CONSTRAINTS ON PLANT RESISTANCE AND TOLERANCE TRADE-OFFS

BY

JOSHUA MILES MESA

DISSERTATION

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Doctoral Committee:

Professor Ken N. Paige, Chair and Director of Research
Professor Ray E. Zielinski
Professor John A. Juvik
Associate Professor Katy D. Heath

ABSTRACT

Plant tissue loss due to herbivory is an important selective force for shaping plant phenotypes (Marquis 1992). Therefore, plants have evolved a variety of mechanisms to mitigate the negative effects of herbivory. Much of what we have learned, to date, on plant responses to their enemies (herbivores and pathogens) have focused on the evolution of chemical and structural traits that reduce or prevent tissue damage by herbivores (resistance) (Simms and Fritz 1990). However, herbivores may also select for traits that allow plants to compensate for tissue loss with little or no detriment in fitness (tolerance) (Painter 1951). Studies have even shown that herbivory leads to, under certain environmental circumstances (Hawkes and Sullivan 2001), an increase in plant reproductive success (overcompensation, i.e., increased fruit and seed production), rather than a decrease.

Plant chemical defense and tolerance to damage is often studied in the context of ecological trade-offs and costs of tolerance (Strauss et al. 2002). It was initially theorized that resistance and tolerance represented two alternative and redundant defense strategies given limited nutrients and energy available in the struggle against hervbivory (van der Meijden et al. 1988). According to this hypothesis, both defensive strategies offer the same fitness benefits (Mauricio et al. 1997). Regardless of the suggestions and assumptions that resistance and tolerance may play redundant roles in plant defense, a recent meta-analysis showed most natural populations appear to be comprised of a mixture of both strategies due to selection for the maintenance of both traits (Leimu and Koricheva 2006).

What has been missing from previous studies was the genetic and molecular underpinnings of plant tolerance and its relationship to the well-characterized molecular pathway involved in chemical defense, i.e., the shikimate pathway. Previous studies in the lab have shown

that the process of endoreduplication is the primary mechanism by which plants compensate for lost tissue following mammalian herbivory (Scholes et al. 2011). Moreover, the gene G6PD1 was also found to play a significant role in the compensatory process, which is the key regulator for the oxidative pentose phosphate pathway (opp) (Siddappaji et al. 2013). This pathway supplies intermediate carbon skeletons for both nucleotides (consistent with the upregulation of DNA with endoreduplication) and for resistance compounds via the shikimate pathway, thus tying both tolerance and resistance within the same pathway.

I utilized *Arabidopsis thaliana* genotypes displaying a range of compensatory responses from under to overcompensation and measured glucosinolate content for each which uncovered a positive association between the two defenses. Recombinant inbred lines from a cross between the overcompensating ecotype Col-4 and the undercompensating Ler-0 of the annual plant *Arabidopsis thaliana* were utilized to assess the relationship between tolerance and resistance. Total glucosinolate content for each ecotype following simulated mammalian herbivory was measured. These data show overcompensating ecotypes also have the highest resistance chemistry. Similarly, the direct association between tolerance and resistance was demonstrated by genetically manipulating the endoreduplication pathway. By overexpressing *ILP1*, a positive regulator of endoreduplication, and thus compensation, glucosinolate production and tolerance was increased in the Col-0 ecotype. This approach allowed us to assess plant resistance-tolerance tradeoffs from a molecular genetic point of view. These results indicate that plant tolerance and resistance pathways are tightly integrated within the oxidative pentose phosphate pathway and may represent a general phenomenon among herbaceous plants given that approximately 90% of herbaceous angiosperms endoreduplicate (Nagl 1976, Sugimoto-Shirasu and Roberts 2003).

Although a causal link has been established between endoreduplication, fitness compensation, and chemical defense, no one has addressed whether insect leaf-feeding can elicit the same compensatory response as removing the apical meristem which lowers the level of auxin and triggers entry into the endocycle. In *Arabidopsis*, wounding has been shown to down-regulate a number of genes that are positively associated with auxin production, suggesting a suppressive effect of insect wound-induced signals, like jasmonic acid, salicylic acid and ethylene, on the auxin signal transduction pathway (Onkokesung et al. 2010). Thus, insect leaf-feeding could trigger endoreduplication by the upregulation of wound-induced signals ostensibly lowering auxin production. Results here support this contention; insect leaf-feeding by *Trichoplusia ni* elicited a compensatory response similar to that elicited by mammalian herbivores - an ecotype-specific response dependent upon the level of endoreduplication. In addition, the interactive effects of mammalian and insect herbivory on each of these inbred lines was assessed to determine whether interactions were additive (pairwise) or nonadditive (diffuse) on fitness compensation (tolerance) and secondary plant metabolite production (resistance). Results show that some ecotypes had non-additive effects of herbivory such as Ler-0 where following clipping plants suffered increased fitness impacts from *T. ni* compared to unclipped plants. Other ecotypes such as CS1906 displayed additive effects of insect and mammalian herbivory.

Despite a failure to detect tradeoffs between the two defenses both defensive strategies utilize carbon skeletons from a shared resource pool in the oxidative pentose phosphate pathway. Therefore, the costs for maintaining both strategies were assessed experimentally. Specifically the cost of resistance in *A. thaliana* was assessed by utilizing a double knockout mutant for two cytochrome P450s CYP79B2 and CYP79B3, key enzymes in the biosynthetic process of indole

glucosinolates, which converts tryptophan to indole-3-acetaldoxime (IAOx). IAOx is then further utilized in the production of indole glucosinolates (Bender and Celenza 2009). Results show that knocking out indole glucosinolate production and thus resistance leads to an increase in the compensatory response compared to wildtype Columbia-0. This shows that despite a positive association there are still physiological costs for maintaining both strategies.

My studies have shown that endoreduplication is an important driver of both compensatory and induced resistance responses following both mammalian and insect herbivory. Moreover, these results showing ecotypic variation in endoreduplication, raises some concerns about drawing conclusions on the impacts of herbivory given that we tend to ignore genotypic variation by collapsing it into an overall mean population response. Through multiple chapters I have shown that when we averaged total seeds and glucosinolates across all plant genotypes, ignoring the genetic variation in endoreduplication among ecotypes of *A. thaliana*, we no longer observe a positive association between the two defenses. This dissertation indicates it will be important to assess the degree of endoreduplication across a population of herbaceous plants prior to measuring fitness and plant resistance responses to herbivory. Thus, additional plant species that endoreduplicate should be investigated to see if this phenomenon is generalizable.

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Chapter 1: General Introduction

There has long been a focus on trade-offs between life history investments in growth, reproduction, and maintenance functions that influence survival and longevity (Reznick and Endler 1982, Reznick et al. 1990, Stearns 1989, Zera and Harshman 2001). The concept of trade-offs is central to almost all arguments of adaptation with such trade-offs (costs) sought to explain variation in investment in traits (Agrawal 2011).We now recognize that defensive functions, such as investments in immunity, play an integral role in the trade-offs that shape organismal evolution (Agrawal 2011, Strauss et al. 2002, Lind et al. 2013). Moreover, the evolution of defenses are known to be among the most diverse and rapidly evolving components of organisms and may dictate everything from species abundance to trait divergence and speciation (Berenbaum 2001, Strauss et al. 2002, Agrawal 2007, Smakowska et al. 2016). Several authors have suggested the existence of constraints on multiple defensive strategies due to limiting resource allocation (Strauss et al. 2002). Such trade-offs have been expected to occur among multiple types of resistance chemicals (Lebreton 1982) and between chemical resistance and plant tolerance (plant regrowth following damage) (van der Meijden 1988), since investment in these antiherbivore defenses is assumed to be costly (Bergelson and Purrington 1996). Here, I propose that plant tolerance and resistance are positively associated, counter to theory, due the joint mediation by the same molecular pathway.

Much of what we have learned, to date, on plant responses to their enemies (herbivores and pathogens) has focused on the evolution of chemical and structural traits that reduce or prevent tissue damage by herbivores (resistance) (Simms and Fritz 1990). Resistance is manifested in an elaborate array of secondary metabolites that function in plant defense against a wide array of microorganisms (viruses, bacteria, fungi) and herbivores (vertebrates, arthropods)

(Lambrix et al. 2001, Wink 1988). The idea that plants respond to herbivores by apportioning resources to resistance traits has been widely established (Ehrlich and Raven 1964). However, herbivory may also select for traits that allow plants to compensate for tissue loss with little or no detriment in fitness (tolerance) (Painter 1951).

Studies have even shown that herbivory leads to, under certain environmental circumstances (Hawkes and Sullivan 2001), an increase in plant reproductive success (overcompensation, i.e., increased fruit and seed production), rather than a decrease. Ecologists first became interested in the potential of overcompensation in the 1970's when multiple authors reported that herbivory may result in an increase rather than decrease in plant growth and fitness in some species (Dyer 1975). These claims were initially dismissed as a result of belowground resources being reallocated to aboveground biomass in perennial plants, resulting in a net fitness decrement (Belsky 1986). However, research by Paige and Whitham (1987) provided the first convincing evidence of overcompensation. Specifically they found an increase in the product of lifetime seed production, germination and seedling survival of over three times that of uneaten controls in the monocarpic biennial scarlet gilia, *Ipomopsis aggregata*, after ungulate herbivores removed 95% or more of the above-ground biomass. The increase in fitness was mostly caused by architectural changes in the plant as multiple flowering stalks resulted after the removal of *I. aggregata's* single inflorescence due to axillary bud break after the release of apical dominance. Furthermore, evidence that overcompensation is a widespread phenomenon has been demonstrated via multiple studies in a variety of plant species including *Ipomopsis arizonica* (Maschinski and Whitham 1989), *Arabidopsis thaliana* (Mauricio et al. 1997), and *Gentianella campestris* (Lennartsson et al. 1997), among many others.

There is also evidence that both tolerance and resistance are genetically variable traits. For example, glucosinolates, secondary metabolites of the *Brassicaceae* and related families, show extensive genetic variation within and among plant species (Daxenbichler et al. 1991). Furthermore, resistance often shows heritable variation within and among populations (Kliebenstein et al. 2001, Windsor et al. 2005). Tolerance is genetically variable as some families' exhibit overcompensation, whereas others express equal to undercompensation (Tiffin and Rausher 1999, Juenger and Bergelson 2000, Weinig et al. 2003). Heritability for tolerance has been shown in one population of *I. aggregata* (Juenger and Bergelson 2000). Moreover, studies comparing historically grazed and ungrazed populations of the plant *G. campestris* show repeatedly grazed populations overcompensate, while historically ungrazed populations undercompensate when grazed (Lennartsson et al. 1997).

Plant chemical defense and tolerance to damage is often studied in the context of ecological trade-offs and costs of tolerance (Strauss et al. 2002). It was initially theorized that resistance and tolerance represented two alternative and redundant defense strategies given limited nutrients and energy available in the struggle against herbivory (van der Meijden et al. 1988). According to this hypothesis, both defensive strategies offer the same fitness benefits (Mauricio et al. 1997). Regardless of the suggestions and assumptions that resistance and tolerance may play redundant roles in plant defense, a recent meta-analysis showed most natural populations appear to be comprised of a mixture of both strategies due to selection for the maintenance of both traits (Leimu and Koricheva 2006). However, our understanding of the joint evolution of tolerance and resistance is still limited, having received attention only in the last 15 years (Fornoni et al. 2004).

Until recently the genetic and mechanistic basis for enhanced growth and reproduction following herbivory was poorly understood. Recent studies have shown that the oxidative pentose phosphate pathway and endoreduplication underlie plant compensatory responses (Scholes and Paige 2011, Siddappaji et al. 2013). The OPP pathway provides carbon skeletons for both nucleotide biosynthesis and plant secondary chemical production thus tying plant tolerance and resistance within the same molecular pathway. I propose that plant tolerance and resistance are positively associated, counter to theory, due the joint mediation by the same molecular pathway. To test this I used a combination of chemical assays for plant resistance traits, flow cytometry for ploidy levels and fitness measures across multiple accessions of *Arabidopsis thaliana* that displayed varying levels of plant compensation. These experiments were followed by manipulative genetic experiments to increase endoreduplication responses in an undercompensating ecotype to both increase tolerance and resistance verifying this association.

I chose *Arabidopsis thaliana*, mouse-ear cress, a small, mostly self-fertizlizing plant in the Brassicaceae family for my studies because it responds to apical damage in a similar way *Ipomopsis* does in response to herbivory (Paige and Whitham 1987). *A. thaliana* is found as a winter annual where seeds germinate in the fall after passing through summer in a dormant state and grow into an overwintering rosette, with stem elongation in the spring (Pigliucci 2002). A variety of species feed upon *A. thaliana* including flea beetles, aphids, leaf miners, caterpillars, and small mammals (Weinig et al. 2003, Van Poecke 2007). *A. thaliana* therefore frequently experiences both leaf and apical meristem damage and has a suite of resistance traits such as trichomes, proteinase inhibitors, and glucosionolates, my compound of interest, that deter and inhibit feeding by herbivores (Beekwilder et al. 2008). Importantly, *A. thaliana* has many

advantages for genome analysis over other plants which have allowed QTL, expression and sequencing analyses that have uncovered the molecular and genetic mechanisms and pathways associated with both plant tolerance and resistance (Scholes and Paige 2011, 2014, Siddappaji et al. 2013, Scholes et al. 2017).

In these studies I specifically proposed to:

1) Characterize the association between plant resistance and tolerance among Recombinant Inbred Lines (RILs) (Lister and Dean, 1993).
    a. Assess resistance and tolerance using a cross of Landsberg *erecta* X Columbia-4, which displays a continuum of compensatory responses given the recent advances in our understanding of the genetic basis of plant tolerance.
    b. Experimentally assess the association found between plant tolerance and resistance utilizing an ILP1-ox mutant which increases endopolyploidy to show causality.
2) Evaluate the plant tolerance/resistance tradeoff in response to insect damage and an assessment of the interactive effects of insect and mammalian damage.
    a. Assess plant compensatory response and glucosinolate induction among 3 accessions following insect leaf damage.
    b. Assess the interactive effects of insect and mammalian damage to determine if plant-herbivore interactions proceed via diffuse or pairwise interactions on fitness compensation and chemical defense.
3) Experimentally assess costs of resistance chemistry using knockout mutants for secondary chemistry on plant tolerance.

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Chapter 2: Molecular Constraints on Resistance-Tolerance Trade-Offs

2.1 Abstract

Plants have numerous mechanisms to cope with the negative effects of herbivory, including plant resistance, structural and chemical traits that reduce damage, and plant tolerance, the ability to compensate for tissues lost. It has been argued that resistance and tolerance represent alternate strategies and thus there should be a tradeoff between resistance and tolerance. However, resistance and tolerance are controlled via the same molecular pathway, the oxidative pentose phosphate pathway and the process of endoreduplication. Endoreduplication is the replication of the genome without mitosis, which leads to an increase in cellular chromosome number. Increasing chromosome number and therefore gene copy number provides a means of increasing gene expression that has been shown to enhance compensation following herbivory. By measuring glucosinolate levels and seed production following the removal of apical dominance in genotypes of *Arabidopsis thaliana* we show that there is a positive association between tolerance and induced chemical defense. Similarly, the direct association between tolerance and resistance is demonstrated by genetically manipulating the endoreduplication pathway. By overexpressing *ILP1*, a positive regulator of endoreduplication, and thus compensation, we experimentally increased glucosinolate production and tolerance in the Col-0 genotype. We suggest that many herbaceous plants that endoreduplicate (~90%) would show a positive relationship between compensation and chemical defense, given that the molecular pathways are shared in common. We discuss these findings in light of contrasting results on measures of tolerance and resistance, given that the true relationship can be masked by ignoring genetic variation in endoreduplication and the timing of chemical measurement.

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2.2 Introduction

Plant tissue loss due to herbivory is an important selective force shaping plant phenotypes (Marquis 1992). Therefore, plants have evolved a variety of mechanisms to mitigate the negative effects of herbivory. Much of what we have learned, to date, on plant responses to their enemies (herbivores and pathogens) have focused on the evolution of chemical and structural traits that reduce or prevent tissue damage by herbivores (resistance) (Simms and Fritz 1990). The idea that plants respond to herbivores by apportioning resources to resistance traits has been widely established (Ehrlich and Raven 1964). However, herbivores may also select for traits that allow plants to compensate for tissue loss (Stowe *et al.* 2000). Plants that can compensate for lost tissue with little or no decrement in fitness are termed tolerant. Attention to the phenomenon of plant tolerance was spurred by studies showing that in some instances plants can respond to plant herbivory with an increase in plant reproductive success (a specialized case of tolerance, termed overcompensation, i.e., increased flower, fruit and seed production) (Paige and Whitham 1987).

As both strategies lower the detrimental effects of herbivory and resources are limited in the environment, models for the joint evolution of resistance and tolerance have predicted a physiological trade-off (van der Meijden *et al.* 1988), i.e., plants employ either resistance or tolerance strategies. Despite this proposed tradeoff there is little empirical data to support this model (Mauricio *et al.* 1997, Muola *et al.* 2010). Furthermore, a recent meta-analysis showed most natural populations appear to be comprised of a mixture of both strategies (Leimu and Koricheva 2006, Núñez-Farfán *et al.* 2007), possibly due to selection for the maintenance of both traits (Leimu and Koricheva 2006, Carmona and Fornoni 2012). For example, selection for both traits could be caused by the joint feeding of specialist and generalist herbivores; specialists that are adapted to feeding on a specific plant are able to circumvent the plants resistance chemistry

which would likely select for tolerance, while generalists are deterred by chemical resistance (Winde and Wittstock 2011, Garrido *et al*. 2012). This deviation in empirical data from the predictions of the models could also arise from certain assumptions in the model about the shape of cost-and-benefit functions, which assume linearity (Fornoni *et al*. 2004). However, a review of the literature shows that costs and benefits of resistance are frequently nonlinear (Bergelson *et al.* 2001).

What has been missing from studies of potential tradeoffs between tolerance and resistance is an understanding of the molecular genetic pathways involved in tolerance (Fornoni 2011) and its relationship to the well-characterized molecular pathway involved in chemical defense, i.e., the shikimate pathway. Our recent studies on the molecular underpinnings of tolerance (with an emphasis on the phenomenon of overcompensation) suggest that plant tolerance and resistance may be positively correlated due to a sharing of the same molecular and genetic pathways, explaining in part the inability to uncover a physiological tradeoff between these two strategies. Specifically, we have uncovered a gene, *GLUCOSE-6-PHOSPHATE-1-DEHYDROGENASE* (G6PD1, At5g35790.1), that plays a major role in controlling the compensatory response in *Arabidopsis thaliana* following the removal of the plant's primary inflorescence (Siddappaji *et al.* 2013). G6PD1 is the central regulatory enzyme in the oxidative pentose phosphate (OPP) pathway that plays a key role in plant metabolism generating NADPH and a variety of metabolic intermediates for biosynthetic processes (Kruger and von Schaewen 2003). G6PD1 supplies intermediate compounds from the OPP pathway into the shikimate pathway for secondary metabolite production for plant chemical defense, such as glucosinolates (Maeda and Dudareva 2012), our compounds of interest here.

Furthermore, we have recently shown that the ability of a plant to increase its ploidy level via endoreduplication leads to rapid regrowth and an increase in fitness following removal of apical dominance, explaining, in part, the phenomenon of overcompensation in plants (Scholes and Paige 2011, Scholes and Paige 2014). Endoreduplication is the replication of the genome without mitosis, which leads to endopolyploidy, an increase in cellular chromosome number (Nagl 1976, Brodsky and Uryvaeva 1977, Melaragno *et al.* 1993). Increasing chromosome number through endoreduplication and therefore gene copy number may provide a means of increasing gene expression of vital genes (such as G6PD1) or gene pathways that promote rapid regrowth rates following herbivory. G6PD1 feeds compounds into the OPP pathway for nucleotide biosynthesis, by the provision of ribose-5-phosphate, necessary for the significant increase in chromosome number via endoreduplication. The increase in DNA content then feeds back on pathways involved in metabolism (e.g., G6PD1) and defense (e.g., glucosinolate production) through increased gene expression (more copies due to increases in endoreduplication following damage) (Scholes *et al.* 2013, Scholes and Paige 2015a,b). Thus, the mechanisms for both plant tolerance and resistance are co-localized within the oxidative pentose phosphate pathway and appear to be molecularly interdependent.

There is also evidence that both tolerance and resistance are genetically variable traits. For example, glucosinolates, secondary metabolites of the Brassicaceae and related families, show extensive genetic variation within and among plant species (Daxenbichler *et al.* 1991) and heritable variation within and among populations (Kliebenstein *et al.* 2001, Windsor *et al.* 2005). Tolerance is genetically variable as some families exhibit overcompensation, whereas others express equal to undercompensation (Siddappaji et al. 2013, Tiffin and Rausher 1999, Juenger and Bergelson 2000, Weinig *et al*. 2003). Heritability for tolerance has been shown in one

population of *Ipomopsis aggregata* (Juenger and Bergelson 2000). Moreover, studies comparing historically grazed and ungrazed populations of the plant *Gentianella campestris* show repeatedly grazed populations overcompensate, while historically ungrazed populations undercompensate when grazed (Lennartsson *et al.* 1997). This suggests that selection has acted upon heritable genetic variation for plant compensation (Lennartsson *et al.* 1997).

Here we assess whether plant tolerance and resistance are, contrary to predictive models, positively correlated due to both strategies being constrained to the same molecular pathway. We specifically assessed plant tolerance in *A. thaliana* as our previous studies established that genotypes of *A. thaliana* differ in their ability to compensate after tissue loss, ranging from lowered fitness after damage (undercompensation; e.g., Landsberg *erecta*, L*er*-0) to increased fitness after damage (overcompensation; e.g., Columbia-4, Col-4) (Siddappaji *et al.* 2013) and we have uncovered the molecular basis for this phenomenon, as noted above (see Siddappaji *et al.* 2013, Scholes and Paige 2014 for more details). We also assessed glucosinolates within each genotype before and following damage to evaluate differences in induction. Furthermore, and more importantly, we tested whether experimental manipulations of endoreduplication and thus fitness compensation causes predictable changes in plant resistance (i.e., glucosinolate production). Our approach allowed us to assess plant resistance-tolerance tradeoffs from a molecular genetic point of view. Our results indicate that plant tolerance and resistance pathways are tightly integrated within the oxidative pentose phosphate pathway and may represent a general phenomenon among herbaceous plants given that approximately 90% of herbaceous angiosperms endoreduplicate (Nagl 1976, Sugimoto-Shirasu and Roberts 2003). We discuss these findings in light of contrasting results from the literature on measures of tolerance and resistance.

2.3 Methods

2.3.1 Study Species

Arabidopsis thaliana, mouse-ear cress, is a small, mostly self-fertilizing plant in the Brassicaceae family. While native to Europe, *A. thaliana* has a wide geographical range spanning Eurasia, North Africa and North America (Ratcliffe 1965, Mitchell-Olds 2001). *A. thaliana* is typically found as a winter annual where seeds of *A. thaliana* germinate in the fall after passing the summer in a dormant state and grow into an overwintering rosette, and following stem elongation in the spring, produce flowers that develop into seed pods known as siliques (Pigliucci 2002). A variety of species including flea beetles, aphids, leaf miners, caterpillars and small mammals such as rabbits (Weinig *et al.* 2003, Van Poecke 2007) feed upon *A. thaliana*. *A. thaliana* thus frequently experiences leaf and apical meristem damage and has a suite of resistance characters such as trichomes, proteinase inhibitors and glucosinolates that deter and inhibit feeding by herbivores (Beekwilder *et al.* 2008).

2.3.2 Glucosinolates

Glucosinolates constitute a large and diverse group of defensive secondary metabolites characteristic of the order Brassicales, which includes *A. thaliana*, our study organism (Müller *et al.* 2010). Glucosinolates (mustard oil glucosides) are nitrogen and sulfur rich natural plant secondary products. More than 120 different glucosinolates, ~40 in *A. thaliana*, have been identified, most of which are classified into three subgroups based on the biosynthetic amino acid precursor, including indole, aliphatic and benzenic glucosinolates (Sønderby *et al.* 2010). In *A. thaliana* most glucosinolate diversity comes from indole and aliphatic glucosinolates (Brown *et al.* 2003). There is also a large body of evidence that glucosinolate breakdown products deter generalist and specialist herbivores on *A. thaliana* (Agrawal and Kurashige 2003) and act in

defense against pathogens (Schlaeppi *et al.* 2010). Upon tissue disruption (e.g. herbivory), glucosinolates stored in the vacuole are mixed with myrosinase (known as the "mustard bomb"), a β-thioglucosidase that is spatially separated in scattered myrosin cells (Ratzka *et al.* 2002). Myrosinase removes the β-glucose moiety from glucosinolates, leading to a variety of toxic breakdown products, such as bioactive nitriles, epithionitriles and isothiocyanates based on reaction conditions such as pH and presence or absence of protein factors such as epithiospecifier proteins (Zhang *et al.* 2006).

2.3.3 Plant Compensation, Resistance and the Role of Endoreduplication

Experimental Design

To assess the relationship between plant compensation and plant resistance, we planted 130-180 individuals each of L*er*-0 and Col-4 and three recombinant inbred lines: CS1906, CS1948 and CS1968. The F2 lines from the cross between L*er*-0 and Col-4 were advanced through inbreeding and single-seed descent for more than eight generations (Lister and Dean 1993) and are available through The Arabidopsis Information Resource (TAIR) Center. All lines were grown in a greenhouse on the campus of the University of Illinois at Urbana-Champaign, under 12 hours of light (~100 $\mu E/m^2/sec$) and dark. Plants were grown individually in 3.5-inch pots using a 9:1 mixture of L1 Sunshine mix and quartz sand. Seeds/seedlings were kept moist during germination, and plants were then watered daily to maintain soil moisture without saturating the soil. Plants were not fertilized. The newly elongating inflorescences of 50 plants of each genotype were randomly clipped, when they reached a height of approximately 6 cm (at 3.5 weeks), leaving approximately 1 cm of inflorescence (comparable to natural mammalian herbivory observed for this species) (Scholes et al. 2017).

At 4.5 weeks, 30 plants of each genotype (15 clipped, 15 unclipped) were analyzed for glucosinolate concentration. At 6.5 weeks 30 additional plants (15 clipped, 15 unclipped) were subsequently analyzed for glucosinolate concentration. These time points were selected based off the labs previous experiments showing that endopolyploidy is not maximized until later in *A. thaliana*'s life cycle (Scholes and Paige 2011). Fresh inflorescence and associated cauline leaf material was taken from both clipped and unclipped plants from each genotype for both time points. Previous studies (Barow 2006) have shown that glucosinolate concentrations vary by organ within *A. thaliana* with inflorescence, cauline leaves and siliques containing higher levels than those of root, stems and rosettes, consistent with predictions of theories on optimal distribution of resistance chemicals (Brown *et al.* 2003). All samples were frozen in liquid nitrogen, and stored at -80°C prior to freeze-drying. Freeze-dried tissues were ground into a fine powder and stored at -20°C prior to glucosinolate analysis. Glucosinolates were extracted from finely ground freeze-dried tissue, converted to desulphoglucosinolates with arylsulfatase and analyzed via high pressure liquid chromatography (HPLC) as described by Brown *et al*. (2003). Specifically, fifty mg of freeze-dried powder and 0.5 mL of 70% methanol were added to 2.5 mL tubes and placed on a heating block at 95°C for 10 min, mixing frequently. Samples were cooled on ice and 0.125 mL glucosinolabin was used as an internal standard and centrifuged at 3,000xg for 10 minutes. The supernatant was saved and the pellet was re-extracted with another 0.5 mL 70% methanol at 95°C for 10 minutes and the two extracts were combined. Protein was subsequently precipitated with 0.15 mL of a 1:1 mixture of 1 M barium acetate and 1 M lead acetate and centrifuged at 12,000xg for 1 min. Each sample was then loaded onto a column containing DEAE Sephadex A-25 resin for desulfatation via arylsulfatase for 18 h and the remaining desulfo-GS eluted. Desulphoglucosinolates were separated on a HPLC system

(Agilent 1100 HPLC system, with a G1311A bin pump, a G1322A vacuum degasser, a G1316A thermostatic column compartment, a G1315B diode array detector and an HP 1100 series G1313A autosampler) with a variable ultraviolet detector set at 229 nm wavelength. Elution of desulphoglucosinolates occurred over 45 minutes with a linear gradient of 0% to 20% acetonitrile in water with a flow rate of 1.0 mL/min. Glucosinolate concentration was established using glucosinalbin as an internal standard, a glucosinolate not found in *A. thaliana*. UV response factors for different glucosinolates were used as determined by Wathelet *et al*. 2001. Indole, aliphatic and total glucosinolates were estimated by adding the 11 most consistently found glucosinolate concentrations within the different lines, 4 of those being indole and the remaining 7 aliphatic.

Upon plant senescence (8 weeks), plants of each genotype were analyzed for fitness (70-120 plants per genotype; half clipped and half unclipped). All plants flowered and produced fruits (siliques) and fitness was measured by the number of siliques and seeds per plant. Our previous studies (Scholes and Paige 2014) have shown that seeds are a good measure of plant fitness as there are no significant differences in seed weight or germination success between clipped and unclipped plants of *A. thaliana*. We measured total silique number for each plant and counted total seed production in 3 randomly selected siliques from each plant. Seeds per silique were averaged per genotype and treatment then multiplied by total silique number for each plant to obtain seed totals per plant. Potential differences in seed production were assessed using Students *t*-tests comparing plants with apical meristem damage to undamaged controls for each line. As noted, fitness was measured on a separate group of plants due to the destructive nature of sampling for glucosinolate analysis. Thus, separate analyses were performed for glucosinolate content (including separate analyses for time points of 4.5 weeks and 6.5 weeks) and fitness

measures. Glucosinolate content was log-transformed prior to statistical analysis to approximate normality. Potential differences in the eleven glucosinolates measured, including overall measures of indole, aliphatic and total glucosinolates, were assessed between clipped and unclipped plants of each genotype, followed by a sequential Bonferonni to maintain a familywise error rate of $\alpha = 0.05$ (Rice 1989).

In addition, we manipulated the plant tolerance pathway via an increase in endoreduplication to measure the effect on glucosinolate content. *INCREASED LEVEL OF POLYPLOIDY1* (*ILP1*; TAIR locus:At5G08550; The Arabidopsis Information Resource, http://www.arabidopsis.org) was selected for use given that it has been shown to increase endoreduplication, resulting in an increase in plant tolerance (such as seed production) (Scholes and Paige 2014). ILP1 induces the endocycle by transcriptionally repressing the 'A2' variety of cyclins (CYCA2; (Imai *et al.* 2006)). CYCA2;3 of the CYCA2 family is a key activator of cyclin-dependent kinase B (CDKB), which is a component of the protein complex that induces cells to undergo mitosis in *A. thaliana* (Boudolf *et al.* 2004). Thus, overexpression of *ILP1* causes further repression of CYCA2 and therefore suppression of mitotic cell division. Endopolyploidy is subsequently induced as S-phase DNA replication continues successively (Imai *et al.* 2006). An *ILP1* overexpression genetic line (*ILP1*-ox) was kindly obtained from the laboratory of Minami Matsui at the RIKEN Yokohama Institute (Plant Functional Genomics Research Group, Plant Science Center; Yokohama, Japan). This genetic line was constructed through the addition of a CaMV 35S promoter to *ILP1* in a Columbia-0 (Col-0) genetic background (Imai *et al.* 2006). *ILP1*-ox has approximately 29× greater expression of *ILP1* than the Col-0 wildtype (Imai *et al.* 2006).

Col-0 was also selected for analysis, and we note that it is related to, yet genetically distinct from, the Columbia-4 genotype used here and in previous studies in our lab (e.g., see (Scholes and Paige 2011). Given the effects of *ILP1*-ox in an undercompensating background, Col-0 (see Scholes and Paige 2014), we sought to test whether an increase in plant tolerance also results in an increase in plant chemical defense, demonstrating cause and effect. Plant tolerance was measured using 25 clipped and 25 unclipped individuals for each genotype as described above. Glucosinolates were measured using 15 clipped and 15 unclipped plants. Cell cycle values were acquired from an earlier experiment in our lab (Scholes and Paige 2014). Compensatory responses and the induction of endoreduplication have been shown to be repeatable (Siddappaji *et al*. 2013, Scholes and Paige 2011, Scholes and Paige 2014, Mesa and Paige unpublished data). Moreover, a two way ANOVA showed that there was no significant difference in the magnitude (% differences clipped relative to unclipped plants) of the compensatory (fitness) responses of Col-0 and *ILP1*-ox ($F=0.338$, $df=1,95$, $p = 0.562$; $F=1.617$, $df=1,95$, $p = 0.207$, respectively) when comparing our results here to those in a previous experiment (Scholes and Paige 2014).

2.4 Results

2.4.1 Tolerance Measures

As expected (based on our previous studies), clipped plants of Col-4 and CS1948 produced significantly greater numbers of seeds than plants that were not clipped, increasing seed production by 51.89% and 48.67%, respectively, ($t_{118} = 14.94$, $p < 0.0001$ and $t_{82} = 13.28$, $p < 0.001$, Fig. 2.1a and b). Clipped plants of L*er*-0 and CS1968, however, displayed significantly lower numbers of seeds compared to unclipped controls, decreasing seed production by 45.84% and 45.53%, respectively ($t_{105} = -11.30$, $t_{89} = -11.91$, both $p < 0.0001$). Clipped plants of CS1906

showed no significant differences in seed production compared to unclipped controls ($t_{69} = 0.35$, $p = 0.727$, Fig.2.1a and b). Siliques of clipped and unclipped plants mirrored total seed production. Thus, we report the ultimate measure of fitness here.

2.4.2 Glucosinolate Content

Plants at 4.5 weeks of age, one week after elongation of the inflorescence and thus clipping, differed in glucosinolate content in Col-4 and L*er*-0. In Col-4, glucosinolates increased by 21.79% for plants that were clipped relative to those that were unclipped ($t_{32} = 3.02$, $p < 0.005$). In L*er*-0, glucosinolates significantly decreased by 16.76% for plants that were clipped relative to those that were not clipped ($t_{31} = -2.77$, $p < 0.01$). No other genotypes displayed significant differences.

At 6.5 weeks of age, 3 weeks after clipping, clipped plants of Col-4 and CS1948 significantly increased glucosinolate content over unclipped controls, with increases of 30.03% and 35.33% , respectively ($t_{28} = 3.77$, $p < 0.001$ and $t_{28} = 4.98$, $p < 0.0001$, Fig. 2.1a and Fig. 2.2a). Although there was a significant increase in glucosinolate production of Col-4 at the 4.5 week time-point, by 6.5 weeks glucosinolate production increased to an even greater extent in Col-4. Clipped plants of L*er*-0 decreased glucosinolate production by 22% though not significantly after sequential Bonferroni adjustment ($t_{28} = -2.18$, $p = 0.0375$). There were no significant differences in glucosinolate production for CS1906 or CS1968.

Breaking glucosinolate compounds into subcategories at 6.5 weeks showed that Col-4, CS1948, and L*er*-0 all differed in indole glucosinolate content (Fig. 2.2b). Specifically, Col-4 and CS1948 showed significant increases (59.04%, $t_{28} = 4.25$, $p < 0.0005$ and 36.2%, $t_{28} = 3.41$, $p < 0.002$, respectively) in indole glucosinolate concentration after clipping. Out of the four indole glucosinolates measured two were significantly upregulated in Col-4 and one in CS1948,

with one glucosinolate, glucosbrassicin, in common (Table S1). Clipped individuals of L*er*-0, however, displayed a decrease (37.96%, $t_{28} = -2.95$, $p < 0.01$) in indole glucosinolate concentration compared to unclipped controls, though not significantly after sequential Bonferroni.

Aliphatic glucosinolate concentration followed a similar pattern, with Col-4 and CS1948 showing significant increases in content (28.45%, $t_{28} = 3.44$, $p < 0.002$ and 35.29%, $t_{28} = 4.78$, $p < 0.0001$, respectively) following clipping and L*er*-0 showing a nonsignificant decrease (20.25% $t_{28} = -1.93$, $p = 0.063$) (Fig. 2.2c). Four aliphatic glucosinolates were upregulated in CS1948 and three in Col-4, two of which were the same (Table S1). Interestingly, one aliphatic glucosinolate was significantly reduced after clipping in Col-4, glucoerucin ($t_{28} = -3.6$, $p < 0.001$). CS1948 also exhibited a lower concentration in glucoerucin after clipping, but not significantly ($t_{28} = -1.13$, $p = 0.27$). No significant differences in either overall indole or aliphatic glucosinolates were found between clipped and unclipped individuals of CS1906 or CS1968 (Fig. 2.2b and c).

2.4.3 Col-0, *ILP1*-ox, Fitness Compensation and Glucosinolate Production

When clipped, wildtype Col-0 undercompensated, displaying significantly lower seed production ($t_{86} = -7.16$, $p < 0.0001$) and a significant decrease in endoreduplication relative to unclipped controls ($t_{215} = -2.34$, $p < 0.05$; Scholes and Paige 2014). The *ILP1* overexpression line, in a Col-0 genetic background (*ILP1*-ox), displayed an increase in compensation from undercompensation to equal compensation with a trend toward overcompensation ($t_{52} = 1.68$, $p = 0.099$) following clipping; the increase in seed production results from the increase in endopolyploidy (see Fig. 2.3). Additionally, Col-0 clipped plants displayed a nonsignificant trend toward lower glucosinolate production ($t_{28} = -2.87$, $p = 0.065$) whereas, *ILP1*-ox showed a significant increase (56.19%) in glucosinolate production following clipping relative to

unclipped controls ($t_{28} = 5.67$, $p < 0.0001$, Fig. 2.3). All individual glucosinolates measured were upregulated, expect for neoglucobrassicin (Table S1).

2.5 Discussion

The potential trade-offs between tolerance and resistance have been studied over the past few decades (van der Meijden *et al.* 1988, Fineblum and Rausher 1995, Mauricio *et al.* 1997, Fornoni *et al.* 2004), but never from an understanding of the molecular genetic pathway(s) involved. Here we show that there is a positive association between plant tolerance and induced chemical defense, in our case glucosinolates. Plants displaying overcompensation showed a significant increase in glucosinolate content at a 6.5 week time point (only the Columbia-4 genotype showed a significant increase at 4.5 weeks). In contrast, equal to undercompensating genotypes showed either no change or a decrease in glucosinolate concentration following simulated herbivory. Based on our results we show that the most tolerant plants had the greatest capability of inducing defensive compounds. This is consistent with our prediction that both resistance and tolerance would be positively associated given that they are co-localized in the oxidative pentose phosphate pathway that controls both tolerance and chemical defense responses.

In addition, we have previously shown that endoreduplication is not only correlated with fitness compensation (Scholes and Paige 2011) but is causally involved in the compensatory response (Scholes and Paige 2014), i.e., by genetically manipulating the endoreduplication pathway, fitness compensation can be altered in the predicted direction. Similarly, the direct association between fitness compensation and plant resistance was clearly demonstrated herein by genetically manipulating the endoreduplication pathway. By overexpressing *ILP1*, a positive regulator of endoreduplication, and thus plant compensation, we experimentally increased

glucosinolate production, and hence resistance, in the Col-0 genotype. This improved Col-0's ability to compensate, changing the response from under to equal-compensation (with a nonsignificant trend toward overcompensation) and significantly increased plant chemical defenses following apical meristem damage.

Although the genotypes used in this study have been removed from their natural setting (and natural selection) for many generations, evidence suggests that our results can still be extrapolated to a natural setting. When we compared our greenhouse findings to those from a recently conducted study of natural populations of *A. thaliana* throughout portions of Europe (Scholes *et al.* 2017) we showed that the degree of fitness compensation corresponds with the degree of endoreduplication (recall that there is a cause-and-effect link between the induction of endoreduplication and tolerance, i.e., fitness compensation, in *A. thaliana* following damage). Specifically, genotypes that equally compensate in greenhouse settings range from a 10% decrease to a 10% increase in cell cycle values (endopolyploidy), whereas undercompensating genotypes generally have a >10% decrease in cell cycle values when damaged. Overcompensating genotypes, by contrast, fall in the range of a 20–45% increase in cell cycle values (Scholes *et al.* 2017). In European populations, that primarily equally compensate, the change in cell cycle values ranged from an 8.66 decrease to a 9.31% increase (Scholes *et al.* 2017). Thus, both laboratory and natural populations still maintain similar response patterns in spite of long-term differences in selection.

Variation in tolerance across genotypes may be related to the frequency of damage a population experiences. For example, we have recently shown a significant positive relationship between tolerance and the frequency of apical meristem damage across natural European populations of A. thaliana (see Figure 3 in Scholes et al. 2017). Further, recent studies comparing

historically grazed and ungrazed populations of *Gentianella campestris* indicate that repeatedly grazed populations can evolve overcompensating tolerance, while ungrazed populations remain completely intolerant (Lennartsson et al. 1997).

There is also considerable sequence variation in G6PD1, with three nonsynonymous substitutions, each causing a change in an amino acid, between Col-4 and Ler-0 that may explain the differential patterns of expression in G6PD1 following apical damage and regrowth and perhaps the differences in compensation (Siddappaji *et al.* 2013). Recall, G6PD1 plays a major role in controlling the compensatory response in *Arabidopsis thaliana* following the removal of the plant's primary inflorescence (Siddappaji *et al.* 2013) and supplies intermediate compounds from the OPP pathway (driven by G6PD1, the central regulatory enzyme in the OPP pathway) into the shikimate pathway for secondary metabolite production for plant chemical defense, such as glucosinolates.

Our results suggest that all herbaceous plants that endoreduplicate (~90%, including annual, biennial and perennial plants) should show a positive relationship between compensation and chemical defense strategies, given that the molecular pathways are shared in common. However, given that the role that endoreduplication plays in fitness compensation was only recently discovered (see Scholes and Paige 2011, Scholes and Paige 2014), and the results of our study here showing cause and effect between chemical defense induction and endoreduplication (based on our experiment manipulating the degree of endoreduplication, described above), it would be easy to mask the positive relationship and in fact experimentally alter the relationship between tolerance and chemical defense depending upon the timing of measurement and the genetic variation in endoreduplication within the population being sampled. For example, when we average glucosinolates and total seeds across all plant genotypes, ignoring the genetic

variation in endoreduplication among genotypes of *A. thaliana*, we observe no relationship between tolerance and resistance, given that we have masked the genotypic effects of endoreduplication (compare Fig. 2.4 where genotypic variation in endoreduplication is ignored to Fig. 2.1 where genotypic variation in endoreduplication is taken into consideration).

In addition, given that the magnitude of chemical induction is dependent on the magnitude of endoreduplication following plant damage and that endoreduplication takes time to reach its peak (in *A. thaliana* about 6.5 weeks following damage), the timing of chemical measurement is important. Many studies (e.g., see Mauricio *et al.* 1997, Strauss *et al.* 2003) measure chemical induction shortly after damage at a time when the process of endoreduplication is just beginning (Scholes and Paige 2011) or the plant has not had sufficient time to induce, e.g., expression analysis on G6PD1, the key regulatory enzyme in the OPP pathway that supplies intermediate compounds into the shikimate pathway for secondary metabolite production for plant chemical defense, showed more than a 1-day delay in upregulation in *A. thaliana* following apical damage (Siddappaji *et al.* 2013). Similarly, analysis of indole and aliphatic glucosinolates in *A. thaliana* showed no significant induction following mechanical wounding of leaves at 4, 24 and 48 hours after damage (Mikkelsen *et al.* 2003). This could lead to high tolerance (assuming high levels of endoreduplication) given that fitness is measured at the end of the growing season (when endoreduplication has had its maximal effect) and low chemical defense given that endoreduplication was not given appropriate time to cause induction or induction was delayed. This could manifest itself as no relationship between tolerance and chemical induction (high tolerance and no significant chemical induction) when in reality there is an overall positive relationship.

In addition, early assessments of chemical resistance may not be reflective of the plants' resistance to damage later in life, when it may matter most. In *A. thaliana*, it seems that maximal defense is at the time when the plant is filling siliques, primarily serving to defend overall plant fitness. Interestingly, clipped plants of the overcompensating Col-4 subjected to insect herbivory by the generalist *Trichoplusia ni* at 6.5 weeks, showed a nonsignificant 7.4% reduction in fitness while undercompensating clipped Ler-0 showed a significant 34% reduction in fitness. Unclipped controls of Col-4 and Ler-0 showed a significant 29.5% and a nonsignificant 17.1% reduction in fitness, respectively. These results are consistent with patterns of endoreduplication and chemical defense (see patterns of endoreduplication in Figure 1a of Scholes and Paige 2014 and total glucosinolates in Figure 2.2a) (Mesa and Paige, Unpublished Results) and support our contention that maximal defense can be important to the plant at the time when the plant is filling siliques.

Furthermore, populations that lack genetic variation, or are sampled from one end of the spectrum by chance alone, and predominantly display decreased levels of endoreduplication following damage will display a decrease in defensive chemistry and tolerance (comparing undamaged controls to damaged plants), sampling only a part of the entire spectrum of an otherwise positive relationship. This can be illustrated by our results on wildtype Col-0 where there is a significant reduction in endoreduplication and hence glucosinolate production and fitness compensation following damage (Fig. 2.3).

Given our results it is difficult to imagine a scenario where plants that endoreduplicate would show a tradeoff between resistance and tolerance as proposed by van der Meijden *et al*. 1988. In fact, tradeoffs between resistance and tolerance are unlikely given that resistance and tolerance are evolutionarily constrained by co-localization in the oxidative pentose phosphate

pathway and the expression of both dictated by the degree of endoreduplication. It is also important to point out that although our studies have focused on apical meristem damage and the induction of endoreduplication in *A. thaliana*, simulated leaf and insect damage (by *Trichoplusia ni*) also elicits the induction of endoreduplication and compensatory responses similar to those observed for apical meristem damage (Mesa and Paige unpublished data).

Despite overall indole, aliphatic and total glucosinolates being upregulated with increasing levels of endoreduplication, in Col-4 there was one aliphatic glucosinolate, glucoerucin, which was produced at lower concentration after damage. Glucoerucin in both clipped and unclipped plants was one of the lowest glucosinolates produced comprising 2.2% of the total glucosinolates in Col-4. Previous studies have shown a similar decrease in glucoerucin following damage which could be due, in part, to a trade-off with other aliphatic glucosinolates such as glucoaraphanin, as glucoerucin serves as a precursor to glucoaraphanin and a multitude of other aliphatic glucosinolates (Beekwilder *et al.* 2008, Kliebenstein *et al.* 2001). Glucoaraphanin was significantly upregulated ($t_{28} = 3.89$, $p < 0.001$) in Col-4 following clipping increasing by 32.63% over the undamaged control. Additionally, glucoaraphanin constitutes a much larger percentage of total glucosinolates making up 64% out of the total glucosinolate content. This illustrates a larger point that while overall endoreduplication and the upregulation of the OPP pathway may provide more substrate for and overall higher glucosinolate production, genotypic variation for secondary modifications can alter which individual glucosinolates are differentially regulated. For example of the 11 glucosinolates measured 6 were upregulated in Col-4 and 5 in CS1948, but only 3 were shared in common with Col-4 and 1 glucosinolate, 3Hydroxypropylglucosinolate, was not produced at all by Col-4. Furthermore, QTL analyses have identified four gene loci responsible for secondary modification of aliphatic glucosinolates

that can alter the structure of glucosinolate profiles after damage (Kiebenstein *et al.* 2001, Sønderby *et al.* 2010). Thus, it is clear that there is both genotypic variation and fine-scale flexibility in structuring chemical defenses in ecological and evolutionary time.

Overall, our results suggest that it will be important to assess the degree of endoreduplication across a population of herbaceous plants prior to measuring fitness and plant resistance responses to herbivory. This will have the effect of removing noise within the system and potentially help avoid drawing erroneous conclusions, assuming that our findings are common among plants that endoreduplicate. Thus, additional plant species that endoreduplicate should be investigated to see if this phenomenon is generalizable. Given that not only glucosinolates but myriad other plant secondary defensive compounds are produced from the shikimate pathway, and given that the majority of herbaceous plants endoreduplicate, it is likely that positive associations between plant compensation and resistance represent the norm.

It is also noteworthy to point out that endoreduplication may serve as a generalized response mechanism for mitigating stress by plastically increasing endopolyploidy in response to a host of environmental factors, other than apical meristem removal, including high light/UV, low temperature, water stress, heavy metals, salt, wounding, pathogens (e.g., fungi, nematodes) and symbiotic biotrophs (e.g., rhizobia, mycorrhizae), among others (see Scholes and Paige 2015a for a review). The integration of the endocycle in the stress response pathway (the OPP and shikimate) provides increases in transcriptional output, metabolism, stress-mitigating compounds, and nucleotypic effects necessary to mitigate a variety of environmental stressors (Scholes and Paige 2015a). Such responses would appear to be advantageous not only for annual and biennial plants but for perennial plants as well, the majority of which endoreduplicate (Barow 2006).

2.6 Acknowledgements

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2.7 Figures

Figure 2.1a) Percent differences in seed and glucosinolate production comparing clipped and unclipped plants for 5 *Arabidopsis thaliana* genotypes. Genotypic responses range from undercompensation (negative percent difference values) to overcompensation (positive percent difference values. Asterisks indicate significance at a familywise error rate of $\alpha = 0.05$ between clipped and unclipped plants. Plus signs indicate marginal ($p < 0.1$) significance.

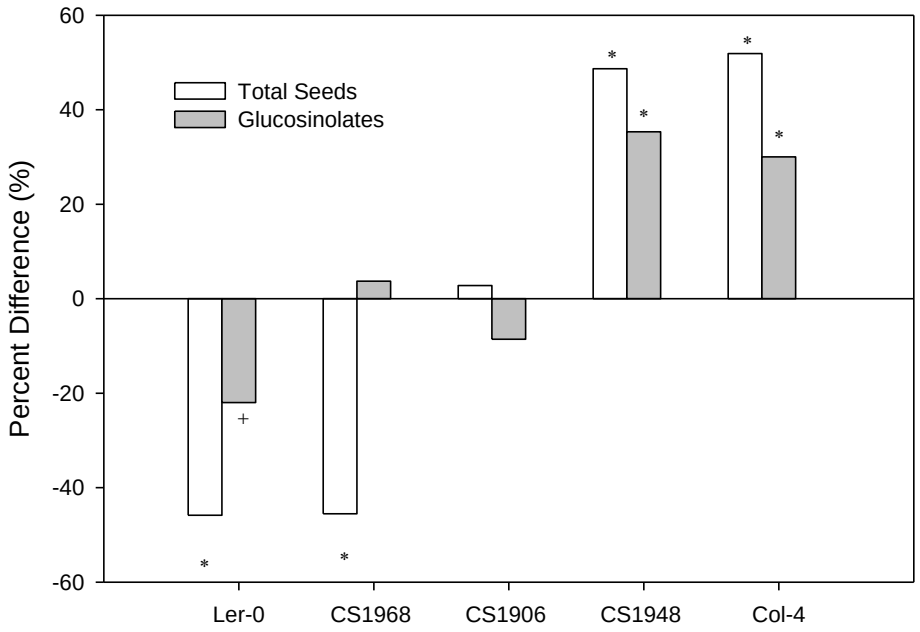


Figure 2.1b) Total seed production comparing clipped and unclipped plants for 5 *A. thaliana* genotypes. Means ± 1 SE are shown. Asterisks indicate significance at a familywise error rate of $\alpha = 0.05$ between clipped and unclipped plants. Plus signs indicate marginal ($p < 0.1$) significance.

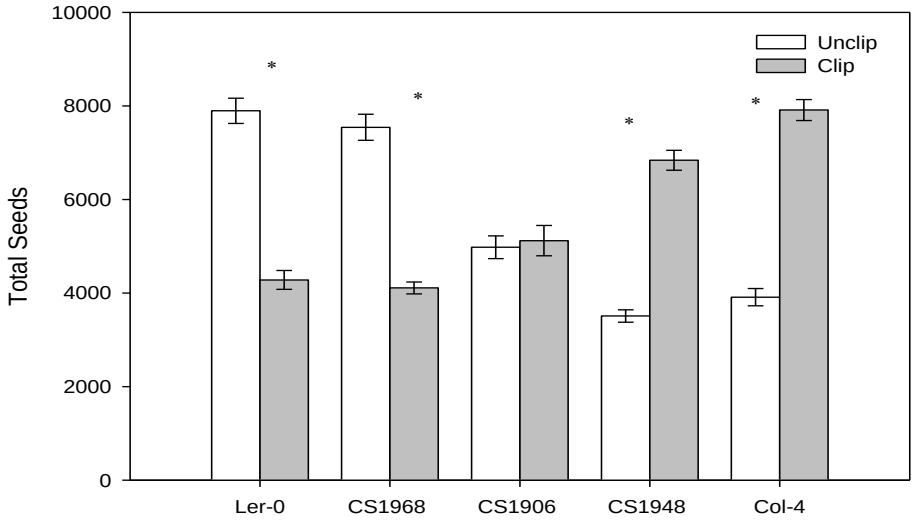


Figure 2.2a) Total glucosinolate content for clipped and unclipped plants for 5 *A. thaliana* genotypes at 6.5 weeks of growth. Means ± 1 SE are shown. Asterisks indicate significance at a familywise error rate of $\alpha = 0.05$ between clipped and unclipped plants. Plus signs indicate marginal ($p < 0.1$) significance.

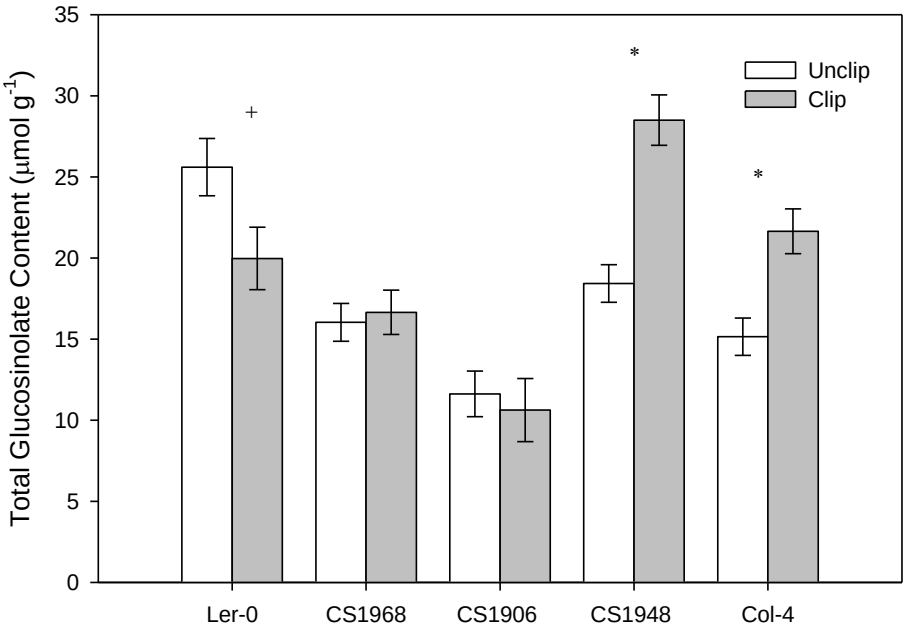


Figure 2.2b) Indole glucosinolate content for clipped and unclipped plants for 5 *A. thaliana* genotypes at 6.5 weeks of growth. Means ± 1 SE are shown. Asterisks indicate significance at a familywise error rate of $\alpha = 0.05$ between clipped and unclipped plants. Plus signs indicate marginal ($p < 0.1$) significance.

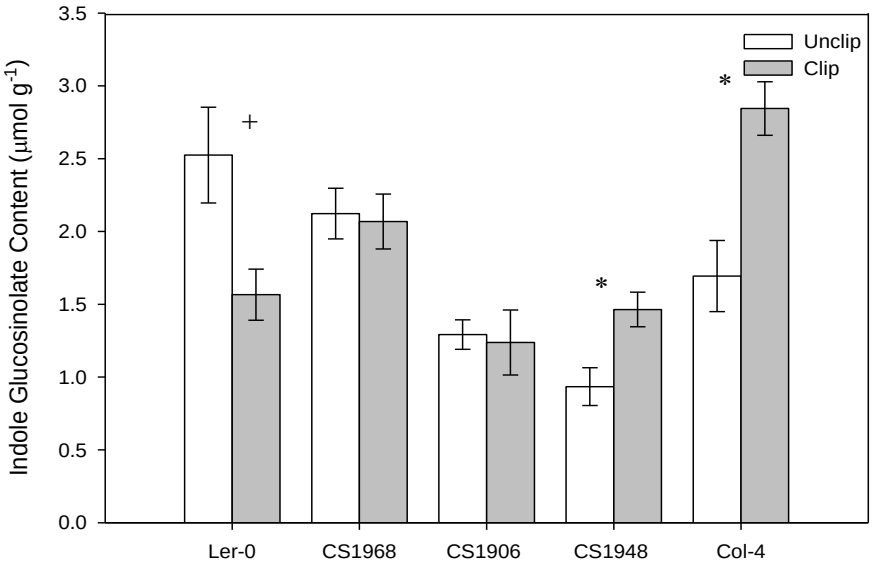


Figure 2.2c) Aliphatic glucosinolate content for clipped and unclipped plants for 5 *A. thaliana* genotypes at 6.5 weeks of growth. Means ± 1 SE are shown. Asterisks indicate significance at a familywise error rate of $\alpha = 0.05$ between clipped and unclipped plants. Plus signs indicate marginal ($p < 0.1$) significance.

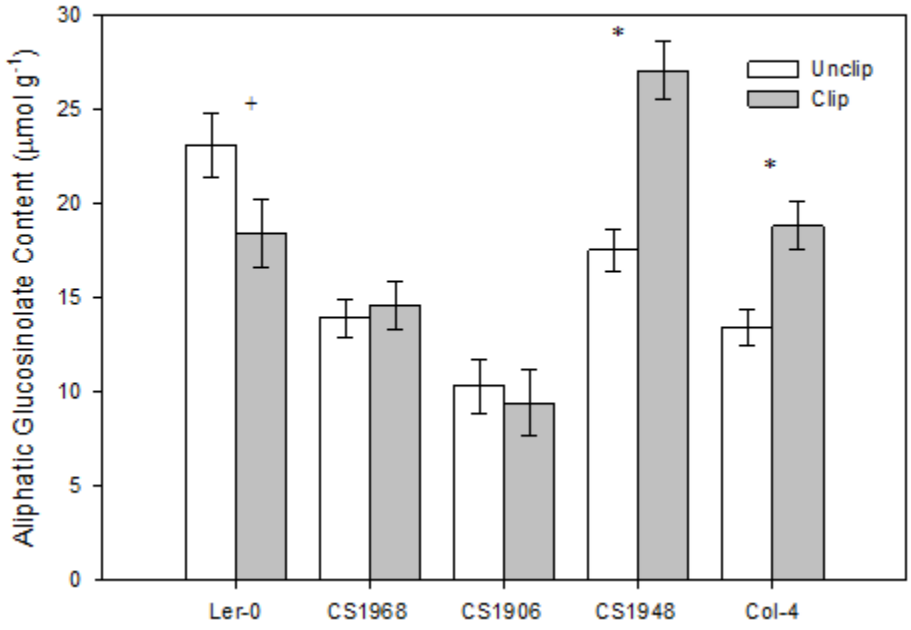


Figure 2.3) Percent difference between clipped and unclipped plants for the Col-0 and *ILP1*-ox mutant. Measures include seeds, glucosinolates and cell cycle values (endopolyploidy). Genotypic responses range from undercompensation (negative percent difference values) to overcompensation (positive percent difference values). Asterisks indicate significance at a familywise error rate of $\alpha = 0.05$. Plus signs indicate marginal ($p < 0.1$) significance. Cell cycle values were generated by a separate study (Scholes and Paige 2014).

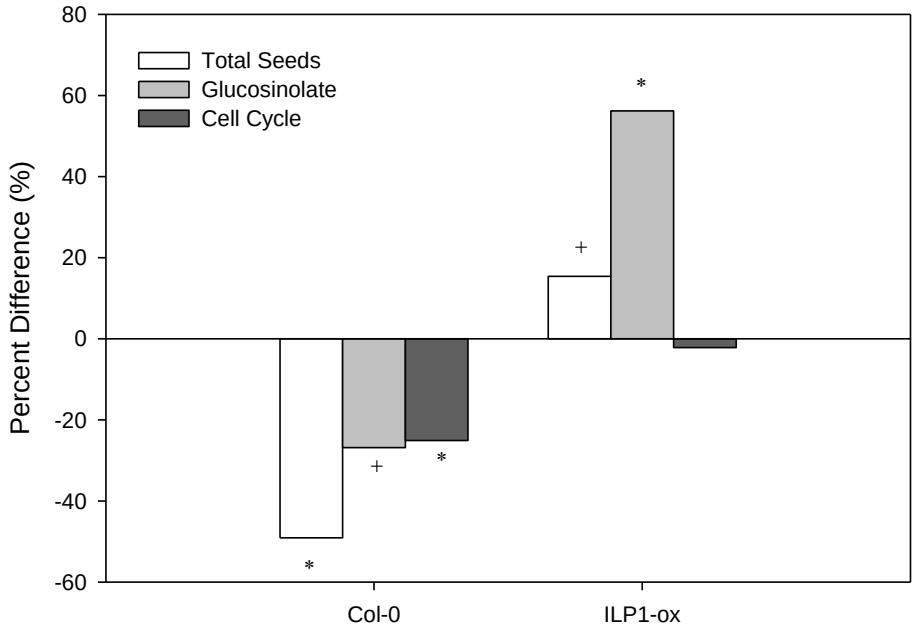
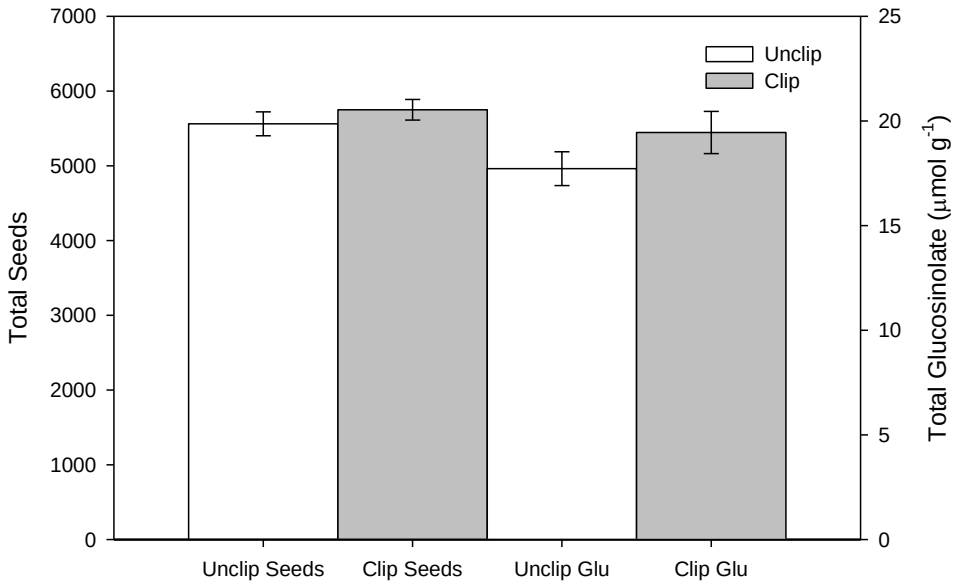


Figure 2.4) Seed totals and total glucosinolates for clipped and unclipped plants of *A. thaliana* averaged across all 5 genotypes. Means $\pm$ 1 SE are shown. Results show that ignoring genetic variation in endoreduplication masks the true association between glucosinolates and tolerance.



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Chapter 3: Individual and Interactive Effects of Herbivory on Plant Fitness: Endopolyploidy as a Driver of Genetic Variation in Tolerance and Resistance

3.1 Abstract

Previous studies have shown a causal link between mammalian herbivory, fitness compensation, and chemical defense; driven by the process of endoreduplication (the replication of the genome without mitosis, leading to endopolyploidy, an increase in cellular chromosome number). Removal of the apical meristem by mammalian herbivores lowers auxin, which triggers entry into the endocycle. Increasing chromosome number through endoreduplication, and therefore gene copy number, provides a means of increasing gene expression promoting rapid regrowth rates, higher defensive chemistry and enhanced fitness. Here we assess whether insect leaf-feeding elicits the same compensatory response as the removal of apical dominance (simulating mammalian herbivory) on *Arabidopsis thaliana*. Insect-feeding has been shown to down-regulate a number of genes that are positively associated with auxin production, suggesting a suppressive effect of insect wound-induced signals, like jasmonic acid, on the auxin signal transduction pathway. Thus, insect leaf-feeding could trigger endoreduplication by the upregulation of wound-induced signals ostensibly lowering auxin production. Results here support this contention; insect leaf-feeding by *Trichoplusia ni* elicited a compensatory response similar to that elicited by mammalian herbivores - an ecotype-specific response dependent upon the level of endoreduplication. Although, feeding by *T. ni* increased chemical defenses above those by mammalian herbivores, there were no concomitant changes in plant fitness.

In addition, the interactive effects of mammalian and insect herbivory on each of these inbred lines was assessed to determine whether interactions were additive (pairwise) or nonadditive (diffuse) on fitness compensation (tolerance) and secondary plant metabolite

production (resistance). The effects of simulated mammalian herbivory (clipping) and insect leaf-feeding by *T. ni* showed a significant ecotype X clipping X *T. ni* interaction on total seed production. Specifically, results indicate that herbivory is diffuse on Ler-0 (i.e., there is a significant clipping X *T. ni* interaction) and pairwise on CS1906 and Col-4 (no significant interaction between clipping and *T. ni* herbivory), albeit, Col-4 is marginally significant and behaves moreso in a diffuse fashion with simulated mammalian herbivory impacting insect feeding behavior through the upregulation of plant chemical defenses. In general, herbivore induced changes in plant quality appear to be responsible for the observed differences in herbivory and fitness compensation.

Overall, insect damage alone leads to a positive association between tolerance and resistance similar to that following mammalian herbivory. Moreover, this association operates by the same mechanism, endoreduplication. Furthermore, dependent upon ecotype, mammalian herbivory alters plant quality for subsequent herbivores leading to diffuse interactions. These studies illustrate the importance of evaluating endoreduplication among plants within a population to avoid masking the positive association between tolerance and resistance and the fitness consequences of multi-herbivore interactions.

3.2 Introduction

Plants have evolved two primary defense strategies to mitigate the negative impact of herbivory, resistance and tolerance. Plant resistance is manifested in an elaborate array of secondary metabolites that function in plant defense against their herbivores as well as structural traits, such as trichomes, thorns, spines, and raphides, that deter herbivores (Lambrix et al. 2001). Herbivores also select for traits that allow plants to maintain fitness in the face of tissue loss. Plant genotypes that can compensate for tissues lost with little or no decrement in fitness relative to those that are undamaged represent such an example and are termed tolerant (see Stowe et al. 2000 for a review). Interest in tolerance was stimulated by empirical studies demonstrating that mammalian herbivory can, under certain circumstances, increase rather than decrease a plant's reproductive success, a phenomenon commonly referred to as overcompensation (Paige and Whitham 1987). Overall, plants show a range of compensatory responses from under-compensation, to equal-compensation to overcompensation both within and among species.

Resistance and tolerance have generally, been regarded as alternate defensive strategies to herbivory, such that there is a trade-off between resistance and tolerance. However, despite a proposed trade-off there is little supportive evidence (Mauricio et al. 1997, Leimu and Koricheva 2006, Nunez-Farfan et al. 2007, Muola et al. 2010). What has been missing from studies of potential trade-offs between tolerance and resistance is an understanding of the molecular genetic pathways involved in tolerance (Fornoni 2011) and its relationship to the well-characterized molecular pathway involved in chemical defense, i.e., the shikimate pathway. Our recent studies on the molecular underpinnings of tolerance (with an emphasis on the phenomenon of overcompensation) have shown that plant tolerance and resistance are positively associated due

to a sharing of the same molecular and genetic pathways, explaining in part the inability to uncover a physiological trade-off between these two strategies (Mesa et al. 2017).

Specifically, we have recently shown that the ability of a plant to increase its ploidy level via endoreduplication leads to rapid regrowth and an increase in fitness following removal of apical dominance, explaining, in part, the phenomenon of overcompensation in plants (Scholes and Paige 2011, 2014, Paige 2018). Endoreduplication is the replication of the genome without mitosis, which leads to endopolyploidy, an increase in cellular chromosome number (Nagl 1976, Brodsky and Uryvaeva 1977, Melaragno et al. 1993). Removal of the apical meristem by mammalian herbivores eliminates production of the plant hormone auxin, leading to a rapid break in dormancy of axillary buds and subsequent stem elongation. High levels of auxin are also known to repress the endocycle, and by contrast, lower levels of auxin trigger an exit from mitotic cycles and an entry into endocycles (Ishida et al. 2010). Thus, there is a direct link between endoreduplication and apical meristem damage. Increasing chromosome number through endoreduplication and therefore gene copy number may provide a means of increasing gene expression of vital genes (such as G6PD1) or gene pathways that promote rapid regrowth rates following herbivory. G6PD1 feeds compounds into the OPP pathway for nucleotide biosynthesis, by the provision of ribose-5-phosphate, necessary for the significant increase in chromosome number via endoreduplication. The increase in DNA content then feeds back on pathways involved in metabolism (e.g., G6PD1) and defense (e.g., glucosinolate production) through increased gene expression (more copies due to increases in endoreduplication following damage; Scholes et al. 2013, Scholes and Paige 2015a, b, Mesa et al. 2017). Thus, the mechanisms for both plant tolerance and resistance are co-localized within the oxidative pentose phosphate pathway and appear to be molecularly interdependent. Furthermore, it is important to

point out that endoreduplication is common in plants, with approximately 90% of herbaceous angiosperms being endopolyploid (Nagl, 1976).

In addition, we have established a clear causal relationship between the process of endoreduplication, fitness compensation following apical meristem damage, and chemical defense. Specifically, the experimental overexpression of ILP1 (Increased Level of Polyploidy1), an endoreduplication enhancer, increases both glucosinolate production and fitness compensation (from undercompensation to equal compensation with a trend toward overcompensation) in a genotype of *A. thaliana* that typically suffers reduced fitness and chemical defense when damaged (Scholes et al. 2014, Mesa et al., 2017).

Although we have shown a causal link between the removal of apical dominance by mammalian herbivores, endoreduplication, fitness compensation, and chemical defense, no one has addressed whether insect leaf-feeding can elicit the same compensatory response as removal of the apical meristem by mammalian herbivores. Removal of the apical meristem lowers the level of auxin and triggers entry into the endocycle. In *Arabidopsis*, wounding has been shown to down-regulate a number of genes that are positively associated with auxin production, suggesting a suppressive effect of insect wound-induced signals, like jasmonic acid, salicylic acid and ethylene, on the auxin signal transduction pathway (Onkokesung et al. 2010). These results suggest that insect leaf-feeding could trigger endoreduplication by the upregulation of jasmonic acid, salicylic acid and ethylene, which ostensibly lowers auxin production. In addition, suppression of basipetal auxin transport (Shi et al. 2006, Wang et al. 2008) and application of auxin to wounds (Baldwin 1989, Baldwin et al. 1997) have been shown to increase and decrease secondary plant defenses, respectively, indicative of a general negative effect of auxin on induced plant defenses against herbivores (Onkokesung et al. 2010). From these observations we

would hypothesize higher defensive chemistry following insect damage, along with higher tolerance, driven by the process of endoreduplication, much as we have seen in some ecotypes following the removal of apical dominance by mammalian herbivores.

In addition, we know little about the interactive effects of mammalian and insect feeding on plant tolerance. Hougen-Eitzman and Rausher (1994) argued, in part, that in multispecies systems plant–herbivore interactions proceed in a diffuse rather than a pairwise (or additive) manner when there are non-additive effects of herbivory on plant fitness. Ecological mechanisms that determine whether the fitness effects of multiple herbivores are non-additive, might include direct interactions among herbivores that could enhance or reduce the effects of each herbivore on plant fitness (Karban 1989, Fritz 1992, Karban and Strauss 1993, Juenger and Bergelson 1998), or herbivore induced changes in plant phenology, architecture or quality which could indirectly alter plant interactions with other herbivores (Schultz and Baldwin 1982, Brown and Weiss 1995, Pilson 1996, Juenger and Bergelson 2001). As noted above, our previous studies on *Arabidopsis thaliana* have shown that the removal of the apical meristem, indicative of mammalian herbivory, leads to lower levels of auxin triggering an exit from the mitotic cycle and an entry into the endocycle. We have also shown that the higher the level of endopolyploidy, the higher the level of fitness compensation and the higher the level of chemical resistance (Mesa et al. 2017). Thus, we would predict that mammalian herbivory would alter plant quality for subsequent insect feeding, but that the magnitude would be dependent upon the level of endopolyploidy and chemical resistance following the removal of apical dominance, which is genotype specific.

Therefore, in this study, we will 1) assess whether insect leaf-feeding elicits the same compensatory response as the removal of apical dominance (simulating mammalian herbivory)

on inbred lines of *Arabidopsis thaliana*, that range from undercompensation to overcompensation and 2) assess the interactive effects of mammalian and insect herbivory on each of these inbred lines to assess whether these interactions are additive (pairwise) or nonadditive (diffuse) on fitness compensation (tolerance) and secondary plant metabolite production (resistance).

3.3 Methods and Materials

3.3.1 System

Arabidopsis thaliana, mouse-ear cress, is a small, predominantly self-fertilizing plant in the Brassicaceae family. While native to Europe, *A. thaliana* has a wide geographical range spanning Eurasia, North Africa and North America (Ratcliffe 1965, Mitchell-olds 2001). Typically *A. thaliana* is found as a winter annual where seeds of *A. thaliana* germinate in the fall after passing the summer in a dormant state and grow into an overwintering rosette. Following stem elongation in the spring, *A. thaliana* produce flowers that develop into seed pods known as siliques (Pigliucci 2002). *A. thaliana* is fed on by a variety of species including flea beetles, aphids, leaf miners, caterpillars, rabbits and other small mammals (Van Poecke 2007, Weinig *et al*. 2003). *A. thaliana* thus frequently experiences leaf and apical meristem damage and has a suite of resistance characters such as trichomes, proteinase inhibitors and glucosinolates which deter and inhibit feeding (Beekwilder *et al*. 2008).

In this study, we used the cabbage looper, *Trichoplusia ni,* for our experiments given that they are commercially available and can be raised in the lab easily, while completing their entire life cycle feeding on *A. thaliana* (Jander et al. 2001). *T. ni* is a generalist herbivore with larvae feeding on a wide variety of plant species including tomatoes, lettuce, alfalfa and *Brassica* species (Shorey et al., 1962). *Brassica* species are characterized by the glucosinolate-myrosinase

system which has been shown to inhibit *T. ni* feeding causing them to lose weight with an ever-increasing concentration of glucosinolates (Jander et al. 2001).

3.3.2 Experimental Design

Compensation and Insect Herbivory

To assess whether insect herbivory alone can elicit a compensatory response similar to that observed by mammalian herbivores we grew 90 plants of each ecotype and subjected half of the plants to feeding by an insect herbivore. Specifically we grew, Ler-0, Col-4 and a recombinant inbred line CS1906, which all differ in their compensatory responses to mammalian herbivory (from undercompensation to equal compensation to overcompensation). Over 100 recombinant inbred lines, including CS1906, were generated from a Ler-0 x Col-4 cross and advanced through single-seed descent for more than eight generations (Lister and Dean 1993). These lines are available through "The Arabidopsis Information Resource (TAIR) Center". All lines were grown in a greenhouse on the campus of the University of Illinois at Urbana-Champaign, under 12 hours of light (~100 $\mu E/m^2/sec$) and dark. Plants were grown individually in 3.5-inch pots using a 9:1 mixture of L1 Sunshine mix and quartz sand. Seeds/seedlings were kept moist during germination, and plants were then watered daily to maintain soil moisture without saturating the soil. Plants were not fertilized.

Trichoplusia ni were purchased from Benzon Research as 1st instar larvae and were fed a diet of cabbage until they reached their 3rd instar (Carlisle, PA, USA). At 3.5 weeks after germination (when the inflorescence of a plant has reached approximately 6 cm in height) three 3rd instar larvae of *T. ni* were allowed to feed on half the plants of each ecotype for 48 hours. *T. ni* larvae were then removed and the plants were allowed to complete their life cycle. At senescence (8 weeks), plants of each ecotype were analyzed for fitness. All plants flowered and

produced fruits (siliques) and fitness was measured by the number of siliques and seeds per plant. Our previous studies (e.g., Scholes and Paige 2011, 2014) have shown that seeds are a good measure of plant fitness as there are no significant differences in seed weight or germination success between clipped and unclipped plants of *A. thaliana*. We measured total silique number for each plant and counted total seed production in three randomly selected siliques of representative length from each plant. Seeds per silique were averaged per plant, ecotype and treatment then multiplied by total silique number for each plant to obtain seed totals per plant.

Additionally, to assess whether herbivory by *T. ni* triggers the induction of endoreduplication, as mammalian herbivory does, we quantified the level of endopolyploidy in plants at the onset of senescence (approximately 6.5 weeks) using flow cytometry. Tissues of 15 undamaged and 15 insect damaged plants of each ecotype were sent to the flow cytometry facility at Iowa State University for analysis where they were prepared by standard protocols (Galbraith et al. 1983). Specifically, fresh tissue was matched for mass and tissue type, chopped with a razor blade, sheared in a nuclear isolation buffer (sodium citrate, 3-morpholinopropane-1-sulphonic acid, magnesium chloride, Triton X-100), filtered for the removal of debris and stained with propidium iodide. Nuclei were analyzed for DNA content via a BD Biosciences (San Jose, CA, USA) FACScanto flow cytometer. Background correction and nuclei population gating were performed using De Novo Software FCS Express (v.3; Los Angeles, CA, USA) to measure the number of nuclei at each ploidy level (2C, 4C, 8C, 16C) for each plant sample. The C here denotes the number of copies of the base genome in a cell. The cell cycle value, calculated as the mean number of endoreduplication cycles per nucleus and therefore an overall measure of endoreduplication (Barow and Meister 2003) was calculated from the number of nuclei at each

level of ploidy for each sample by the equation: Cycle value = (0 x n_{2c} + 1 x n_{4c} + 2 x n_{8c} + 3 x n_{16c}) / (n_{2c} + n_{4c} + n_{8c} + n_{16c}), where the cycle value is the sum of the number of nuclei at each ploidy level multiplied by the number of endocycles required to achieve each level of ploidy, divided by the total number of nuclei measured. Cycle values range from zero, which indicates that all nuclei are diploid, to three, indicating that all nuclei are 16C.

Lastly, at 6.5 weeks 30 additional plants (15 damaged, 15 undamaged) of each ecotype were analyzed for glucosinolate concentration. Fresh inflorescence and associated cauline leaf samples were frozen in liquid nitrogen, and stored at -80°C prior to freeze-drying with a lyophilizer. Freeze-dried tissues were ground into a fine powder and stored at -20°C prior to glucosinolate analysis. Glucosinolates were extracted from finely ground freeze-dried tissue, converted to desulphoglucosinolates with arylsulfatase and analyzed via high pressure liquid chromatography (HPLC) as described by Brown et al. (2003). Specifically, fifty mg of freeze-dried powder and 0.5 mL of 70% methanol were added to 2.5 mL tubes and placed on a heating block at 95°C for 10 min, mixing frequently. Samples were cooled on ice and centrifuged at 3,000xg for 10 minutes. The supernatant was saved and the pellet was re-extracted with another 0.5 mL 70% methanol at 95°C for 10 minutes and the two extracts were combined. Protein was subsequently precipitated with 0.15 mL of a 1:1 mixture of 1 M barium acetate and 1 M lead acetate and centrifuged at 12,000xg for 1 min. Each sample was then loaded onto a column containing DEAE Sephadex A-25 resin for desulfatation via arylsulfatase for 18 h and the remaining desulfo-GS eluted. Desulphoglucosinolates were separated on a HPLC system (Agilent 1100 HPLC system, with a G1311A bin pump, a G1322A vacuum degasser, a G1316A thermostatic column compartment, a G1315B diode array detector and an HP 1100 series G1313A autosampler) with a variable ultraviolet detector set at 229 nm wavelength. Elution of

desulphoglucosinolates occurred over 45 minutes with a linear gradient of 0% to 20% acetonitrile in water with a flow rate of 1.0 mL/min. Glucosinolate concentration was established using glucosinalbin as an internal standard, a glucosinolate not found in *A. thaliana*. UV response factors for different glucosinolates were used as determined by Wathelet et al. 2001.

Interactive effects of mammalian and insect herbivory

To assess the interactive effects of mammalian (clipping) and insect herbivory, we planted 100 individuals each of Ler-0, Col-4 and the recombinant inbred line CS1906. These plants were grown in the same conditions as outlined above. The newly elongating inflorescences of half of the plants of each ecotype were randomly clipped, when they reached a height of approximately 6 cm (at 3.5 weeks), leaving approximately 1 cm of inflorescence (comparable to natural mammalian herbivory observed for this species) (Scholes et al. 2017). This clipping treatment imposed on *A. thaliana* is comparable to natural apical damage observed throughout its native range and stimulates similar changes in architectural traits following damage (Scholes and Paige 2011, 2014; Scholes et al. 2017).

At approximately 5.5 weeks after germination during flowering and silique production two 3rd instar larvae of *T. ni* were placed on half of the plants, 25 clipped and 25 unclipped. The remaining 25 clipped and 25 unclipped plants were utilized as no insect controls. Prior to placing *T. ni* on the plants larvae were weighed to assess starting weight and were then allowed to feed on the plants for 48 hours and then reweighed. One larvae was marked with a small black dot to independently keep track of each for post weighing. Overall growth was calculated by subtracting final growth minus starting growth. Following plant senescence (8 weeks) all plants of each ecotype and treatment were analyzed for fitness. All plants flowered and produced

siliques and fitness was measured by the number of siliques and seeds per plant following the protocols listed previously.

Statistical Analyses

Potential differences in seed production, glucosinolate production and cell cycle values were assessed using an analysis of variance and Type III sums of squares to assess tolerance, resistance and the relationship between tolerance and resistance following *T. ni* herbivory. Factors included ecotype, *T. ni* herbivory and their interaction. Independent tests were performed as different sets of plants were used for each variable assessed (total seed production, cell cycle value and glucosinolate concentration, see Tables 3.S 1-3). Tests for seed production were run on log-transformed data to equalize variances. Differences among treatments within ecotypes were determined using Tukey pairwise comparisons.

Likewise, differences in seed production for the interactive effects of herbivory were assessed using analysis of variance and Type III sums of squares among three factors (ecotype, clipping, *T. ni* herbivory) and their interactions. Ecotypic differences between clipping and *T. ni* herbivory and their interactions were assessed using Tukey pairwise comparisons. All data for both experiments were analyzed using Systat 13.0.

The potential for diffuse interactions, or non-additivity, is indicated by significant statistical interactions between herbivores in an analysis-of-variance model in which each herbivore is considered a treatment factor (Hougen-Eitzman and Rausher, 1994, Wise and Sacchi, 1996, Anderson and Paige 2003, Strauss et al. 2005). Pairwise interactions or additive effects between a plant and its herbivores would be indicated by the absence of statistical interactions between herbivores on plant fitness, whereas, diffuse interactions would be indicated

by a significant interaction between clipping and *T. ni* herbivory within an ecotype. (Hougen-Eitzman and Rausher, 1994, Wise and Sacchi, 1996, Strauss et al. 2005).

3.4 Results

3.4.1 Compensation and Insect Herbivory

Ecotypes differed in total seed production ($p < 0.001$) in their response to *T. ni* herbivory ($p < 0.001$) (Table 3.S1). Specifically, a post hoc Tukey test revealed that Ler-0 significantly undercompensated following insect herbivory with a 38% decrease in total seed production when compared to undamaged controls ($p < 0.001$) (Fig. 3.1a), whereas, Col-4 significantly overcompensated following herbivory by *T. ni* with a 52% increase in seed production ($p < 0.001$). Lastly, CS1906 equally compensated with no significant difference between undamaged controls and insect damaged plants ($p = 0.97$). Furthermore, ecotypes differed in their cell cycle values ($p =0.020$) and overall plants that were fed on by *T. ni* differed in their cell cycle values compared to undamaged controls ($p<0.001$) with a marginally significant ecotype x *T. ni* interaction ($p = 0.106$) (Table 3.S2). Specifically, Ler-0 did not differ significantly in endopolyploidy following insect damage ($p = 0.998$) (Fig. 3.1a). Col-4 significantly increased in endopolyploidy following insect herbivory ($p = 0.010$) consistent with an increase in plant seed production. Lastly, CS1906 cell cycle values did not significantly differ when comparing *T. ni* fed plants and uneaten controls ($p = 0.177$).

We additionally found that ecotypes differed in their glucosinolate concentrations ($p < 0.001$) and plants fed on by *T. ni* significantly differed in glucosinolate content compared to uneaten controls ($p < 0.031$) (Table 3.S3). Moreover, we found a significant ecotype x *T. ni* interaction ($p = 0.024$). Ler-0 did not differ significantly in glucosinolates between those fed on by *T. ni* and uneaten controls ($p = 0.959$) (Fig. 3.1a). In contrast, glucosinolates increased in the

Col-4 ecotype following *T .ni* herbivory ($p < 0.01$). Lastly, CS1906 also did not differ significantly in glucosinolates after insect damage ($p = 0.999$). These data taken together indicate that insect feeding leads to similar induction of endoreduplication and resistance chemistry as seen with mammalian damage, ranging from low levels of endoreduplication and resistance chemistry in undercompensating ecotypes to high levels of endoreduplication and resistance chemistry in overcompensating ecotypes (see Fig 3.1a and b for a comparison of insect and simulated mammalian herbivory).

We also found that insect herbivory led to higher induction of glucosinolates compared to simulated mammalian herbivory. Specifically, in Col-4 glucosinolate concentration was significantly higher following insect herbivory compared to simulated mammalian herbivory ($t_{27} = 4.25$, $p < 0.001$) (insect 29.7 ± 3.7 vs mammalian 21.6 ± 1.4 µmol/g). Ler-0 also experienced significantly higher glucosinolate induction than simulated mammalian herbivory ($t_{24} = 3.43$, $p < 0.01$) (insect 33.24 ± 3.7 vs mammalian 19.9 ± 1.9 µmol/g). CS1906 did not differ significantly in glucosinolate induction between insect and mammalian damage ($t_{20} = 1.86$, $p > 0.05$).

3.4.2 Interactive Effects of Herbivory

Ecotypes differed significantly ($p < 0.001$) in total seed production and their responses to different types of damage (*T. ni* versus clipping) (Table 3.S4, Fig 3.2). The main effect of clipping was marginally significant ($p = 0.082$), but more importantly ecotypes differed significantly in their responses to simulated mammalian damage (ecotype x clipping) ($p < 0.001$) with Ler-0 undercompensating, CS1906 equally compensating and Col-4 overcompensating. Additionally, there was a significant effect of *T. ni* herbivory, decreasing total seed production across all ecotypes ($p < 0.001$). However, there was a marginal ecotype x *T. ni* effect ($p = 0.08$), indicating some ecotypes suffered greater negative fitness impacts than others (Fig. 3.2).

We also found a significant ecotype x clipping x *T. ni* interaction ($p = .015$), indicating that ecotypes differ in their responses to herbivore interactions (Table 3.S4). Additionally, we found that *T. ni* growth differed on ecotypes ($p < 0.001$) and we observed a significant ecotype x clipping interaction ($p < 0.001$) (Table 3.S5). We also found a significant clipping x *T. ni* interaction in Ler-0 (Table 3.S6) indicative of diffuse interactions. Specifically, Ler-0 unclipped plants did not differ in their seed production comparing insect damaged plants to undamaged controls. Clipped plants suffered a significant decrease in seed production from simulated mammalian damage alone ($p < 0.001$) and an additional 62% decrease in seed production following insect damage ($p < 0.001$) (Fig. 3.2). Likewise, *T. ni* gained 57% more weight on clipped Ler-0 in comparison to unclipped controls ($p < 0.001$) (Fig. 3.3). In contrast, Col-4 overcompensated from clipping alone and the interactive effects of clipping and *T. ni* herbivory while unclipped plants accrued a significant decrease following *T. ni* damage ($p < 0.05$) (Fig. 3.2). *T. ni* also grew significantly less on clipped Col-4 than on unclipped control plants ($p < 0.05$), with a 46% decline in weight on clipped plants compared to unclipped plants (Fig. 3.3). We also found a marginally significant clipping X *T. ni* interaction ($p = 0.107$; Table 3.S8) in Col-4, perhaps indicative of diffuse interactions. Lastly, CS1906 did not differ among any of these treatments (clipping, *T. ni* herbivory or their interaction; Fig. 3.2) and *T. ni* growth did not differ between clipped and unclipped plants ($p = 0.971$) (Fig. 3.3). The lack of any statistical interaction between clipping and *T. ni* suggest that ecological interactions are pairwise in CS1906 ($p = 0.984$, Table 3.S7).

3.5 Discussion

3.5.1 Compensation and Insect Herbivory

Tolerance caused by mammalian herbivory has been viewed as mechanistically different from damage caused by leaf-feeding insects (e.g., Strauss et al. 2001, Strauss et al. 2003). Mammalian herbivory often entails the removal of the apical meristem leading to increased branching and increased flower and fruit production (Paige and Whitham 1987, Paige 1992). In contrast, insect leaf-feeding, purportedly, does not result in the release of lateral meristems and concomitant increases in flower and fruit production (Strauss et al. 2003). Our results here, however, show that insect leaf-feeding alone can elicit a compensatory response similar to that elicited by the removal of the apically dominant meristem, typical of mammalian herbivores. We show that insect leaf-feeding in some ecotypes of *A. thaliana* can lead to the release of axillary meristems (both basal meristems and meristems off the primary meristem) and the production of new growth, dependent upon the ecotype-specific level of endoreduplication, which is triggered by the suppression of auxin production (Ishida et al. 2010, Onkokesung et al. 2010).

We specifically show that Col-4, which overcompensates for simulated mammalian herbivory, also overcompensated for insect leaf herbivory; insect feeding showed significant increases in seed production (52% increase), endopolyploidy (18% increase) and glucosinolate production (48% increase). Simulated mammalian herbivory showed an identical response in both seed production (52% increase) and endopolyploidy (18% increase), and a significant 30% increase in glucosinolate production compared to undamaged controls. CS1906 which equally compensates for simulated mammalian herbivory also equally compensated for insect damage; seed production, endopolyploidy and glucosinolate production showed no significant difference following insect feeding or simulated mammalian herbivory compared to their undamaged

controls. In contrast, Ler-0 which undercompensates following simulated mammalian herbivory also significantly undercompensated following insect feeding; seed production significantly decreased by 38% following insect feeding with no significant differences in endopolyploidy or glucosinolate production, whereas, simulated mammalian herbivory significantly decreased seed production by 43% with no significant difference in endopolyploidy, and only a marginally significant 22% decrease in glucosinolate production when compared to their undamaged controls (see Figures 3.1a and 3.1b for a comparison).

The primary difference between insect and mammalian herbivory is that the magnitude of glucosinolate induction was significantly higher following insect herbivory in comparison to simulated mammalian herbivory in Col-4 and Ler-0. Specifically, Col-4 increased glucosinolate production by 48% following insect damage, whereas, simulated mammalian herbivory lead to only a 30% increase in glucosinolate production. Similarly, Ler-0 showed a non-significant 10% increase following insect herbivory and a marginally significant 22% decrease following simulated mammalian herbivory. These additional increases (above those generated from endopolyploidy alone) likely result from insect feeding that upregulate jasmonic acid leading to the synthesis of secondary metabolites that act in plant defense (McConn et al. 1997, Rasmann et al. 2012). Alternatively, these differences may be due to the lack of mammalian regurgitant in the clipping experiments, although no significant differences in fitness compensation have been observed between natural mammalian herbivory and simulated herbivory in our previous studies (e.g., Paige and Whitham 1987, Paige 1992). Comparative studies of chemical induction have not been conducted comparing natural and simulated mammalian herbivory. However, there were no differences in the magnitude of seed production – both insect and mammalian herbivory on Col-4 increased by 52%, for CS1906 there were no statistical changes in seed production and

for Ler-0 seed production decreased by 38 and 43% for insect and mammalian herbivory, respectively. Thus, the increase in glucosinolate production by insect feeding did not translate into fitness differences among the ecotypes, comparing insect to simulated mammalian herbivory. Overall, these results indicate that the mode of herbivory does not significantly alter the relationship between plant tolerance and resistance and that insect leaf-feeding triggers the endocycle in a fashion similar to that caused by the removal of apical dominance, lowering auxin production (Paige 2018).

Although there is genetic variation in endoreduplication/endopolyploidy among ecotypes of *A. thaliana*, the causes of such variation are unknown. The tremendous allelic diversity in cell cycle regulators (cyclins and cyclin-dependent kinases) may be the key in explaining such variation in endopolyploidy (Dewitte et al., 2007; Scholes and Paige, 2015, Paige 2018). In addition, one might expect allelic variation within genes in the OPP pathway to affect the level of endoreduplication and hence the degree of fitness compensation and chemical defense. For example, a mutation that decreases the expression of G6PD1 could limit nucleotide synthesis and perhaps the degree of endopolyploidy. For example, there is considerable sequence variation in G6PD1, with three nonsynonymous substitutions, each causing a change in an amino acid, between Col-4 and Ler-0 that may explain the differential patterns of expression in G6PD1 following damage and regrowth and perhaps the differences in compensation (Max Planck Institute for Developmental Biology, POLYMORPH Project, http://polymorph-lark20.weigelworld.org/cgi-bin/retrieve_cds_snp. cgi) (Siddappaji et al. 2013). Furthermore, the molecular mechanisms specifying the number of endocycles within a cell are also unknown (Paige 2018).

Our results, showing ecotypic variation in endoreduplication, raises some concerns about drawing conclusions on the impacts of herbivory given that we tend to ignore genotypic variation by collapsing it into an overall mean population response. For example, our previous studies (Mesa et al. 2017) on simulated mammalian herbivory showed that when we averaged total seeds and glucosinolates across all plant ecotypes, ignoring the genetic variation in endoreduplication among five ecotypes of *A. thaliana*, we observed no differences in fitness or chemical resistance between damaged and undamaged plants. Similar results were found in this study; when averaging all three ecotypes and comparing insect damaged to undamaged plants, the average population level response was one of overcompensation and an associated increase in glucosinolate production. In reality, ecotypes ranged from undercompensation to equal-compensation to overcompensation along with a correlated change in glucosinolate production (i.e., the higher the level of endoreduplication, and hence fitness, the higher the level of glucosinolate production given that fitness and chemical defense are co-localized within the same genetic pathway, the oxidative pentose phosphate pathway, and both driven by endoreduplication). Such results are likely of general importance given that approximately 90% of all herbaceous angiosperms endoreduplicate (Nagl 1976).

These results could also have important applied significance, given that insect feeding alone may lead to overcompensation and increases in plant resistance. For example, many biological control agents include insect herbivores that often times have limited effect on invading plant populations (Louday 1983, McFadyen 1998, Garren and Strauss 2009). In fact, there are a few studies showing that biological control agents can stimulate and increase growth and fitness in invasive plant populations (Schat and Blossey 2005, Russell-Mercier and Sargen 2015, Thomsen and Sargent 2017). If successfully invading plants endoreduplicate, showing a

positive association between plant resistance and tolerance as we have found, this could explain why many plant species are so successful at invading and may be a promising avenue for investigation.

3.5.2 Interactive Effects of Herbivory

The effects of simulated mammalian herbivory (clipping) and insect leaf-feeding by *T. ni* showed a significant ecotype X clipping X *T. ni* interaction on total seed production (Table 4.S4). Specifically, results indicate that herbivory is diffuse on Ler-0 (i.e., there is a significant clipping X *T. ni* interaction) and pairwise on CS1906 and Col-4 (no significant interaction between clipping and *T. ni* herbivory), albeit, Col-4 is marginally significant at the 0.107 level and behaves moreso in a diffuse fashion with simulated mammalian herbivory impacting insect feeding behavior (see discussion below, Figure 3.2).

From a mechanistic perspective, herbivore induced changes in plant quality are likely responsible for the observed differences in herbivory and fitness compensation. Both Col-4 and Ler-0 undergo significant changes in plant chemistry following mammalian herbivory tied to patterns of endoreduplication (the higher the level of endoreduplication the higher the level of glucosinolate production; a causal relationship) (Mesa et al. 2017). The overcompensating ecotype, Col-4, significantly increased glucosinolate production following simulated mammalian damage leading to a severe reduction in *T. ni* feeding. While clipped Col-4 plants did not suffer any significant loss in seed production after *T. ni* feeding, as a result of increased resistance chemistry, unclipped Col-4 accrued a significant loss in fitness with significantly lower chemical induction (see Mesa et al. 2017, Fig. 2a). In contrast, the undercompensating ecotype, Ler-0 which shows a significant reduction in glucosinolates following mammalian damage allowed *T. ni* to feed uninhibited (Mesa et al. 2017), incurring a significant reduction in total seed

production. CS1906 on the other hand displayed pairwise herbivory with no significant interactions between insect and mammalian herbivory. CS1906 equally compensates for mammalian damage and does not have altered resistance chemistry levels so both clipped and unclipped plants responded similarly to *T. ni* herbivory.

Overall, *T. ni* growth patterns reflect the levels of glucosinolate production following mammalian damage, with higher glucosinolate levels resulting in less plant tissue loss from feeding, significant reductions in caterpillar weight gain and likely lower egg production and reduced survivorship. These results illustrate an important point, i.e., that genotypic or lineage effects should not be ignored when studying the interactive effects of herbivory. For example, if we focus only on the clipping by *T. ni* interaction in the analysis (see Table 3.S4), we erroneously conclude that the impacts on fitness is pairwise. However, when ecotype is added into the model we uncover patterns of both diffuse and pairwise effects on fitness as each ecotype alters the degree of palatability differentially following mammalian herbivory, which is also dependent upon the degree of endoreduplication following damage.

In conclusion, we found that insect damage alone leads to a positive association between tolerance and resistance (from undercompensating ecotypes to overcompensating ecotypes) similar to that following mammalian herbivory. Moreover, this association operates by the same mechanism, endoreduplication. Furthermore, we found that depending on ecotype mammalian herbivory alters plant quality for subsequent herbivores leading to diffuse interactions. These studies illustrate the importance of evaluating endoreduplication among plants within a population to avoid masking the positive association between tolerance and resistance and the fitness consequences of multi-herbivore interactions.

3.6 Figures

Figure 3.1A) Percent differences in seed, glucosinolate and cell cycle values for three *Arabidopsis thaliana* ecotypes. Asterisks indicate significance at $\alpha = 0.05$ between plants fed on by *T. ni* and undamaged controls.

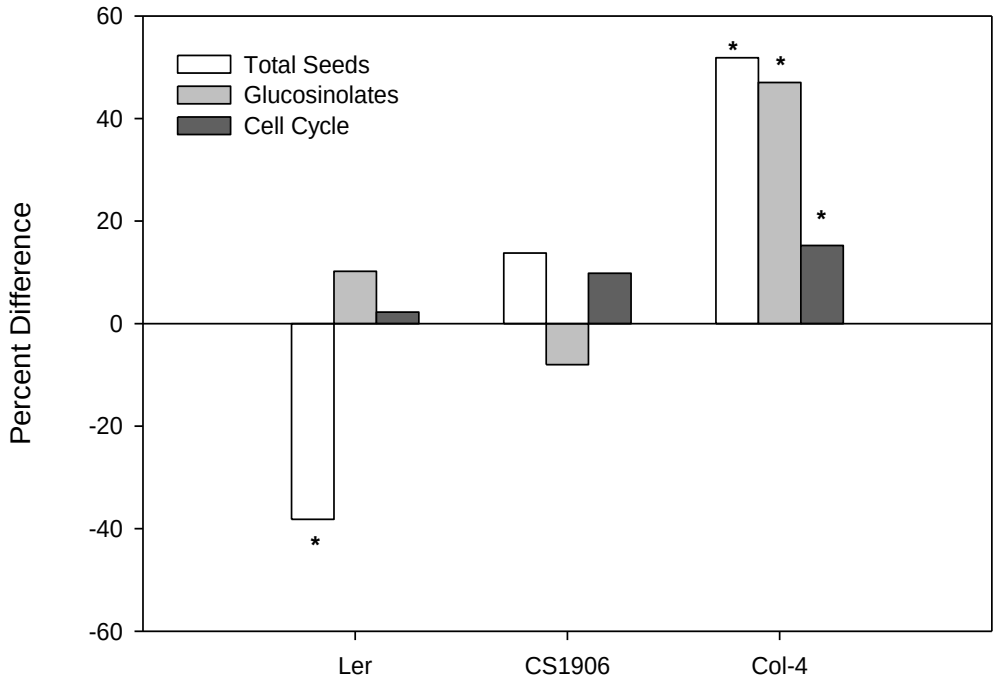


Figure 3.1B) Percent differences in seed, glucosinolate and cell cycle values for three *Arabidopsis thaliana* ecotypes. Asterisks indicate significance at $\alpha = 0.05$ between clipped and unclipped controls.

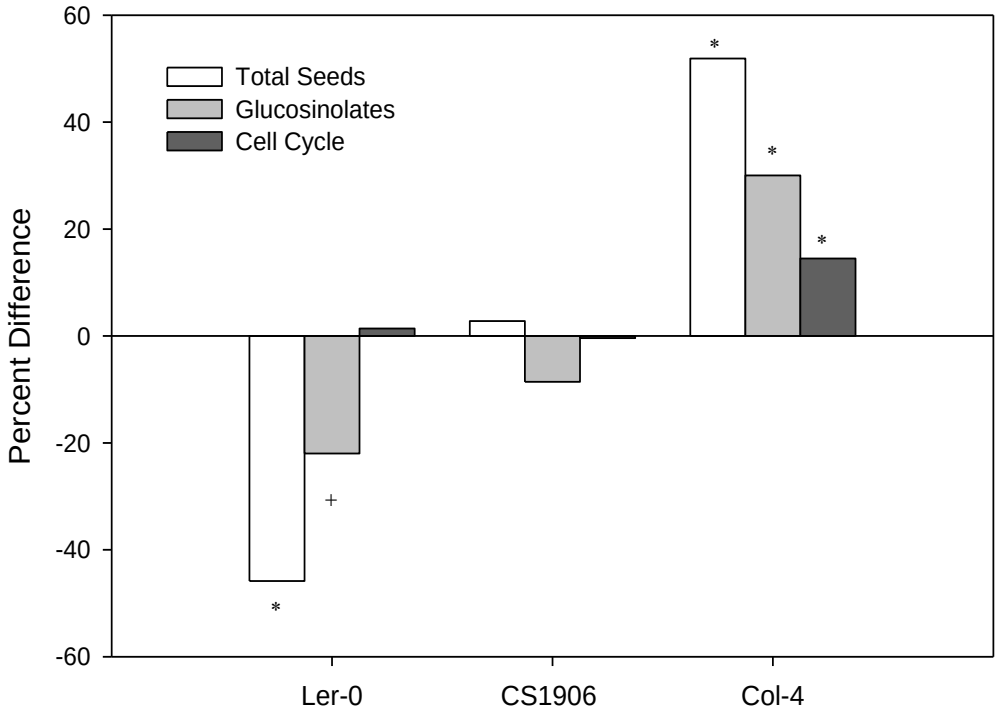


Figure 3.2) Seed totals for three ecotypes comparing unclipped and clipped plants with *T. ni* damage and undamaged controls. Means ± SE are shown. Letter indicate which values are different within ecotype.

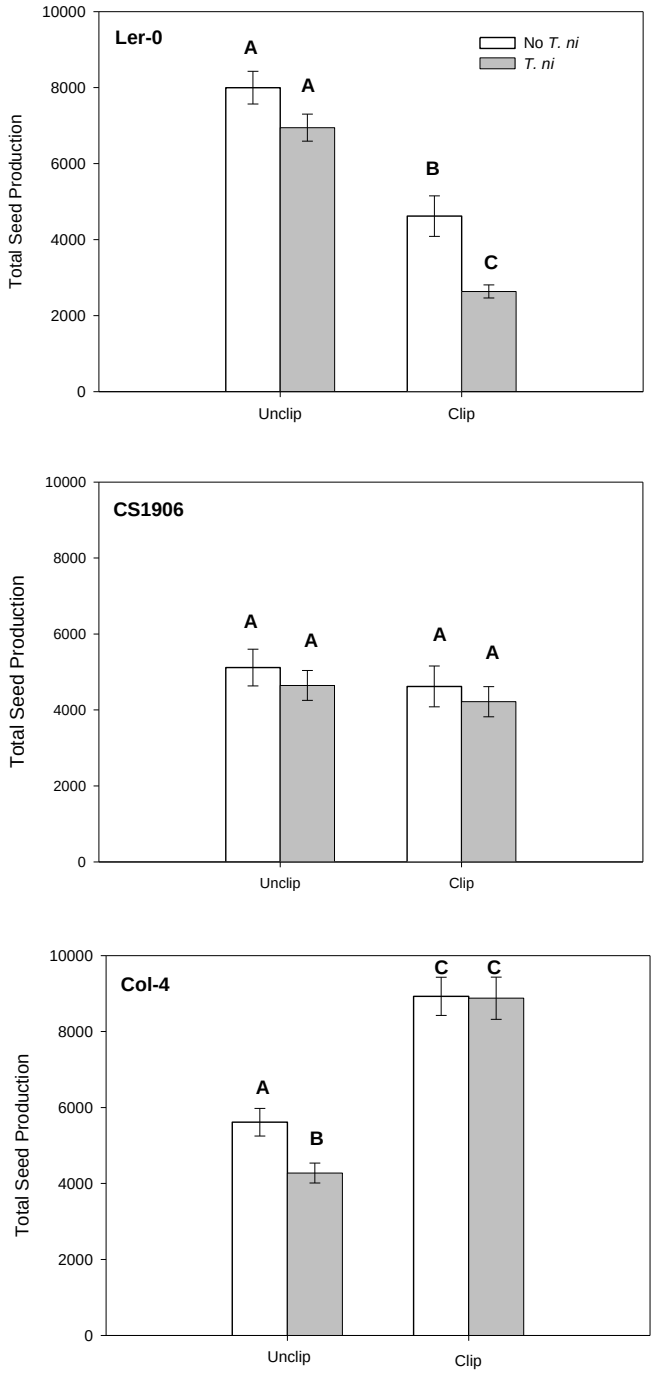
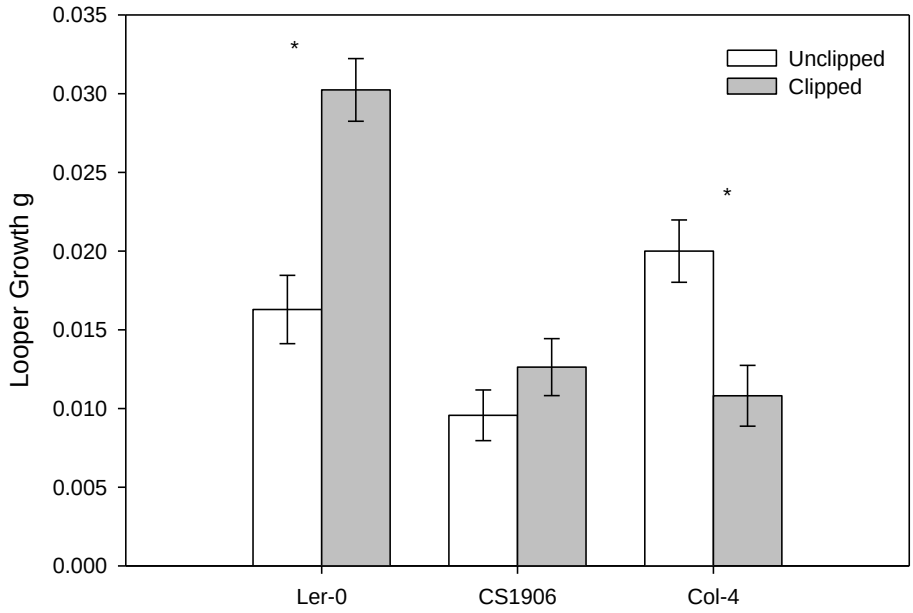


Figure 3.3) *T. ni* growth on clipped and unclipped controls for three ecotypes. Means ± SE are shown. Asterisks indicate significant differences at $\alpha = 0.05$ between clipped and unclipped plants.



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Chapter 4: Molecular Constraints on Tolerance-Resistance Trade-Offs: Is There a Cost?

4.1 Abstract

Plants possess myriad defenses against their herbivores, including constitutive and inducible chemical compounds and regrowth strategies known as tolerance. Recent studies have shown that plant tolerance and resistance are positively associated given they are co-localized in the same molecular pathway, the oxidative pentose phosphate pathway. However, given that both defensive strategies utilize carbon skeletons from a shared resource pool in the oxidative pentose phosphate pathway there could still be costs in maintaining both strategies. Here we investigate whether there were costs to maintaining both strategies by utilizing a double knockout of Cytochrome P450 genes, cyp79B2 and cyp79B3, key enzymes in the biosynthetic process of indole glucosinolates. These mutant plants are devoid of any indole glucosinolates thus reducing plant resistance. Results show that knocking out indole glucosinolate production and thus resistance leads to an increase in fitness compensation in the undercompensating wild-type Columbia-0 following damage. These results show that in spite of a positive relationship between plant tolerance and resistance there are still costs in maintaining both plant defenses. We discuss the mechanistic basis of these costs and associated experiments to understand these trade-offs.

4.2 Introduction

There has long been a focus on trade-offs (costs) between life history investments in growth, reproduction, and maintenance functions that influence survival and longevity (Reznick and Endler 1982, Reznick et al. 1990, Stearns 1989, Zera and Harshman 2001). We now recognize that defensive functions and their associated costs, such as investments in resistance, play an integral role in the trade-offs that shape organismal evolution (Agrawal 2011, Strauss et al. 2002, Lind et al. 2013). Moreover, plants possess multiple types of defenses against herbivores, including constitutive and inducible chemical compounds, structural traits (collectively, resistance) and regrowth/fitness strategies (tolerance). Multiple defense mechanisms have been hypothesized to be costly for a plant, as investment in one anti-herbivore defense is assumed to reduce resources available for other defenses and growth and reproduction (Strauss et al. 2002). Therefore, constraints on multiple defensive strategies due to resource allocation costs should manifest as a negative association between the defenses. Such a trade-off has been predicted to occur between plant tolerance (fitness compensation) and resistance chemistry (van der Meijden 1989). If a tradeoff exists it would have important evolutionary ramifications because it could result in selection leading either to maximal resistance or maximal tolerance, but not both (Fineblum and Rausher 1995). However, a recent meta-analysis on plant resistance and tolerance found no overall negative association between these two traits (Koricheva et al. 2004). In fact, we recently found a positive causal association between plant resistance and tolerance due to joint mediation of both within the same molecular pathway (Mesa et al. 2017).

We have uncovered a gene, Glucose-6-Phosphate-1-Dehydrogenase (G6PD1, At5g35790.1) by using a combination of transcriptomic, quantitative trait loci mapping, and

candidate gene knockout and complementation studies in *Arabidopsis thaliana* recombinant inbred lines (showing the full range of fitness compensation from under to overcompensation) and their parents, Landsberg *erecta* (an undercompensating genotype) and Columbia-4 (an overcompensating genotype). G6PD1 plays a major role in controlling the compensatory response in *Arabidopsis thaliana* following the removal of the plant's primary inflorescence (Siddappaji et al. 2013). G6PD1 is the central regulatory enzyme in the oxidative pentose phosphate (OPP) pathway that plays a key role in plant metabolism generating NADPH and a variety of metabolic intermediates for biosynthetic processes (Kruger and von Schaewen 2003). G6PD1 supplies intermediate compounds from the OPP pathway into the shikimate pathway for secondary metabolite production for plant chemical defense, such as glucosinolates (Maeda and Dudareva 2012).

We have also shown that following the removal of apical dominance, phenotypically plastic increases in ploidy level via endoreduplication leads to rapid regrowth and an increase in fitness, explaining, in part, the phenomenon of overcompensation in plants (Scholes and Paige, 2011, 2014). Endoreduplication is the replication of the genome without mitosis, which leads to endopolyploidy, an increase in cellular chromosome number (e.g., see Nagl, 1976). Removal of the apical meristem by herbivores eliminates production of the plant hormone auxin, leading to a rapid break in dormancy of axillary buds and subsequent stem elongation (Ishida et al., 2010). High levels of auxin are also known to repress the endocycle, and by contrast, lower levels of auxin trigger an exit from mitotic cycles and an entry into endocycles. Insect leaf-feeding also can trigger endoreduplication by the upregulation of jasmonic acid, which also lowers auxin production (Machado et al., 2013) and can lead to overcompensation in some ecotypes of

Arabidopsis (Mesa and Paige, unpublished data). Thus, there is a direct link between endoreduplication and plant damage.

Increasing chromosome number through endoreduplication and therefore gene copy number may provide a means of increasing expression of vital genes (e.g., see Bourdon et al., 2012) (such as G6PD1) or genetic pathways that promote rapid regrowth rates following herbivory. G6PD1 feeds compounds into the OPP pathway for nucleotide biosynthesis, by the provision of ribose-5-phosphate, necessary for the significant increase in chromosome number via endoreduplication. The increase in DNA content then feeds back positively on pathways involved in metabolism (e.g., G6PD1) and defense (e.g., glucosinolate production) through increased gene expression (more copies due to increases in endoreduplication following damage; Scholes and Paige, 2014, 2015). An increase in total cellular DNA content through endoreduplication also leads to extensive cell growth via cell expansion (Melaragno et al., 1993). Growth by cell division along with growth by cell expansion through endoreduplication may be faster than growth by cell division alone (Barow, 2006). Rapid growth and development following the removal of apical dominance may be enhanced by maximizing nutrient transport (with fewer plasmodesmata), protein synthesis (with more copies of DNA), and light and water absorption (with larger cell size and storage capacity) (Lee et al., 2009). Importantly, the experimental overexpression of ILP1 (Increased Level of Polyploidy1), an endoreduplication enhancer, increases glucosinolate production and compensation (from undercompensation to equal compensation with a trend toward overcompensation) in a genotype of *A. thaliana* that typically suffers reduced fitness when damaged (Mesa et al., 2017), demonstrating a causal relationship between the process of endoreduplication, fitness compensation, and chemical defense.

Overall, these results translate into a positive relationship between plant tolerance and resistance (Mesa et al. 2017). Despite the molecular constraints that lead to a positive association between tolerance and resistance, the lack of a trade-off does not preclude the possibility of a cost in maintaining both strategies, because of the fact that both defensive strategies utilize carbon skeletons from a shared resource pool in the oxidative pentose phosphate pathway.

Most studies that have detected costs of resistance have utilized signaling hormones such as jasmonic acid to further increase resistance chemicals and measure the effects on fitness (Heil and Baldwin 2002). However, methodological issues can arise from pleiotropic effects of defense hormones with physiological and morphological changes unrelated to resistance (Creelman and Mullet 1997). This is especially problematic as plant defense hormones have antagonistic effects on the plant growth hormone auxin (Onkokesung et al. 2010).

Here, we specifically assess the cost of resistance in *A. thaliana* by utilizing a double knockout mutant for two cytochrome P450s, key enzymes in the biosynthetic process of indole glucosinolates, which convert tryptophan to indole-3-acetaldoxime (IAOx). IAOx is then further used in the production of indole glucosinolates (Bender and Celenza 2009). Specifically, we assessed whether knocking out indole glucosinolate production and thus resistance leads to an increase in fitness compensation in the undercompensating wild-type Columbia-0.

4.3 Methods

4.3.1 System

Arabidopsis thaliana, mouse-ear cress, is a small, mostly self-pollinating plant in the Brassicaceae family. While native to Europe, *A. thaliana* has a wide geographical range spanning Eurasia, North Africa and North America (Ratcliffe 1965, Mitchell-olds 2001). Typically *A.*

thaliana is found as a winter annual where seeds of *A. thaliana* germinate in the fall after passing the summer in a dormant state and grow into an overwintering rosette, and following stem elongation in the spring, produce flowers that develop into seed pods known as siliques (Pigliucci 2002). *A. thaliana* is fed on by a variety of species including flea beetles, aphids, leaf miners, caterpillars and rabbits (Van Poecke 2007, Weinig *et al*. 2003). *A. thaliana* thus frequently experiences leaf and apical meristem damage and has a suite of resistance characters such as trichomes, proteinase inhibitors and glucosinolates which deter and inhibit feeding by herbivores (Beekwilder *et al*. 2008).

4.3.2 Glucosinolates

Glucosinolates constitute a large and diverse group of defensive secondary metabolites characteristic of the order Brassicales, which includes *A. thaliana*, our organism of study (Muller *et al*. 2010). Glucosinolates (mustard oil glucosides) are nitrogen and sulfur rich natural plant secondary products that consist of a sulfonated oxime and a β-thioglucose moiety, but differ in side chain structures (Pfalz *et al.* 2009). There have been ~40 glucosinolates found in *Arabidopsis*, out of the 120 glucosinolates identified, most of which are classified into three subgroups based on the biosynthetic amino acid precursor, those subgroups being indole, aliphatic and benzenic (Sonderby *et al*. 2010). Indole and aliphatic glucosinolates constitute most of the diversity of glucosinolates in *A. thaliana* (Brown *et al*. 2003). Indole glucosinolates, our chemicals of interest, are composed of four individual compounds: glucobrassicin, 4-methoxy-glucobrassicin, neoglucobrassicin and 4-hydroxyglucobrassicin.

Many studies have shown that glucosinolate breakdown products deter generalist and specialist herbivores on *A. thaliana* (e.g., Agrawal and Kurashige 2003) and act in defense against pathogens (Schlaeppi *et al*. 2010). Upon herbivory, glucosinolates stored in the vacuole

are mixed with the enzyme myrosinase (known as the "mustard bomb"), a β-thioglucosidase that is separated in scattered specialist cells known as myrosin cells (Ratzka *et al*. 2002). Myrosinase cleaves the β-glucose moiety from glucosinolates, leading to a variety of toxic breakdown products, such as bioactive nitriles, epithionitriles and isothiocyanates based on reaction conditions and protein factors such epithiospecifier proteins (Zhang *et al*. 2006).

To assess the possible costs between plant tolerance and resistance 100 seeds of Col-0 and *cyp79B2 cyp79B3* double mutant lines were planted and grown. While single Cytochrome P450 (*cyp)* mutant lines show little deficiency in the ability to produce indole glucosinolates, double mutants are completely devoid of any indole glucosinolates and camelixin (Glawischnig *et al*. 2004; Zhao *et al*. 2002). Moreover, the *cyp* double mutants do not differ in their aliphatic glucosinolate levels from wildtype plants (Muller et al. 2010). In *A. thaliana*, indole glucosinolate synthesis involves a catalyzed conversion of tryptophan to indole-3-acetaldoxime (IAOx) carried out by two cytochrome P450s, *cyp79B2* and *cyp79B3* (Hull *et al*. 2000, Celenza *et al* 2005). IAOx is further catalyzed through four subsequent reactions to form glucobrassicin, the most abundant indole glucosinolate found in *A. thaliana* (Bender and Celenza 2009). Glucobrassicin can then be further modified to the other three indole glucosinolates found in *A. thaliana* with modified indole rings (Pfalz *et al.* 2009). The *cyp79B2 cyp79B3* double mutant line was kindly obtained from the laboratory of John Celenza at Boston University (Department of Biology, Boston, Massachusetts). Lines were grown in a greenhouse on the campus of the University of Illinois, Champaign, under 12 hours of light (~100 $\mu E/m^2/sec$) and dark. Plants were grown individually in 3.5-inch pots using L1 Sunshine mix. Seeds/seedlings were kept moist during germination, and plants were then watered daily to maintain soil moisture without saturating the soil. Plants were not fertilized. When inflorescences reached 6 cm, about 3.5

weeks, 50 plants of each ecotype were randomly clipped, leaving approximately 1 cm of inflorescence (comparable to natural mammalian herbivory (Scholes *et al*. 2016).

At 6.5 weeks, 30 plants of Col-0 (15 clipped, 15 unclipped) were analyzed for indole glucosinolate concentration. Inflorescence material was taken from both clipped and unclipped plants. In addition, the level of indole glucosinolates were assessed on a couple of clipped and unclipped samples of the cyp79B2 cyp79B3 double mutant line to verify that there were in fact, undetectable levels of indole glucosinolates, consistent with Zhao et al. 2002. All samples were frozen in liquid nitrogen, and stored at -80C prior to freeze-drying. Freeze-dried tissues were ground into a fine powder and stored at -20C prior to glucosinolate analysis. Glucosinolates were extracted from finely ground freeze-dried tissue, converted to desulphoglucosinolates with arylsulfatase and analyzed via high pressure liquid chromatography (HPLC) as described by Brown *et al*. (2003). Freeze-dried powder 50mg and 0.5 mL of 70% methanol were added to 2.5 mL tubes and placed on a heating block at 95C for 10 min, mixing frequently. Samples were cooled on ice and 0.125 mL glucosinolabin was used as an internal standard and centrifuged at 3,000xg for 10 minutes. The supernatant was saved and the pellet was re-extracted with another 0.5 mL 70% methanol at 95C for 10 minutes and the two extracts were combined. Protein was subsequently precipitated with 0.15 mL of a 1:1 mixture of 1 M barium acetate and 1 M lead acetate and centrifuged at 12,000xg for 1 min. Each sample was then loaded onto a column containing DEAE Sephadex A-25 resin for desulfation via arylsulfatase for 18 h and the remaining desulfo-GS eluted. Desulphoglucosinolates were separated on a HPLC system (Agilent 1100 HPLC system, with a G1311A bin pump, a G1322A vacuum degasser, a G1316A thermostatic column compartment, a G1315B diode array detector and an HP 1100 series G1313A autosampler) with a variable ultraviolet detector set at 229 nm wavelength. Elution of

desulphoglucosinolates occurred over 45 minutes with a linear gradient of 0% to 20% acetonitrile in water with a flow rate of 1.0 mL/min. Glucosinolate concentration was established using glucosinalbin as an internal standard, a glucosinolate not found in *A. thaliana*. UV response factors for different glucosinolates were used as determined by Wathelet *et al*. (2001). Indole glucosinolates were estimated by adding the four indole glucosinolates observed in Col-0.

Upon plant senescence (8 weeks), plants of both ecotypes were analyzed for fitness (50 plants per ecotype; 25 clipped, 25 unclipped). Fitness measures included the number of siliques, seeds per plant, and the average seed weight. Our previous studies have shown that seeds are a good measure of plant fitness as there are no significant differences in germination success between clipped and unclipped plants of *A. thaliana*. We measured total silique number for each plant and counted total seed production in 3 randomly selected siliques from each plant. Seeds per silique were averaged per ecotype and treatment then multiplied by total silique number for each plant to obtain seed totals per plant. Average seed weights were measured by weighing 50 seeds per plant from 10 plants per ecotype x treatment group. Each weight measurement was then divided by the number of seeds to yield average seed weight for each ecotype x treatment group. Additionally, we measured rosette diameter at time of senescence for Col-0 and *cyp79B2 cyp79B3*.

Potential differences in composite seed and glucosinolate production were assessed using an analysis of variance and Type III sums of squares with two treatment factors (genotype and clipping). Rosette diameter was used as a covariate to adjust for differences in plant size. In addition, we regressed total seed production on rosette diameter of *A. thaliana* for both Col-0 and cyp79B2 cyp79B3 double mutant lines to justify using rosette diameter as an appropriate covariate in the model (see Fig. 4.S1). Independent tests were performed as different sets of

plants were used for each variable assessed. Differences among treatments within genotypes were determined using Tukey pairwise comparisons.

4.4 Results

Genotypes differed in composite seed production ($p < 0.001$) in their response to simulated mammalian herbivory ($p < 0.001$) (Table 4.S1). Specifically, a post hoc Tukey test revealed Col-0 significantly undercompensated with a 55% decrease in composite seed yield when comparing damaged plants to undamaged controls ($p < 0.001$). In contrast, the *cyp79B2 cyp79B3* double mutant with severely impaired resistance significantly overcompensated ($p < 0.001$) following damage with a 30.92% increase in seed production (Fig. 4.1A). Results remained the same following adjustment with rosette size run as a covariate to adjust composite seed yield means (Fig. 4.1B). Results show that there is a linear relationship between composite seed yield and rosette size justifying rosette size as an appropriate covariate in our analysis (Fig. 4.S1).

Genotypes also differed in indole glucosinolate concentrations ($p < 0.001$) in their response to simulated mammalian herbivory ($p < 0.001$) (Table 4.S2). Unclipped Col-0 plants produced on average 1.35 μmol g^{-1} of total indole glucosinolates. Col-0 displayed a 41.38% decrease ($p < 0.03$) in indole glucosinolates following removal of the apical meristem. We also confirmed that the knockout mutants did not produce any indole glucosinolates as shown by Zhao et al. 2002.

4.5 Discussion

In 1989, van der Meijden *et al*. proposed that plant resistance and tolerance should be alternative defense strategies given limited resources in an environment. However, we have recently shown that both resistance and tolerance are positively and causally entwined within the

same molecular pathway (the oxidative pentose-phosphate pathway leading directly into the shikimate pathway). By measuring glucosinolate levels and seed production following the removal of apical dominance in ecotypes of *Arabidopsis thaliana* (that range the entire spectrum of seed production from undercompensating to equally compensating to overcompensating), we have shown that there is a positive association between tolerance and induced resistance (Fig. S2, from Mesa et al. 2017). Similarly, the causal relationship between tolerance and resistance was recently demonstrated by genetically manipulating the endoreduplication pathway. By overexpressing ILP1 (Increased Level of Polyploidy 1), a positive regulator of endoreduplication, we have experimentally increased glucosinolate production and seed production in the undercompensating wild-type Col-0 ecotype (Fig. 4.S3, from Mesa et al. 2017).

Here we show that despite a positive relationship between the two defense strategies there is still a cost in maintaining them. Our results show that knocking out the indole glucosinolate biosynthetic pathway via a *cyp79B2 cyp79B3* double mutant in a Col-0 background results in plants overcompensating after tissue damage with clipped plants having significantly higher total seed production compared to the undercompensating wild-type Col-0. As both resistance and tolerance are controlled via the oxidative pentose-phosphate pathway (Scholes and Paige 2015, Mesa *et al*. 2017) knocking out production of indole glucosinolates allowed more resources to be shunted towards tolerance. These results support the early theoretical framework of van der Meijden (1988), who suggested that resources limit plant defensive responses.

Knockout mutants for indole glucosinolate biosynthesis also have reduced levels of the growth regulator indole-3-acetic acid (IAA) as indole-3-acetaldoxime (IAOx) is used as an intermediate of both indole glucosinolates and IAA biosynthesis (Zhao *et al*. 2002, Celenza et al. 2005). Effects of the downregulation of auxin can be seen in this experiment as unclipped plants

of *cyp79B2cyp79B3* were significantly smaller than wild-type plants (Fig 4.2; note, that we found the same statistical pattern whether we adjusted for size differences or not in this experiment, see Fig. 4.1A and B). Zhao et al. (2002) found similar results, both rosettes and elongating cotyledons were smaller in the *cyp79B2cyp79B3* double knockouts and auxin was downregulated. Plants, however, are not entirely deficient in IAA due to redundancy of IAA pathways (Quirino et al. 1999, Ljung et al. 2001, Zhao et al. 2002) and this may have restricted unclipped plants from entering the endocycle (auxin is the gate-keeper for entering the endocycle). However, clipped knockout rosettes were as large as the two wild-type treatments and these plants overcompensated. These results suggest that the level of compensation is constrained by the level of resources available in one of two ways. Either resource availability alone constrains the compensatory ability or resources limit the level of endopolyploidy achieved following the removal of apical dominance in Col-0, or perhaps they both play an important role. It is interesting to note that nutrient deprivation through inhibition of the insulin-signaling pathway in *Drosophila* can block endoreplication (Britton et al. 2002, Lee et al. 2009). Thus, the trade-off uncovered here may result from unblocking endoreduplication. We are currently growing additional plants to assess the level of endopolyploidy and the effects of fertilization on compensation in the Cytochrome P450 double knockout mutant of Col-0 in light of these results. In addition, bypassing the shikimate pathway, may lead to an increase in the production of glucose 6 phosphate dehydrogenase 1 (G6PD1), that plays a key role in compensation and primary metabolism (Siddappaji et al. 2013), ultimately leading to an increase in plant growth and reproduction. There is also the possibility that blockage of the shikmate pathway increases the availability of translational machinery (e.g., ribosomes) leading to an increase in the compensatory response (Caveney et al. 2017) through endoreduplication.

As noted above, unclipped Cytochrome P450 double knockout plants displayed lower levels of auxin production resulting in smaller plant size, given that auxin is the primary growth hormone in plants. While we have shown the efficacy of utilizing knockout mutants in measuring plant defense tradeoffs we were unable to avoid the pleiotropic effects that have afflicted earlier work on plant defense tradeoffs (Creelman and Mullet 1997, Wasternack and Parthier 1997). However, given the interdependence of indole glucosinolates and auxin production it would be difficult to uncouple the two. Perhaps a knockout mutant within the aliphatic glucosinolate pathway, which is derived from methionine would suffice in avoiding pleiotropic effects on auxin.

In spite of a positive relationship between tolerance and resistance in *Arabidopsis thaliana*, we have shown that plant resistance carries a significant cost in tolerance, suppressing the degree of compensation. Our results are currently unclear as to the mechanistic basis of this resource based trade-off. What we need to do moving forward, is to address whether endoreduplication is a general mechanism for explaining patterns of tolerance and resistance and tolerance/resistance trade-offs. We know that endoreduplication is common in plants, with approximately 90% of herbaceous angiosperms being endopolyploid (Nagl, 1976). We also know that there is genetic variation for compensation/tolerance across numerous plant species. For example, some families exhibit overcompensating tolerance, whereas others express incomplete tolerance (e.g., see Tiffin and Rausher, 1999; Siddappaji et al., 2013). What is unknown is the degree to which plant species are genetically/genotypically plastic in terms of the level of endoreduplication following herbivory (Paige 2018) and more generally how common the association is between endoreduplication, tolerance and plant resistance. Therefore, there is a need for studies on other species to determine whether there is a positive association between the

degree of fitness compensation following herbivory, resistance and the level of endopolyploidy, and ultimately, a causal relationship, as we have shown in *A. thaliana*.

4.6 Figures

Figure 4.1A) Composite seed production for clipped and unclipped plants for Col-0 and *cyp79B2 cyp79B3* double mutant lines. Shown are means ± 1 SE. Asterisks indicate significance at a familywise error rate of $\alpha = 0.05$ for clipped and unclipped plants.

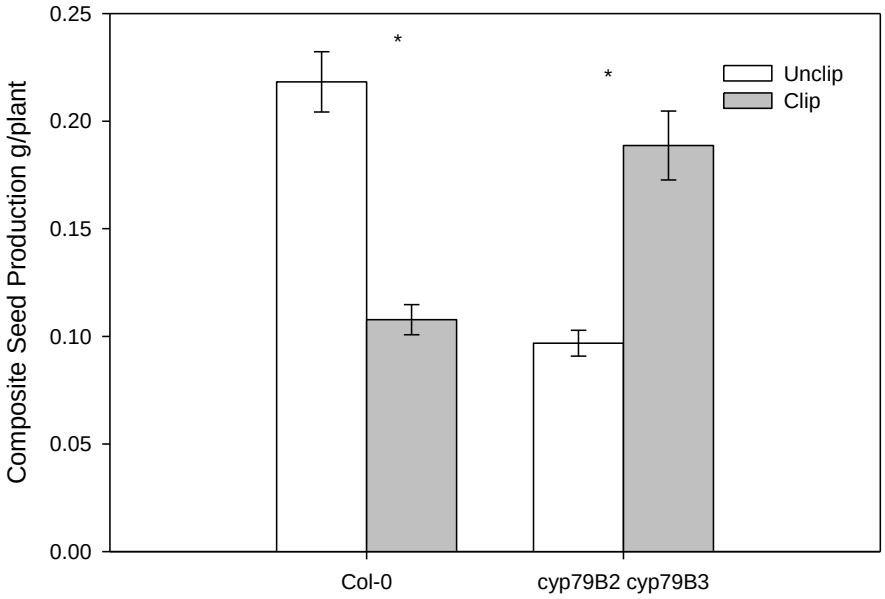


Figure 4.1B) Composite seed production for clipped and unclipped plants for Col-0 and *cyp79B2 cyp79B3* double mutant lines adjusted for rosette size of 5.4 as a covariate. Shown are means ± 1 SE. Asterisks indicate significance at a familywise error rate of $\alpha = 0.05$ for clipped and unclipped plants.

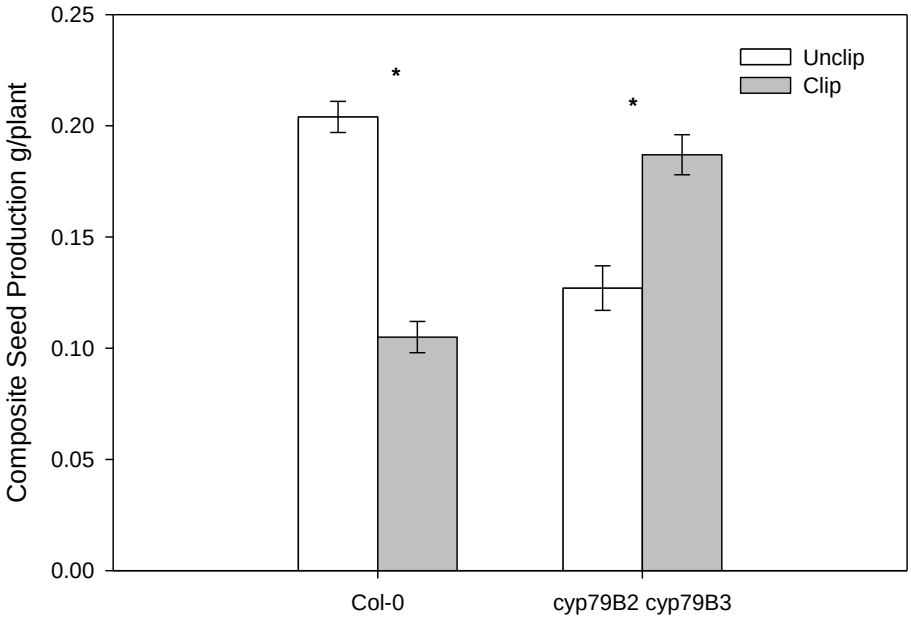
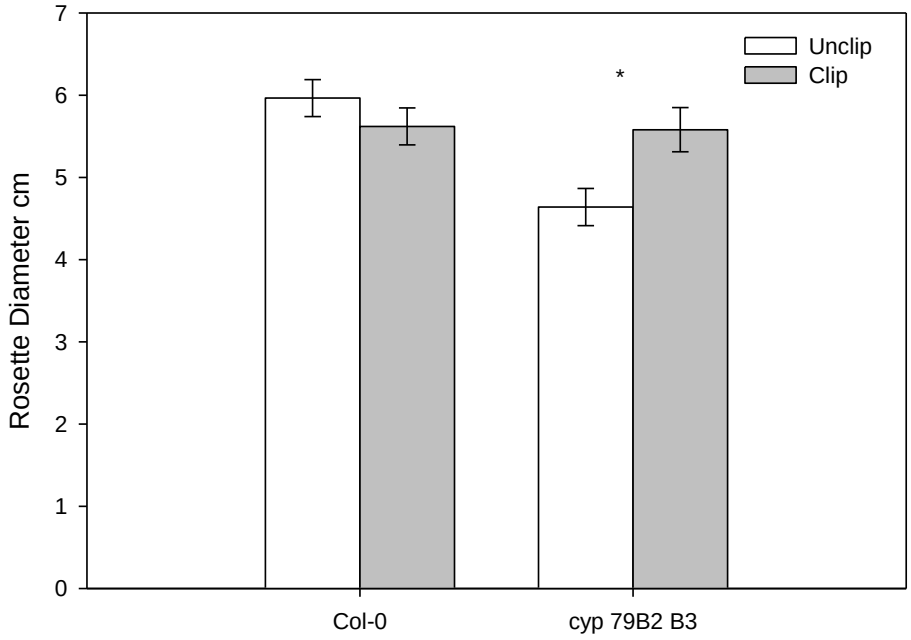


Figure 4.2) Rosette diameter for clipped and unclipped Col-0 and *cyp79B2 cyp79B3* double mutant lines. *cyp79B2 cyp79B3* clipped were significantly larger than unclipped plants. Shown are means ± 1 SE. Asterisks indicate significance at a familywise error rate of $\alpha = 0.05$.



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Chapter 5: General Conclusion

Plants being sessile organisms have evolved multiple strategies to defend against their natural enemies. As these defense strategies co-occur within the same plant this allows an opportunity to test fundamental concepts of biological trade-offs. Trade-offs have been expected to occur among the two primary defenses, tolerance and resistance (van der Meijden 1988), however researchers have been unable to detect a negative association between the two defenses (Koricheva et al. 2004). The focus of my dissertation studies has been on uncovering the association between plant tolerance and resistance based on the underlying molecular basis and illustrating evolutionary constraints on trade-offs.

In chapter 2 I was able to show that in *Arabidopsis thaliana* plant tolerance and resistance are positively and causally associated. By experimental manipulation of the endoreduplication pathway, the mechanism of plant compensation, we were able to increase both tolerance and resistance validating our hypothesis that both defenses are constrained by the same molecular pathway, the oxidative pentose phosphate pathway. Importantly, we found that by ignoring genetic variation in endoreduplication we were able to mask the positive relationship between tolerance and resistance. Instead when treating our samples as a population mean, we found intermediate levels of both strategies, a result most often seen in the literature (Koricheva et al. 2004). It is therefore, important to assess the degree of endoreduplication across a population of herbaceous plants prior to measuring fitness and plant resistance responses to herbivory, especially since ~90% of herbaceous plants endoreduplicate (Nagl 1976, Sugimoto-Shirasu and Roberts 2003).

In chapter 3 I showed that insect leaf herbivory lead to a similar positive association between tolerance and resistance as seen with mammalian damage. Importantly, insect damage

leaves the apical meristem intact and does not cut off the source of auxin production as with mammalian damage, a growth hormone that prevents entry into the endocycle (Ishida et al. 2010). However, insect damage induces defense hormones such as jasmonic acid which interacts antagonistically with auxin, thus allowing entry into the endocycle and for compensation via endoreduplication to occur (Onkokesung et al. 2010). In addition, the interactive effects of mammalian and insect herbivory on each of these inbred lines was assessed to determine whether interactions were additive (pairwise) or nonadditive (diffuse) on fitness compensation (tolerance) and secondary plant metabolite production (resistance). Following initial mammalian damage plant tissue palatability changed to co-occurring insect herbivores. Overcompensating ecotypes increased resistance chemistry making them unpalatable to insect herbivores, allowing plants to retain their increased levels of fruit and seed production by lowering insect growth. Conversely, undercompensating ecotypes accrued a further reduction in seed production because initial mammalian damage decreased resistance chemistry making them more palatable to insect herbivores. These results show that dependent on ecotype the interactive effects of mammalian and insect herbivory are pairwise and diffuse. Ler-0 had reduced glucosinolates following simulated mammalian herbivory leading to increased damage from insect herbivores indicating diffuse interactions. CS1906 and Col-4 both displayed pairwise interactions, although Col-4 following simulated mammalian herbivory increased resistance chemistry reducing insect damage.

In chapter 4 I was able to further manipulate the plant defense pathways via genetic knockouts to show that while plant tolerance and resistance are positively associated that plant defense still comes at a cost. As mentioned earlier, theoretical models have assumed that plant defenses are costly and that it would lead to a trade-off between multiple defenses (van der

Meijden et al. 1988). Here we show that theoretical assumptions are correct and that defense is costly, but constraints on the evolution of tolerance and resistance prevents a negative association from evolving, at least in *A. thaliana*. Specifically, I used a knockeout mutant of two cytochrome P450s (*cyp79B2 cyp79B3*), key enzymes in the biosynthetic process of indole glucosinolates, which converts tryptophan to indole-3-acetaldoxime (IAOx). IAOx is then further used in the production of indole glucosinolates (Bender and Celenza 2009). This resulted in resource reallocation from resistance chemistry to compensation and increased plant tolerance from undercompensation seen in the wildtype Col-0 to overcompensation in the double knockout mutant.

Although my results among others show that there is genetic variation in endoreduplication among plant genotypes and species, the cause of such variation is unknown. Allelic diversity in cell cycle regulators (cyclins and cyclin dependent kinases) may play an important role in explaining this variation (Dewitte et al. 2007, Scholes and Paige 2015). Additional variation in endoreduplication may arise from allelic diversity in the opp pathway. Moreover, the role of natural selection in maintaining variation in endopolyploidy within and across taxa needs to be experimentally addressed. For example, can endopolyploidy and its associated induction threshold evolve via natural selection in relation to differences among herbivores or herbivory level? If so, what are the associated molecular changes or targets of selection?

It is also noteworthy that endoreduplication may serve as a generalized response mechanism for mitigating stress by plastically increasing endopolyploidy in response to a host of environmental factors, other than herbivory, including high light/UV, low temperature, water stress, heavy metals, salt, wounding, pathogens (e.g., fungi, nematodes), and symbiotic biotrophs

(e.g., rhizobial bacteria, mycorrhizal fungi), among others (see Scholes and Paige [2015] for a review). The endocylce provides increases in transcriptional output, metabolism, stress-mitigating compounds and nucleotypic effects necessary to mitigate a variety of environmental stressors. Thus, plasticity in cellular ploidy may be important in the response to environmental stress by providing a mechanism for organisms to fine-tune themselves to their local environment via control at the level of individual cells. Nonetheless, with limited exception, few experimental studies have been directed toward the role of endoreduplication in ecological, evolutionary, or environmental contexts, clearly opening new avenues for inquiry.

Overall, across my dissertation studies I found that it is imperative to measure endoreduplicaton across a plant population otherwise one might come to erroneous conclusions as to the nature of defense trade-offs if compensation via endoreduplication is a general phenomenon. In addition, given that the magnitude of chemical induction is dependent on the magnitude of endoreduplication following plant damage and that endoreduplication takes time to reach its peak (in *A. thaliana*, about 6.5 weeks following damage), the timing of chemical measurement is important. Many studies (e.g., see Mauricio et al. 1997, Strauss et al. 2003) measure chemical induction shortly after damage at a time when the process of endoreduplication is just beginning (Scholes and Paige 2011). Furthermore, the mode of herbivory does not appear to account for the discrepancies in the literature as both mammalian and insect herbivores lead to positive associations between the two defenses. Therefore, it is important to assess the degree of endoreduplication across a population and through time, to remove noise within the system and avoid drawing potentially erroneous conclusions, assuming that our findings are common among plants that have the capability to endoreduplicate. An important next step is to evaluate additional plant species that endoreduplicate to see if this

phenomenon is generalizable. Given that a myriad of other plant secondary defensive compounds are produced from the shikimate pathway, and that most herbaceous plants endoreduplicate, it is likely that positive associations between tolerance and resistance represent the norm.

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Supplemental Material

Table 2.S1) Independent *t*-tests for all individual glucosinolates between clipped and unclipped plants for each genotype. N/A indicates the glucosinolate was not found in that genotype and asterisks indicate significance at a familywise error rate of $\alpha = 0.05$ between clipped and unclipped plants. Compounds are as follows (1 = 3Hydroxypropylglucosinolate, 2 = Glucoiberin, 3 = Glucoraphanin, 4 = Glucoalyssin, 5 = Gluconapin, 6 = 4-Hydroxyglucobrassicin, 7 = Glucoerucin, 8 = 8-Methylsulfinyloctyl glucosinolate, 9 = Glucobrassicin, 10 = 4-Methoxyglucobrassicin, 11 = Neoglucobrassicin).

	Statistic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
L*er*-0	*t*	-2.61	-3.39	-1.1	N/A	-0.43	-1.99	N/A	-0.22	-0.84	-1.26	-5.91
	p	0.0145	0.0021*	0.2805	N/A	0.6692	0.0564	N/A	0.8230	0.4094	0.2164	0.000002*
	df	28	28	28	N/A	28	28	N/A	28	28	28	28
CS1968	*t*	2.9	-0.77	-0.13	N/A	-.38	-0.24	-2.33	1.91	1.47	-2.55	-2.19
	p	0.007*	0.4490	0.8995	N/A	0.7046	0.8095	0.0271	0.0658	0.1527	0.016*	0.0373
	df	28	28	28	N/A	28	28	28	28	28	28	28
CS1906	*t*	N/A	-0.09	-.46	0.07	-0.65	-0.77	-3.2	-1.42	0.56	-2.11	-2.4
	p	N/A	0.9259	0.6475	0.9418	0.5221	0.4470	0.0037*	0.1672	0.5796	0.0449	0.0241
	df	N/A	25	25	25	25	25	25	25	25	25	25
CS1948	*t*	7.18	1.33	4.15	5.06	5.75	2	-1.13	0.60	3.01	1.73	-1.59
	p	0.00000008*	0.1956	0.00028*	0.00002*	0.000003*	0.0555	0.2676	0.5520	0.0054*	0.0939	0.1231
	df	28	28	28	28	28	28	28	28	28	28	28

Table 2.S1 - Continued

Col-4	***t***	**N/A**	**5.92**	**3.89**	**4.26**	**1.37**	**2.15**	**-3.61**	**-0.94**	**4.32**	**3.7**	**-0.06**
	p	N/A	0.000002*	0.0005*	0.0002*	0.1821	0.0405*	0.0012*	0.3554	0.0002*	0.0009*	0.9522
	df	N/A	28	28	28	28	28	28	28	28	28	28
Col-0	*t*	N/A	-1.25	-1.32	-1.90	-1.19	-2.61	-2.25	-3.11	-2.84	-3.41	-2.48
	p	N/A	0.2204	0.1968	0.0684	0.2435	0.0144	0.0323	0.0043*	0.0083	0.0020*	0.0196
	df	N/A	28	28	28	28	28	28	28	28	28	28
***ILP1*-ox**	*t*	N/A	5.52	5.50	5.52	3.91	5.01	4.04	3.77	6.54	5.98	1.87
	p	N/A	0.000007*	0.000007*	0.000007*	0.0005*	0.00003*	0.0004*	0.0008*	0.0000004*	0.000002*	0.0720
	df	N/A	28	28	28	28	28	28	28	28	28	28

Figure 3.S1) Differences between undamaged and *T. ni* damaged plants for total seed, glucosinolate and cell cycle values for three ecotypes. Asterisks indicate significant differences at $\alpha = 0.05$ between clipped and unclipped plants.

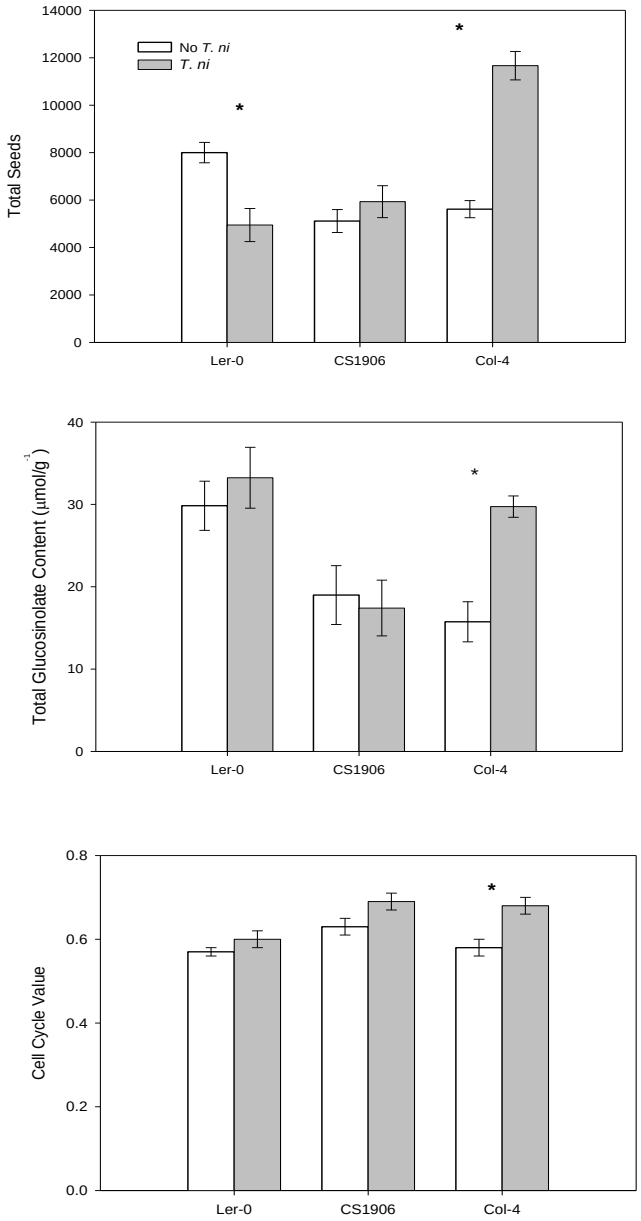

Figure 3.S2) Differences between unclipped and clipped damaged plants for total seed, glucosinolate and cell cycle values for three ecotypes. Asterisks indicate significant differences at $\alpha = 0.05$ between clipped and unclipped plants.

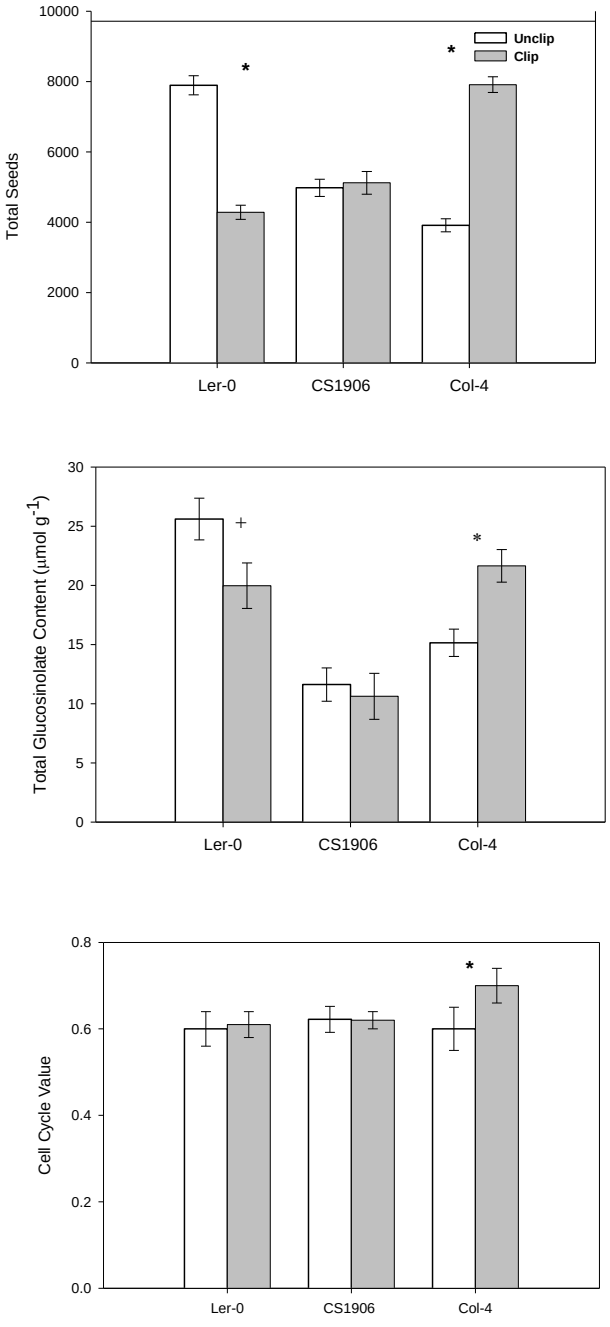

Table 3.S1) Analysis of Variance for Seed Total for *T. ni* compensation experiment

Source	Type III SS	df	Mean Squares	F-Ratio	p-Value
ECOTYPE	4.329	2	2.164	12.838	0.000
T. ni	0.159	1	0.159	0.942	0.334
ECOTYPE*T.ni	9.508	2	4.754	28.197	0.000
Error	19.556	116	0.169		

Table 3.S2) Analysis of Variance for Cell Cycle Values for *T. ni* compensation experiment

Source	Type III SS	df	Mean Squares	F-Ratio	p-Value
ECOTYPE	0.055	2	0.028	4.106	0.020
T. ni	0.089	1	0.089	13.129	0.000
ECOTYPE*T.ni	0.031	2	0.016	2.304	0.106
Error	0.567	84	0.007		

Table 3.S3) Analysis of Variance for Glucosinolates for *T. ni* compensation experiment

Source	Type III SS	df	Mean Squares	F-Ratio	p-Value
ECOTYPE	1,830.963	2	915.481	10.153	0.000
T. ni	440.089	1	440.089	4.881	0.031
ECOTYPE*T. ni	713.550	2	356.775	3.957	0.024
Error	5,500.093	61	90.165		

Table 3.S4) Analysis of variance for Seed Total for herbivore interaction experiment

Source	Type III SS	df	Mean Squares	F-Ratio	p-Value
ECOTYPE	4.464	2	2.232	22.698	0.000
CLIPPING	0.300	1	0.300	3.053	0.082
T. ni	1.619	1	1.619	16.464	0.000
ECOTYPE*CLIPPING	15.589	2	7.794	79.269	0.000
ECOTYPE**T. ni***	0.505	2	0.252	2.565	0.080
CLIPPED**T. ni***	0.043	1	0.043	0.432	0.512
ECOTYPE*CLIPPING**T. ni***	0.850	2	0.425	4.321	0.015
Error	18.682	190	0.098		

Table 3.S5) Analysis of variance for *T. ni* growth on clipped and unclipped plants

Source	Type III SS	df	Mean Squares	F-Ratio	p-Value
ECOTYPE	0.005	2	0.003	19.782	0.000
CLIPPING	0.000	1	0.000	2.093	0.149
ECOTYPE*CLIPPING	0.005	2	0.002	17.855	0.000
Error	0.028	209	0.000		

Table 3.S6) Analysis of variance for Ler-0 Seed Total for herbivore interaction experiment.

Source	Type III SS	df	Mean Squares	F-Ratio	p-Value
Clipping	9.422	1	9.422	109.982	.000
T. ni	1.824	1	1.824	21.292	.000
Clipping* T. ni	.676	1	.676	7.896	.006
Error	5.740	67	.086		

Table 3.S7) Analysis of variance for CS1906 Seed Total for herbivore interaction experiment.

Source	Type III SS	df	Mean Square	F-Ratio	p-Value
Clipping	.078	1	.078	.554	.460
T. ni	.103	1	.103	.728	.398
Clipping* T. ni	5.904E-5	1	5.904E-5	.000	.984
Error	7.204	51	.141		

Table 3.S8) Analysis of variance for Col-4 Seed Total for herbivore interaction experiment.

Source	Type III SS	df	Mean Square	F-Ratio	p-Value
Clipping	6.265	1	6.265	78.607	.000
T. ni	.351	1	.351	4.405	.039
Clipping * T. ni	.213	1	.213	2.669	.107
Error	5.738	72	.080		

Table 4.S1) Analysis of Covariance for composite seed yield.

Source	Type III SS	df	Mean Square	F-ratio	p-Value
Rosette	.306	1	.306	137.793	.000
Genotype	.000	1	.000	.116	.734
Clipping	.012	1	.012	5.343	.022
Genotype x Clipping	.191	1	.191	85.977	.000
Error	.296	133	.002		

Table 4.S2) Analysis of Variance for indole glucosinolates.

Source	Type III SS	df	Mean Squares	F-Ratio	p-value
Genotype	51.437	6	8.573	17.457	.0001
Clipping	1.198	1	1.198	2.439	.120
Genotype x Clipping	27.769	6	4.628	9.424	.0001
Error	94.781	193	.491		

Figure 4.S1) Regression of rosette diameter and total seeds of *A. thaliana* for both Col-0 and cyp79B2 cyp79B3 double mutant lines. Data show a significant positive relationship between the two variables.

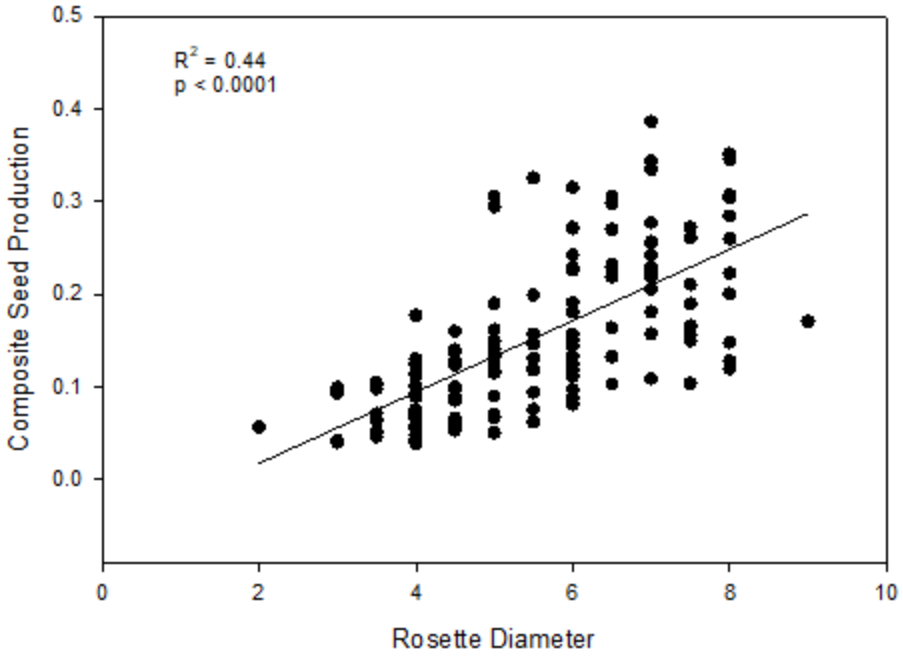

Figure 4.S2) Percent differences in composite seed yield and indole glucosinolate production comparing clipped and unclipped plants for 7 *Arabidopsis thaliana* genotypes. Genotypic responses range from undercompensation (negative percent difference values) to overcompensation (positive percent difference values. Asterisks indicate significance at a familywise error rate of $\alpha = 0.05$ between clipped and unclipped plants. Plus signs indicate marginal ($p < 0.1$) significance.

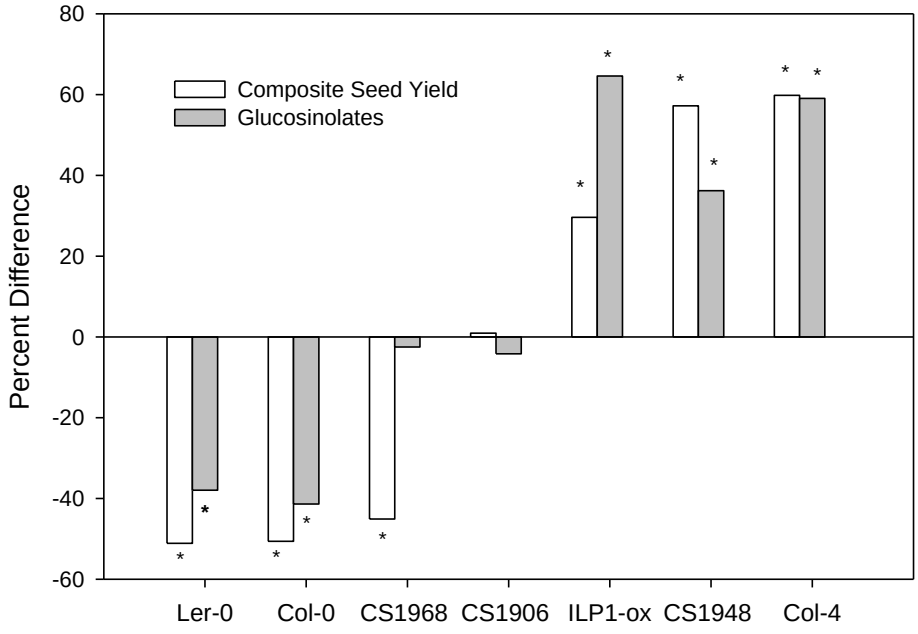

Figure 4.S3) Percent difference between clipped and unclipped plants for the Col-0 and *ILP1*-ox mutant. Measures include seeds, glucosinolates and cell cycle values (endopolyploidy). Genotypic responses range from undercompensation (negative percent difference values) to overcompensation (positive percent difference values). Asterisks indicate significance at a familywise error rate of $\alpha = 0.05$. Plus signs indicate marginal ($p < 0.1$) significance. Cell cycle values were generated by a separate study (Scholes and Paige 2014).