

The correlation of higher total SA concentrations with susceptibility to herbivory suggests an ecological cost of maintaining a high level of free SA in plant tissues. Herbivory is known to have a negative effect on plant fitness (Schoonhoven et al., 2005), likewise, decreased herbivory in plants with high constitutive glucosinolate levels, or other secondary compounds, has also been reported (Mauricio, 1998; Agrawal & Kurashige, 2003). Ecological costs of high SA levels are likely mediated by defense pathway crosstalk between SA and JA (Traw et al., 2003; Cipollini et al., 2004; Thaler et al., 2002a, b, 2012), whereby, low SA levels may allow for increased expression of JA-mediated defenses making a plant more resistant to herbivores, but simultaneously resulting in greater susceptibility to pathogens (Todesco et al., 2010). The metabolic cost of high SA levels may be counterbalanced by increased resistance to biotrophic pathogens and phloem-sucking insects such as aphids.

Insect herbivores reduce plant fitness both directly by removing tissues and indirectly by making plants less competitive relative to neighboring plants (Mothershead & Marquis, 2000). Plants, in turn, produce physical traits such as trichomes and chemical traits such as secondary compounds that reduce feeding by herbivores (Walters, 2011). Plants with greater allocation to these defensive traits suffer less

damage and compete better when herbivores are abundant. When multiple species are compared within natural communities, however, it is generally observed that while some individual species have high allocation to these defensive traits, other species have consistently lower allocation (Gilbert, 2002). Given the benefits of high resistance, why is it that some plant species within a community possess predictably low allocation to defense against insect herbivores? Among the explanations that have received the greatest attention are tradeoffs with other beneficial traits, phylogenetic constraint and low apparency (Feeny, 1976). Indeed, expressing defense against one enemy often makes a plant more susceptible to attacks from other enemies (Strauss et al., 2002; Karban, 2011). Tradeoffs represent opposing interactions between two traits and can occur due to hormonal negative crosstalk (Karban & Baldwin 1998), ecological interactions between plants and multiple enemies (Groen et al., 2013), and resource-based limitations (Bazzaz et al 1987). Hormonal negative crosstalk refers to interactions among the major plant hormones involved in plant stress responses, jasmonic acid (JA) and salicylic acid (SA) and has been demonstrated in several studies (Thaler et al., 2002a,b; Traw et al., 2003; Spoel et al., 2003; Cipollini et al., 2004; Peng et al., 2007). The fact that spring-flowering species in this study have lower SA levels and greater resistance to *P. rapae* herbivory may reflect pressure from other environmental factors such as infection by pathogens or competition from neighboring plants.

Phylogenetic analysis based on the work of Koch et al. (2001) showed that these ten mustard species can be grouped into four fully-resolved clades. Analysis of *P. rapae* behavior and performance with respect to clade showed that while adult females have a clear preference for the exclusively summer-flowering Clade 1 (Figure 4.6G), this preference was not reflected in larval herbivory rate (Figure 4.6B) or RGR (Figure 4.6C). The coevolution of insects, especially butterflies, and plants has long been the subject of evolutionary theory (Ehrlich & Raven, 1964; Braby & Tueman, 2006; Janz, 2011). Thus, it is expected that phylogenetic relationships will have an influence on female oviposition choice,

as was indeed seen in this study. However, it is somewhat surprising that this relationship was not reflected in larval performance.

Pieris rapae caterpillars feed widely on plants in the family Brassicaea and the caterpillars of this species have become an important agricultural pest (Lasota & Kok, 1989; Capinera, 2001). Previous work has found that *P. rapae* caterpillar numbers are higher later in the growing season (Gaines & Kok, 1995; Maltais et al., 1998) and that early-flowering cultivars of broccoli are host to lower numbers of caterpillars of various species when compared to later-flowering cultivars (Vail et al., 1991). My work provides a physiological explanation for this observation. The five spring-flowering species in this study have lower constitutive SA concentrations and greater resistance to herbivory. These lower SA levels preclude the inhibition of JA-dependent defenses via negative crosstalk. With higher JA-dependent defenses, these plants will support lower numbers of insects than summer-flowering species with higher SA concentrations. The lower herbivory rate that I observed for the two species with the highest levels of SA may possibly be explained by a different effect of SA on plant defenses. Recent reports have shown that, in some cases, induced levels of SA has a direct negative effect on larval growth and performance (Raju et al., 2009; Akbar et al., 2012).

In conclusion, this work is the first to show that constitutive SA values influence insect feeding performance. Furthermore, I show that differences in seasonal flowering times are correlated with *P. rapae* larval performance. The underlying mechanism, however, is still unclear, as constitutive SA levels did not show a significant correlation with flowering time. Further research in this area will increase our understanding of plant defense chemistry and will influence agricultural practices with the goal of increasing crop resistance to insect herbivores.

5. INTERSPECIFIC VARIATION IN CROSSTALK AMONG MUSTARDS IN RESPONSE TO EXOGENOUS SA AND *TRICHOPLUSIA NI* FEEDING

In the following chapter, I describe a study I conducted that investigates the evolutionary conservation of negative signal crosstalk between the salicylic acid and jasmonic acid pathways. A chemical SA analog was applied to five mustard species and its effects on larval *Trichoplusia ni* relative growth rate were assessed.

INTRODUCTION

Plants are constantly attacked by numerous enemies and have evolved a multitude of chemical defenses as a result. These defenses prevent damage from most enemies and reduce the damage caused by species able to overcome these defenses. These defenses, however, have fitness costs (Agrawal et al., 2002; Strauss et al., 2002). Plants, thus balance the costs and potential benefits of investing in chemical defenses by producing some defenses constitutively and other defenses only when induced by a particular enemy. Current thinking is that plants fine-tune expression of their defensive chemicals with a sophisticated signaling network mediated by important hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (Clarke et al, 2000; Mewis et al., 2005).

The plant hormone JA is a key regulator of plant defenses against chewing insect herbivores (Kessler & Baldwin, 2002; Cipollini et al., 2004). JA is typically induced by insect damage leading to the expression of many defense-related genes (De Vos, 2005). Glucosinolates (GS) are defensive compounds produced by plants in the order Brassicales and stored in vacuoles in a biologically inactive

form until the plant is attacked by an herbivore (Rodman et al., 1996; Halkier & Gershenzon, 2006). Upon tissue damage by a chewing insect, storage compartments for GS and their separately-stored activating enzymes, myrosinases, are disrupted bringing these compounds into contact and resulting in the formation of toxic isothiocyanates, nitriles and other compounds (Björkman, 1976; Kjær, 1976; Halkier & Gershenzon, 2006; Rask et al., 2000). However, insect damage also induces production of GS resulting in increased tissue concentrations after an attack (Halkier & Gershenzon, 2006; Agrawal, 1998, 2000; Agrawal et al., 1999, 2002; Agrawal & Kurashige, 2003; Mattiacci et al., 2001; Traw, 2002; Traw & Dawson, 2002). GS are effective defensive compounds, slowing the growth of not only generalist insect herbivores, but also adapted specialist insects such as *Pieris rapae* caterpillars (Stowe, 1998; Agrawal & Kurashige, 2003). Variation in GS content has been shown to vary among (Daxenbichler et al., 1991) and within (Kliebenstein et al., 2001; Gols et al., 2008; Van Leur et al., 2008; Schranz et al., 2009; Agerbirk et al., 2010) species. Furthermore, the susceptibility of some mustard species to certain insects and resistance to others indicates differences in defense compound profile. *Barbarea vulgaris*, for example is resistant to herbivory by the crucifer-specialist diamondback moth, *Plutella xylostella* (Idris & Grafius, 1996), and *Capsella bursa-pastoris* is highly resistant to a different Brassicaceae specialist, larvae of the cabbage white butterfly, *Pieris rapae* (personal observation).

Salicylic acid (SA) is a plant hormone responsible for activating defenses against biotrophic pathogens and phloem-feeding insects, such as aphids. Infection with a biotrophic pathogen will cause an increase in SA levels, both at the site of infection (hypersensitive response) and systemically throughout the plant (systemic acquired resistance; Vlot et al., 2009). However, expression of defense compounds is bioenergetically expensive and can have fitness costs for the plant if expressed when not needed (Agrawal et al., 2002; Strauss et al., 2002; Traw, 2002; Cipollini et al., 2003; Bingham & Agrawal, 2010). Therefore, the simultaneous expression of both SA- and JA-mediated defenses is prevented in many plant species through negative crosstalk between these pathways. This is thought to be a cost-

saving mechanism that allows for fine-tuning of defenses for optimal protection and fitness (Thaler et al., 2002a,b, 2012; Traw et al., 2003; Cipollini et al., 2004; Taylor et al., 2004; Spoel et al., 2003; Kunkel & Brooks, 2002; Pieterse & Van Loon, 2004; Peng et al., 2007). Inhibition of JA-mediated defenses by SA reduces the amount of protease inhibitors, glucosinolates and other insect-deterring defenses produced by plants (e.g. Cipollini et al., 2004). However, it is not known to what extent different species of plants exhibit crosstalk and whether this influences insect herbivore performance. Therefore, to determine how SA inhibition of JA-mediated defenses varies in closely-related mustard species I measured the relative growth rate (RGR) of *Trichoplusia ni* caterpillars on plants with and without the application of exogenous SA. I then analyzed GS concentrations to determine if crosstalk inhibition of defenses against insects was caused by reduction in GS levels brought on by increased SA concentration.

MATERIALS AND METHODS

Mustard Plants and *Trichoplusia ni*

Seeds were collected from the following locations: *Draba verna* from Squaw Valley Park in Allegheny County Pennsylvania; *Barbarea vulgaris* and *Lepidium campetre* from a roadside in Ithaca NY; *Lesquerella fendleri* from the USDA, Peoria, IL; *Boechera stricta* was a kind gift from Thomas Mitchell-Olds. Seeds were sown in Pro-Mix BX soil (Premier Tech, Quakertown, PA) in 36-well flats and placed in a 4°C cold-room for 3 d of cold stratification. Flats were then transferred to an environmentally-controlled growth chamber at the University of Pittsburgh with constant conditions of 22°C, 12h day-night cycle and 350µmol $m^{-2}sec^{-1}$ light provided by a 1:1 mixture of sodium and metal halide lamps. All plants were grown simultaneously, watered as needed, fertilized every 10 d and moved at least once per week within the growth chamber to minimize positional effects. After 4 weeks of growth, whole plants

were harvested by cutting at the base with a razor blade, placing in a coin envelope and immediately flash-frozen in liquid nitrogen. Plant tissue was then transferred to a -80°C freezer until further assay.

Trichoplusia ni eggs were obtained from Benzon Research Inc. (Carlisle, Pennsylvania, USA). Upon hatching, caterpillars were placed in artificial diet cups consisting of wheat germ obtained from Benzon Research Inc. and kept at room temperature.

Plant Treatments and Insect Challenge

Six weeks after germination plants in the acibenzolar-S-methyl (ASM) treatment group were sprayed to runoff with 200 mg/L Actigard (Syngenta, Wilmington, Delaware, USA) dissolved in distilled water while plants in the control group were sprayed with a similar amount of distilled water. Two days after treatment, first and second instar *T. ni* caterpillars were size-matched and weighed to the microgram in groups of five on a MX5 Microbalance (Mettler Toledo) and placed on each plant. Plants were then enclosed in hair nets secured with pipe cleaners at the rim of each pot. Plants were then returned to the growth chamber. Caterpillars were reweighed individually after four days on the plants. *Camelina microcarpa* plants treated with ASM began to turn yellow and senesce before the end of the experiment. Therefore, results from these plants were not included in the data analysis.

Measurement of Glucosinolate Concentration

Leaf samples were taken from two plants of each treatment for all species for analysis of GS concentration by HPLC. All leaf samples were stored in a -80^{o}C freezer prior to analysis. Samples were removed from the freezer and immediately placed in a lyophilizer for 2d to freeze-dry the tissue. Glucosinolate extraction followed the methods of Traw et al. (2003). Approximately 50-90 mg of dry leaf tissue was weighed, pulverized and GS extracted in boiling 70% methanol (Agerbirk et al., 2001).

Samples were loaded into open columns packed with 0.1 g DEAE Sephadex A-25 (Pharmacia Inc.) to desulphate GS (Hugentobler & Renwick (1995). GS were analyzed on a Hewlett-Packard Model 1100 HPLC system equipped with an autosampler, a 4.5 × 15-cm C-18 column (Luna, Phenomenex Corp.), and diode-array detector. The HPLC solvent was run at 1 mL/min according to the following program: 100% water for 2 min, followed by a linear change to 20% acetonitrile for 5 min, 35% acetonitrile for 15 min, and 100% acetonitrile for 18 min. Total GS concentration was calculated as the sum of the areas of the top five peaks in each sample.

Statistical Analysis

Initial weights were calculated as the average of five first-instar caterpillars. For final weights, individual caterpillars were weighted to the microgram. The relative growth rate of the caterpillars was calculated as Ln mg per day:

RGR = (ln (final weight) – ln (initial weight))/day

Since five caterpillars were placed on five plants of each species and each treatment, a nested ANOVA was used to calculate F- and P-values for differences in treatment groups. A few caterpillars escaped, but in most cases four or five caterpillars were recovered for the second weight measurement on day 4.

RESULTS

All plant species tested supported *T. ni* caterpillar growth and survival. However, there was a significant difference in the performance of *T. ni* caterpillars as measured by the relative growth rate (RGR) of the caterpillars over a four-day period ($F_{4,111}$=63.94, $P < 0.001$, Figure 5.1). In general, there was a significant

difference in weight gained by *T. ni* caterpillars when plants were sprayed with ASM compared to control plants sprayed with water only (Figure 5.1, Table 5.1). Three of the five plant species tested supported greater RGR of *T. ni* caterpillars when the plants were treated with ASM compared to control plants, while a fourth species, *Boechera stricta* showed the same trend, but the difference was not statistically significant. Surprisingly, control *Lesquerella fendleri* plants supported significantly greater growth of *T. ni* caterpillars when compared to ASM-treated *L. fendleri*, which is in direct contrast to results seen on the other plant species.

Significant differences between treatment groups were not seen in leaf glucosinolate concentration (Figure 5.2, Table 5.2). In general control and ASM-treated plants that endured caterpillar feeding had higher leaf concentrations of GS, but these differences were not significant. There were no differences in GS concentration seen between control and ASM-treated plants that experienced four days of *T. ni* caterpillar feeding.

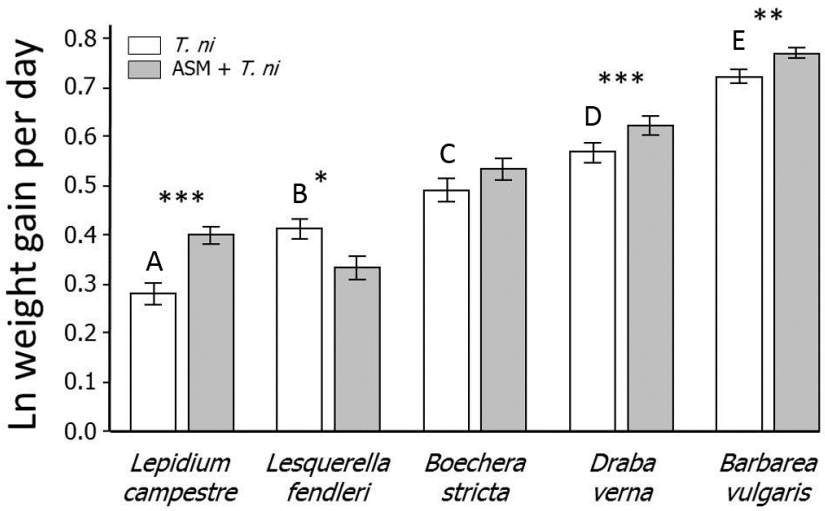

Figure 5.1. Mean (+/- SEM) relative growth rate of first instar *Trichoplusia ni* caterpillars feeding on five different mustard species. White bars indicate plants challenged with *T. ni* caterpillars with no pretreatment. Gray bars indicate plants first pretreated with 200mg/L Actigard (Syngenta) then challenged with first instar *T. ni* caterpillars. Significant differences at P = 0.05 in control groups between species are indicated by the absence of shared letters. Significant differences between control and treatment groups within a species are indicated by asterisks: *P < 0.05; **P < 0.01; ***P < 0.001.

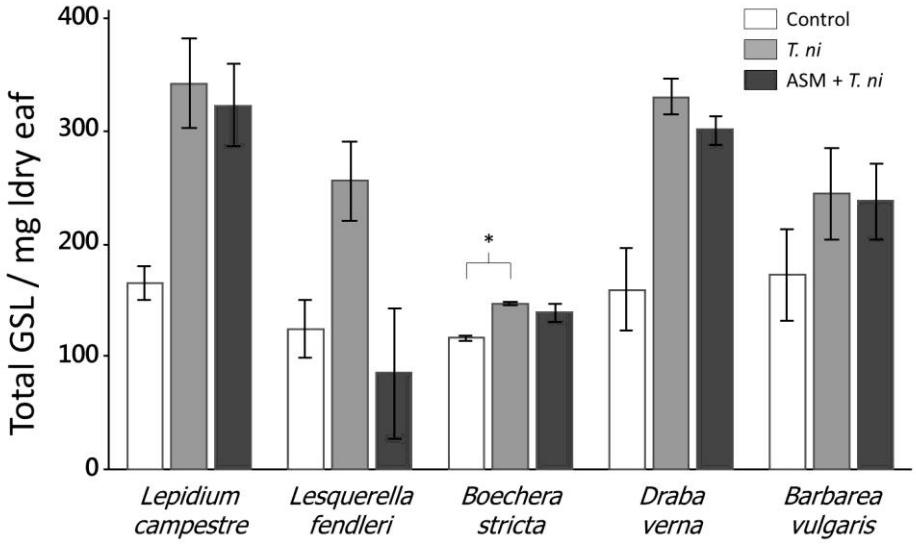

Figure 5.2. Mean (+/- SEM) concentration of top five glucosinolates of dry leaf tissue. White bars indicate control plants not challenged with *Trichoplusia. ni* caterpillars. Gray bars indicate plants challenged with *T. ni* caterpillars with no pretreatment. Black bars indicate plants first pretreated with 200mg/L Actigard (Syngenta) then challenged with first instar *T. ni* caterpillars. *P < 0.05.

Table 5.1. *Trichoplusia ni* caterpillar relative growth rate on five species of mustards. P-values were determined using a fully nested general linear model.

Species	Ctrl RGR	SE	ASM RGR	SE	F	P
Lepidium campestre	0.286	0.021	0.400	0.017	37.63	0.000
Lesquerella fendleri	0.413	0.020	0.333	0.024	4.47	0.043
Boechera stricta	0.491	0.023	0.534	0.023	2.85	0.100
Draba verna	0.569	0.021	0.624	0.020	16.18	0.000
Barbarea vulgaris	0.724	0.015	0.772	0.012	9.75	0.004

Table 5.2. P-values for differences in glucosinolate concentration among treatments in mustard leaves of five different species.

	P-value		
Species	No Tx vs Ctrl	No Tx vs ASM	Ctrl vs ASM
Lepidium campestre	0.151	0.159	0.784
Draba verna	0.149	0.173	0.387
Barbarea vulgaris	0.423	0.429	0.914
Boechera stricta	0.049	0.221	0.501
Lesquerella fendleri	0.204	0.638	0.239

DISCUSSION

In this study I investigated the conservation of defense signaling negative crosstalk between the SA pathway and defenses targeted at insect herbivores. Application of SA to plants is known to activate defenses against biotrophic pathogens and inhibit defenses against insect herbivores (Cipollini et al., 2004; Traw et al., 2003; Thaler et al., 2002a, b). Therefore, I applied SA to five mustard species to investigate the extent that SA inhibition JA-mediated defenses is shared among species. The goal of such research is to find a shared mechanism of defense signaling to help inform the development of a general theory of the coevolution of plants and their insect herbivores. I found that ASM strongly reduced plant defenses against *T. ni* caterpillars in only three of the five mustard species (*Lepidium campestre*, *Draba verna*, *Barbarea vulgaris*) tested. These results were indicated by significantly greater caterpillar growth on plants treated with the SA analog ASM. A fourth species (*Boechera stricta*) supported a nonsignificant increase in larval growth, while the fifth species (*Lesquerella fendleri*), remarkably, showed the opposite trend. *L. fendleri* plants treated with ASM and then challenged with *T. ni* caterpillars showed greater resistance to feeding as demonstrated by reduced caterpillar growth. These results are surprising considering that negative crosstalk has been demonstrated in species from several different plant families (Thaler et al., 2012). If ASM did indeed induce the SA pathway in *L. fendleri* as in other plants, these results show that divergent evolution of defense-pathway crosstalk has occurred in this species. However, without testing expression levels of genes induced by SA, I cannot be certain that *L. fendleri* recognized ASM as SA and responded with induction of the SA pathway.

First instar caterpillars of the generalist species *T. ni* successfully fed on each of the five species in this study for at least four days. However, significant differences were seen in caterpillar growth on the control plants of the different species. These results indicate that either the plants have differing nutritional values for these caterpillar, or they possess different types or amounts of growth-inhibiting

defensive compounds. Both possibilities could serve as explanations for these results. Slansky & Feeny (1977) reported that leaf nitrogen content was correlated with growth of *P. rapae* caterpillars on mustard plants, but GS content had no effect. However, other work has indicated that GS concentration in leaves does significantly reduce growth of both *P. rapae* and *Plutella xylostella* caterpillars (Agrawal, 2000). I therefore measured GS concentration of healthy plants and plants that had been fed upon for four days by *T. ni* caterpillars. Total GS content of the leaves was not correlated with the performance of the caterpillars. While the plants did show a general trend of increase GS concentration after feeding, these results were not statistically significant. Furthermore, I surprisingly found that, although, treating the plants with ASM resulted in increased caterpillar growth in three of the species, it had no significant effect on total GS concentration in plant leaves that had been fed upon for four days. A rapid loss of effect of ASM and return to normal plant chemistry can be ruled out, because application of is known to affect plant signaling for up to two weeks, after which time reapplication is recommended by the manufacturer.

In conclusion, negative crosstalk was intact in three of the five species used in this study. An explanation for the significant results seen in caterpillar performance cannot be attributed to total GS concentration in the leaves. Therefore, another mechanism, such as decreased production of proteinase inhibitors in ASM-treated plants, may be responsible for these results. A fourth species had a nonsignificant response, while the fifth species showed enhanced resistance to herbivory upon treatment with ASM. Results of studies such as this can be used by ecologists to develop theories of the evolution of plant defenses and it can also be used by plant breeders to develop more resistant varieties of crops.

6: CONCLUSIONS

In this thesis I first asked whether varying constitutive defense hormone levels among plant species correlate with the performance of insect herbivores. To address this question, I compared ten mustard species from a ruderal community in the Northeastern United States in common garden experiments and found that the five spring-flowering mustards (*Capsella bursa-pastoris, Draba verna, Cardamine impatiens, Barbarea vulgaris, and Arabidopsis thaliana*) as a group received significantly less herbivory and supported lower RGR of *Pieris rapae* larvae relative to five summer-flowering mustards (*Sisymbrium altissimum*, *Brassica nigra, Sinapis arvense, Lepidium campestre,* and *Arabis canadensis*). I then asked whether the differences that I observed could be explained by the underlying leaf SA concentrations and to what extent the patterns reflected the phylogenetic relationships among the species. I found that the species with the lowest leaf constitutive SA concentrations (*Capsella bursa-pastoris, Draba verna,* and *Cardamine impatiens*) were the most resistant to herbivory and supported the lowest larval relative growth rates. The highest herbivory and relative growth rates occurred in species with intermediate concentrations of leaf constitutive SA. Total SA concentration, which includes both free and sugar-conjugated SA, exhibited a stronger relationship with larval herbivory and performance than did the free SA component alone. In oviposition tests, the three most preferred species by gravid *Pieris rapae* females were all summer annuals (*Sisymbrium altissimum*, *Brassica nigra,* and *Sinapis arvense*). Oviposition rates were correlated significantly with phylogenetic groupings, whereas larval herbivory, larval relative growth rates and leaf constitutive SA concentrations were not. I conclude that low constitutive SA concentrations may benefit plants by reducing herbivory and larval growth rates of *Pieris rapae* feeding on the plants. These findings may also help explain how and why *Pieris rapae* outbreaks

occur in agricultural crops. In the second study, I extended this work in two directions, asking 1) whether exogenous application of SA increases susceptibility of plants to insect damage and 2) whether the effect would be observed in a generalist herbivore, the cabbage looper, *Trichoplusia ni* (Chapter 5).

Together, these two studies showed that both endogenous and exogenous SA were associated with changes in insect performance. Previous studies had shown that induction of SA suppresses plant resistance to herbivores. My results overall strongly supported this pattern. However, I did find several interesting exceptions. Constitutive SA concentrations exhibited a parabolic relationship with insect performance, whereby insect performance peaked at intermediate constitutive concentrations of SA. Extreme low and extreme high levels of SA were associated with resistance. This is a novel pattern that had not been described previously in the literature. Indeed, this is the first study that has linked any variation in constitutive SA with the performance of a leaf chewing herbivore. By and large, the exogenous application of SA caused increased susceptibility of the plants to insect feeding. Here, the one exception was *Lesquerella fendleri*. This species became more resistant to insect feeding after the SA treatment.

In future research, additional defense traits beyond those that I measured should also be included. To verify that the defense pathways are indeed being upregulated or downregulated, it would be useful to analyze the expression of key genes in both the SA and JA pathways. Several members of the *PATHOGENESIS RELATED* and *WRKY* gene families are activated by SA activity and are commonly used as markers for induction of the SA pathway. Measuring expression levels of these genes after application of ASM would verify whether induction of the SA pathway did indeed occur in each plant species tested. Likewise, several JA-responsive genes, such as *COI1*, *MYC2* and several members of the *JAZ* family, are commonly used as markers for induction of JA pathway signaling. These marker genes are good surrogates for JA signaling since measuring JA concentration itself requires gas

chromatography/mass spectrometry and is therefore time consuming and expensive. Measuring the response of these genes to either hormone application or insect damage would indicate if the JA pathway was effected by the experimental treatment. This would help to reveal the source of variation in response between species and allow for the assessment of the conservation of these signaling pathways.

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