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Mining the plant–herbivore interface with a leafmining *Drosophila* of *Arabidopsis*

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Abstract

Experimental infections of *Arabidopsis thaliana* (*Arabidopsis*) with genomically characterized plant pathogens such as *Pseudomonas syringae* have facilitated the dissection of canonical eukaryotic defence 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 defence 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 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, defence-related mutants, and hormone and chitin-treated plants revealed significant differences in host preference and variation in larval performance across *Arabidopsis* accessions. The jasmonate and glucosinolate pathways in *Arabidopsis* are important in mediating quantitative resistance against *S. flava*, and priming with jasmonate or chitin resulted in increased resistance. Expression of xenobiotic detoxification genes was reduced in *S. flava* larvae reared on *Arabidopsis* jasmonate signalling mutants and increased in plants pretreated with chitin. These results and future research directions are discussed in the context of developing a genetic model system to analyse insect–plant interactions.

Keywords: *Arabidopsis*, *Drosophila*, gene expression, herbivory, jasmonate, model system

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Introduction

Metazoan parasites (*sensu* Price 1980), including most herbivorous insects, comprise a majority of lineages of eukaryotic 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 nonmicrobial 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 crosstalk 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 signalling molecules. Whether these similarities are attributable to common ancestry or

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convergence is a topic of considerable debate (Ausubel 2005; Staal & Dixelius 2007; Leulier & Lemaitre 2008), a debate made more complex because of functional constraints on the basic structure of parasite elicitor molecules, which restricts form and function of host PRRs.

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 (Dangl & Jones 2001; Jones & Dangl 2006; Bittel & Robatzek 2007; 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; Zhang *et al.* 2002; Reese *et al.* 2007) 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 defence 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 defence pathways in the plant (Dong *et al.* 1991; Rahme *et al.* 1995). Importantly, pathogen virulence factors and host defence 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 (Bergelson *et al.* 2001; Mauricio *et al.* 2003; Allen *et al.* 2004). *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 defence pathways and herbivore countermeasures. Here, we describe the first stage of the 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 (Yang *et al.* 2007; Trinh *et al.* 2008). 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). Fortuitously, 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 (Hering 1951; Collinge & Louda 1989).

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 *c.* 225 million years (Scott *et al.* 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 nigrita*, 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 co-evolutionary 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 19th 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 *Scaptomyza apicalis* (Hardy) and *Scaptomyza 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, Capparacecae, Resedaceae, and Tropaeloaceae, although it has also been reported from pea (Fabaceae: *Pisum sativum*) (Máca 1972). *Scaptomyza flava* is capable of killing host plants (Bjorksten *et al.* 2005). It was first reported in the United States on *Arabidopsis* in the year 1900 (Chittenden 1902). *Scaptomyza flava* completes the development on *Arabidopsis* and several other members of the Brassicaceae in the wild, and its reproductive behaviour 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* defence mutants, across *Arabidopsis* accessions (including adult preference and larval performance) and plants pretreated with elicitors of a defence response. Finally, we tested how *S. flava* xenobiotic metabolism gene expression is mediated by the presence/absence of individual host defence genes.

Materials and methods

Placing Scaptomyza 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* (*Drosophila grimshawi*, *Drosophila mojavensis*, *Drosophila 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*, *Scaptomyza 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 defence

compounds or stress (Toba & Aigaki 2000; Matzkin *et al.* 2006; Mittapalli *et al.* 2007a,b,c; Li *et al.* 2008). The five genes were (*Drosophila melanogaster* annotations) as follows: *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 the 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 microlitres 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 as follows(5′–3′): GstD1F2 (5′-TGAAGATYAATCCYCAGCACAC-3′), GstD1R2 (5′-RGCCCAGTTCTCATCCCAG-3′), MgstlF1 (5′-TACRTTCTGGRCCGGTGT-3′), MgstlR1 (5′-CACAAAGAAGGMGAGCGCA-3′), PeritrophinAF1 (5′-CAYACCGAGAACTGYGATCA-3′), PeritrophinAR1 (5′-CGAASACCTTRTCMTCGC-3′), PHGPxF1 (5′-GGMAACGATGTCTCBCTGG-3′), PHGPxR1 (5′-TGGGATCGGTRGTGGGTG-3′), Sod-1F2 (5′-GCSAAGGGCACHGTTTTCT-3′), Sod-1R2 (5′-CAACDGTGCGTCCRATAATG-3′), RpL32F1 (5′-CCAGCATACMGGCCCAAG-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′-TAAACTTCAGGGTGACCAAAAAATCA-3′). The second set was comprised of Ben (5′-GCTACTACATAATAKGTATCATG-3′) (Brady *et al.* 2000) and Jerry (5′-CAACATTTATTTTGATTTTTTGG-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 uL. 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 (downloaded from http://tree.bio.ed.ac.uk/software/seal/) 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

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). *Drosophila 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 (Bosco *et al.* 2007; Gregory & Johnston 2008).

Characterization of the Scaptomyza flava *life cycle and performance across* Arabidopsis *accessions, elicitor pretreated plants and defence mutants*

To characterize the life cycle of *S. flava* on *Arabidopsis*, we surface sterilized and cold-stratified wild-type (Col-0) seeds in 0.1% agarose for 3 days at 4 °C and sowed them in Jiffy #7 peat pellets. These were placed under plastic domes to facilitate germination on a 16 h

light:8 h dark cycle [using Gro-Lux (Sylvania, Danvers, MA, USA) 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 4 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 h, 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 (LAM) assay was not used.

As a measure of larval performance across *Arabidopsis* genotypes, we used a 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. Wild-type (Col-0) plants were placed with the *S. flava* colony for 12 h 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 h. 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^2 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 to quantify the relationship between LAM and development time. To estimate the 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 defence-related mutants in the Col-0 genetic background for LAM and/or development time, including the jasmonate (JA) signalling 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 pretreated plants with 1 mM methyl JA (MeJA) dissolved in ethanol or sterile water + ethanol using the same method (*sensu* Cui *et al.* 2002) and measured LAM as above. To test the effect of herbivore-associated molecular patterns on insect performance, we also pretreated plants with 10 or 100 μg/mL crab-shell chitin (Sigma) or sterile water 24 h 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*$_{pro}$*:GUS* (β-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-methyl glucosinolate; 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, Indianapolis, IN, USA). The reverse primer used was p81F2R3 (5′-CGCGGATCCT-GAGTGAAAATGGTGGATGG-3′) and introduced a *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 $CYP81F2_{pro}$*: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 $CYP81F2_{pro}$*:GUS* plants were either placed with ovipositing female flies for 24 h, 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 h, removed and then assayed for GUS activity as described in Denoux *et al.* (2008) and observed using a Discovery V12 microscope (Zeiss, Thornwood, NY, USA). Specifically, 10 mL 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 5 min and incubated at 37 °C for 4 h. 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* Scaptomyza flava *herbivory*

Reactive oxygen species, including hydroxyl radical (OH·) and superoxide (O_2^-), serve to defend the plant against natural enemies directly as biocidal molecules and as signalling 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 colour. In brief, 4-week-old Col-0 plants were exposed to adult female flies and leaves were harvested 48 h 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 h 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 (i) 4-week-old Col-0 (control) plants or *jar1-1* plants and (ii) 4-week-old Col-0 plants in which three basal leaves had been infiltrated as above with 10–100 μg/mL of crab shell chitin or water (control), 24 h 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 h, 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 °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 GstD1rtF2 (TCGGATTCCTGA-ACACCTTC), GstD1rtR2 (GTAGCCAGCAACCTCG-AAAG), which amplified a 112-bp fragment of GstD1, PerAF1 (GCGATCAGTTTTTCCTCTGC), PerAR1 (GG-CCCAATTGTAGTTGCAGT), which amplified a 112-bp fragment of Peritrophin A, RpL32rtF1 (GGACCGT-TATGCCAAGTTGT), and RpL32rtR1 (CGCTTGTTG-GAACCATAACC), 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, Hercules, CA, USA) 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 3 min at 95 °C followed by 45 cycles of 95 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s. Reactions were performed in triplicate on a MyiQ BioRad real-time thermocycler and single colour detection system in 25-μL reaction volumes with 5 μL of template. We created a standard 10-fold dilution ser-

ies to test the 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 Scaptomyza flava *accessions and culturing of* Scaptomyza 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 4-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 h light:8 h dark cycle (using Gro-Lux bulbs) at 22 °C. After about 1 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 S1, Supporting 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 Scaptomyza 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 438-bp fragment of *GstD1*, a 248-bp fragment of *Mgstl*, a 486-bp 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 data set of 1891 bp to be compared among *S. flava* and the 12 sequenced *Drosophila* species. Results from the maximum-likelihood multigene phylogenetic analysis in Garli, limited to the 12 sequenced *Drosophila* species, place *S. flava* sister to *Drosophila 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; Remsen & DeSalle 1998; Kwiatowski & Ayala 1999) (Fig. S1, Supporting information). 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. nigrita* (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. nigrita* in a clade with all other *Scaptomyza* species and embedded within the *Drosophila* genus (Fig. S2, Supporting information). The subgenus *Drosophila* in which *Scaptomyza* is known to be embedded was not re-covered as monophyletic.

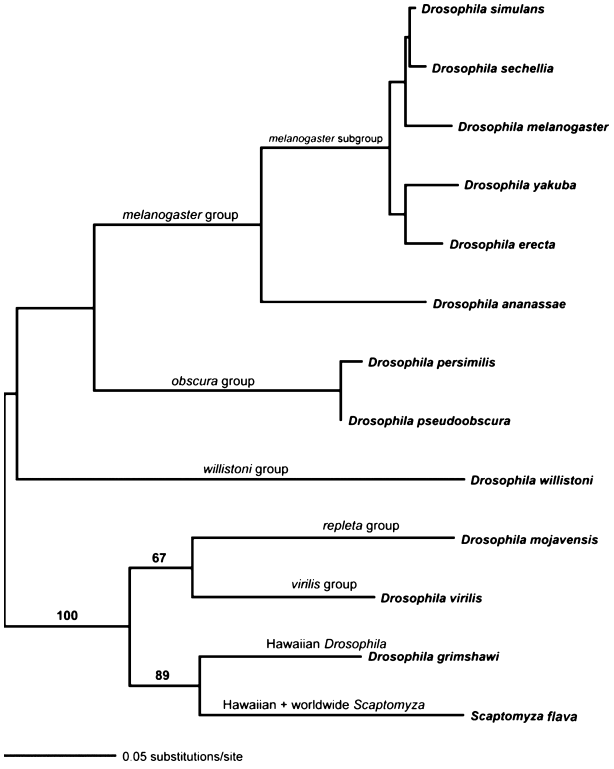

Fig. 1 Maximum-likelihood phylogram placing *Scaptomyza flava* in relation to the 12 *Drosophila* genomic models based on 1891 bp comprising five partial detoxification enzyme genes (Cu, Zn *Sod1*, *GstD1*, *Mgstl*, *Peritrophin A*, *PHGPx*). The tree was rooted at the midpoint. *Scaptomyza flava* is placed sister to *Drosophila grimshawi* and is embedded with the subgenus *Drosophila*. Bootstrap replicates are shown only for subgenus *Drosophila*.

Estimation of genome size

Using propidium iodide flow cytometry (see Materials and methods), we estimated the genome size of *S. flava* females and males to be 0.3 pg [±0.004 SDM (standard deviation of the mean)] or 293 Mb (±4.24 SDM) and 0.29 pg (±0.01 SDM) or 287 Mb (±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 Scaptomyza flava *life cycle and performance across* Arabidopsis *accessions*

The life cycle of *S. flava* was completed in approximately 3 weeks in our growth chamber conditions (Fig. 2). Specifically, 20 Col-0 plants were exposed to >200 ovipositing females for 24 h. These females created, on average, 64.5 (±37.85) feeding punctures and laid 3.15 (±1.46) eggs/plant, from which 42 adults emerged. Average egg-adult development time was 20.52 days (±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 h of oviposition and in most cases created a serpentine mine in the direction of the midvein, tunnelled 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 h (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 (honestly significant difference), $F = 4.334$, d.f. = 4, $P < 0.01$] and laid significantly more eggs in Tsu-0 than in the other accessions (ANOVA and Tukey's HSD, $F = 4.83$, d.f. = 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 4 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^2 ± 65.31 SDM) than on Col-0 (132.78 mm^2 ± 66.56 SDM) plants over a 4-day interval after transfer. Total development time (egg to adult

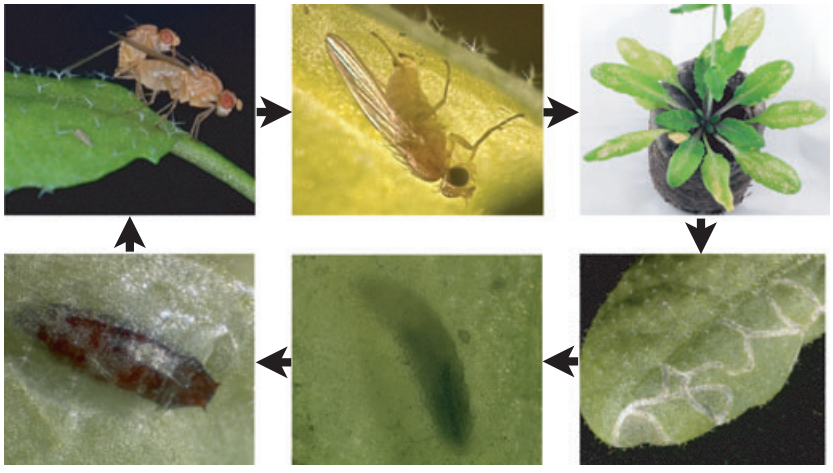

Fig. 2 Life cycle of *Scaptomyza flava* on *Arabidopsis thaliana*. From upper left, clockwise: A pair mating on a leaf, a female creating a feeding puncture with her dentate ovipositor, feeding punctures and initial mines on leaves, a serpentine mine of a first instar larva in a leaf, a blotch mine of 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.

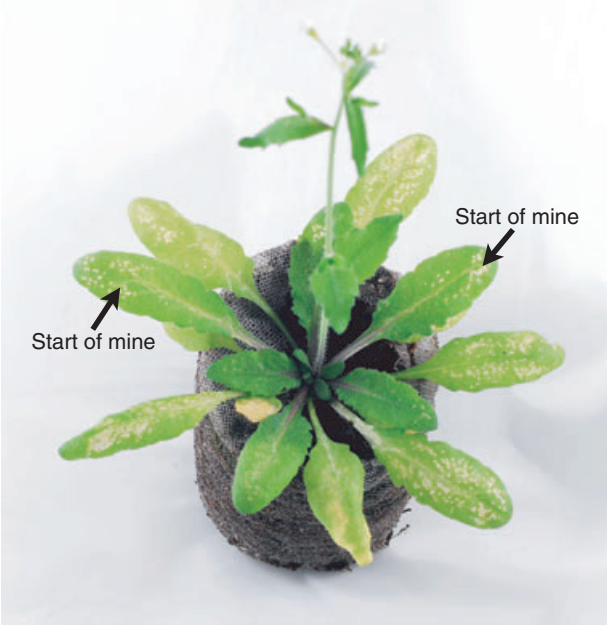

Fig. 3 *Arabidopsis thaliana* plant exposed to *Scaptomyza flava* females for 24 h. Larvae were allowed to develop for another 48 h before being photographed. Feeding punctures and leaf mines of first instar larvae are clearly visible in the leaf laminae (arrows). Leaf mines begin in the distal portion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

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 ± 1.08 SDM) than larvae reared on Col-0 (average number of days = 21.35 ± 1.55 SDM) (Fig. 5).

Characterization of the Scaptomyza flava *life cycle and performance on* Arabidopsis *elicitor pretreated plants and defence-related mutants*

We screened *Arabidopsis* JA-dependent defence mutants or plants pretreated with the defence-related hormone MeJA or the defence-related elicitor crab shell chitin 24 h prior to adding larvae, using the LAM assay in which larvae fed for 48 h after transfer from Col-0 nurse plants (Fig. 6). *Scaptomyza flava* larvae consumed significantly more leaf tissue on the canonical JA signalling–deficient mutant *coi1-1* and the JA biosynthetic mutant *cyp794a1*, consistent with the well-studied role of JAs in insect defence responses (Jander & Howe 2008). A similar trend was observed with a *jar-1* mutant, although the increase in LAM was not statistically significant, consistent with the observation that *jar-1* mutants are not completely deficient in JA signalling (Suza & Staswick 2008). Correspondingly, *S. flava*

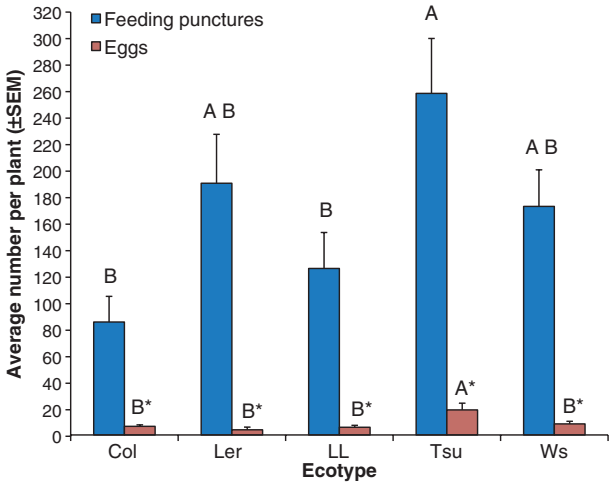

Fig. 4 Feeding punctures and eggs laid across five accessions of *Arabidopsis thaliana*. Flies created significantly more feeding punctures and laid significantly more eggs on Tsu-0 than on Col-0. Different letters indicate statistically significant differences between treatments, and asterisks indicate statistical comparisons for number of eggs.

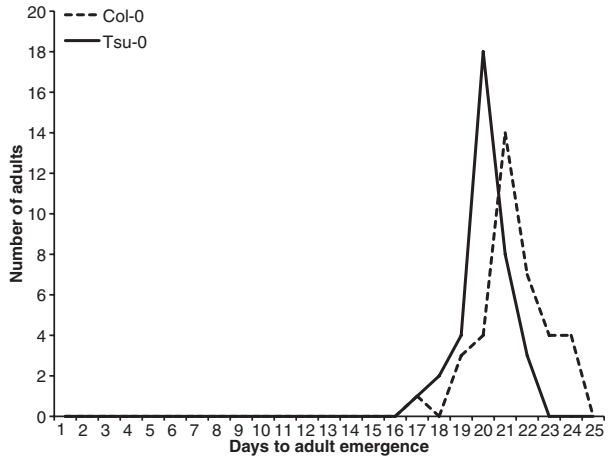

Fig. 5 Development time differences in flies reared on Col-0 vs. Tsu-0 *Arabidopsis thaliana* accessions. Total counts of flies emerging from Col-0 and Tsu-0 plants. Larvae were transferred from Col-0 nurse plants in the second instar into Col-0 and Tsu-0 plants (one larva/plant) and allowed to develop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

larvae consumed less leaf tissue on plants pretreated (sprayed) with MeJA. Larvae consumed the same amount of tissue on *jin1*-2 mutants as wild type. 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

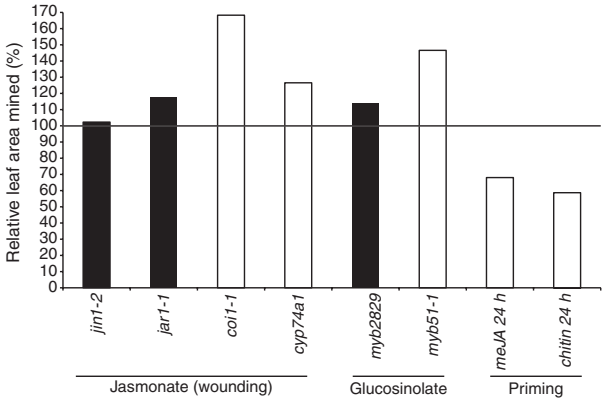

Fig. 6 Leaf area mined (LAM) of *Scaptomyza flava* larvae reared on *Arabidopsis thaliana* jasmonate (JA) pathway defence mutants or plants pretreated with methyl JA (MeJA) (1 mM) or chitin (10–100 μg/mL) relative to LAM in larvae reared in wild-type (Col-0) plants. The horizontal bar is set to 100%, which is the amount fed by larvae reared in wild-type (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 h. Results represent independent experiments; larvae reared on Col-0 were always run alongside each experimental assay; sample sizes ranged from 8 to 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 LAM between mutant or pretreatment and wild-type were found in *coi1-1*, $P < 0.01$; *cyp74a1*, $P < 0.05$, *myb51-1*, $P < 0.0001$, 1 mM MeJA pretreatment, $P < 0.05$; chitin pretreatment, $P < 0.05$. White bars indicate statistically significant differences; black bars indicate no significant difference between treatments.

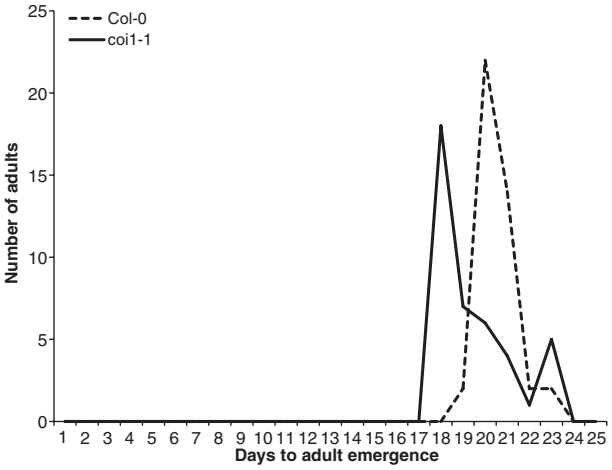

Fig. 7 Development time differences in *Scaptomyza flava* flies reared on Col-0 vs. *coi1-1 Arabidopsis thaliana* plants [loss of jasmonate (JA) signaling]. Total counts of flies emerging over a 25-day period from Col-0 and *coi1-1* plants. Plants were placed with ovipositing females at the same time, removed and larvae were allowed to develop naturally. Development time was significantly shorter in larvae reared on *coi1-1* than on Col-0 ($P < 0.001$). Loss of JA signaling increases feeding rate and development time in *S. flava*.

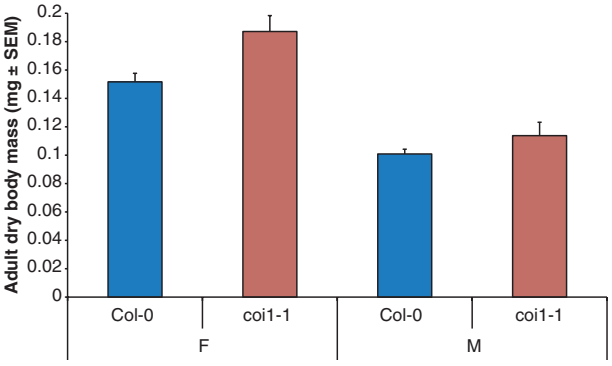

Fig. 8 Adult body mass differences in flies reared on Col-0 vs. *coi1-1 Arabidopsis thaliana* plants [loss of jasmonate (JA) signaling]. Plants were placed with ovipositing *Scaptomyza flava* females at the same time, removed and larvae were allowed to develop naturally. Body mass of males and females 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 signalling leads to larger adult body mass in *S. flava*.

in aliphatic glucosinolate biosynthesis and susceptible to other herbivores. Finally, plants sprayed with chitin, which is known to elicit a plant defence 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 signalling 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 (±0.11 SDM) on *coi1*-1 vs. 11.13 days (±0.09 SDM) on Col-0. Pupae eclosed as adults on average 1.06 (±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) vs. *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 h, 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 $CYP81F2_{pro}$*:GUS* plants in response to adult female feeding punctures, and GUS activity was detected immediately around feeding punctures. In $CYP81F2_{pro}$*:GUS* plants exposed to ovipositing females, GUS activity was strongest around feeding punctures with eggs (Fig. 9). In $CYP81F2_{pro}$*:GUS* plants with larvae transferred from Col-0 plants that had been allowed to feed for 24 h, GUS activity was strong along the midvein 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* Scaptomyza flava *herbivory*

Leaves of plants placed with ovipositing female flies for 48 h 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

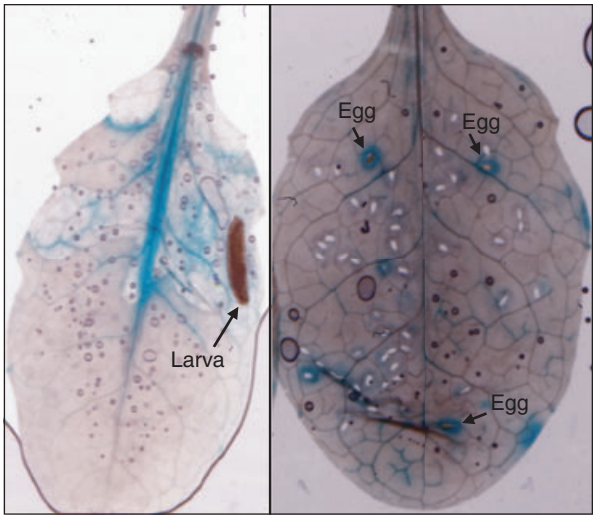

Fig. 9 Activation of the indolic glucosinolate modifier *CYP81F2* gene in the *Arabidopsis thaliana promoter*:GUS reporter line when attacked by *Scaptomyza flava* adult females and larvae for 24 h. Strong induction occurs around eggs (right photo) and in mines (left photo), but less so around simple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

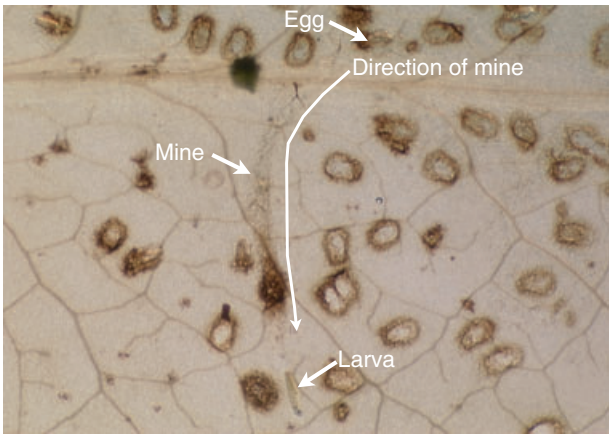

Fig. 10 DAB staining of hydrogen peroxide production (stained brown) in *Arabidopsis thaliana* leaves left with adult female *Scaptomyza flava* flies for 48 h. Note the staining around feeding punctures and in the walls of the mine formed by the first instar larva that hatched during this experiment. DAB, 3,3′-diaminobenzidine tetrahydrochloride.

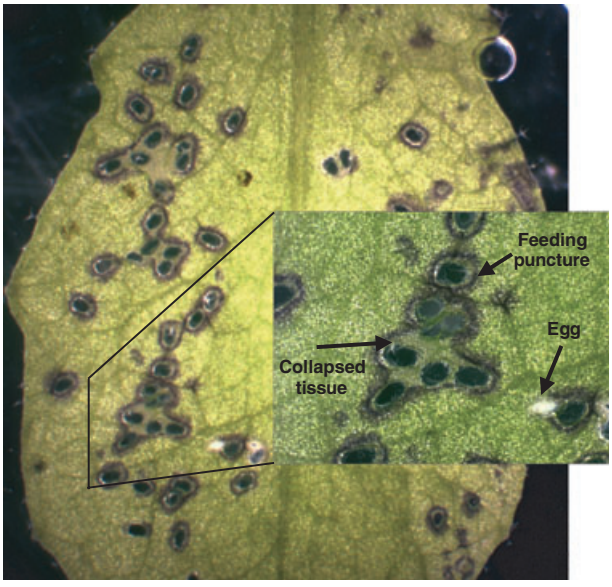

Fig. 11 Nitro blue tetrazolium staining of superoxide radicals (stained blue) around feeding punctures, but not eggs in *Arabidopsis thaliana* that were exposed to *Scaptomyza flava* females for 48 h. Collapsed tissue (apparently dead and not producing superoxide) in areas of high puncture density is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

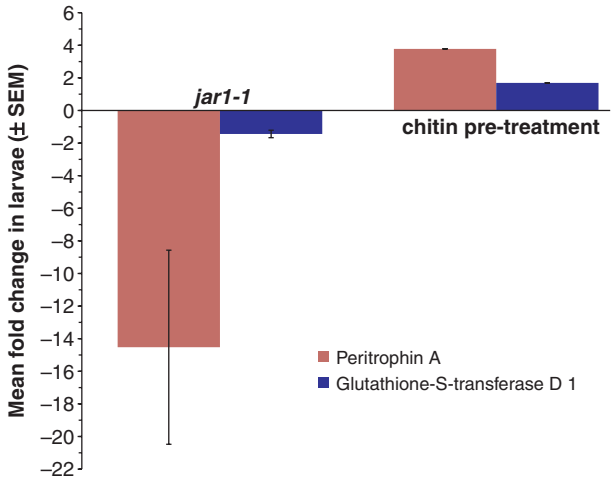

Fig. 12 Real-time quantitative PCR of two candidate xenobiotic metabolism genes (*GstD1* and *Peritrophin A*) in second instar larvae of *Scaptomyza flava* relative to a control gene (*RpL32*). Larvae were reared for 48 h in (i) jasmonate (JA) pathway loss of function mutant plants shown to be susceptible to herbivory (*jar1-1*) and in wild-type *Arabidopsis thaliana* plants and (ii) wild-type plants in which three lower leaves were infiltrated with 10–100 μg/mL crab shell chitin 24 h prior to adding larvae or in wild-type plants pretreated with sterile water. Values are in relation to expression levels of larvae reared in wild-type controls. Relative to larvae reared in wild-type plants, larvae showed lower expression of *GstD1* and *Peritrophin A* when reared in the JA pathway mutant and higher expression when reared in plants pretreated with chitin.

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 downregulation of transcripts of *GstD1* and *Peritrophin A* relative to larvae reared in wild-type (Col-0) plants, normalized with *RpL32* transcript abundance (Fig. 12). Larvae reared on wild-type plants pretreated with chitin exhibited upregulation of transcripts of *GstD1* and *Peritrophin A* relative to larvae reared in wild-type plants pretreated with the water control, normalized with *RpL32* transcript abundance (Fig. 12).

Discussion

A postgenomic 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/Boechera* spp.-associated herbivore systems (Kliebenstein *et al.* 2001a; Ratzka *et al.* 2002; Wittstock *et al.* 2004; Broekgaarden *et al.* 2007; Vogel *et al.* 2007; Wheat *et al.* 2007). However, functional genomic studies at the plant–insect interface have mostly emphasized the plant side of the equation (Ramsey *et al.* 2007; Zheng & Dicke 2008; The International Aphid Genomics Consortium 2010; Whiteman & Jander 2010). This imbalance is likely to change rapidly in the postgenomic 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 postgenomics 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 defence-response pathways. For example, recombinant inbred populations can be used to identify quantitative trait loci underlying resistance/susceptibility (Jander *et al.* 2001; Kliebenstein *et al.* 2001a,b; Zhang *et al.* 2006; Bidart-Bouzat & Kliebenstein 2008). 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 signalling 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 Scaptomyza 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 *Drosophila 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 centre 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* (*c.* 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 *Drosphila*, but similar to that of some of the *Drosophila* species already sequenced.

Characterization of the Scaptomyza flava *life cycle and performance across* Arabidopsis *accessions, elicitor pretreated plants and defence mutants*

Scaptomyza flava completes its life cycle on *Arabidopsis* accession Col-0 in approximately 21 days at 22 °C. This is a longer interval than the development time for *D. melanogaster* and other nonleafmining *Scaptomyza*, but perhaps not surprising given that larvae must cope with a dynamic and inducible host defence 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 defence pathways. While saprophytic and microbe-feeding *Drosophila* must also encounter host plant defence molecules, they are not typically living in or on organs of a host plant that can mount a defence 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.

Scaptomyza 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 towards the midvein, travelling 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 signalling 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 behaviour 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 the 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 *Arabidopsis thaliana*, averaging 20 days from egg to adult (Whiteman *et al.* unpublished). 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* defence mutants and histochemical assays revealed, most importantly, that the JA pathway and the glucosinolate pathway are important in mediating a full defence 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 (*coi1-1*, *jar1-1* and *cyp74a1*) were susceptible to *S. flava*. Moreover, pretreating wild-type plants with MeJA 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 *coi1-1* plants. This suggests that *Arabidopsis* defences modulated by JA 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 wild type. 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* defences 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 (Fraenkel 1959). However, there is growing evidence that specialists also pay a cost associated with feeding on these plant toxins (Agrawal & Kurashige 2003), despite harbouring unique pathways for coping with them (Wittstock *et al.* 2004). It would interesting to know whether the glucosinolate detoxification pathways in *S. flava* are similar to those in the specialist or generalist Lepidoptera.

Plants pretreated with chitin also showed resistance to *S. flava* larvae, consistent with the fact that chitin elicits a major defence response in *Arabidopsis* and other plants, including production of chitinases and protease inhibitors (Zhang *et al.* 2002) that are ecologically important plant defences 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 because of 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 because 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 tissue.

In summary, the screen of defence mutants, the indolic glucosinolate reporter line, the plants pretreated with MeJA 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 defence gene, *JAR1*, and how pretreatment 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 wild type, the JA pathway–deficient mutant *jar1-1* and in plants pretreated 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 midgut 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 wild-type (Col-0) plants and upregulated in larvae reared on plants pretreated with chitin relative to those reared on plants pretreated 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 wild-type plants. Thus, anti-herbivore defences are likely to be less robust in this mutant than in wild-type plants and the expression data from these two genes are a reflection of this. Conversely, larvae reared on chitin pretreated plants ate less than larvae reared on water pretreated 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,b,c), suggesting that general patterns and pathways might underpin insect resistance against plant defences.

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 defence 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 defence 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 behavioural, 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.

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NKW's research aims to uncover the ecological genetic and evolutionary bases of host–parasite interactions, including the evolution of host specialization. His new laboratory group at the University of Arizona is using the *Scaptomyza* lineage as a model for understanding the evolution of herbivory and host specificity as well as the genomic and neuronal correlates of those life histories.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Maximum-likelihood phylogram placing *Scaptomyza flava* in relation to *Drosophila* species based on 282 bp of the superoxide dismutase 1 (*Sod1*) nuclear gene. The tree was rooted with the drosophilid *Chymomyza amoena*. *Scaptomyza flava* is placed in the lineage with the other *Scaptomyza* species, a clade recovered with high support. Although the monophyly of the Hawaiian *Drosophila* is not recovered, there is little support in this tree for deeper nodes within the subgenus *Drosophila*, likely due to the fact that the analysis is of 282 bp of a single gene.

Fig. S2 Maximum-likelihood phylogram (with 100 bootstrap replicates) placing *Scaptomyza flava* in relation to the 12 completely sequenced *Drosophila* species and seven *Scaptomyza* species based on 942 bp of the cytochrome oxidase *c* subunit I (*COI*) mitochondrial gene (in some cases, <942 bp of *COI* was available). The tree was rooted with a sequence from *Anopheles gambiae*. *Scaptomyza flava* is placed in the lineage with the other seven *Scaptomyza* species and is most closely related to the mustard specialist *S. nigrita* from North America in this analysis. Although the monophyly of the subgenus *Drosophila* is not recovered, this mitochondrial gene evolves rapidly at second and third position sites. *Drosophila mojavensis* is placed within the *Scaptomyza* clade, but this is likely to the fact that it is on long branch. However, *Scaptomyza* and *D. mojavensis* are both in the subgenus *Drosophila*.

Video S1 A looped video of a female *Scaptomyza flava* creating a feeding puncture on the underside of an *Arabidopsis thaliana* leaf. Note that the fly spins around and imbibes the wound exudates.

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