



Published in final edited form as:

*Mol Ecol.* 2011 March ; 20(5): 995–1014. doi:10.1111/j.1365-294X.2010.04901.x.

## Mining the plant-herbivore interface with a leafmining

### *Drosophila* of *Arabidopsis*

Noah K. Whiteman<sup>1,2</sup>, Simon C. Groen<sup>1</sup>, Daniela Chevasco<sup>1</sup>, Ashley Bear<sup>1</sup>, Noor Beckwith<sup>1</sup>, T. Ryan Gregory<sup>3</sup>, Carine Denoux<sup>4</sup>, Nicole Mammarella<sup>4</sup>, Frederick M. Ausubel<sup>4</sup>, and Naomi E. Pierce<sup>1</sup>

<sup>1</sup> Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts 02478, USA

<sup>2</sup> Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona 85721, USA

<sup>3</sup> Department of Integrative Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

<sup>4</sup> Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114, USA

### Abstract

Experimental infections of *Arabidopsis thaliana* (*Arabidopsis*) with genomically characterized plant pathogens such as *Pseudomonas syringae* have facilitated dissection of canonical eukaryotic defense pathways and parasite virulence factors. Plants are also attacked by herbivorous insects, and the development of an ecologically relevant genetic model herbivore that feeds on *Arabidopsis* will enable the parallel dissection of host defense and reciprocal resistance pathways such as those involved in xenobiotic metabolism. An ideal candidate is *Scaptomyza flava*, a drosophilid fly whose leafmining larvae are true herbivores that can be found in nature feeding on *Arabidopsis* and other crucifers. Here we describe the eukaryotic life cycle of *S. flava* on *Arabidopsis*, and use multiple approaches to characterize the response of *Arabidopsis* to *S. flava* attack. Oviposition choice tests and growth performance assays on different *Arabidopsis* ecotypes, defense-related mutants, and hormone and chitin-treated plants revealed significant differences in host preference and variation in larval performance across *Arabidopsis* accessions. The jasmonate (JA) and glucosinolate pathways in *Arabidopsis* are important in mediating quantitative resistance against *S. flava*, and priming with JA or chitin resulted in increased resistance. Expression of xenobiotic detoxification genes was reduced in *S. flava* larvae reared on *Arabidopsis* JA signaling mutants, and increased in plants pre-treated with chitin. These results and future research directions are discussed in the context of developing a genetic model system to analyze insect/plant interactions.

### Keywords

*Arabidopsis*; *Drosophila*; gene expression; herbivory; jasmonate; model system

## Introduction

Metazoan parasites (*sensu* Price 1980), including most herbivorous insects, comprise a majority of lineages of life on the planet (Poulin & Morand 2000). Parasites dominate food web links (Lafferty *et al.* 2006) and are highly relevant to agriculture, medicine and conservation biology. There are two principal guilds of non-microbial eukaryotic macroparasites (May & Anderson 1979), one that attacks animals, such as cestodes (tapeworms) and the other that attacks plants, such as leafmining insects. The research communities studying each guild are diverse, but there is a notable lack of cross talk in the literature. This is surprising considering that the two communities are studying similar phenomena, but perhaps can be explained by the fact that the hosts are phenotypically and evolutionary distinct. Despite the ancient timing of their evolutionary coalescence, plant and animal lineages have much in common with respect to innate immunity (Keen *et al.* 2000). This is particularly true for pattern recognition receptors (PRRs) and some signaling molecules. Whether these similarities are due to common ancestry or convergence is a topic of considerable debate (Ausubel 2005; Leulier & Lemaitre 2008; Staal & Dixelius 2007), a debate made more complex because of functional constraints on the basic structure of parasite elicitor molecules, which restricts form and function of host pattern recognition receptors.

From the parasite's side of the equation, arthropods, fungi, and helminths (parasitic worms, including nematodes, flukes and tapeworms) possess common and extremely potent elicitors of host innate immune pathways, often called microbial, pathogen, or herbivore associated molecular patterns: MAMPS/PAMPS/HAMPS (Bittel & Robotzek 2007; Dangel & Jones 2001; Jones & Dangel 2006; Mithofer & Boland 2008), although there are mechanisms for recognition of self-damage in plants as well (Heil 2009). One such example of an elicitor that is both a MAMP and a HAMP is chitin (Miya *et al.* 2007; Wan *et al.* 2008), which is derived from fungi and macroparasites. Chitin, a biopolymer of N-acetyl-beta-D-glucosamine is not present in vertebrates or plants, but is otherwise abundant in the environment. Chitin is a major constituent of arthropod exoskeleton, fungal cell wall, and helminth eggshell, pharynx and/or cuticle. Infiltration of chitin oligomers into vertebrate or plant tissue causes massive and sometimes analogous innate immune responses, including upregulation of chitinases (Escott & Adams 1995; Reese *et al.* 2007; Zhang *et al.* 2002) that are detrimental to parasite development (Lawrence & Novak 2006), and likely cause allergies in humans. Thus, elicitors such as chitin that are common to parasites of animals and plants can be recognized by and cause parallel immune responses in plant and animal hosts.

### ***Arabidopsis* as a model host**

Our understanding of eukaryotic defense pathways has been revolutionized by the application of genetic and genomic tools developed for the model plant *Arabidopsis* (Brassicales: Brassicaceae) (Jones *et al.* 2008). The availability of genetically tractable microbial pathogens was key to identifying pathogen virulence genes/effectors and their cognate receptors and defense pathways in the plant (Dong *et al.* 1991; Rahme *et al.* 1995). Importantly, pathogen virulence factors and host defense pathways identified by studying host-pathogen interactions in *Arabidopsis* have proven to be ubiquitous across a wide variety of pathogen and plant species, respectively. Without a genetically tractable plant and corresponding genetically tractable pathogens, characterizing resistance and virulence pathways would have been extremely difficult. Importantly, studies of *Arabidopsis*-pathogen interactions also generated fundamental insights into co-evolutionary mechanisms (Ehrlich & Raven 1965) in pathogens and hosts (Mauricio *et al.* 2003; Allen *et al.* 2004; Bergelson *et al.* 2001). *Arabidopsis* is also an important model for ecology and evolutionary biology because of its genetic tractability (Mitchell-Olds 2001). It is surprising, therefore, that there

is currently no genomically characterized or genetically tractable model arthropod parasite of *Arabidopsis*. This impedes our ability to characterize the mechanistic interactions between plant defense pathways and herbivore countermeasures. Here, we describe the first stage of development of a genetically tractable insect herbivore of *Arabidopsis*.

### The leafminer *Scaptomyza flava*

The Drosophilidae are potentially an excellent lineage to survey for potential model herbivores given the large number of species and great variation in life history (Markow & O'Grady 2005) and the fact that the genomes of 12+ species from across the family tree have been completely sequenced (Clark *et al.* 2007). The ideal model would be closely related to genomically characterized and genetically tractable species such as the fly, *Drosophila melanogaster* (Diptera: Drosophilidae, subgenus *Sophophora*), yet would need to naturally feed on *Arabidopsis* or its relatives in the wild to be ecologically relevant. Genetic tools, including classical and transgenic tools, available for *D. melanogaster* have facilitated unprecedented insight into human biology and pathogenesis, including canonical detoxification pathways that are relevant to both herbivorous insects and humans (Trinh *et al.* 2008; Yang *et al.* 2007). However, *D. melanogaster* is not an herbivore but instead feeds on microbes living on decaying plant tissue or on fungi, like most other drosophilids (Markow & O'Grady 2005). Fortunately, there is a lineage within the Drosophilidae called *Scaptomyza* that contains a number of herbivorous species (Hackman 1959; Máca 1972). Larvae of these insects are leafminers, consuming leaves internally (endophages). Adult females feed on plants by making punctures with their ovipositors and feeding on wound exudates (Collinge & Louda 1989; Hering 1951).

Extant leafminers comprise an ecologically, evolutionarily and economically important guild from four holometabolous insect orders, including Coleoptera, Diptera, Hymenoptera, and Lepidoptera (Hespenheide 1991). There are approximately 10,000 described species with leafmining larvae (Connor & Taverner 1997). Leafminers have been parasitizing angiosperms for at least 100 million years (Opler 1973, Labandeira *et al.* 1994) and gymnosperms for a minimum of ca. 225 million years (Scott 2004). Within the Diptera, leafmining evolved independently as many as 25 times (Labandeira 2003). Leafminers have a particularly intimate association with their host plants because they must live inside leaves. They experience significantly lower pathogen attack rates than their free living relatives, but also suffer significantly higher rates of parasitoid attack (Connor & Taverner 1997). The ecological impact of leafmining flies on their hosts can be dramatic; for example, the drosophilid *Scaptomyza nigrata*, a leafminer of the native mustard *Cardamine cordifolia* (Brassicaceae) in the mountains of western North America, has been shown to be the major factor influencing the spatial distribution of its host plant (Louda & Rodman 1996) and has a significant effect on fitness in individual plants (Louda & Collinge 1992). Leafminers are also models for studying sympatric speciation in insects (Tavormina 1982) as well as for studying coevolutionary interactions over deep time because the mines are often diagnosable to the family or even genus level, allowing for fossil calibrations of phylogenetic hypotheses (Lopez-Vaamonde *et al.* 2006).

We focused our attention on *Scaptomyza flava* (Fallen), a foliar herbivore (leafminer) of wild *Arabidopsis* and other mustards in North America and Eurasia (Chittenden 1902; Mitchell-Olds 2001). *Scaptomyza flava* was first described by Fallen in 1823 as *Drosophila flava*, but was later placed in the new drosophilid genus *Scaptomyza* by Hardy in 1849. This 19<sup>th</sup> century taxonomy no longer reflects our current understanding of relationships in the Drosophilidae. All recent phylogenetic hypotheses based on DNA sequence and morphological data unequivocally show that the *Scaptomyza* lineage, comprising 272 described species, is nested phylogenetically within the genus *Drosophila* and within the paraphyletic subgenus *Drosophila* (O'Grady & Desalle 2008). The taxonomy of this group

is confused (Hackman 1959; Martin 2004) and the same species has also been called *S. apicalis* (Hardy) and *S. flaveola* (Meigen). This fly is widely distributed in the Holarctic and has been introduced into New Zealand, where it is a major pest of mustards (Martin 2004). Immatures of *S. flava sensu lato* species are usually found in plant species in the order Brassicales, including the families Brassicaceae, Capparaceae, Resedaceae, and Tropaeoaceae, although it has also been reported from pea (Fabaceae: *Pisum sativum*) (Máca 1972). *Scaptomyza flava* is capable of killing host plants (Bjorksten 2005). It was first reported in the U.S. on *Arabidopsis* in the year 1900 (Chittenden 1902). *Scaptomyza flava* completes development on *Arabidopsis* and several other members of the Brassicaceae in the wild, and its reproductive behavior has been well described (Shakeel et al. 2009).

The idea that a *Scaptomyza* species could be utilized as a genomic model herbivore of *Arabidopsis* was suggested in the literature by Mitchell-Olds (2001). He noted that *Scaptomyza* species attacked *Arabidopsis* and relatives in Europe and that because of its close relationship to members of the subgenus *Drosophila* (for example an *S. pallida* P-element is active in *D. melanogaster*; (Simonelig & Anxolabehere 1991), the tools of *Drosophila* and *Arabidopsis* could both be leveraged to study plant-insect interactions in a powerful new way. It is important to note that the genome of *S. flava* has not yet been sequenced.

In this study, we describe the development of the fly *S. flava* as a model parasite of *Arabidopsis*. We took advantage of tools that had been developed from both *Drosophila* and *Arabidopsis* genomic resources. We first characterized the life cycle of *S. flava* on *Arabidopsis* and placed it in a phylogenetic context. We then quantified variation in *S. flava* performance across a set of canonical *Arabidopsis* defense mutants, across *Arabidopsis* accessions (including adult preference and larval performance) and plants pretreated with elicitors of a defense response. Finally, we tested how *S. flava* xenobiotic metabolism gene expression is mediated by the presence/absence of individual host defense genes.

## Materials and Methods

### Placing *S. flava* in a phylogenetic context

The lineage *Scaptomyza* has been placed within the subgenus *Drosophila* and appears to be monophyletic based on limited sampling (O'Grady & Desalle 2008). Because *S. flava* was never previously included in a DNA sequence-based phylogeny, we used genomes of the three completely sequenced *Drosophila* species in the subgenus *Drosophila* (*D. grimshawi*, *D. mojavensis*, *D. virilis*) to design PCR primers for five nuclear protein coding genes present as single copies (orthologs) in the 12 completely sequenced genomes to be used in a phylogenetic analysis for placing *S. flava* in an evolutionary context. We also PCR amplified and sequenced a fragment of the mitochondrial gene *COI* (encoding cytochrome c oxidase subunit I) from *S. flava* and another species of North American *Scaptomyza*, *S. nigrita*, collected as leafminers from *Cardamine cordifolia* plants in Gothic, Colorado in 2009 (Collinge & Louda 1989). These species were placed in a phylogeny with *COI* sequences from the 12 completely sequenced species and partial or fully overlapping sequences of *COI* from seven other *Scaptomyza* species (O'Grady & Desalle 2008). Orthologous *COI* fragments from the seven other *Scaptomyza* species and the 12 completely sequenced *Drosophila* species were downloaded from GenBank (O'Grady & Desalle 2008).

The five nuclear genes are also candidate xenobiotic metabolism genes whose expression has been shown in other dipteran species to be modulated by plant defense compounds or stress (Li et al. 2008; Matzkin et al. 2006; Mittapalli et al. 2007a; Mittapalli et al. 2007b; Mittapalli et al. 2007c; Toba & Aigaki 2000). The five genes were (*D. melanogaster* annotations): *GstD1* (encoding glutathione S-transferase D1, a canonical Phase II

detoxification enzyme), *Mgstl* (encoding a presumptive microsomal glutathione S-transferase-like, a canonical Phase II detoxification enzyme), *Peritrophin A* (encoding peritrophin A, a chitin binding structural component of peritrophic membrane involved in physical and biochemical protection of midgut epithelium), *PHGPx* (encoding phospholipid hydroperoxidase glutathione peroxidase, an antioxidant enzyme), and *Sod1* (encoding copper/zinc superoxide dismutase 1, a cytoplasmic antioxidant enzyme). We also designed PCR primers for *RpL32* (encoding ribosomal protein 32, typically used as a control in *D. melanogaster* expression studies; e.g., Carpenter *et al.* 2009). Notably, the gene *Sod1* has been successfully used to estimate evolutionary relationships within the family Drosophilidae (Kwiatowski *et al.* 1994; Kwiatowski & Ayala 1999). Importantly, a phylogeny including *Sod1* sequences across the family and from *Scaptomyza* species showed that the lineage was nested within the Hawaiian *Drosophila* (Kwiatowski & Ayala 1999).

We amplified these six genes and the *RpL32* control from cDNA isolated from larvae and adults that had fed on *Arabidopsis* plants. To isolate RNA, fresh larvae and adults were collected from plants and crushed with a mortar and pestle in 1.5 µL Eppendorf tubes following the RNeasy Mini Kit (Qiagen) protocol. Fifteen µL of RNA was used for cDNA synthesis. Primers for the nuclear genes were designed by aligning sequences in Clustal X (Larkin *et al.* 2007) and then submitted to Primaclade (Gadberry *et al.* 2005), which identifies appropriate priming sites. Primers were (5'-3'): GstDIF2 (5'-TGAAGATYAATCCYCAGCACAC-3'), GstDIR2 (5'-RGCCCAGTTCTCATCCCAG-3'), MgstlF1 (5'-TACRTTCTGGRCCGGTGT-3'), MgstlR1 (5'-CACAAAGAAGGMGAGCGCA-3'), PeritrophinAF1 (5'-CAYACCGAGAAGACTGYGATCA-3'), PeritrophinAR1 (5'-CGAASACCTTRTCMTCGC-3'), PHGPxF1 (5'-GGMAACGATGTCTCBCTGG-3'), PHGPxR1 (5'-TGGGATCGGTRGTGGGTG-3'), Sod-1F2 (5'-GCSAAGGGCACHGTTTTTCT-3'), Sod-1R2 (5'-CAACDGTGCGTCCRATAATG-3'), RpL32F1 (5'-CCAGCATAACMGCCCAAG-3'), RpL32R (5'-TTCTGCATCAGCAGCACC-3'). We amplified two fragments of *COI* from *S. flava* and *S. nigrita* using previously published primers. The first set (Folmer *et al.* 1994) was comprised of LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3'). The second set was comprised of Ben (5'-GCTACTACATAATAKGTATCATG-3') (Brady *et al.* 2000) and Jerry (5'-CAACATTTATTTTGG-3') (Simon *et al.* 1994). PCR conditions followed Whiteman *et al.* (2007). The annealing temperature for all PCR reactions was 55°C, and reaction volumes were 25 µL. Sequencing was carried out using BigDye terminator sequencing on both strands using the above primers (Whiteman *et al.* 2007). Contigs from double-stranded sequences were aligned in Se-AL with orthologs of each gene from the 12 sequenced *Drosophila* species. The *RpL32* locus was excluded from phylogenetic analysis because of in-dels in the alignment. None of the sequences contained stop codons. Sequences were subjected to BLAST and in all cases the orthologous *D. grimshawi* genes were the best hits.

We then performed an unrooted maximum likelihood bootstrap search (100 replicates) on the concatenated nuclear gene alignment in Garli (Zwickl 2006). Because of the availability of *Sod1* sequences from a variety of drosophilid species (Kwiatowski *et al.* 1994), including *Scaptomyza* from Hawaii and North America, we also performed a maximum likelihood phylogenetic and bootstrap (100 replicates) analysis in RaxML (Stamatakis *et al.* 2008), using default parameters on an alignment including representatives of drosophilids from previously published studies (Kwiatowski *et al.* 1994; Remsen & DeSalle 1998) and the 12 completely sequenced models. The tree was rooted with *Chymomyza amoena*, which is placed outside of *Drosophila* (O'Grady & Desalle 2008). We then performed another



RaxML search using the *COI* alignment comprising *S. flava* and seven other *Scaptomyza* species, rooting the tree with the mosquito *Anopheles gambiae*.

### Estimation of genome size

In order to determine *S. flava*'s suitability for future genomic sequencing and analysis, we estimated genome size using propidium iodide staining of brain tissue (Gregory & Johnston 2008). *D. melanogaster* Oregon R strain females were used as a reference (0.18 pg genome=175 Mb) and five male and five female adult *S. flava* were assayed and mean size in pg was estimated. These values were compared to those from the 12 sequenced *Drosophila* species (Gregory & Johnston 2008; Bosco *et al.* 2007).

### Characterization of the *S. flava* life cycle and performance across *Arabidopsis* accessions, elicitor pre-treated plants and defense mutants

To characterize the life cycle of *S. flava* on *Arabidopsis*, we surface sterilized and cold-stratified wildtype (Col-0) seeds in 0.1% agarose for three days at 4°C and sowed them in Jiffy #7 peat pellets. These were placed under plastic domes to facilitate germination on a 16 hour light:8 hour dark cycle (using Gro-Lux bulbs) at 22°C, 50% relative humidity, in a Harris walk-in growth chamber at the Museum of Comparative Zoology, Harvard University. Plants were watered every third day and thinned to one plant per pot at day seven. Plants were allowed to grow for four weeks and were then used for experiments at the end of the fourth week. These growing conditions are typical for all experiments described below. Environmental conditions were identical for rearing *S. flava*.

### Oviposition preference and larval performance

For a subset of performance experiments, we allowed females to create feeding punctures and oviposit in leaves of arrayed plants in the fly colony. This was a choice test in which equal numbers of control and experimental plants were randomly placed throughout the bottom of the cage for 24 hours, plants were removed and feeding punctures and eggs were counted on each leaf. This relatively rough measure was used to give an indication of whether the number of feeding punctures predicted the number of eggs laid per leaf. In most cases, larvae were allowed to develop normally and a development time assay was used (below) to test for larval performance, in cases where the leaf area mined assay was not used.

As a measure of larval performance across *Arabidopsis* genotypes, we used a leaf area mined (LAM) assay, a development time (egg to pupa or egg to adult) assay or a body mass assay (adults mass taken upon emergence). We followed the LAM assay as described elsewhere (Gratton & Welter 1998), in which a single larvae is taken from a nurse plant and placed into an experimental or control plant and allowed to feed. Leaf area removed over a time interval is then recorded. Wildtype (Col-0) plants were placed with the *S. flava* colony for 12 hours to allow female flies to feed and oviposit freely. These plants were removed and placed in a tray under plastic domes to synchronize hatching for 48 hours. Larvae were removed from these nurse plants and placed on leaves of control (as described below for each experiment) and experimental plants, one larva per plant, on the same developmentally staged leaf. Larvae were allowed to feed for 2–4 days (depending on the experiment) and leaves were clipped off and digital photos were taken of each leaf while placed flat (on their abaxial surfaces) on a sheet of graph paper with a 1 mm<sup>2</sup> grid. LAM was calculated using the ImageJ freeware (<http://rsbweb.nih.gov/ij/>). In one experiment (performance on Tsu-0 vs. Col-0), leaves were photographed while still attached to the plant and then larvae were allowed to develop to adult eclosion in order to quantify the relationship between LAM and development time. To estimate development time, the larval transfer procedure was used as above, except that individual plants were placed singly in clear acrylic boxes (known as

‘magenta boxes’) with mesh lids. Each day after transfer, plants were examined for the presence of a pupa or an adult and the date and time of pupation or eclosion was recorded. We also allowed larvae to develop to adulthood from the choice experiments and calculated development time as above and in some cases weighed adults upon emergence. Notably, although larvae do consume all tissues within leaves, the LAM assay may be less informative than a weight gain analysis, for example.

### **Arabidopsis accessions and mutants used**

We initially screened for variation in LAM of larvae across five *Arabidopsis* accessions, including Landsberg erecta-0 (Ler-0), Columbia-0 (Col-0), Llagostera-0 (Ll-0), Wassilweskija (Ws-0) and Tsu-0. A large difference in LAM was found between Tsu-0 and Col-0, and we pursued more detailed development time studies on these two accessions as described above. We then screened a battery of defense-related mutants in the Col-0 genetic background for LAM and/or development time, including the JA signaling loss of function mutants *coronatine insensitive 1 (coi1-1*, non-glabrous), *jasmonate insensitive 1 (jin1-2)*, *jasmonate resistant 1 (jar1-1)*, the allene oxide synthase mutant (*cyp74a1*) the aliphatic glucosinolate deficient mutants *myb28myb29* and the indolic glucosinolate deficient *myb51-1*.

To test the effects of plant hormone induction on insect performance, we pre-treated plants with 1mM methyl JA (MeJA) dissolved in ethanol or sterile water+ethanol using the same method (*sensu* Cui *et al.* 2002), and measured LAM as above. In order to test the effect of herbivore associated molecular patterns on insect performance, we also pre-treated plants with 10 or 100 µg/mL crab-shell chitin (Sigma) or sterile water 24 hours prior to adding larvae using blunt syringes as described in Cui *et al.* (2002) (data from the 10 and 100 µg/mL were later pooled) and measured LAM as above.

### **Cloning of CYP81F2 promoter regions and transgenic line construction**

We studied potential induction of indolic glucosinolate biosynthetic genes after leafminer attack by using a *CYP81F2<sub>pro</sub>:GUS* (beta-glucuronidase) reporter line, which was constructed as in Millet *et al.* (2010) (see Supporting Information). *CYP81F2* is involved in the biosynthesis and accumulation of the indole glucosinolates (4-hydroxy-indole-3-yl-methyl and 4-methoxy-indole-3-yl-methylglucosinolate; (Pfalz *et al.* 2009) and in callose deposition (Clay *et al.* 2009; Millet *et al.* 2010). Moreover, this gene is the major gene involved in the metabolic regulation of indolic glucosinolates in *Arabidopsis* (Pfalz *et al.* 2009). The promoter regions of *CYP81F2* (2 kb) were amplified using Expand High Fidelity polymerase(Roche). The reverse primer used was p81F2R3 (5'-CGCGGATCCTGAGTGAAAATGGTGGATGG-3') and introduced an *Bam*HI site. The forward primer used was p81F2L (5'-CGCTCTAGACACGATCAAATCCAAAAGCA-3') and introduced an *Xba*I site. The PCR product was cut with *Bam*HI and *Xba*I and ligated into the multiple cloning sites of pBI101(Jefferson *et al.* 1987), which confers resistance to kanamycin. The *CYP81F2<sub>pro</sub>:GUS* constructs obtained were sequenced and transformed into *Agrobacterium tumefaciens* strain GV3101(Koncz & Schell 1986). Transgenic *Arabidopsis* Col-0 plants were generated using standard floral dip transformation methods and progeny selected on kanamycin as described previously (Clough & Bent 1998).

Four week old *CYP81F2<sub>pro</sub>:GUS* plants were either placed with ovipositing female flies for 24 hours, removed and then assayed for GUS activity, or second instar larvae were transferred from Col-0 nurse plants and placed on leaves of the *GUS* reporter line, allowed to feed for 24 hours, removed and then assayed for GUS activity as described in Denoux *et al.* (2008) and observed using a Discovery V12 microscope (Zeiss). Specifically, ten milliliters of GUS substrate (50 mM sodium phosphate, pH 7, 10 mM EDTA, 0.5 mM

$K_4[Fe(CN)_6]$ , 0.5 mM  $K_3[Fe(CN)_6]$ , 0.5 mM X-Gluc, and 0.01% Silwet L-77) was poured around each leaf in a tube. Leaves were vacuum-infiltrated for five minutes and incubated at 37°C for 4 hours. Leaves were then fixed with a 3:1 ethanol:acetic acid solution at 4°C overnight and placed in 95% ethanol. Finally, leaves were cleared in lactic acid and photographed using a Zeiss Discovery V12 microscope.

### Interactions between reactive oxygen species in *Arabidopsis* and *S. flava* herbivory

Reactive oxygen species, including hydroxyl radical ( $OH\bullet$ ) and superoxide ( $O_2^-$ ), serve to defend the plant against natural enemies directly as biocidal molecules and as signaling molecules, through the hypersensitive response. In *Arabidopsis*, we asked whether an ROS precursor, hydrogen peroxide ( $H_2O_2$ ) is produced in response to leafminer attack and leaf puncturing by adult female flies. In this assay, we stained leaves with 3,3'-diaminobenzidine tetrahydrochloride (DAB) following the protocol in Clarke (2009). Hydrogen peroxide polymerizes DAB and produces a brown color. In brief, four-week old Col-0 plants were exposed to adult female flies and leaves were harvested 48 hours thereafter. In separate experiments, third instar larvae were removed from Col-0 nurse plants and were placed into small holes in petioles, made with forceps. Larvae were left for 48 hours and then leaves were assayed for  $H_2O_2$ . Leaves were cleared with ethanol and placed under a dissecting light microscope for imaging. We also used nitro blue tetrazolium (NBT) staining to determine specifically whether superoxide was present after wounding, using the same protocol as above, except that clearing with ethanol was not required.

### Candidate genes for leafminer xenobiotic metabolism and quantitative, real time PCR

We transferred larvae in the second instar (hatching was synchronized) from Col-0 nurse plants to (1) four week old Col-0 (control) plants or *jar1-1* plants and (2) four week old Col-0 plants in which three basal leaves had been infiltrated as above with 10–100  $\mu$ g/mL of crab shell chitin or water (control), 24 hours prior to adding larvae to systemic (non-infiltrated) leaves. Larvae (N=5–7 late second instar larvae were used for each of three biological replicates for each treatment) were allowed to feed for 48 hours and leaves were detached and LAM measured as above. Larvae were extracted from leaves with forceps, and frozen immediately in liquid nitrogen and stored at  $-80^\circ\text{C}$  until RNA extractions were initiated. The candidate xenobiotic metabolism genes *GstD1* and *Peritrophin A* and the control gene *RpL32* were amplified from *S. flava* cDNA and used in a gene expression analysis. Primers for real-time quantitative PCR for each of the genes (5'-3') were: *GstD1*rtF2 (TCGGATTCTGAACACCTTC), *GstD1*rtR2 (GTAGCCAGCAACCTCGAAAG), which amplified a 112bp fragment of *GstD1*, *PerAF1* (GCGATCAGTTTTCTCTGC), *PerAR1* (GGCCCAATTGTAGTTGCAGT), which amplified a 112 bp fragment of *Peritrophin A*, *RpL32*rtF1 (GGACCGTTATGCCAAGTTGT), and *RpL32*rtR1 (CGCTTGTTGGAACCATAACC), which amplified a 117 bp fragment of *RpL32* and spanned an exon junction. RNA was isolated as described above and was reverse transcribed using iScript Reverse Transcriptase (Bio-Rad) using the manufacturer's protocol. *Peritrophin A*, *GstD1* and *RpL32* cDNAs were amplified using iQ SYBR green Supermix (Bio-Rad) with primers above. The amplification protocol was three minutes at 95°C followed by 45 cycles of 95°C for 10 seconds, 55°C for 30 seconds and 72°C for 30 seconds. Reactions were performed in triplicate on a MyiQ BioRad real time thermocycler and single color detection system in 25  $\mu$ L reaction volumes with 5  $\mu$ L of template. We created a standard 10-fold dilution series to test efficiency of primers and conducted a melting curve analysis to determine the number of products amplified. Relative abundance of transcripts was calculated following (Pfaffl 2001) and technical and biological replicates were averaged.



## Results

### Collection of wild *S. flava* accessions and culturing of *S. flava* on *Arabidopsis*

We collected leafminers from wild mustards (*Barbarea vulgaris*) in Cambridge and Belmont, Massachusetts in June-August 2007 and again in 2008. Leaves or whole plants were brought into the laboratory and placed in containers with water (leaves) or soil (plants) and larvae were allowed to complete development in mesh cages. Adult flies were identified unequivocally as *S. flava* using taxonomic keys (Hackman 1959; Baechli *et al.* 2004). Females have distally blunt, dentate ovipositor guides. Adult flies were aspirated out of the rearing cages and placed with four-week old *Arabidopsis* plants of accession Columbia (Col-0), which is the background of most mutant and transgenic lines used by the *Arabidopsis* community. Cages were placed in a walk-in growth chamber on a 16 hour light: 8 hour dark cycle (using Gro-Lux bulbs) at 22°C. After about one week, female flies began piercing leaves of *Arabidopsis* from the abaxial surface of the leaves with their dentate ovipositors and were observed to drink the exudates from these wounds (Video 1 in Supplementary Information). The *S. flava* colony was subsequently allowed to feed and develop *ad libitum* on *Arabidopsis*. Several isofemale lines were initiated after *S. flava* lines were established on *Arabidopsis* in the laboratory.

### Placing *S. flava* in a phylogenetic context

Using PCR, we obtained the following amplification products from cDNA isolated from the 2008 *S. flava* collection propagated on *Arabidopsis*: a 438bp fragment of *GstD1*, a 248 bp fragment of *Mgstl*, a 486bp fragment of *Peritrophin A*, a 347 bp fragment of *PHGPx*, and a 372 bp fragment of *Sod1* which are deposited in GenBank (accession numbers HQ000008-HQ000014). This resulted in a concatenated dataset of 1891 bp to be compared among *S. flava* and the 12 sequenced *Drosophila* species. Results from the maximum likelihood multi-gene phylogenetic analysis in Garli, limited to the 12 sequenced *Drosophila* species, place *S. flava* sister to *D. grimshawi*, with high bootstrap support (Fig. 1), which is entirely consistent with the placement of the *Scaptomyza* lineage as sister to the Hawaiian *Drosophila* radiation (O'Grady & Desalle, 2008). Importantly, the topology shown in Fig. 1 that included the 12 models is otherwise identical to the analysis including all orthologous protein coding genes in the genome (Clark *et al.* 2007). We also carried out phylogenetic analysis using the partial *S. flava* Cu, Zn *Sod1* sequence and corresponding *Sod1* sequences from 27 other drosophilid species, including three *Scaptomyza* species (Kwiatowski *et al.* 1994; Kwiatowski & Ayala 1999; Remsen & DeSalle 1998) (Supporting Information Figure 1). This analysis places *S. flava* within the *Scaptomyza* lineage with high bootstrap support. We also obtained a 942 bp fragment of the mitochondrial *COI* gene from *S. flava* and a 330 bp fragment from *S. nigrata* (nested within the 942 bp fragment from *S. flava*) and these are deposited in GenBank (accession numbers HM991724 and HM991725). The resulting phylogeny was relatively unresolved, but placed *S. flava* sister to *S. nigrata* in a clade with all other *Scaptomyza* species and embedded within the *Drosophila* genus (Supporting Information Figure 2). The subgenus *Drosophila* in which *Scaptomyza* is known to be embedded, was not recovered as monophyletic.

### Estimation of genome size

Using propidium iodide flow cytometry (see Materials and Method), we estimated the genome size of *S. flava* females and males to be 0.3 pg ( $\pm 0.004$  SDM) or 293 Mb ( $\pm 4.24$  SDM) and 0.29 pg ( $\pm 0.01$  SDM) or 287 Mb ( $\pm 11.84$  SDM), respectively. The average genome size for female *S. flava* was slightly smaller than that of *D. virilis* (0.34 pg for males and females), which has the largest genome size among the 12 completely sequenced species.

### Characterization of the *S. flava* life cycle and performance across *Arabidopsis* accessions

The life cycle of *S. flava* was completed in roughly three weeks in our growth chamber conditions (Fig. 2). Specifically, twenty Col-0 plants were exposed to >200 ovipositing females for 24 hours. These females created, on average, 64.5 ( $\pm 37.85$ ) feeding punctures and laid 3.15 ( $\pm 1.46$ ) eggs/plant, from which 42 adults emerged. Average egg-adult development time was 20.52 days ( $\pm 0.86$  SDM). The relationship between feeding puncture number and eggs laid per plant individual was positive and significant (Pearson's  $r = 0.6$ , two-sided  $P < 0.01$ ). Larvae typically hatched within 48 hours of oviposition and in most cases, created a serpentine mine in the direction of the midvein, tunneled along the midvein and continued feeding in the direction of the petiole (Fig. 3). Once at the petiole, larvae continued to feed and moved back into the lamina. Larvae completed three larval instars and remained in the same leaf or plant during development, but occasionally moved between leaves and plants. Pupation occurred within the leaves or in the soil.

To evaluate adult female preference for *Arabidopsis* accessions, a colony of flies was simultaneously exposed to individual plants of four *Arabidopsis* accessions for 24 hours (Col-0, N=10; Ler-0, N=9; Ll-0, N=10; Tsu-0, N=9; Ws-0, N=12) in an oviposition and feeding choice assay. Females created significantly more feeding punctures per plant in Tsu-0 than in Ll-0 and Col-0 (ANOVA and Tukey's HSD,  $F=4.334$ ,  $df=4$ ,  $P < 0.01$ ) and laid significantly more eggs in Tsu-0 than in the other accessions (ANOVA and Tukey's HSD,  $F=4.83$ ,  $df=4$ ,  $P < 0.01$ ) (Fig. 4).

We focused on comparisons between Tsu-0 and Col-0 because of the clear preference females had for Tsu-0 relative to the Col-0 accession and because of previous reports of susceptibility of Tsu-0 and resistance of Col-0 to specialist chewing herbivores (Pfalz *et al.* 2007). We tested whether larvae consumed leaf tissue at a faster rate when reared on Tsu-0 than when reared on Col-0 and whether development time (egg to adult eclosion) was faster when larvae were reared on Tsu-0 than on Col-0. Single larvae were transferred from Col-0 nurse plants at day four of development to Col-0 (N=37) and Tsu-0 (N=36) experimental plants and leaves were digitally photographed four days later for LAM calculations. LAM was inversely and significantly correlated with egg to adult development time (Pearson's  $r = -0.35$ ,  $P < 0.01$ ) across plants. Larvae consumed significantly more leaf tissue (one-tailed t-test,  $P < 0.00001$ ) on Tsu-0 (average=200.23 mm<sup>2</sup>  $\pm 65.31$  SDM) than on Col-0 (132.78 mm<sup>2</sup>  $\pm 66.56$  SDM) plants over a four-day interval after transfer. Total development time (egg to adult emergence) was on average, 1.27 days shorter (one-tailed t-test,  $P < 0.00001$ ) in larvae reared on Tsu-0 (average number of days=20.08  $\pm 1.08$  SDM) than larvae reared on Col-0 (average number of days=21.35  $\pm 1.55$  SDM) (Fig. 5).

### Characterization of the *S. flava* life cycle and performance on *Arabidopsis* elicitor pre-treated plants and defense-related mutants

We screened *Arabidopsis* jasmonate-dependent defense mutants or plants pre-treated with the defense-related hormone MeJA or the defense-related elicitor crab shell chitin 24 hours prior to adding larvae, using the LAM assay in which larvae fed for 48 hours after transfer from Col-0 nurse plants (Fig. 6). *S. flava* larvae consumed significantly more leaf tissue on the canonical JA signaling deficient mutant *coil-1* and the JA biosynthetic mutant *cyp794a1*, consistent with the well-studied role of jasmonates in insect defense responses (Jander & Howe 2008). A similar trend was observed with a *jar-1* mutant, although the increase in leaf area mined was not statistically significant, consistent with the observation that *jar-1* mutants are not completely deficient in JA signaling (Suza & Staswick 2008). Correspondingly, *S. flava* larvae consumed less leaf tissue on plants pre-treated (sprayed) with methyl JA. Larvae consumed the same amount of tissue on *jin1-2* mutants as wildtype. It was not surprising that the transcription factor mutant *myb51-1*, which is deficient in

indole glucosinolate biosynthesis, was also significantly more susceptible to *S. flava* herbivory, and a similar trend was observed with the *myb28myb29* double mutant, which is deficient in aliphatic glucosinolate biosynthesis and susceptible to other herbivores. Finally, plants sprayed with chitin, which is known to elicit a plant defense response against fungal pathogens and is a major component of insect exoskeletons, also exhibited resistance to *S. flava* herbivory.

We further explored the interaction between the loss of JA signaling and *S. flava* performance by assaying development time and body mass of emerged adults. We first exposed Col-0 (N=20 plants) and *coi1-1* (N=20) as above, and recorded number of eggs laid, days until pupation and date of adult eclosion. Flies reared on *coi1-1* pupated significantly earlier (one-sided t-test,  $P < 0.00001$ ), pupating at an average of 10.29 days ( $\pm 0.11$  SDM) on *coi1-1* versus 11.13 days ( $\pm 0.09$  SDM) on Col-0. Pupae eclosed as adults on average 1.06 ( $\pm 1.73$  SDM) days earlier (one-sided t-test,  $P < 0.001$ ) than those reared on Col-0 (Fig. 7). There was no significant difference in survivorship of larvae when reared on Col-0 (24 eggs laid, 15 adults emerged) versus *coi1-1* (25 eggs laid, 17 adults emerged) (Chi-square test,  $P > 0.05$ ). In a separate experiment, we exposed Col-0 (N=27 plants) and *coi1-1* (N=34 plants) to ovipositing female flies for 24 hours, removed the plants, allowed larvae to develop and measured body mass of males and females. Females (one-sided t-test,  $P < 0.01$ ) and males (one-sided t-test,  $P < 0.05$ ) weighed significantly more when reared from egg to adult on *coi1-1* plants than on Col-0 plants (Fig. 8).

As shown in Fig. 6, the transcription factor mutants, *myb51* and *myb28myb29*, were more susceptible to *S. flava* herbivory. Consistent with this result, the indole glucosinolate pathway gene *CYP81F2* was induced in leaves of *CYP81F2<sub>pro</sub>:GUS* plants in response to adult female feeding punctures and GUS activity was detected immediately around feeding punctures. In *CYP81F2<sub>pro</sub>:GUS* plants exposed to ovipositing females, GUS activity was strongest around feeding punctures with eggs (Fig. 9). In *CYP81F2<sub>pro</sub>:GUS* plants with larvae transferred from Col-0 plants that had been allowed to feed for 24 hours, GUS activity was strong along the mid-vein of the leaf, in the petiole, and in smaller veins in areas that had been damaged by *S. flava* larvae.

### Interactions between reactive oxygen species in *Arabidopsis* and *S. flava* herbivory

Leaves of plants placed with ovipositing female flies for 48 hours showed evidence of feeding punctures, and in some cases first instar larvae formed serpentine mines. Staining with DAB revealed  $H_2O_2$  production around the periphery of most feeding punctures (Fig. 10). DAB staining also revealed  $H_2O_2$  production along the walls of a mine formed by a neonate larva. Staining superoxide with NBT revealed similar patterns around feeding punctures (Fig. 11), superoxide was observed around the peripheries of most feeding punctures. Leaves stained with NBT also revealed collapsed and apparently dead tissue in several areas of high feeding puncture density. This tissue did not stain blue and appeared desiccated, similar to the plant hypersensitive response that is elicited by *R*-gene mediated resistance to bacterial, fungal, and viral pathogens. Eggs were observed within some feeding punctures, but no superoxide was observed immediately around them.

### Candidate genes for leafminer xenobiotic metabolism and quantitative, real-time PCR

Larvae reared on the JA deficient *jar1-1* mutant exhibited down-regulation of transcripts of *GstD1* and *Peritrophin A* relative to larvae reared in wildtype (Col-0) plants, normalized with *RpL32* transcript abundance (Fig. 12). Larvae reared on wildtype plants pre-treated with chitin exhibited upregulation of transcripts of *GstD1* and *Peritrophin A* relative to larvae reared in wildtype plants pretreated with the water control, normalized with *RpL32* transcript abundance (Fig. 12).

## Discussion

### A post-genomic plant-arthropod parasite system

Studies of plant-herbivore interactions that integrate genetic, functional and ecological approaches include the parsnip webworm-parsnip system (e.g., Berenbaum 1983; Zangerl & Berenbaum 2003; Li *et al.* 2004), the wild tobacco-sphinx moth system (e.g., Lou & Baldwin 2003; Steppuhn *et al.* 2008; Zavala *et al.* 2008), and the *Arabidopsis/Boechea* spp.-associated herbivore systems (Kliebenstein *et al.* 2001a; Ratzka *et al.* 2002; Wheat *et al.* 2007; Wittstock *et al.* 2004; Vogel *et al.* 2007; Broekgaarden *et al.* 2007). However, functional genomic studies at the plant-insect interface have mostly emphasized the plant side of the equation (Zheng & Dicke 2008; Ramsey *et al.* 2007; The International Aphid Genomics Consortium 2010; Whiteman & Jander 2010). This imbalance is likely to change rapidly in the post-genomic era, as the genomes of many other herbivorous species become available. Nonetheless, *Drosophila* species are the best-characterized insects from a genomics point of view, and the most easily manipulated from a functional genetics perspective, tools that took decades to develop. Thus, an herbivore that is situated phylogenetically within the *Drosophila* clade and that feeds on *Arabidopsis* is ideal and highly tractable in a post-genomics context.

Here, we show that the drosophilid fly, *Scaptomyza flava*, is a promising model herbivore of *Arabidopsis*. Because this species feeds on *Arabidopsis* in the wild, both in Europe (Mitchell-Olds 2001) and in North America (Chittenden 1902), it is also ecologically relevant (Harvey *et al.* 2007). It is readily cultured on mustards in the laboratory, including on *Arabidopsis* accessions and associated mutants, and interacts with *Arabidopsis* in a similar manner to other chewing herbivores (e.g., Jander *et al.* 2001; Cui *et al.* 2002). The tools of *Arabidopsis* can thus be used to study how the fly interacts with specific host plant defense-response pathways. For example, recombinant inbred populations can be used to identify quantitative trait loci underlying resistance/susceptibility (Bidart-Bouzat & Kliebenstein, 2008; Jander *et al.* 2001; Kliebenstein *et al.* 2001a; Kliebenstein *et al.* 2001b; Zhang *et al.* 2006). Moreover, because *S. flava* is nested phylogenetically within the subgenus *Drosophila*, genomic tools and eventually, genetic tools, from this model insect group can be leveraged. In a proof-of-principle study, we used a PCR-based approach to clone several putative xenobiotic metabolism genes from *S. flava* using orthologous genes from the 12 *Drosophila* species from which primers were designed, followed by expression studies of the genes in larvae reared in an *Arabidopsis* jasmonic acid signaling mutant and plants treated with chitin, a potential HAMP. The results of these studies show that *S. flava* is interacting with *Arabidopsis* like a typical chewing herbivore. In summary, the *Arabidopsis* – *S. flava* system can take advantage of the suite of genomic and genetic tools developed for both *Arabidopsis* and *Drosophila*.

### Phylogenetic context and genome size of *S. flava*

In a five gene phylogeny that included the 12 sequenced *Drosophila* species, we found that *S. flava* is phylogenetically nested within the subgenus *Drosophila* (*Drosophila* is paraphyletic) and sister to *D. grimshawi*, consistent with the placement of *Scaptomyza* in previous studies as sister to the Hawaiian *Drosophila* radiation (O'Grady & Desalle, 2008). Based on multiple lines of phylogenetic evidence, including near complete mitochondrial genomes (O'Grady & Desalle, 2008) and taxonomically broad sampling, it is hypothesized that the *Scaptomyza* lineage arose in Hawaii and some individuals secondarily dispersed to the mainland. Hawaii is the center of drosophilid diversity and >1000 species are found there (Kaneshiro 1997). Of the 272 species of *Scaptomyza*, 161 (60%) live only in Hawaii. The remaining 40% are found elsewhere around world. Little is known about the natural history of *Scaptomyza* in Hawaii, although larval ecologies are diverse. For example, larvae

of some species feed on microflora associated with morning glory (*Ipomaea* spp.) and on spider egg sacs (Magnacca *et al.* 2008).

Before genome sequencing can begin, it is necessary to quantify genome size of *S. flava*. Our analyses indicate that the *S. flava* genome is approximately 290 Mb in size, considerably larger than that of *D. melanogaster* (ca. 175 Mb), but smaller than the largest *Drosophila* species sequenced to date, *D. virilis* (>300 Mb). Differences between male and female *S. flava* are likely due to the fact that males have a Y chromosome and females two X chromosomes. The Y is inferred to be smaller than the X in this case. Thus, the genome size of *S. flava* is relatively large in the context of other *Drosophila*, but similar to that of some of the *Drosophila* species already sequenced.

### **Characterization of the *S. flava* life cycle and performance across *Arabidopsis* accessions, elicitor pre-treated plants and defense mutants**

*S. flava* completes its life cycle on *Arabidopsis* accession Col-0 in approximately 21 days at 22°C. This is a longer interval than development time for *D. melanogaster* and other non-leafmining *Scaptomyza*, but perhaps not surprising given that larvae must cope with a dynamic and inducible host defense response while feeding and developing in living plants that actively defend themselves. Moreover, we have shown that variation in development time is quantitatively related to the specific inducible host plant defense pathways. While saprophytic and microbe-feeding *Drosophila* must also encounter host plant defense molecules, they are not typically living in or on organs of a host plant that can mount a defense response. Future studies could examine the genetic basis and genomic consequences of this transition from a microbe-feeding ancestor (Markow & O'Grady, 2005) to a truly herbivorous, parasitic lifestyle.

*S. flava* adult females readily created feeding punctures and oviposited on all *Arabidopsis* lines to which they were exposed, including various accessions, knockout mutants and reporter lines. The fact that number of feeding punctures and number of eggs laid was positively related suggests that oviposition preference and larval performance could be linked in this species. When an egg hatches in the distal parts of the lamina, a larva typically creates mines toward the midvein, traveling along it and ending up in the petiole. The adaptive value of this is unknown, but leafmining moths have long been known to create 'green windows' in senescing autumn leaves that are rich in photosynthate (Giron *et al.* 2007). Thus, leafminers are hypothesized to manipulate host plant physiology to their benefit. Plant cytokinins are elevated in these regions and are associated with senescence and photosynthesis activity generally, and were recently shown to be potentially synthesized by *Wolbachia* endosymbionts of the leafminers (Kaiser *et al.* 2010). It is possible that the leafminers are physically disrupting plant signaling by mechanically damaging vessels that could transport signals to and from the leaf. Alternatively, leafminers could take refuge in the thicker-walled petioles during parasitoid attack, considering that leafminers are highly susceptible to parasitoids (Connor & Taverner 1997), and approximately 50% of wild *S. flava* pupae that we isolated were parasitized by *Dacnusa* braconid wasps. Leafminer feeding behavior could also be stimulated by glucosinolates, which are higher along the midvein than other areas of the leaf (Shroff *et al.* 2008). These hypotheses are not mutually exclusive and can be readily tested using the abundant genetic and physiological tools available for *Arabidopsis*.

We found that females preferred feeding and ovipositing on the accession Tsu-0 relative to Col-0. Larvae consumed more leaf tissue when feeding on Tsu-0 in the LAM assay than when feeding in Col-0 and development time was also shorter when flies were reared in Tsu-0 plants than in Col-0 plants. Thus, in this case oviposition preference and larval performance appear to be linked, consistent with the 'naïve adaptationist' hypothesis



(Levins & MacArthur 1969), whereby selection acts to maximize oviposition on plants where larvae perform the best (Via 1984). Linkage of these phenotypes seems reasonable, but surprisingly is not always found in other herbivore species. Moreover, the implications for preference-performance correlation or anti-correlation have important implications for our understanding of host switching and sympatric speciation (Berlocher & Feder 2002). We have collected *S. flava* from *Barbarea vulgaris*, *Brassica* sp., *Lepidium* sp., and *Alliaria petiolata* in New England and larval transfers to *Cakile* sp. resulted in successful adult eclosion, suggesting that the *S. flava* lineage we are studying is specialized on mustards, but can feed on a variety of Brassicaceae (Beilstein *et al.* 2006). Preliminary data suggest that development time on *Barbarea verna* is equivalent to that on *A. thaliana*, averaging 20 days from egg to adult (Whiteman *et al.* unpublished data). Because there is a correlation between these two traits in *S. flava* using *Arabidopsis* accessions, this could be a useful system with which to explore the genetic basis of this preference-performance correlation as well as the genetic basis of the traits in the host plant driving these differences. In leafminers, there appears to be more variation within a species in host plant suitability than in free-living herbivores (Mattson *et al.* 1988). Leafminers are typically restricted to a single leaf or host plant, so there should be strong selective pressure on females to choose plant individuals (genotypes) in which their larvae perform best. Importantly, Tsu-0 has been shown to be more susceptible to specialist caterpillars (*Pieris* spp. and *Plutella xylostella*) than Col-0 (Pfalz *et al.* 2007), which is resistant. Conversely, Tsu-0 has been shown to be more resistant to the biotrophic (requiring living tissue) gram negative plant pathogenic bacterium *Pseudomonas syringae* than Col-0, in an *avrRpt2* dependent fashion (Mauricio *et al.* 2003). Tsu-0 is also resistant to the biotrophic pathogen *Plasmodiophora brassicae* (Alix *et al.* 2007) and Cauliflower mosaic virus (Agama *et al.* 2002).

However the linkage between adult preference and larval performance might be influenced by the following factors: (i) leafminers might sometimes change leaves during their development, (ii) variations at the microscales can allow insects to adapt their nutritional intakes even if they are restricted within a small leaf area, (iii) leaves are a very dynamic resource and the nutritional quality of leaves experienced by laying females most likely differs with that of the nutritional environment experienced by developing larvae, (iv) leafminers might be able to manipulate the host plant physiology thus limiting the impact of the initial leaf composition.

Our screens of the *Arabidopsis* defense mutants and histochemical assays revealed, most importantly, that the JA pathway and the glucosinolate pathway are important in mediating a full defense response against *S. flava* attack, a response typical for most chewing herbivores (Beekwilder *et al.* 2008; Jander & Howe 2008). Induction of indolic glucosinolates is regulated by the JA pathway (Halkier & Gershenzon 2006), and biosynthetic and regulatory mutants of the JA pathway (*coil-1*, *jar1-1* and *cyp74a1*) were susceptible to *S. flava*. Moreover, pre-treating wildtype plants with methyl JA resulted in induced resistance to *S. flava*. LAM was greater, egg-adult development time was shorter, and body mass of adults was higher when individuals were reared on *coil-1* plants. This suggests that *Arabidopsis* defenses modulated by JAs provide protection against *S. flava* attack, and the glucosinolate mutant assays are consistent with this. However, the *jin1-2* mutant was not susceptible to herbivory, in contrast to the findings of Dombrecht *et al.*, (2007). On the other hand, our results are consistent with those of Lorenzo *et al.* (2004), in which all *jin1* mutant alleles tested showed a slight, but reproducible increase in resistance against necrotrophic pathogens. The gene *jin1* encodes the AtMYC2 transcription factor, which regulates expression of two branches of the JA pathway. One branch is related to pathogen infection and the other to wounding. JIN1 expression can have different effects on these two branches of the JA pathway. Thus, this mutant expresses a more complex phenotype than the other JA mutants assayed. Larvae consumed more tissue when reared on the indolic pathway

regulatory mutant *myb51-1* than on wildtype. The GUS reporter assays of *CYP81F2* activation revealed that the indolic glucosinolate pathway was induced upon *S. flava* attack, either by ovipositing females or by larvae. The finding of strong induction around eggs, but not simple punctures by females, is consistent with studies of pierid butterflies that showed that *Arabidopsis* defenses are elicited by egg cement (Little *et al.* 2007). These data indicate that there is an elicitor on or in *S. flava* eggs or in the reproductive tract of female flies to which *Arabidopsis* responds by inducing *CYP81F2* gene expression.

The glucosinolate-dependent result is somewhat surprising given that *S. flava* is a mustard specialist that is presumably adapted to the presence of toxic glucosinolates *sensu* Frankel (1959). However, there is growing evidence that specialists also pay a cost associated with feeding on these plant toxins (Agrawal & Kurashige 2003), despite harboring unique pathways for coping with them (Wittstock *et al.* 2004). It would be interesting to know whether the glucosinolate detoxification pathways in *S. flava* are similar to those in the specialist or generalist Lepidoptera.

Plants pre-treated with chitin also showed resistance to *S. flava* larvae, consistent with the fact that chitin elicits a major defense response in *Arabidopsis* and other plants, including production of chitinases and protease inhibitors (Zhang *et al.* 2002) that are ecologically important plant defenses against insects (Lawrence & Novak 2006).

Assays for reactive oxygen precursors and species showed that *Arabidopsis* produced a reactive oxygen burst and hydrogen peroxide burst after attack by *S. flava* females and after larval feeding. Hydrogen peroxide was produced in leaf tissues actively being mined suggesting that these molecules are also reaching the insect gut where they can be highly damaging (Collins *et al.* 2010). Staining with NBT revealed no superoxide produced within collapsed tissues in areas surrounded by dense feeding punctures of adult females. This suggests that the plant is initiating a type of hypersensitive response that results in local cell death and subsequent desiccation of the areas affected. This resulted in death of eggs due to desiccation, although this was not quantified. Egg desiccation could be a very simple, effective method of killing *S. flava*, containing possible further damage. A hypersensitive response in plants against insects has been reported previously (Fernandes 1990). Interestingly, NBT staining revealed little superoxide around eggs, which could be due to the fact that punctures are made before eggs are laid and eggs are pushed into the leaf area around the wound (the lumen of the wound) causing very little tissue damage.

In summary, the screen of defense mutants, the indolic glucosinolate reporter line, the plants pretreated with methyl JA or chitin and the reactive oxygen species assays, all revealed that full resistance against *S. flava* is modulated by the JA pathway, the glucosinolate pathway and potentially the reactive oxygen burst in *Arabidopsis*. Future studies from the plant's perspective might examine transcriptional, metabolomic and proteomic responses to *S. flava* attack in *Arabidopsis*.

Finally, we studied the absence of a major plant defense gene, *JAR1*, and how pre-treatment of the plant with the MAMP and potential HAMP, chitin, influenced the regulation of candidate xenobiotic metabolism genes in the leafminer. Quantitative expression readouts were obtained in larvae reared on wildtype, the JA-pathway deficient mutant *jar1-1* and in plants pre-treated with chitin or water. Readouts were obtained of two candidate xenobiotic metabolism genes, *GstD1* and *Peritrophin A* and a housekeeping gene *RpL32*. *GstD1* is an important detoxification enzyme in *Drosophila* species involved in pesticide resistance, including to DDT (Tang, Tu, 1994; Trinh *et al.* 2010), as well as resistance to cactus secondary metabolites (Matzkin *et al.* 2006), caffeine and nicotine (Trinh *et al.* 2010). *Peritrophin A* is a structural, chitin-binding protein integral to the peritrophic membrane (a

semi-permeable chitinous matrix lining the midgut of most insects). It is significantly upregulated in response to xenobiotics in Hessian fly reared on resistant wheat (Mittapalli et al. 2007c). More generally, the peritrophic membrane serves to protect the mid-gut epithelium from microorganisms, mechanical damage, large plant secondary metabolites and reactive oxygen species. Importantly, the peritrophic membrane serves as a physical antioxidant in herbivorous insects, protecting the epithelium from hydroxyl radicals and reducing hydrogen peroxide in the midgut of *Helicoverpa zea* (Summers, Felton, 1998).

We found that these two candidate xenobiotic metabolism genes were both downregulated in larvae reared on the JA mutant *jar1-1* relative to those reared on wildtype (Col-0) plants, and upregulated in larvae reared on plants pre-treated with chitin relative to those reared on plants pre-treated with water. These results are consistent with the hypothesis that the two genes are involved in xenobiotic metabolism. The JA mutants are susceptible to pathogens and herbivores, and larvae reared on them feed more and develop more quickly than on wildtype plants. Thus, anti-herbivore defenses are likely to be less robust in this mutant than in wildtype plants and the expression data from these two genes are a reflection of this. Conversely, larvae reared on chitin pre-treated plants ate less than larvae reared on water pre-treated plants, consistent with microarray data showing that a suite of potential anti-herbivore genes are induced upon chitin treatment. Moreover, infiltrating with chitin causes significant increases in protease inhibitors in leaves of tomato (Walker-Simmons & Ryan 1984). These are the first such gene expression studies to our knowledge on leafminers, and are a useful point of comparison to similar studies on the galling Hessian fly. Upregulation of glutathione transferases and peritrophins was observed when larvae were reared on incompatible relative to compatible wheat lines (Mittapalli et al. 2007a; Mittapalli et al. 2007b; Mittapalli et al. 2007c), suggesting that general patterns and pathways might underpin insect resistance against plant defenses.

In conclusion, we have placed *S. flava* in a phylogenetic context, developed performance assays for adults and larvae, quantified variation in *S. flava* adult preference and larval performance across accessions, identified that the JA and glucosinolate pathways are important in defense against *S. flava*, and showed that candidate xenobiotic metabolism gene expression is dependent on the activation or disruption of JA pathway in *Arabidopsis*. Species in the genus *Scaptomyza* exhibit among the most varied ecologies and distributions of any in the Drosophilidae, including several species that feed on mustards in the wild, and possibly independent colonization of mustards. Because *Scaptomyza* species are most likely derived from microbe-feeding ancestors and now show considerable variation in life history, this lineage also provides a useful context in which to explore the mechanistic basis of a transition to herbivory in insects, as well as the functional basis of host specificity. Subsequent research currently being conducted utilizes a reverse genomics approach, relying on next generation sequencing, to characterize the transcriptome of *S. flava* in response to the presence or absence of canonical plant defense pathways. This approach is more powerful than a candidate gene approach because there is no ascertainment bias and it does not rely on *a priori* information. Sequencing the full genome of *S. flava* will facilitate the analysis of behavioral, molecular genetic, and physiological interactions between *S. flava* and *Arabidopsis*, and open the door to comparative studies with other *Scaptomyza* species. Developing transgenic *S. flava* flies (e.g., for RNAi experiments) and conducting transgenesis experiments will also be essential in studying the function of putative genes important in herbivory in general and detoxifying glucosinolates in particular. This system will also be useful for studying three-way interactions between plants, insects and pathogens such as *Pseudomonas syringae* because each actor is genomically tractable.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

NKW was supported by a Kirschstein National Research Service Award (NRSA) from the National Institutes of Health (F32AI069732 to NKW), FMA was supported by a grant from the National Science Foundation (grant MCB-0519898 to FMA) and a grant from the National Institutes of Health (grant GM48707 to FMA), and NEP was supported by a grant from the National Science Foundation (grant SES-0750480 to NEP). NWK, SCG, FAM and NEP were supported by a grant from the Harvard University's Provost Fund for Interfaculty Collaboration and a grant from the Harvard University Science and Engineering Committee Seed Fund for Interdisciplinary Science. We thank Jen Bush for advice on culturing of *Arabidopsis*, Andrew Brownjohn, Benjamin Goldman-Huertas, Sondra Lavigne, Christopher Meehan, and Matan Shelomi for assistance with laboratory work and culturing of *S. flava*. We thank Mark Beilstein for advice on the phylogenetic analyses and William Gelbart, Patrick O'Grady, and Therese Markow for useful discussions of *Drosophila* biology. We thank Gabe Miller for photographs of *Arabidopsis*. We thank Paul H. Williams for telling NEP about leafmining flies of *Arabidopsis* in a meeting hosted by Ed and Kyoko Jones as part of the Japan Fulbright Masters Teacher Program in 2004.

## References Cited

- Agama K, Beach S, Schoelz J, Leisner SM. The 5' third of Cauliflower mosaic virus Gene VI Conditions Resistance Breakage in *Arabidopsis* Ecotype Tsu-0. *Phytopathology*. 2002; 92:190–196. [PubMed: 18943093]
- Agrawal AA, Kurashige NS. A role for isothiocyanates in plant resistance against the specialist herbivore *Pteris rapae*. *J Chem Ecol*. 2003; 29:1403–1415. [PubMed: 12918924]
- Alix K, Lariagon C, Delourme R, Manzanares-Dauleux MJ. Exploiting natural genetic diversity and mutant resources of *Arabidopsis thaliana* to study the *A. thaliana*-*Plasmodiophora brassicae* interaction. *Plant Breeding*. 2007; 126:218–221.
- Allen RL, Bittner-Eddy PD, Grenville-Briggs LJ, et al. Host-parasite coevolutionary conflict between *Arabidopsis* and downy mildew. *Science*. 2004; 306:1957–1960. [PubMed: 15591208]
- Ausubel FM. Are innate immune signaling pathways in plants and animals conserved? *Nat Immunol*. 2005; 6:973–979. [PubMed: 16177805]
- Baechli, G.; Vilela, CR.; Escher, SA.; Saura, A. *The Drosophilidae (Diptera) of Fennoscandia and Denmark*. Brill: Leiden; 2004.
- Beekwilder J, van Leeuwen W, van Dam N, et al. The impact of the absence of aliphatic glucosinolates on insect herbivory in *Arabidopsis*. *PLoS One*. 2008; 3:e2068.
- Beilstein MA, Al-Shehbaz A, Kellogg EA. Brassicaceae phylogeny and trichome evolution. *American Journal of Botany*. 2006; 93:607–619.
- Berenbaum M. Coumarins and caterpillars: A case for coevolution. *Evolution*. 1983; 37:163–179.
- Bergelson J, Kreitman M, Stahl EA, Tian D. Evolutionary dynamics of plant R-genes. *Science*. 2001; 292:2281–2285. [PubMed: 11423651]
- Berlacher SH, Feder JL. Sympatric speciation in phytophagous insects: moving beyond controversy? *Annual Review of Entomology*. 2002; 47:773–815.
- Bidart-Bouzat MG, Kliebenstein DJ. Differential levels of insect herbivory in the field associated with genotypic variation in glucosinolates in *Arabidopsis thaliana*. *J Chem Ecol*. 2008; 34:1026–1037. [PubMed: 18581178]
- Bittel P, Robatzek S. Microbe-associated molecular patterns (MAMPs) probe plant immunity. *Curr Opin Plant Biol*. 2007; 10:335–341. [PubMed: 17652011]
- Bjorksten TA, Robinson M, La Salle J. Species composition and population dynamics of leafmining fleas and their parasitoids in Victoria. *Australian Journal of Entomology*. 2005; 44:186–191.
- Bosco G, Campbell P, Leiva-Neto JT, Markow TA. Analysis of *Drosophila* species genome size and satellite DNA content reveals significant differences among strains as well as between species. *Genetics*. 2007; 177:1277–1290. [PubMed: 18039867]

- Brady, SG.; Gadau, J.; Ward, PS. Systematics of the ant genus *Camponotus* (Hymenoptera: Formicidae): a preliminary analysis using data from the mitochondrial gene cytochrome oxidase I. In: Austin, AD.; Downton, M., editors. Hymenoptera: Evolution, Biodiversity and Biological Control. CSIRO Publishing; Collingwood: 2000. p. 131-139.
- Broekgaarden C, Poelman EH, Steenhuis G, Voorrips RE, Dicke M, Vosman B. Genotypic variation in genome-wide transcription profiles induced by insect feeding: *Brassica oleracea*-*Pieris rapae* interactions. BMC Genomics. 2007; 8:239. [PubMed: 17640338]
- Carpenter, J.; Hutter, S.; Baines, JF.; Roller, J.; Saminadin-Peter, SS., et al. The Transcriptional Response of *Drosophila melanogaster* to Infection with the Sigma Virus (Rhabdoviridae); PLoS ONE. 2009. p. e6838
- Chittenden FH. Some insects injurious to vegetable crops. Bulletin of the US Department of Agriculture, Division of Entomology. 1902
- Clark AG, Eisen MB, Smith DR, et al. Evolution of genes and genomes on the *Drosophila* phylogeny. Nature. 2007; 450:203–218. [PubMed: 17994087]
- Clarke, JD. Phenotypic Analysis of Arabidopsis Mutants: Diaminobenzidine Stain for Hydrogen Peroxide. Cold Spring Harb Protoc. 2009.
- Clay NK, Adio AM, Denoux C, Jander J, Ausubel FM. Glucosinolate metabolites required for an *Arabidopsis* innate immune response. Science. 2009; 5910:95–101. [PubMed: 19095898]
- Clough SJ, Bent AF. Floral dip: a simplified method for *Agrobacterium* mediated transformation of *Arabidopsis thaliana*. Plant J. 1998; 16:735–43. [PubMed: 10069079]
- Collinge SK, Louda SM. *Scaptomyza nigrita* Wheeler (Diptera: Drosophilidae), a leaf miner of the native crucifer, *Cardamine cordifolia* A. Gray (Bittercress). Journal of the Kansas Entomological Society. 1989; 62:1–10.
- Collins RM, Afzal M, Ward DA, et al. Differential proteomic analysis of *Arabidopsis thaliana* genotypes exhibiting resistance or susceptibility to the insect herbivore, *Plutella xylostella*. PLoS One. 2010; 5:e10103. [PubMed: 20386709]
- Connor EF, Taverner MP. The evolution and adaptive significance of the leaf-mining habit. Oikos. 1997; 79:6–25.
- Cui J, Jander G, Racki LR, et al. Signals involved in *Arabidopsis* resistance to *Trichoplusia ni* caterpillars induced by virulent and avirulent strains of the phytopathogen *Pseudomonas syringae*. Plant Physiol. 2002; 129:551–564. [PubMed: 12068100]
- Dangl JL, Jones JD. Plant pathogens and integrated defence responses to infection. Nature. 2001; 411:826–833. [PubMed: 11459065]
- Denoux C, Galletti R, Mammarella N, Gopalan S, Werck D, De Lorenzo G, Ferrari S, Ausubel FM, Dewdney J. Activation of defense response pathways by OGs and Flg22 elicitors in *Arabidopsis* seedlings. Mol Plant. 2008; 1:423–445. [PubMed: 19825551]
- Dombrecht B, Xue GP, Sprague SJ, Kirkegaard JA, Ross JJ, Reid JB, Fitt GP, Sewelam N, Schenk PM, Manners JM, Kazan K. MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. Plant Cell. 2007; 19:2225–2245. [PubMed: 17616737]
- Dong X, Mindrinos M, Davis KR, Ausubel FM. Induction of *Arabidopsis* defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. Plant Cell. 1991; 3:61–72. [PubMed: 1824335]
- Ehrlich PR, Raven PH. Butterflies and plants: a study in coevolution. Evolution. 1965; 18:586–603.
- Escott GM, Adams DJ. Chitinase activity in human serum and leukocytes. Infect Immun. 1995; 63:4770–4773. [PubMed: 7591134]
- Fernandes GW. Hypersensitivity: a neglected plant resistance mechanism against insect herbivores. Environmental Entomology. 1990; 19:1173–1182.
- Frankel G. The raison d'être of secondary plant substances. Science. 1959; 129:1466–1470. [PubMed: 13658975]
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994; 3:294–297. [PubMed: 7881515]
- Gadberry MD, Malcomber ST, Doust AN, Kellogg EA. Primateclade--a flexible tool to find conserved PCR primers across multiple species. Bioinformatics. 2005; 21:1263–1264. [PubMed: 15539448]



- Giron D, Kaiser W, Imbault N, Casas J. Cytokinin-mediated leaf manipulation by a leafminer caterpillar. *Biol Lett*. 2007; 3:340–343. [PubMed: 17412674]
- Gratton C, Welter SC. Oviposition preference and larval performance of *Liriomyza helianthi* (Diptera: Agromyzidae) on normal and novel host plants. *Environmental Entomology*. 1998; 27:926–935.
- Gregory TR, Johnston JS. Genome size diversity in the family Drosophilidae. *Heredity*. 2008; 101:228–238. [PubMed: 18523443]
- Hackman W. On the genus *Scaptomyza* Hardy (Dipt., Drosophilidae) with descriptions of new species from various parts of the world. *Acta Zoologica Fennica*. 1959; 97:3–73.
- Halkier BA, Gershenzon J. Biology and biochemistry of glucosinolates. *Annu Rev Plant Biol*. 2006; 57:303–333. [PubMed: 16669764]
- Harvey J, Leotien W, Benkirane M, Duyts H, Wagenaar R. Nutritional suitability and ecological relevance of *Arabidopsis thaliana* and *Brassica oleracea* as foodplants for the cabbage butterfly, *Pieris rapae*. *Vegetatio*. 2007; 189:117–126.
- Heil M. Damaged-self recognition in plant herbivore defence. *Trends Plant Sci*. 2009; 14:356–363. [PubMed: 19540148]
- Hering, EM. Biology of leaf miners. Junk-Gravenhage; Berlin: 1951.
- Hespenheide HA. Bionomics of leaf-mining insects. *Annual Review of Entomology*. 1991:535–560.
- The International Aphid Genomics Consortium. Genome sequencing of the pea aphid *Acyrtosiphon pisum*. *PLoS Biology*. 2010; 8:e1000313. [PubMed: 20186266]
- Jander G, Cui J, Nhan B, Pierce NE, Ausubel FM. The TASTY locus on chromosome 1 of *Arabidopsis* affects feeding of the insect herbivore *Trichoplusia ni*. *Plant Physiol*. 2001; 126:890–898. [PubMed: 11402216]
- Jander G, Howe G. Plant interactions with arthropod herbivores: state of the field. *Plant Physiol*. 2008; 146:801–803. [PubMed: 18316632]
- Jefferson RA, Kavanagh TA, Bevan MW. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J*. 1987; 6:3901–3907. [PubMed: 3327686]
- Jones AM, Chory J, Dangl JL, et al. The impact of *Arabidopsis* on human health: diversifying our portfolio. *Cell*. 2008; 133:939–943. [PubMed: 18555767]
- Jones JD, Dangl JL. The plant immune system. *Nature*. 2006; 444:323–329. [PubMed: 17108957]
- Kaiser W, Huguet E, Casas J, Commin C, Giron D. Plant green-island phenotype induced by leaf-miners is mediated by bacterial symbionts. *Proc Biol Sci*. 2010; 277:2311–2319. [PubMed: 20356892]
- Kaneshiro KY. Perkins' legacy to evolutionary research on Hawaiian Drosophilidae, Diptera. *Pacific Science*. 1997; 51:450–461.
- Keen N, Staskawicz B, Mekalanos J, Ausubel F, Cook RJ. Pathogens and hosts: the dance is the same, the couples are different. *Proc Natl Acad Sci U S A*. 2000; 97:8752–8753. [PubMed: 10922030]
- Kliebenstein DJ, Kroymann J, Brown P, et al. Genetic control of natural variation in *Arabidopsis* glucosinolate accumulation. *Plant Physiol*. 2001a; 126:811–825. [PubMed: 11402209]
- Kliebenstein DJ, Lambrix VM, Reichelt M, Gershenzon J, Mitchell-Olds T. Gene duplication in the diversification of secondary metabolism: tandem 2-oxoglutarate-dependent dioxygenases control glucosinolate biosynthesis in *Arabidopsis*. *Plant Cell*. 2001b; 13:681–693. [PubMed: 11251105]
- Koncz C, Schell J. The promoter of the T<sub>L</sub>-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol Gen Genet*. 1986; 204:383–396.
- Kwiatowski J, Skarecky D, Bailey K, Ayala FJ. Phylogeny of *Drosophila* and related genera inferred from the nucleotide sequence of the Cu, Zn Sod gene. *J Mol Evol*. 1994; 38:443–454. [PubMed: 7545938]
- Kwiatowski J, Ayala FJ. Phylogeny of *Drosophila* and related genera: Conflict between molecular and anatomical analyses. *Mol Phylogenet Evol*. 1999; 13:319–328. [PubMed: 10603260]
- Labandeira, CC. Fossil history and evolutionary ecology of Diptera and their associations with plants. In: Weigmann, B.; Yeates, D., editors. *The Evolutionary Biology of Flies*. Columbia University Press; New York: 2003. p. 217-273.

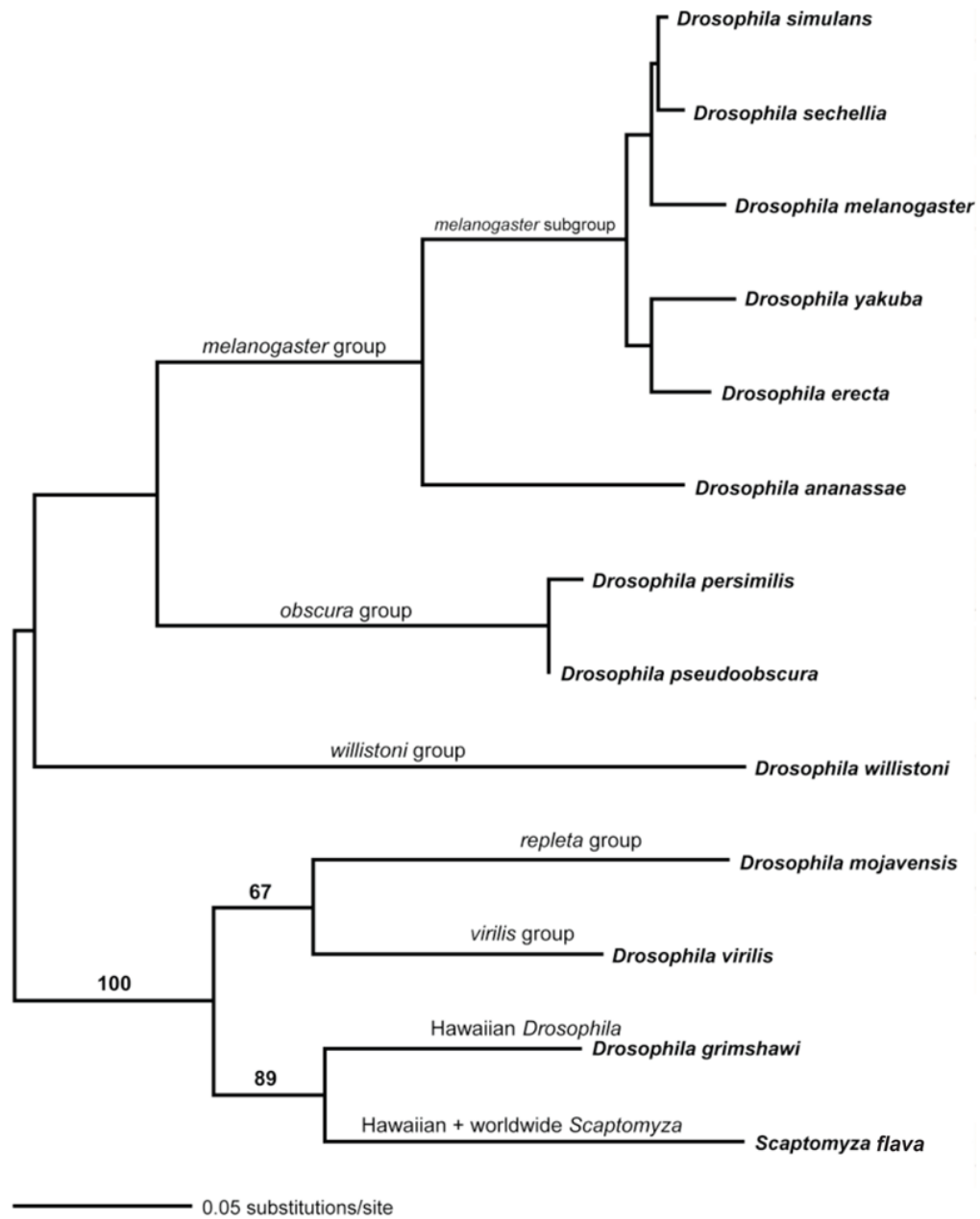
- Labandeira CC, Dilcher DL, Davis DR, Wagner DL. Ninety-seven million years of angiosperm-insect association: paleobiological insights into the meaning of coevolution. *Proc Natl Acad Sci U S A*. 1994; 91:12278–12282. [PubMed: 11607501]
- Lafferty KD, Dobson AP, Kuris AM. Parasites dominate food web links. *Proc Natl Acad Sci U S A*. 2006; 103:11211–11216. [PubMed: 16844774]
- Larkin MA, Blackshields G, Brown NP, et al. Clustal W and Clustal X version 2.0. *Bioinformatics*. 2007; 23:2947–2948. [PubMed: 17846036]
- Lawrence SD, Novak NG. Expression of poplar chitinase in tomato leads to inhibition of development in colorado potato beetle. *Biotechnol Lett*. 2006; 28:593–599. [PubMed: 16614898]
- Leulier F, Lemaitre B. Toll-like receptors--taking an evolutionary approach. *Nat Rev Genet*. 2008; 9:165–178. [PubMed: 18227810]
- Levins R, MacArthur RH. An hypothesis to explain the incidence of monophagy. *Ecology*. 1969; 50:910–911.
- Li HM, Buczkowski G, Mittapalli O, et al. Transcriptomic profiles of *Drosophila melanogaster* third instar larval midgut and responses to oxidative stress. *Insect Mol Biol*. 2008; 17:325–339. [PubMed: 18651915]
- Li W, Zangerl AR, Schuler MA, Berenbaum MR. Characterization and evolution of furanocoumarin-inducible cytochrome P450s in the parsnip webworm, *Depressaria pastinacella*. *Insect Mol Biol*. 2004; 13:603–613. [PubMed: 15606809]
- Little D, Gouhier-Darimont C, Bruessow F, Reymond P. Oviposition by pierid butterflies triggers defense responses in *Arabidopsis*. *Plant Physiol*. 2007; 143:784–800. [PubMed: 17142483]
- Lopez-Vaamonde C, Wikstrom N, Labandeira C, et al. Fossil-calibrated molecular phylogenies reveal that leaf-mining moths radiated millions of years after their host plants. *J Evol Biol*. 2006; 19:1314–1326. [PubMed: 16780532]
- Lorenzo O, Chico JM, Sánchez JJ, Solano R. *JASMONATE-INSENSITIVE1* encodes a MYC transcriptional factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. *The Plant Cell*. 2004; 16:1938–1950. [PubMed: 15208388]
- Lou Y, Baldwin IT. *Manduca sexta* recognition and resistance among allopolyploid *Nicotiana* host plants. *Proc Natl Acad Sci USA*. 2003; 100:14581–14586. [PubMed: 14530394]
- Louda SM, Collinge SK. Plant resistance to insect herbivores: A field test of the environmental stress hypothesis. *Ecology*. 1992; 73:153–169.
- Louda SM, Rodman JE. Insect herbivory as a major factor in the shade distribution of a native Crucifer (*Cardamine cordifolia* A. Gray, Bittercress). *Journal of Ecology*. 1996; 84:229–237.
- Máca J. Czechoslovak species of the genus *Scaptomyza* Hardy (Diptera, Drosophilidae) and their bionomics. *Acta Entomologica Bohemoslov*. 1972; 69:119–132.
- Magnacca KN, Foote D, O'Grady PM. A review of the endemic Hawaiian Drosophilidae and their host plants. *Zootaxa*. 2008; 1728:1–58.
- Markow TA, O'Grady PM. Evolutionary genetics of reproductive behavior in *Drosophila*: connecting the dots. *Annu Rev Genet*. 2005; 39:263–291. [PubMed: 16285861]
- Martin NA. History of an invader, *Scaptomyza flava* (Fallen, 1823) (Diptera: Drosophilidae). *New Zealand Journal of Zoology*. 2004; 31:27–32.
- Mattson, WJ.; Lawrence, RK.; Haack, RA.; Herms, DA.; Charles, PJ. Defensive strategies of woody plants against different insect-feeding guilds in relation to plant ecological strategies and intimacy of association with insects. In: Mattson, WJ.; Levieux, J.; Bernard-Dagan, C., editors. *Mechanisms of Woody Plant Defenses Against Insects*. 1988.
- Matzkin LM, Watts TD, Bitler BG, Machado CA, Markow TA. Functional genomics of cactus host shifts in *Drosophila mojavensis*. *Mol Ecol*. 2006; 15:4635–4643. [PubMed: 17107489]
- Mauricio R, Stahl EA, Korves T, et al. Natural selection for polymorphism in the disease resistance gene *Rps2* of *Arabidopsis thaliana*. *Genetics*. 2003; 163:735–746. [PubMed: 12618410]
- May RM, Anderson RM. Population biology of infectious diseases: Part II. *Nature*. 1979; 280:455–461. [PubMed: 460424]
- Millet, YA.; Danna, CH.; Clay, NK., et al. *Plant Cell*. Vol. 22. Innate immune responses activated in *Arabidopsis* roots by microbe-associated molecular patterns; p. 973-990.

- Mitchell-Olds T. *Arabidopsis thaliana* and its wild relatives: a model system for ecology and evolution. *Trends in Ecology and Evolution*. 2001; 16:693–700.
- Mithofer A, Boland W. Recognition of herbivory-associated molecular patterns. *Plant Physiol*. 2008; 146:825–831. [PubMed: 18316636]
- Mittapalli O, Neal JJ, Shukle RH. Antioxidant defense response in a galling insect. *Proc Natl Acad Sci U S A*. 2007a; 104:1889–1894. [PubMed: 17261812]
- Mittapalli O, Neal JJ, Shukle RH. Tissue and life stage specificity of glutathione S-transferase expression in the Hessian fly, *Mayetiola destructor*: implications for resistance to host allelochemicals. *J Insect Sci*. 2007b; 7:1–13. [PubMed: 20307234]
- Mittapalli O, Sardesai N, Shukle RH. cDNA cloning and transcriptional expression of a peritrophin-like gene in the Hessian fly, *Mayetiola destructor* [Say]. *Arch Insect Biochem Physiol*. 2007c; 64:19–29. [PubMed: 17167751]
- Miya A, Albert P, Shinya T, et al. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. *Proc Natl Acad Sci U S A*. 2007; 104:19613–19618. [PubMed: 18042724]
- O’Grady P, Desalle R. Out of Hawaii: the origin and biogeography of the genus *Scaptomyza* (Diptera: Drosophilidae). *Biol Lett*. 2008; 4:195–199. [PubMed: 18296276]
- Opler PA. Fossil Lepidopterous leaf mines demonstrate the age of some insect-plant relationships. *Science*. 1973; 179:1321–1323. [PubMed: 17835937]
- Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001; 29:e45. [PubMed: 11328886]
- Pfalz M, Vogel H, Kroymann J. The gene controlling the indole glucosinolate modifier I quantitative trait locus alters indole glucosinolate structures and aphid resistance in *Arabidopsis*. *Plant Cell*. 2009; 21:985–999. [PubMed: 19293369]
- Pfalz M, Vogel H, Mitchell-Olds T, Kroymann J. Mapping of QTL for resistance against the crucifer specialist herbivore *Pieris brassicae* in a new *Arabidopsis* inbred line population, Da(1)-12xEi-2. *PLoS One*. 2007; 2:e578. [PubMed: 17593977]
- Poulin R, Morand S. The diversity of parasites. *Q Rev Biol*. 2000; 75:277–293. [PubMed: 11008700]
- Price PW. Evolutionary biology of parasites. *Monogr Popul Biol*. 1980; 15:1–237. [PubMed: 6993919]
- Rahme LG, Stevens EJ, Wolfort SF, et al. Common virulence factors for bacterial pathogenicity in plants and animals. *Science*. 1995; 268:1899–1902. [PubMed: 7604262]
- Ramsey JS, Wilson AC, de Vos M, et al. Genomic resources for *Myzus persicae*: EST sequencing, SNP identification, and microarray design. *BMC Genomics*. 2007; 8:423. [PubMed: 18021414]
- Ratzka A, Vogel H, Kliebenstein DJ, Mitchell-Olds T, Kroymann J. Disarming the mustard oil bomb. *Proc Natl Acad Sci U S A*. 2002; 99:11223–11228. [PubMed: 12161563]
- Reese TA, Liang HE, Tager AM, et al. Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature*. 2007; 447:92–96. [PubMed: 17450126]
- Remsen J, DeSalle R. Character congruence of multiple data partitions and the origin of the Hawaiian Drosophilidae. *Mol Phylogenet Evol*. 1998; 9:225–235. [PubMed: 9562982]
- Scott AC, Anderson JM, Anderson HM. Evidence of plant-insect interactions in the Upper Triassic Molteno Formation of South Africa. *Journal of the Geological Society*. 2004; 161:401–410.
- Shakeel M, He XZ, Martin NA, Hanan A, Wang Q. Diurnal periodicity of adult eclosion, mating and oviposition of the European leafminer. *Scaptomyza flava* (Fallén) (Diptera: Drosophilidae). 2009; 62:8–85.
- Shroff R, Vergara F, Muck A, Svatos A, Gershenzon J. Nonuniform distribution of glucosinolates in *Arabidopsis thaliana* leaves has important consequences for plant defense. *Proc Natl Acad Sci U S A*. 2008; 105:6196–6201. [PubMed: 18408160]
- Simon C, Frati F, Bechenbach A, Crespi B, et al. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequence and compilation of conserved polymerase chain reaction primers. *Ann Entomol Soc Am*. 1994; 87:651–701.
- Simonelig M, Anxolabehere D. A P element of *Scaptomyza pallida* is active in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A*. 1991; 88:6102–6106. [PubMed: 1648729]

- Staal J, Dixelius C. Tracing the ancient origins of plant innate immunity. *Trends Plant Sci.* 2007; 12:334–342. [PubMed: 17644465]
- Stamatakis A, Hoover P, Rougemont J. A rapid bootstrap algorithm for the RAxML Web servers. *Syst Biol.* 2008; 57:758–771. [PubMed: 18853362]
- Steppuhn A, Schuman MC, Baldwin IT. Silencing jasmonate signalling and jasmonate-mediated defenses reveals different survival strategies between two *Nicotiana attenuata* accessions. *Mol Ecol.* 2008; 17:3717–3732. [PubMed: 18662222]
- Summers CB, Felton GW. Peritrophic envelope as a functional antioxidant. *Archives of Insect Biochemistry and Physiology.* 1998; 32:131–142.
- Suza W, Staswick P. The role of JAR1 in jasmonoyl-L-isoleucine production in *Arabidopsis* wound response. *Planta.* 2008; 227:1221–1232. [PubMed: 18247047]
- Tang AH, Tu C-PD. Biochemical characterization of *Drosophila* glutathione S-transferase D1 and D21: *Drosophila* DDT dehydrochlorinase. *Journal of Biological Chemistry.* 1994; 269:27876–27884. [PubMed: 7961718]
- Tavormina SJ. Sympatric genetic divergence in the leaf-mining insect *Liriomyza brassicae* (Diptera: Agromyzidae). *Evolution.* 1982; 36:523–534.
- Toba G, Aigaki T. Disruption of the microsomal glutathione S-transferase-like gene reduces life span of *Drosophila melanogaster*. *Gene.* 2000; 253:179–187. [PubMed: 10940555]
- Trinh K, Andrews L, Krause J, et al. Decaffeinated coffee and nicotine-free tobacco provide neuroprotection in *Drosophila* models of Parkinson's disease through an NRF2-dependent mechanism. *J Neurosci.* 2010; 30:5525–5532. [PubMed: 20410106]
- Trinh K, Moore K, Wes PD, et al. Induction of the phase II detoxification pathway suppresses neuron loss in *Drosophila* models of Parkinson's disease. *J Neurosci.* 2008; 28:465–472. [PubMed: 18184789]
- Via S. The quantitative genetics of polyphagy in an insect herbivore. I. Genotype-environment interaction in larval performance on different host plant species. *Evolution.* 1984; 38:881–895.
- Vogel H, Kroymann J, Mitchell-Olds T. Different transcript patterns in response to specialist and generalist herbivores in the wild *Arabidopsis* relative *Boechera divaricarpa*. *PLoS One.* 2007; 2:e1081. [PubMed: 17957263]
- Walker-Simmons M, Ryan CA. Protease inhibitor synthesis in tomato leaves: Induction by chitosan oligomers and chemically modified chitosan and chitin. *Plant Physiol.* 1984; 76:787–790. [PubMed: 16663925]
- Wan J, Zhang XC, Neece D, et al. A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in *Arabidopsis*. *Plant Cell.* 2008; 20:471–481. [PubMed: 18263776]
- Wheat CW, Vogel H, Wittstock U, et al. The genetic basis of a plant-insect coevolutionary key innovation. *Proc Natl Acad Sci U S A.* 2007; 104:20427–20431. [PubMed: 18077380]
- Whiteman NK, Kimball RT, Parker PG. Co-phylogeography and comparative population genetics of the threatened Galapagos hawk and three ectoparasite species: ecology shapes population histories within parasite communities. *Mol Ecol.* 2007; 16:4759–4773. [PubMed: 18028178]
- Whiteman NK, Jander. Genome-enabled research on the ecology of plant-insect interactions. *Plant Physiol.* 2010; 154:475–478. [PubMed: 20921167]
- Wittstock U, Agerbirk N, Stauber EJ, et al. Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proc Natl Acad Sci U S A.* 2004; 101:4859–4864. [PubMed: 15051878]
- Yang J, McCart C, Woods DJ, et al. A *Drosophila* systems approach to xenobiotic metabolism. *Physiol Genomics.* 2007; 30:223–231. [PubMed: 17488889]
- Zangerl AR, Berenbaum M. Phenotype matching in wild parsnip and parsnip webworms: causes and consequences. *Evolution.* 2003; 57:806–815. [PubMed: 12778550]
- Zavala JA, Giri A, Jongsma MA, Baldwin IT. Digestive duet: midgut digestive proteinases of *Manduca sexta* ingesting *Nicotiana attenuata* with manipulated proteinase inhibitor expression. *PLoS ONE.* 2008; 3:e2008.
- Zhang B, Ramonell K, Somerville S, Stacey G. Characterization of early, chitin-induced gene expression in *Arabidopsis*. *Mol Plant Microbe Interact.* 2002; 15:963–970. [PubMed: 12236603]

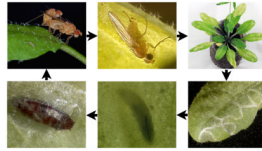
- Zhang Z, Ober JA, Kliebenstein DJ. The gene controlling the quantitative trait locus EPITHIOSPECIFIER MODIFIER1 alters glucosinolate hydrolysis and insect resistance in *Arabidopsis*. *Plant Cell*. 2006; 18:1524–1536. [PubMed: 16679459]
- Zheng S-J, Dicke M. Ecological genomics of plant-insect interactions: From gene to community. *Plant Physiology*. 2008; 146:812–817. [PubMed: 18316634]
- Zwickl, DJ. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. University of Texas; Austin: 2006.



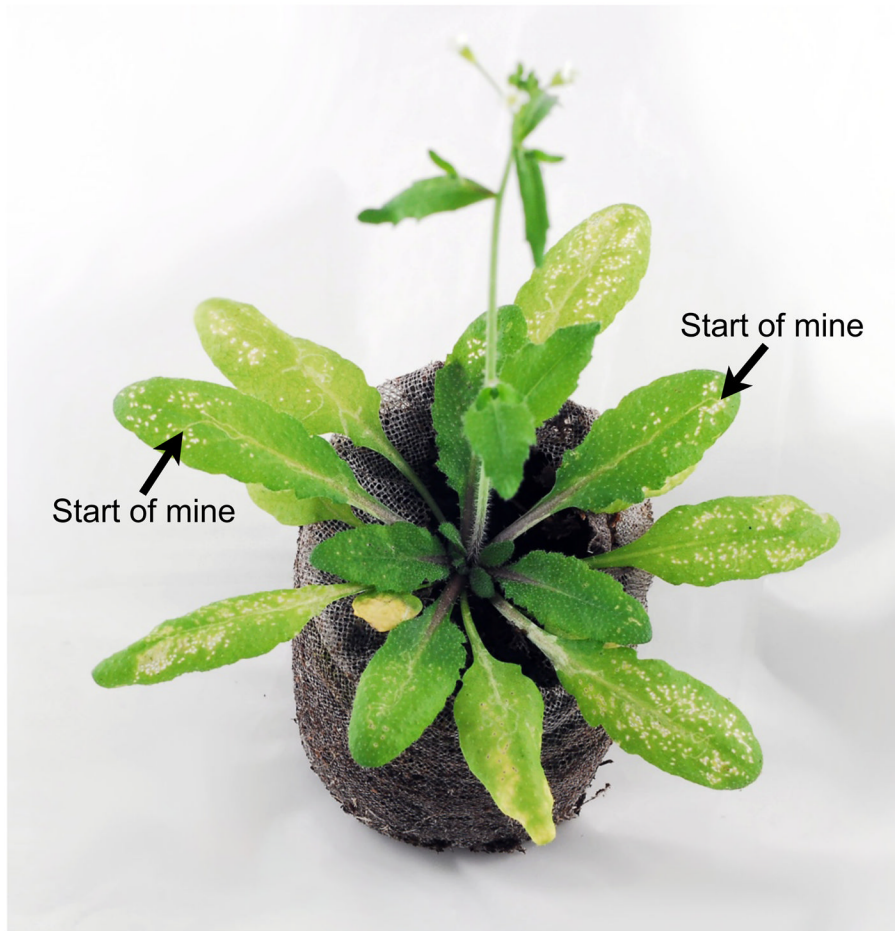


**Figure 1.**

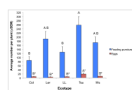
Maximum-likelihood phylogram placing *Scaptomyza flava* in relation to the 12 *Drosophila* genomic models based on 1891 bp comprising five partial detoxification enzyme genes (Cu, Zn *Sod1*, *GstD1*, *Mgstl*, *Peritrophin A*, *PHGPx*). The tree was rooted at the midpoint. *S. flava* is placed sister to *D. grimshawi* and is embedded with the subgenus *Drosophila*. Bootstrap replicates are shown only for subgenus *Drosophila*.



**Figure 2.** Life-cycle of *Scaptomyza flava* on *Arabidopsis thaliana*. From upper left, clockwise: A pair mating on a leaf, a female creating a feeding puncture with her dentate ovipositor, feeding punctures and initial mines on leaves, a serpentine mine of a first instar larva in a leaf, a blotch mine of a second or third instar larva, a puparium under the epidermis of a leaf. Egg to adult development time is 21 days at 22°C.

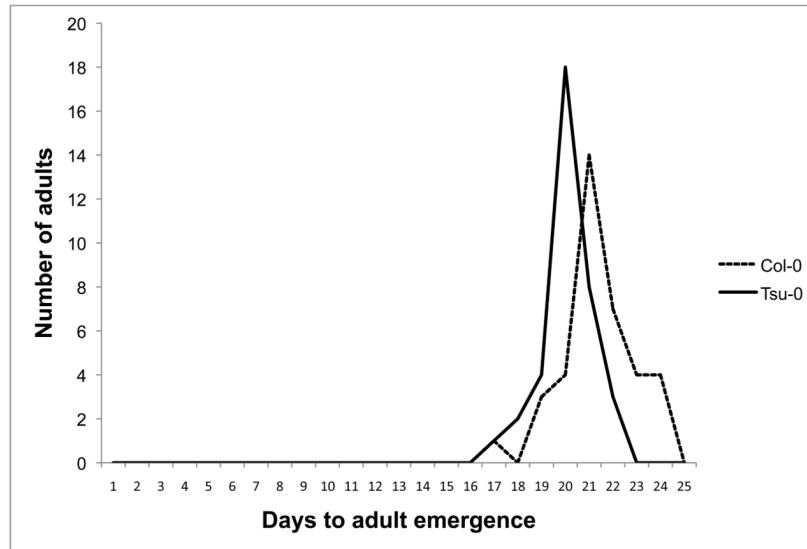


**Figure 3.** *Arabidopsis thaliana* plant exposed to *Scaptomyza flava* females for 24 hours. Larvae were allowed to develop for another 48 hours before being photographed. Feeding punctures and leaf mines of first instar larvae are clearly visible in the leaf laminae (arrows). Leaf mines begin in the distal portion of the leaves, and larvae move toward the midvein, parallel to it, and end up in the petiole. Chlorosis (yellowing) of attacked leaves is apparent.



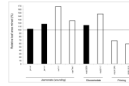
**Figure 4.**

Feeding punctures and eggs laid across five accessions of *Arabidopsis thaliana*. Flies created significantly more feeding punctures and laid significantly more eggs on Tsu-0 than on Col-0. Different letters indicate statistically significant differences between treatments and asterisks indicate statistical comparisons for number of eggs.

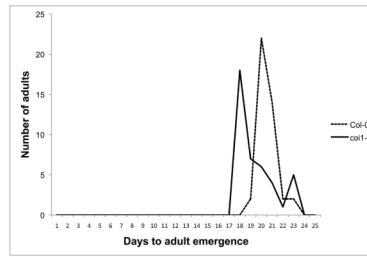


**Figure 5.** Development time differences in flies reared on Col-0 vs. Tsu-0 *Arabidopsis thaliana* accessions. Total counts of flies emerging from Col-0 and Tsu-0 plants. Larvae were transferred from Col-0 nurse plants in the second instar into Col-0 and Tsu-0 plants (one larva/plant) and allowed to develop until eclosion. Development time was significantly shorter in larvae reared on Tsu-0 than on Col-0.



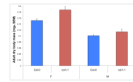
**Figure 6.**

Leaf area mined of *Scaptomyza flava* larvae reared on *Arabidopsis thaliana* jasmonate pathway defense mutants or plants pre-treated with methyl JA (1 mM) or chitin (10–100 µg/ml) relative to LAM in larvae reared in wildtype (Col-0) plants. The horizontal bar is set to 100%, which is the amount fed by larvae reared in wildtype (Col-0) plants in each independent experiment. Larvae were transferred from Col-0 nurse plants in the second instar into experimental and control plants and allowed to feed for 48 hours. Results represent independent experiments; larvae reared on Col-0 were always run alongside each experimental assay; sample sizes ranged from 8–24 individual larvae in the same number of plants of each genotype per treatment. JA signaling and glucosinolates are required for full resistance to *S. flava* attack. Significant differences in leaf area mined between mutant or pre-treatment and wildtype were found in: *coi1-1*,  $P < 0.01$ ; *cyp74a1*,  $P < 0.05$ , *myb51-1*,  $P < 0.0001$ , 1mM methyl JA pre-treatment,  $P < 0.05$ ; chitin pre-treatment,  $P < 0.05$ ). White bars indicate statistically significant differences, black bars indicate no significant difference between treatments.

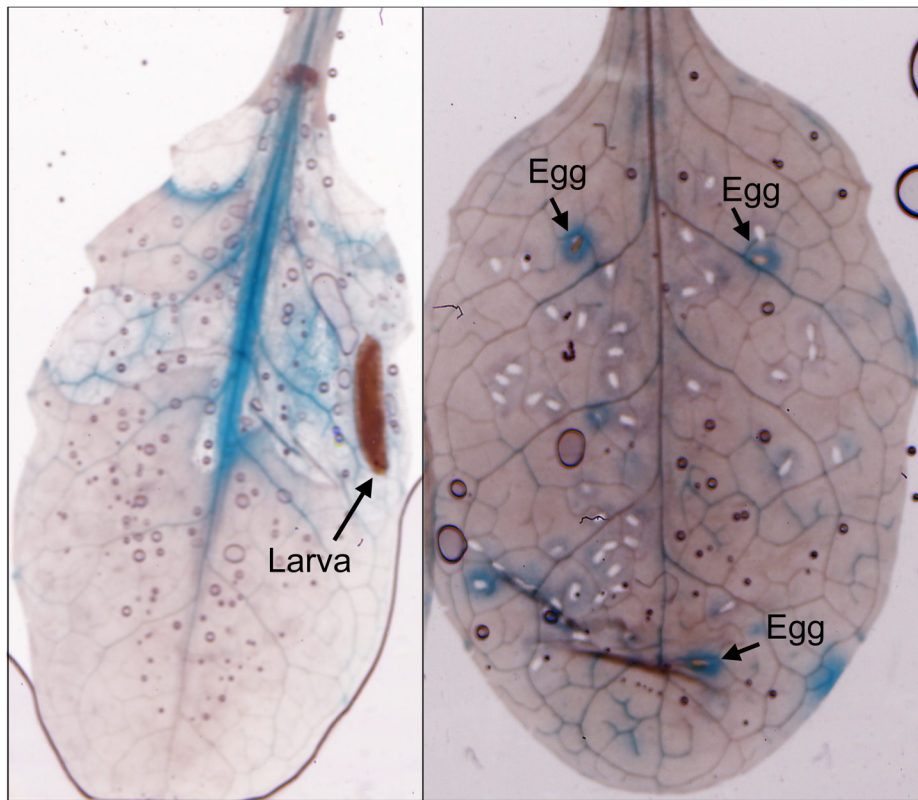


**Figure 7.**

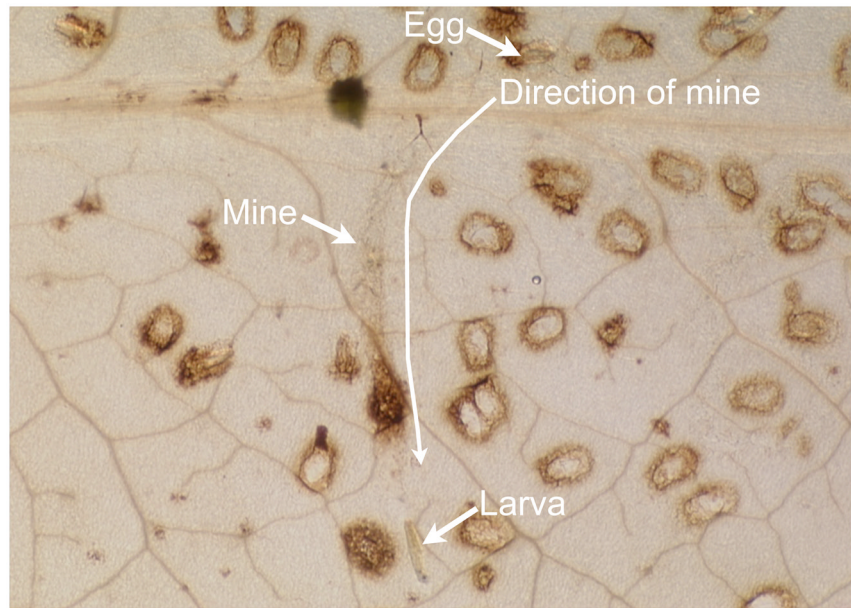
Development time differences in *Scaptomyza flava* flies reared on Col-0 vs. *coi-1* *Arabidopsis thaliana* plants (loss of JA signaling). Total counts of flies emerging over a 25 day period from Col-0 and *coi-1* plants. Plants were placed with ovipositing females at the same time, removed and larvae were allowed to develop naturally. Development time was significantly shorter in larvae reared on *coi-1* than on Col-0 ( $P < 0.001$ ). Loss of JA signaling increases feeding rate and development time in *S. flava*.

**Figure 8.**

Adult body mass differences in flies reared on Col-0 vs. *coi1-1* *Arabidopsis thaliana* plants (loss of JA signaling). Plants were placed with ovipositing *Scaptomyza flava* females at the same time, removed and larvae were allowed to develop naturally. Body mass of males and females was significantly larger (one-sided t-test,  $P < 0.01$  for males, one-sided t-test,  $P < 0.05$  for females) when reared on *coi1-1* than on Col-0. Loss of JA signaling leads to larger adult body mass in *S. flava*.

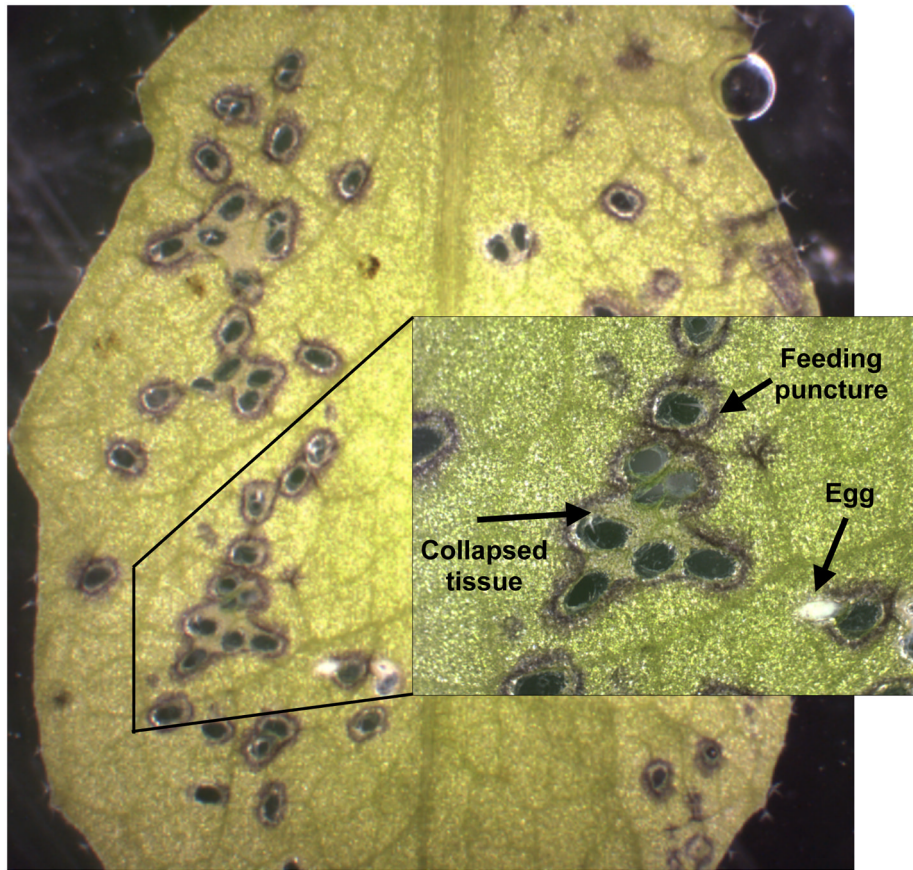


**Figure 9.** Activation of the indolic glucosinolate modifier *CYP81F2* gene in the *Arabidopsis thaliana* promoter:GUS reporter line when attacked by *Scaptomyza flava* adult females and larvae for 24 hours. Strong induction occurs around eggs (right photo) and in mines (left photo), but less so around simple feeding punctures from adult females (right). This indicates that larval elicitors may be present that activate *CYP81F2* and the indolic glucosinolate pathway.

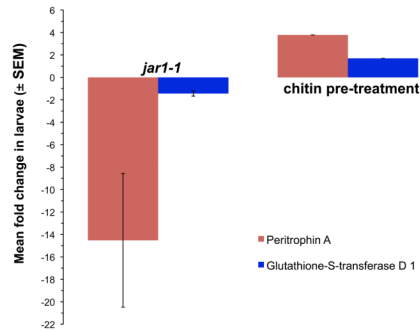


**Figure 10.** DAB staining of hydrogen peroxide production (stained brown) in *Arabidopsis thaliana* leaves left with adult female *Scaptomyza flava* flies for 48 hours. Note the staining around feeding punctures and in the walls of the mine formed by the first instar larva that hatched during this experiment.





**Figure 11.** NBT staining of superoxide radicals (stained blue) around feeding punctures, but not eggs in *Arabidopsis thaliana* that were exposed to *Scaptomyza flava* females for 48 hours. Collapsed tissue (apparently dead and not producing superoxide) in areas of high puncture density is indicative of a hypersensitive response. Dead eggs were observed in these areas in leaves, and presumably were killed from desiccation.



**Figure 12.**

Real time quantitative PCR of two candidate xenobiotic metabolism genes (*GstD1* and *Peritrophin A*) in second instar larvae of *Scaptomyza flava* relative to a control gene (*RpL32*). Larvae were reared for 48 hours in (1) JA pathway loss of function mutant plants shown to be susceptible to herbivory (*jar1-1*) and in wildtype *Arabidopsis thaliana* plants and (2) wildtype plants in which three lower leaves were infiltrated with 10–100  $\mu\text{g}/\text{mL}$  crab shell chitin 24 hours prior to adding larvae or in wildtype plants pre-treated with sterile water. Values are in relation to expression levels of larvae reared in wildtype controls. Relative to larvae reared in wildtype plants, larvae showed lower expression of *GstD1* and *Peritrophin A* when reared in the JA pathway mutant and higher expression when reared in plants pre-treated with chitin.