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Genetic basis of octanoic acid resistance in *Drosophila sechellia*: functional analysis of a fine-mapped region

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Abstract

Drosophila sechellia is a species of fruit fly endemic to the Seychelles islands. Unlike its generalist sister species, *D. sechellia* has evolved to be a specialist on the host plant *Morinda citrifolia*. This specialization is interesting because the plant's fruit contains secondary defense compounds, primarily octanoic acid (OA), that are lethal to most other Drosophilids. Although ecological and behavioral adaptations to this toxic fruit are known, the genetic basis for evolutionary changes in OA resistance are not. Prior work showed that a genomic region on chromosome 3R containing 18 genes has the greatest contribution to differences in OA resistance between *D. sechellia* and *D. simulans*. To determine which gene(s) in this region might be involved in the evolutionary change in OA resistance, we knocked-down expression of each gene in this region in *D. melanogaster* with RNA interference (RNAi) (i) ubiquitously throughout development, (ii) during only the adult stage, and (iii) within specific tissues. We identified three neighboring genes in the *Osiris* family, *Osiris 6 (Osi6)*, *Osi7*, and *Osi8*, that lead to decreased OA resistance when ubiquitously knocked-down. Tissue specific RNAi, however, showed that decreasing expression of *Osi6* and *Osi7* specifically in the fat body and/or salivary glands increased OA resistance. Gene expression analyses of *Osi6* and *Osi7* revealed that while standing levels of expression are higher in *D. sechellia*, *Osi6* expression is significantly downregulated in salivary glands in response to OA exposure, suggesting that evolved tissue-specific environmental plasticity of *Osi6* expression may be responsible for OA resistance in *D. sechellia*.

Introduction

Insects are among the most abundant and diverse group of organisms on the planet, with plant-feeding insects making up the majority of described species (Price *et al.* 1980; Strong *et al.* 1984; Jolivet 1992; Bernays & Chapman 1994). Most phytophagous insect species are specialists and feed on a small number of related plant species (Eastop 1973; Price *et al.* 1980; Mitchell 1981; Ehrlich & Murphy 1988; Jolivet 1992; Bernays & Chapman 1994). Specialization is the result of host-specific adaptations that are generally related to differences in plant chemistry. Because these adaptations occur commonly, are key for ecological adaptation and fundamental for ecosystem function, adaptations of phytophagous insect species to novel host plants are model traits for adaptive evolution in nature (Via 1999; Dambroski *et al.* 2005). Typical adaptive phenotypes associated with host-plant specialization include resistance to plant secondary defense compounds as well as preference behaviors associated with locating the new food source (Jaenike 1987; Via 1990; Futuyma 1991). Despite years of research we still know little about the genetic basis of such adaptive traits.

Fruit flies in the genus *Drosophila* are an excellent model for understanding the evolution of adaptive traits associated with insect-host plant associations because of the incredible diversity of food sources used by these species and their frequent shifts between food sources (Matzkin *et al.* 2006; Linz *et al.* 2013; Matzkin 2014). The well-studied *Drosophila melanogaster* supercomplex contains both generalist and specialist species, allowing dissection of the genetic basis of host transitions. The generalist species in this group include *D. melanogaster*, *D. simulans*, and *D. mauritiana*, which feed on the rotting fruit of several species of plants. Nested within this group of generalist species is a single derived specialist species, *D. sechellia*, which is endemic to the Seychelles islands and feeds almost exclusively on a single host plant: *Morinda citrifolia* (Matute & Ayroles 2014).

Specialization on *M. citrifolia* is interesting because the fruit of the plant contains toxic defense compounds that are lethal to most other species of *Drosophila.* The primary toxin produced by *M. citrifolia* is octanoic acid (OA), a medium chain fatty acid comprising 58% of the total volatile compounds in the fruit (Moreteau *et al.* 1994; Farine *et al.* 1996; Amlou *et al.* 1998; Legal *et al.* 1999). OA concentration varies during the ripening process with peak toxicity at full ripening (Legal *et al.* 1994), and is detoxified over time by microorganisms, opening up the niche to the other *Drosophila* species (R'Kha *et al.* 1991; Matute & Ayroles 2014). Because both adult and larval stages of *D. sechellia* are resistant to the OA levels present during the

highest peak in toxicity (Jones 1998, 2001), *D. sechellia* appears to have achieved a reproductive advantage through minimization of competition by being able to utilize the food source during an earlier time in the fruit's development.

Because the primary defense compound in the fruit is OA, it is used as a proxy for resistance studies in *Drosophila*. Resistance to OA varies among species in the *Drosophila melanogaster* species supercomplex—*D. simulans* and *D. mauritiana* are both less resistant to OA than *D. melanogaster*, and all three species are markedly less resistant than *D. sechellia*, which shows tolerance to extremely high levels of OA (Amlou *et al.* 1997; Jones 1998). In addition to resistance to OA, *D. sechellia* differs from sister taxa by a suite of associated derived traits including increased egg production in the presence of, attraction to, and oviposition site preference for *M. citrifolia* (R'Kha *et al.* 1991; Jones 2004; Matsuo *et al.* 2007) and a recent study suggests that a derived change in the catecholamine regulatory protein *Catsup* in *D. sechellia* and the presence of L-DOPA in *M. citrifolia* fruit has facilitated the specialization of *D. sechellia* on its toxic host (Lavista-Llanos *et al.* 2014). However, the specific genes involved in resistance of *D. sechellia* to OA remain unknown.

Genetic analyses of OA resistance in *D. sechellia* adults suggest that it is not highly polygenic with five chromosomal regions mapped that contribute to variation in this trait, including a single region of large-effect on chromosome 3R (91A-93D) that explains ~15% of the difference between *D. simulans* and *D. sechellia* (Jones 1998, 2005; Huang & Erezyilmaz 2015). A recent study using introgression to move *D. sechellia* genomic regions conferring OA resistance into a *D. simulans* genetic background further narrowed this resistance locus to a single 170kb region containing 18 genes (Hungate *et al.* 2013). The genes in this region have a variety of predicted functions including three odorant binding proteins (obp): *Obp83cd*, *Obp83ef*, and *Obp83g*; and nine *Osiris* genes which are biologically and molecularly uncharacterized but predicted to be transmembrane proteins localized to the endo-lysosomal system and potentially involved in the dosage-sensitive triple lethal locus (Dorer *et al.* 2003; Shah *et al.* 2012). Interestingly, it appears that evolved resistance to OA has likely evolved through parallel evolution in both *D. sechellia* and a newly identified island population of *D. yakuba* that has evolved OA resistance. In this study they performed a population genomics scan for differentiation between island noni specialist and mainland generalist populations of *D. yakuba* and among the strongest differentiation peaks was the same genomic region implicated in *D. sechellia* OA resistance by

introgression mapping(Yassin *et al.* 2016, Hungate *et al.* 2013).

To identify the strongest candidate gene(s) in this region that contribute to OA resistance in *D. sechellia* and potentially in parallel in *D. yakuba*, we used RNA interference (RNAi) in *D. melanogaster* to functionally test each gene in a mapped resistance region (Hungate *et al.* 2013) for a role in OA resistance. Using two different RNAi screens of genes in this region, one knocking down each gene's expression ubiquitously throughout development and the other knocking down each gene's expression only in adults, we found that three genes, *Osiris 6* (*Osi6*), *Osiris 7* (*Osi7*), and *Osiris 8 (Osi8)* significantly reduced resistance to the toxic effects of OA when their expression was reduced. Tissue specific knockdowns, however, showed that decreasing expression of *Osi6* and *Osi7* specifically in the fat body and salivary glands increased OA resistance. The results of these functional tests together with species, tissue and environment-specific expression profiles, and sequence analyses suggest that derived tissue-specific toxin-induced gene expression changes of *Osi6* play an important role in OA resistance in *D. sechellia.*

Methods

Fly strains and maintenance

Strains of four species of *Drosophila* were used in this study: *D. melanogaster* (Canton S, Oregon R, *Zhr* (full genotype: XYS.YL.Df(1)Zhr), z30, 14021-0231.36 (*dm3*), w1118, a balanced ubiquitous GAL4 driver line (*actin*-GAL4/CyO), a GeneSwitch-GAL4 driver (*Tubulin*-P[Switch]), Wang *et al.* 1994), and a tissue specific driver line (elav-GAL4)), *D. simulans* (Tsimbazaza, 14021-0251.195), *D. mauritiana* (14021-0241.60) and *D. sechellia* (14021-0428.25, 14021-0428.08, 14021-0428.27, 14021-0428.07, 14021-0428.03). Additional *D. melanogaster* UAS-RNAi lines from the Vienna *Drosophila* UAS-RNAi Center (Dietzl *et al.* 2007), (VDRC# 60000, 102518, 42725, 18814, 40807, 33967, 7552, 5738, 33970, 9606, 43404, 26791, 42612, 5747, 102392, 44545, 8475, 5753, for full genotypes see Table S1). Tissue-specific GAL4 drivers and a line carrying a mutant allele of *Obp83g* were obtained from the Bloomington Stock Center (Stock# 30843, 30844, 6357, 6870, 8180, 58515, for full genotypes see Table S1). All flies were reared on cornmeal medium using a 16:8 light:dark cycle at 25℃.

Octanoic acid resistance assay

Flies used in resistance assays were generated by crossing 3 virgin female with 3 male flies to control offspring larval density. For RNAi experiments, virgin female *actin*-GAL4/CyO, *Tubulin*-P[Switch]-GAL4 or tissue-specific GAL4 lines were crossed to UAS-RNAi males and all resultant progeny were aged to 1-4 days post eclosion. Flies were then anesthetized with CO_2 and separated by sex in all crosses and balancer chromosome (CyO) associated phenotypes in crosses with *actin*-GAL4/CyO or #8765. Flies were then allowed to revive in empty fly vials (Genesee Scientific) at a density of 10 flies per vial for 1.5 hours. Flies were then transferred into experimental vials containing 3.25g *Drosophila* instant media mix (Carolina Biological) supplemented with ≥99% octanoic acid (Sigma) and homogenized to produce food with 0.5-1.2% OA. GeneSwitch crosses were reared at room temperature and F_1 offspring were aged between 1-3 days. Aged flies were then transferred to fresh fly food mixed with mifepristone (RU486 Sigma, St. Louis) from a stock solution of 10 mg/ml in 100% EtOH to a final concentration of 10μg/ml overnight for 24 hours. Flies were then immediately used in the OA assay. The number of individuals "knocked down" (a fly was counted as "knocked down" when the individual was no longer able to walk or fly) was determined every five minutes for 60 minutes.

Mixed effect Cox regression analysis

A semi-parametric Cox proportional-hazard model was used to test the risk of OA exposure during gene knockdown using a mixed effect Cox model (Cox 1972; Fox 2002) in the *coxme* package in R (R Development Core Team 2011). We report the regression coefficient, β, that when exponentiated gives the relative hazard in the treatment group, for RNA-induced knockdown flies (UAS-RNAi/GAL4) against the parental UAS-RNAi line as reference. The sibling controls (UAS-RNAi/CyO) were also graphed against the UAS-RNAi parental reference line. Only when knocked-down flies (UAS-RNAi/GAL4) were significantly different from both parental lines (UAS-RNAi and GAL4) and their respective sibling controls (UAS-RNAi/CyO) were knocked-downs deemed significant. Vial number and day were included in the model as random effects, and sex was used as a multiplicative interaction variable:

```
coxme(Surv(Time,Status)~Genotype*Sex+(1|Date)+(1|Vial),data=RNAi,ties=c("efron")).
```

The proportional hazard Cox regression, *coxph*, package was used to plot survivorship (percent) after 60 minutes to visualize variation in fly survival using different OA concentrations ranging from 0.5% to 1.2% in 1-4 day old female *D. melanogaster* (*actin*-GAL4/CyO) individuals (Cox 1972; Hertz-Picciotto & Rockhill 1997). The *coxph* package was also used to graphically represent proportional hazards within and between species as survival curves with 95% confidence intervals. Sample sizes for knockdown experiments are shown in Table S2 and Table S3.

Gene expression analyses in Drosophila

Quantitative reverse transcriptase PCR (qRT-PCR) was used to measure gene expression levels for *Osi6, Osi7, Osi8* and the housekeeping gene *Alpha Tubulin 84B* (*αTub84B*). RNA was isolated from 0-4 day old whole adult flies (*D. melanogaster* (14021-0231.36), *D. simulans* (14021-0251.195), *D. sechellia* (14021-0428.25,), *and D. mauritiana* (14021-0241.60)) that were transferred post-eclosion to experimental vials containing 3.25g *Drosophila* instant media mix (Carolina Biological, control food) or vials supplemented with ≥98% octanoic acid (Sigma) and homogenized to produce food with 0.2% OA. This concentration was chosen as it prevented mortality and allowed for collection of sensitive species while still having obvious behavioral effects on the flies. Flies were then aged for three days in this treatment, anesthetized with CO_2 and snap frozen whole, or dissected into tubes containing 10 heads (abbreviated NS), intestine (abbreviated IN), salivary glands and associated tissue (abbreviated SG), and fat body and associated dorsal abdominal cuticle (Krupp & Levine 2010) (abbreviated FB) and kept at -80°C until use. RNA was extracted from each pool of flies or tissues using the Promega SV total RNA extraction system with modified protocol (Promega, Coolon *et al.* 2013). cDNA was synthesized from total RNA using T(18) primers and Superscript II (Invitrogen) according to manufacturer recommendations. qRT-PCR was performed on an Applied Biosystems StepOne Plus thermocycler. For each sample, Applied Biosystems PowerUp SYBR Green Master Mix (25µl) was mixed with 0.5µl GoTaq DNA Polymerase, 9.5µl nuclease-free water, and 10µl cDNA and split into five reactions containing 9µl each. Once split, gene specific primers (Table S4) were added (0.5µl each) for a total volume of 10µl per reaction. Cycling conditions for PCR were the same for all genes except for different annealing temperatures: 50°C for 2 minutes followed by

95°C for 2 min, followed by 50 cycles of 95°C for 15 sec, annealing temp (56°C for *Osi6*, *Osi7* and *Osi8*, 63°C for *αTub84B*) for 30 sec and 72°C for 30 sec. Melt curves were generated for each reaction to ensure specificity. Threshold cycle (C_T) values were generated for each reaction based on entry into log phase amplification during PCR. For *Osi6*, *Osi7* and *Osi8*, ΔC_T values were generated by correcting each against the housekeeping gene *αTub84B* (ΔC_T = GOI C_T - *αTub84B* C_T. Four biological replicates were run for each sample type and T tests were performed to evaluate statistical significance. For comparisons between flies or tissues from flies reared on control food and food containing OA, $\Delta\Delta C_T$ values were generated by subtracting control – OA for each sample and T tests were performed testing against zero.

All other measures of gene expression were obtained from prior studies (Graveley *et al.* 2011; Brown *et al.* 2014; Coolon *et al.* 2014). Levels of gene expression quantified using RNA-seq on *D. melanogaster* (*y[1]; cn[1] bw[1] sp[1]*) across development (larvae, pupae, adult) (Graveley *et al.* 2011), *D. melanogaster* (*Oregon R*) in response to various perturbations (chemical exposure to cadmium, copper, zinc, caffeine, paraquat, as well as extended cold, cold shock, heat shock) (Brown *et al.* 2014) and *D. melanogaster* (*Oregon R*) tissue-specific expression levels (larval fat body, larval salivary gland, pupal nervous system (abbreviated CNS), pupal fat body, adult intestine, adult carcass and adult head) (Brown *et al.* 2014) were obtained from data generated by the modENCODE project.

Sequence analyses: synonymous and nonsynonymous changes

Coding sequences (CDS) for *Osi6*, *Osi7*, and *Osi8* were downloaded from FlyBase (St. Pierre *et al.* 2014) for the *Drosophila* species with sequenced genomes. Sequence was absent for the *D. simulans* ortholog of *Osi7* from the Flybase genome build, so we used recently published genomic sequence data from the *Tsimbazaza* isofemale line of *D. simulans* (Coolon *et al.* 2014; McManus *et al.* 2014). Sequence for the *D. mauritiana* orthologs of *Osi6*, *Osi7*, and *Osi8* was determined for the CDS by Sanger sequencing performed by the University of Michigan DNA Sequencing Core. Sequences were aligned with GENEIOUS software (Biomatters Ltd.) and synonymous and nonsynonymous sequence changes were identified. Sequences of *Osi6/7/8* in *D. melanogaster*, *D. simulans*, and *D. sechellia* were confirmed with Sanger sequencing. Additional lines of *D. sechellia* were analyzed with Sanger sequencing to determine if derived differences in *D. sechellia* were fixed.

Results

Quantifying octanoic acid resistance in Drosophila

The resistance of *Drosophila sechellia* to the toxic effects of *M. citrifolia* fruit and its primary toxin OA are well documented; however, the assay by which toxicity is measured (e.g. exposure to OA vapor, OA in instant media food, and natural OA in *M. citrifolia* fruit) and the concentration of OA (0.1-100%) used varies considerably among studies (Moreteau *et al.* 1994; Farine *et al.* 1996; Amlou *et al.* 1997; Legal *et al.* 1999; Hungate *et al.* 2013). To control the concentration of OA each fly experienced in the mortality assay, we exposed flies to OA mixed into food. To determine the optimal concentration of OA to use for resistance experiments, we assayed 1-3 day old adult female *D. melanogaster* (*actin*-GAL4/CyO) for mortality associated with exposure to five concentrations of OA (0.5-1.2%, Figure 1A). We found that mortality increased with increasing OA concentrations (Figure 1A). To allow both increases and decreases from a baseline OA associated mortality to be detected, we chose to use 1.2% OA in all subsequent experiments, at which approximately 50% mortality was observed within 60 minutes (Figure 1A).

To quantify differences in OA resistance among the members of the *D. melanogaster* species group (*D. melanogaster*, *D. simulans*, *D. sechellia*, and *D. mauritiana*), we performed mortality assays at a concentration of 1.2% OA (Figure 1B). The four species tested form distinct groups with the five *D. sechellia* lines most resistant and having more than 80% survival at 60 minutes. Both tested *D. simulans* lines were the least resistant and 100% death was observed within 20 minutes of exposure. Finally, the one *D. mauritiana* and the six *D. melanogaster* lines tested had intermediate resistance and for each line some individuals survived the entire 60 minutes of exposure to OA. Resistance varied considerably within species among lines of both *D. sechellia* and *D. melanogaster* (Figure S1A). We also observed differences between the sexes with females more resistant than males in some cases (Figure S1B) and therefore sex was included in all subsequent statistical models for this reason.

We used a Cox regression model to analyze the data from the survivorship curves (Cox 1972). To quantify the relative resistance of each line when exposed to OA, we used regression

coefficients representing relative survival (-β) where all lines were compared to a reference *D. melanogaster* line (w^{1118} *v60000*, Figure 1C). The patterns observed in the survival curves are recapitulated in this analysis with *D. sechellia* lines having the greatest relative survival (-β = 5.4 to 2.3), *D. melanogaster* (-β = 1.4 to -0.8) and *D. mauritiana* (-β = -0.5) intermediate and *D. simulans* having the lowest relative survival (-β = -1.5 to -1.7) and each species is significantly different from each other species ($P < 0.03$ in all cases, Figure 1C).

Ubiquitous RNAi knockdown of Osi6 *and* Osi7 *altered OA resistance*

To test the functional role of genes in the mapped OA resistance region (Hungate *et al.* 2013) on OA resistance, we used RNAi to knock down expression of each gene in *D. melanogaster* and performed OA resistance assays. RNAi was performed by crossing transgenic lines that express hairpin RNAs under the control of the yeast upstream activating sequence (UAS) (Dietzl *et al.* 2007) with a line that ubiquitously expresses GAL4 driven by the *cis*-regulatory sequence from *Actin 5C* resulting in knockdown in every life stage and cell type. Hairpin-UAS RNAi lines were available for 17 of the 18 genes in the mapped resistance region with only *Odorant Binding Protein 83g* (*Obp83g*) not available. Because the OA mortality assay is sensitive to genetic background in *D. melanogaster* (Figure 1C) our experimental design included a sibling control for each line tested. To do this we crossed each homozygous RNAi-UAS line to a line expressing *actin*-GAL4 from chromosome 2 that was heterozygous over a dominantly marked balancer chromosome (*CyO*) which produced both knockdown (RNAi-UAS/*actin*-GAL4) and control (RNAi/*CyO*) progeny. We confirmed that the presence of the *CyO* balancer chromosome had no effect on survival by comparing *actin*-GAL4/*CyO* to homozygous *actin*-GAL4. To identify the effect of knockdown on OA associated mortality, we compared knockdown individuals to sibling controls with a mixed effects Cox proportional hazards model (Cox 1972; Hertz-Picciotto & Rockhill 1997). Using this approach we found that only two genes significantly altered OA resistance when knocked down, *Osi6* and *Osi7*, which both significantly decreased resistance to OA (-β = -2.65, $P = 2 \times 10^{-5}$; -β = -2.8, $p = 7 \times 10^{-7}$, Figure 2A). Because only 17/18 genes in the mapped resistance region were available as RNAi-UAS lines, we tested the final gene, *Obp83g*, using an available mutant line and found that it did not have a significant effect on OA resistance (-β = -2.0, $P = 0.1$, Figure S2).

Stage-specific RNAi knockdown of Osi6 *and* Osi8 *altered OA resistance*

Ubiquitous knockdown of genes with RNAi may have indirect consequences on OA associated mortality because most genes are pleiotropic and are important for other biological or developmental processes. When we investigated developmental expression patterns for *Osi6*, *Osi7* from ModENCODE data (Graveley *et al.* 2011), we found that *Osi6* and *Osi7* have similar temporal expression patterns and that both were highly expressed with cyclic expression throughout development, increasing and decreasing in expression during each major developmental stage (Figure S3A). Within the first 24 hours of development, *Osi6* and *Osi7* are the most active with peak expression occurring at 16 hours of embryonic development. They maintain this cyclic pattern throughout the larval and pupal stages, and show very low expression in adult *D. melanogaster*, consistent with a possible developmental role for these genes.

Interestingly, *Osi6* and *Osi7* knockdown led to large reductions in the number of progeny that survive to adulthood (note reduced sample size despite setting 10-50X the crosses for these lines, Table S3), which confirm important developmental roles of these two genes. To bypass developmental defects associated with ubiquitous knockdown, we performed stage-specific knockdown for the same 17 genes described above. Using the Gene-Switch system we induced knockdown only in adult individuals immediately prior to and during OA exposure. The Gene-Switch system we used is a hormone induced *Tubulin*-P[Switch] GAL4 driver consisting of a modified chimeric GAL4 gene (Gene-Switch) that encodes the GAL4 DNA binding domain, the human progesterone receptor ligand-binding domain, and the activation domain from human protein *p65*. The chimeric molecule only becomes active in the presence of the synthetic antiprogestin, mifepristone (RU486), and when active binds to the UAS sequence to activate transcription of the RNA hairpin, knocking-down expression of that gene only when RU486 is provided (Osterwalder *et al.* 2001; Roman *et al.* 2001).

Using this inducible, stage-specific knockdown system, RNAi was performed for the genes in the mapped resistance region by crossing each RNAi-UAS line to the *Tubulin*-P[Switch] GAL4 line and comparing sibling offspring from this cross with and without knockdown (+/- RU486). We found that knockdown of *Osi6* ($-\beta = -2.02$, $P = 0.016$) and *Osi8* ($-\beta = -2.13$, $P = 0.0015$) resulted in significant decreases in resistance compared to hormone unexposed siblings. Interestingly and in contrast to ubiquitous knockdown described above, *Osi7* had no effect in this experiment ($-\beta = 0.19$, $P = 0.77$, Figure 2B). To rule out an effect of RU486

alone we tested the response of *w1118*, the genetic background the RNAi-UAS lines were made in, to RU486 in the OA mortality assay and found no effect ($-\beta = -0.47$, $P = 0.58$).

Synonymous and nonsynonymous changes in Osi6, Osi7 *and* Osi8 *genes*

Knockdown with RNAi identified three candidate genes, *Osi6*, *Osi7*, and *Osi8* that may play a role in OA resistance. All three genes are in the *Osiris* gene family, members of which are characterized by an endoplasmic reticulum signal sequence, a transmembrane domain, a number of endocytic signaling motifs, an AQXLAY motif, and a pair of 5' cysteine residues (Dorer *et al.* 2003; Shah *et al.* 2012; Lee *et al.* 2013). Beyond these sequence-based predictions, *Osi6*, *Osi7* and *Osi8* all remain functionally uncharacterized. Protein coding sequence changes in these genes could contribute to the evolution of OA resistance at this locus so we identified derived sequence differences in the *D. sechellia* orthologs of *Osi6*, *Osi7*, and *Osi8* that could have functional consequences on OA resistance. To do this, we aligned the coding sequences of these genes from *D. sechellia*, *D. simulans*, *D. mauritiana* and *D. melanogaster*. The *D. sechellia* ortholog of *Osi6* contains 5 derived synonymous and no derived nonsynonymous changes (Table 1). The *D. sechellia* ortholog of *Osi7* also had 5 synonymous changes and no fixed derived nonsynonymous changes. Finally, the ortholog of *Osi8* had 4 synonymous and 2 nonsynonymous derived sequence differences in *D. sechellia.* This rules out changes in the amino acid sequence of *Osi6* and *Osi7* from contributing to the resistance phenotype in *D. sechellia* and suggests that changes in regulatory sequences and therefore gene expression cause any functional differences that might exist between *D. sechellia* and other species at these loci, while protein coding changes at *Osi8* may contribute to its possible role in OA resistance.

Osi6, Osi7, Osi8 *expression varies by species, tissue and developmental stage*

To investigate how gene expression of *Osi6*, *Osi7* and *Osi8* may have evolved, we used qRT-PCR to measure relative mRNA levels in *D. sechellia*, *D. mauritiana*, *D. simulans* and *D. melanogaster* (Figure 3) hereafter referred to as gene expression. *Osi6* and *Osi7* gene expression was highest in *D. sechellia*, intermediate in *D. melanogaster*, and lowest in *D. simulans* and *D. mauritiana*. *Osi6* expression in *D. sechellia* was significantly higher than *D. melanogaster* ($P = 0.02$), *D. simulans*, ($P = 0.001$) and *D. mauritiana* ($P = 3.4 \times 10^{-5}$). *Osi6* expression in *D. melanogaster* was similar to *D. simulans* ($P = 0.08$) and significantly higher than *D. mauritiana*

(P = 0.049). *D. simulans* and *D. mauritiana* had indistinguishable expression of *Osi6* (P = 0.37). Similar to that observed for *Osi6*, we found that *Osi7* was expressed significantly higher in *D. sechellia* than the other three species ($P < 9.1 \times 10^{-6}$ in each comparison) but there was no difference between any of the other species ($P > 0.05$ in each case, Figure 3). There were also no observed differences in expression of *Osi8* among all tested species ($P > 0.05$ in all cases). Interestingly, the pattern of differential expression of *Osi6* and *Osi7* between species matches the pattern observed for species-specific octanoic acid resistance (Figures 1B,C, 3). These whole-fly measures of gene expression strongly suggest that changes in gene regulation confer OA resistance. However, they do not provide information about the tissues where gene expression changes are important for this trait-change.

To better understand how these genes may affect OA resistance, we obtained tissue-specific gene expression data from *Osi6, Osi7*, and *Osi8* in *D. melanogaster* (Brown *et al.* 2014). We found that *Osi6* and *Osi7* are primarily expressed in the fat body, salivary gland, intestine and central nervous system and *Osi8* is expressed in the fat body and nervous system (Figure S3B). These data from *D. melanogaster* suggest the primary tissues where *Osi6, Osi7* and *Osi8* are expressed but much of the data were collected from larval and pupal life stages where expression may or may not be linked to adult OA resistance traits.

Tissue-specific knockdown of Osi6, Osi7 *and* Osi8

Ubiquitous and stage-specific knockdown screens reduced expression of *Osi6, Osi7* and *Osi8* in every cell throughout the entire organism. However, available gene expression data indicated that these genes likely have expression domains restricted to particular tissues. Because each gene's functional contribution to OA sensitivity might be localized in one or more specific tissues and these tissue-specific effects may differ, we used GAL4-driver lines that have documented tissue-specific GAL4 expression and crossed these strains to the same RNAi-UAS lines described above to knockdown *Osi6, Osi7* and *Osi8* in specific tissues identified by the expression analyses (Figure S3B). The tissues targeted by these experiments were: 1) nervous system (NS), 2) salivary glands (SG), 3) fat body (FB) and 4) intestine (IN). There was no effect of *Osi8* knockdown in any tested tissue ($P > 0.05$ in all cases, Figure 4A). Knockdown of *Osi7* in the salivary glands ($-\beta = 2.081$, $P_{SG} = 0.026$) and fat body ($-\beta = 2.985$, $P_{FB} = 0.013$) significantly increased resistance to OA in *D. melanogaster* (Figure 4A). Similarly, knockdown

of *Osi6* in the salivary glands significantly increased resistance to OA (-β = 3.655, P_{SG} = 0.022 and all other *Osi6* or *Osi7* tissue-specific knockdown resulted in OA resistance that was unchanged. As an additional control we also performed knockdown of *Osi5*, a gene not found to influence OA resistance in ubiquitous or stage-specific knockdown, in all the same tissues and again found no significant effects on OA resistance ($P > 0.05$ in each case, Figure S4). Surprisingly, tissue-specific knockdown in the salivary gland (*Osi6* and *Osi7*) and fat body (*Osi7*) had an opposite effect from that observed for ubiquitous knockdown (Compare Figure 2A, B and Figure 4A). Ubiquitous knockdown lead to reduced resistance and tissue-specific knockdown caused increased resistance, therefore making these individuals more similar to the level of resistance observed in *D. sechellia.*

Tissue-specific knockdown of *Osi6* and *Osi7* caused increased OA resistance suggesting that these genes may have tissue-specific reductions in expression in *D. sechellia* as compared to *D. simulans*. To investigate tissue-specific expression for *Osi6*, *Osi7* and *Osi8* we used qRT-PCR to measure gene expression levels in the same tissues used for RNAi (nervous system, salivary glands, fat body and intestine) dissected from adult *D. simulans* and *D. sechellia* individuals (Figure 4B). We found that *Osi6* and *Osi7* are expressed at higher levels in *D. sechellia* than in *D. simulans* when averaged across all tissues (P_{Osi6} = 0.009, P_{Osi7} = 0.031) consistent with that observed for expression levels in whole flies (Figure 3). We did not however, observe any significant differences between expression levels in any of the tissues tested individually in either *D. simulans* or *D. sechellia* ($P > 0.05$ in all cases, Figure 4B) suggesting that the higher expression observed in whole flies was caused by some untested tissue(s).

Osi6 *gene expression response to OA exposure*

Tissue-specific reductions in expression with RNAi in *D. melanogaster* led to increased OA resistance yet there are no significant decreases in gene expression in specific tissues for *Osi6*, *Osi7* or *Osi8* in *D. sechellia*. It is possible however; that *Osi6*, *Osi7* and/or *Osi8* have gene expression decreases in response to OA exposure that may confer resistance to the toxin. Consistent with this idea, *Osi6* and *Osi7* were shown to respond to external stressors in *D. melanogaster* (Brown *et al.* 2014) (Figure S3C). To determine whether *Osi6*, *Osi7* and *Osi8* are responsive to OA exposure we treated adult flies for 3 days in non-lethal OA conditions (0.2% OA) and used qRT-PCR to measure differential gene expression in response to OA exposure as

compared to control in *D. sechellia*, *D. melanogaster*, *D. simulans* and *D. mauritiana* (Figure 5A). Exposure to OA caused a significant increase in expression of *Osi6* ($P = 0.033$) and *Osi7* ($P = 0.009$) in *D. simulans* consistent with their greater sensitivity to OA exposure. While no other species showed significant ($P > 0.05$ in all cases) responses to OA exposure for *Osi6, Osi7* or *Osi8, D. sechellia* did have small decreases for both *Osi6* and *Osi7* in response to OA (Figure 5A). To investigate whether *Osi6, Osi7* and *Osi8* have tissue-specific differential expression in response to OA exposure masked in whole animal expression quantification, we dissected adult tissues from OA exposed and control *D. simulans* and *D. sechellia* flies and measured gene expression with qRT-PCR (Figure 5B). Significant salivary gland-specific reduction of *Osi6* expression was observed for *D. sechellia* ($P = 0.04$) and a significant fat body-specific increase in *Osi6* expression was observed in *D. simulans* ($P = 0.04$). Additionally, we observed marginally significant increased expression of *D. simulans Osi7* in response to OA exposure ($P = 0.07$). Therefore, tissue-specific expression plasticity is consistent with and explains the expression levels observed in whole flies (Figure 5A vs. 5B) and is consistent with observed RNAi phenotypes (Figures 2A, 2B, 4A).

Discussion

Host specialization in phytophagous insects is a classical model of adaptive evolution. Research on these systems allows for new understanding of normal ecosystem function and identifies new strategies for control of agricultural crop pests. Understanding the genetic basis of ecological adaptations to novel habitats is needed, yet only a few such case studies exist. Here we investigated the genetic basis of *D. sechellia* adaptation to feed almost exclusively on the toxic fruit of the *M. citrifolia* plant. To do this we used RNAi in *D. melanogaster* to functionally test individual genes for a role in resistance to OA, the primary toxin in *M. citrifolia*. We focused this screen on a fine mapped region known to contribute to OA resistance containing 18 genes (Hungate *et al.* 2013) ultimately identifying three candidate genes; *Osi6, Osi7,* and *Osi8* that affect OA resistance in *D. melanogaster*. Because this work was done in a heterologous host, and some crosses contain individuals with balancer chromosome differences, we performed crosses utilizing geneswitch-GAL4 where all individuals are genotypically identical and differ only in

the expression of RNAi knockdown in adult stages controlled by hormone exposure. We then followed these experiments with tissue-specific RNAi and ultimately analyzed DNA sequence and expression profiles of *Osi6, Osi7* and *Osi8* in different species, tissues, and environmental contexts. Together with a recently published population genomics scan between an island noni specialist and mainland generalist populations of *D. yakuba* that found the *Osiris* cluster was among the strongest differentiation peaks (Yassin *et al.* 2016), it seems likely that the *Osiris* gene cluster is responsible for the mapped resistance in both *D. sechellia* and possibly *D. yakuba*. Furthermore, we believe the *Osiris* gene family may represent a new family of genes insects may utilize in the evolution of defense against chemicals in the environment (Whiteman *et al.* 2012; Whiteman & Gloss 2016), and if true represents a novel mechanism for evolved chemical resistance that warrants further study.

Two of these candidate genes (*Osi6* and *Osi7*) have tissue-specific effects on OA resistance in *D. melanogaster* and sequence analyses of these genes found no protein coding differences derived in *D. sechellia.* Furthermore, derived species specific changes in *Osi6* and *Osi7* gene expression and tissue- and OA environment-specific changes in *Osi6* gene expression in *D. sechellia* suggest that regulatory changes at this locus are at least partially responsible for this phenotypic change. Interestingly, when *Osi6* is ubiquitously knocked down it drives decreased resistance to OA, yet upon knockdown in the salivary glands causes increased resistance to OA in *D. melanogaster*. Because standing levels of *Osi6* and *Osi7* are significantly higher in *D. sechellia* it is possible that increased expression in some untested tissue(s) may confer resistance while decreases in other tissues (salivary glands) can also confer resistance to OA. Increases and decreases in expression leading to identical phenotypic outcomes is not uncommon as demonstrated in studies of several genes including Tbx6 in zebrafish segmentation (Windner *et al.* 2015), *SIR4* in yeast gene silencing (Marshall *et al.* 1987), SRPK1 in AKT activation and human cancer (Wang *et al.* 2014). Finally, and most relevant to our findings, increases and decreases in Optineurin in mammals leads to disrupted protein trafficking in the endomembrane system, perhaps analogous to the role *Osiris* genes may play in OA resistance (Park *et al.* 2010).

In addition to the phenotypic outcomes we described in this manuscript, we have also observed other traits associated with knockdown of *Osi6* and *Osi7* during other developmental stages. For example, when either *Osi6* or *Osi7* are knocked down ubiquitously during embryonic

development, almost complete lethality is observed with only a few surviving progeny. We have also observed a wing phenotype associated with knockdown of *Osi6* during larval development. Because we see differences in the phenotypic outcome for these genes depending on developmental stage, tissue and environmental context, these results suggest that great care must be taken for functional tests of such pleiotropic genes, which likely represents the majority of the genome, because null mutants and even ubiquitous knockdown can result in phenotypes that may not represent the individual contributions of the different cellular functions of genes in complex organisms and in complex environments.

Genetic mapping of the trait to this narrow window of the genome that contains no transcription factors suggests that the role of *Osi6* in OA resistance is likely to be a consequence of *cis*-regulatory differences, and this may be an example of how the modular nature of these types of changes can avoid possible negative effects of pleiotropic coding mutations and allow the evolution of novel traits. Additionally, while our RNAi data indicate that increased OA resistance can be achieved through tissue-specific reductions in *Osi6* in the salivary gland or *Osi7* in the salivary gland or fat body, it appears the path that evolution took in *D. sechellia* was through changes in *Osi6* expression in the salivary gland. To determine the precise mutations and mechanisms responsible for the role of *Osi6* in OA resistance we will next assay *Osi6* gene function in *D. sechellia* directly using transgenic approaches and recent advances in CRISPR technology (Gratz *et al.* 2013).

Osiris *family genes and a role in detoxification*

The *Osiris* gene family remains almost completely uncharacterized, but amino acid alignments of the 24 genes in this group identified conserved motifs including a single transmembrane domain suggesting that *Osi6*, *Osi7*, and *Osi8* are localized in the membrane (Shah *et al.* 2012). Additionally, sequence-based annotation identified an endoplasmic reticulum signal sequence at the N-terminus and a number of endocytic signaling motifs that suggest these genes are involved in the endo-lysosomal system in some way (Dorer *et al.* 2003; Shah *et al.* 2012; Lee *et al.* 2013). The only member of this gene family that has been functionally characterized is *Osi21* and a recent study found that *diehard4*, a $norpA^{P24}$ suppressor, is encoded by *Osi21* (Lee *et al.* 2013). Detailed molecular analysis showed that *Osi21* has a critical role in membrane homeostasis between the endosomes and lysosomes in eye cells. Loss of *Osi21* shifts

the membrane balance of the endo-lysosomal system towards the lysosome through reduction in the number and size of late endosomes and concurrent proliferation of the number of lysosomes. No effects on golgi complexes or early endosomes were detected.

Using a line expressing an *Osi21*-GFP fusion protein, Lee *et al.* (2013) found that *Osi21* functions directly on the endo-lysosomal system and *Osi21* co-localizes with late endosome markers. Ultimately, they found that *Osi21* acts as a negative regulator of late endosomal membrane traffic to the lysosome and that loss of *Osi21* function facilitates toxin traffic towards the lysosome and eventual lysosomal degradation of that toxin (Lee *et al.* 2013). Extreme sequence similarity between *Osi21* and *Osi6, Osi7* and *Osi8* (Dorer *et al.* 2003) suggests they may have similar cellular functions. If *Osi6, Osi7* and *Osi8* do function as negative regulators of lysosomal degradation of toxins, reductions in their expression level in *D. sechellia* could promote OA resistance. Consistent with this hypothesis, we found that *Osi6* was expressed at much lower levels in the salivary gland of *D. sechellia* in response to OA exposure than in sister species, further suggesting that this may be the mechanism conferring resistance. Additionally, derived nonsynonymous mutations in *Osi8* may lead to reduced or altered protein function that could also provide resistance independent of gene expression regulation.

Beyond the role *Osiris* genes play in evolved toxin resistance in *D. sechellia*, a recent study described a newly isolated island population of *D. yakuba* that has evolved OA resistance upon specialization to feed on *M. citrifolia* with one of the strongest differentiation peaks between OA resistant and OA sensitive populations overlapping the *Osiris* cluster. In fact, the highest peak of differentiation was found to be 10kb upstream of *Osi6*, potentially indicating a *cis*-regulatory element of *Osi6* is also responsible for this trait change in *D. yakuba* (Yassin *et al.* 2016). This strongly suggests convergent evolution has taken place in these two island populations in response to the same selective pressure. While OA resistance in both species is polygenic, there is significant overlap of the QTL for these traits (Yassin *et al.* 2016) suggesting there may not be many loci that when mutated could confer resistance to this toxin. Because so few cases of convergent evolution have a defined molecular basis, it seems that the data outlined here in conjunction with those published by Yassin *et al.* (2016) allow new questions to be asked in this system about the level at which the trait changes in these two species may be convergent. At the level of specific molecular variants, do these traits show molecular parallelism (Whiteman & Gloss 2016)? Are these traits convergent at the level of molecular phenotypes including

derived tissue and environment specific gene expression levels like those we report here? Are these traits convergent on the same gene but driven by different mutations and molecular mechanisms? To address these questions and many more we must now focus on detailed dissection of the molecular function of *Osi6*, *Osi7* and *Osi8* in *D. sechellia* and sister species to determine the precise mutations and mechanisms by which they may contribute to OA resistance.

Author Contributions

J.M.A.L., S.M.L., P.J.W. and J.D.C designed the experiments and J.M.A.L., S.M.L., J.M.A., E.C.M., and L.A.S. performed the experiments. J.M.A.L. and J.D.C. performed statistical tests. J.D.C., S.M.L. and J.M.A.L. wrote the text and prepared the figures and J.M.A.L., S.M.L., J.M.A., E.C.M., L.A.S., P.J.W. and J.D.C edited the article.

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Data Accessibility

All *Osiris* gene sequences generated for this study can be found in GENBANK under accession numbers KT388094- KT388098. All octanoic acid resistance data and qPCR data generated in this study are archived at Dryad (doi:10.5061/dryad.s0s60).

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Figure legends

Figure 1: Measuring OA associated relative survival with survivorship curves. Survival curves from the OA resistance assay are shown (**A**) for *D. melanogaster* (*w1118*) across an OA concentration gradient and (**B**) in different *Drosophila* species, showing a representative line of *D. sechellia* (red), *D. melanogaster* (green), *D. mauritiana* (orange) and *D. simulans* (blue); for data from all lines see Figure S1A. Dotted lines in (**B**) represent 95% confidence intervals from a Cox regression model. (**C**) Relative survival is shown as $-\beta$ from the Cox regression model for different strains of each species relative to a baseline from the *D. melanogaster w1118* line. Error bars represent 2 standard error of the mean (2SE).

Figure 2: Relative survival of RNAi targeting genes in a region associated with OA resistance. Relative survival (-β) from a Cox regression model comparing (**A**) ubiquitous knockdown (*Act5c*-GAL4/UAS-RNAi) to line-specific baseline of sibling controls (CyO/UAS-RNAi) and (**B**) stage specific knockdown in *Tubulin*-P[Switch]/UAS-RNAi individuals RU486 compared to a baseline of no RU486 addition. Error bars represent 2SE and asterisks indicate significant differences ($P < 0.05$).

Figure 3: *Osiris* candidate gene expression profiles.
Relative gene expression levels for *Osi6, Osi7* and *Osi8* were measured in 1-4 day old whole adult *D. sechelllia* (red), *D. melanogaster* (green), *D. mauritiana* (orange) and *D. simulans* (blue) with qRT-PCR. Normalized relative expression is shown ($2^{-\Delta C_T}$), error bars represent standard error and results of significance tests are shown with letters indicating overlapping 95% confidence intervals when letters are shared between bars and significant differences when they are not shared ($P < 0.05$).

Figure 4: Relative survival of tissue-specific RNAi knockdown of *Osi6, Osi7* and *Osi8* in response to OA exposure.
(**A**) Relative survival (-β) comparing tissue-specific knockdown (tissue-specific GAL4/UAS-RNAi) to a baseline of the UAS-RNAi line used. Both tissue-specific knockdown (black) and parental controls (tissue-specific GAL4, grey) are shown. Error bars represent 2SE and asterisks indicate significant differences ($P < 0.05$) between tissue-specific knockdown and both parental controls. (**B**) Gene expression levels for *Osi6, Osi7* and *Osi8* were measured in 1-4 day old adult dissected tissues from *D. sechelllia* (red) and *D. simulans* (blue) with qRT-PCR. Tissues quantified were head/nervous system (NS), salivary gland and associated tissue (SG), fat body lining the dorsal abdominal cuticle (FB) and intestine (IN). Normalized relative expression is shown ($2^{-\Delta C_T}$), error bars represent standard error and asterisks indicate significant differences ($P < 0.05$).

Figure 5: *Osi6, Osi7 and Osi8* gene expression response to OA exposure in whole flies and dissected tissues. (**A**) Gene expression levels for *Osi6, Osi7* and *Osi8* were measured in 1-4 day

old whole adult *D. sechelllia* (red), *D. melanogaster* (green), *D. mauritiana* (orange) and *D. simulans* (blue) with qRT-PCR comparing flies grown in standard media with flies grown in the presence of 0.2% OA. Normalized relative expression was determined (ΔC_T) and used to calculate differential expression between flies grown in the presence or absence of OA ($\Delta\Delta C_T$). (**B**) Differential expression ($\Delta\Delta C_T$) was determined as in **A** for dissected tissues from adult *D. sechellia* (red) and *D. simulans* (blue) flies grown in the presence or absence of OA. Tissues quantified were head/nervous system (NS), salivary gland and associated tissue (SG), fat body lining the dorsal abdominal cuticle (FB) and intestine (IN). Error bars represent standard error and asterisks indicate significant differences ($P < 0.05$).

Tables

Table 1: Summary of fixed derived sequence differences in *D. sechellia.*

Table 1:

Gene	Synonymous	Nonsynonymous	Length
Osi6	5	0	939
Osi7	5	0	867
Osi8	4	2	825

Figure 1

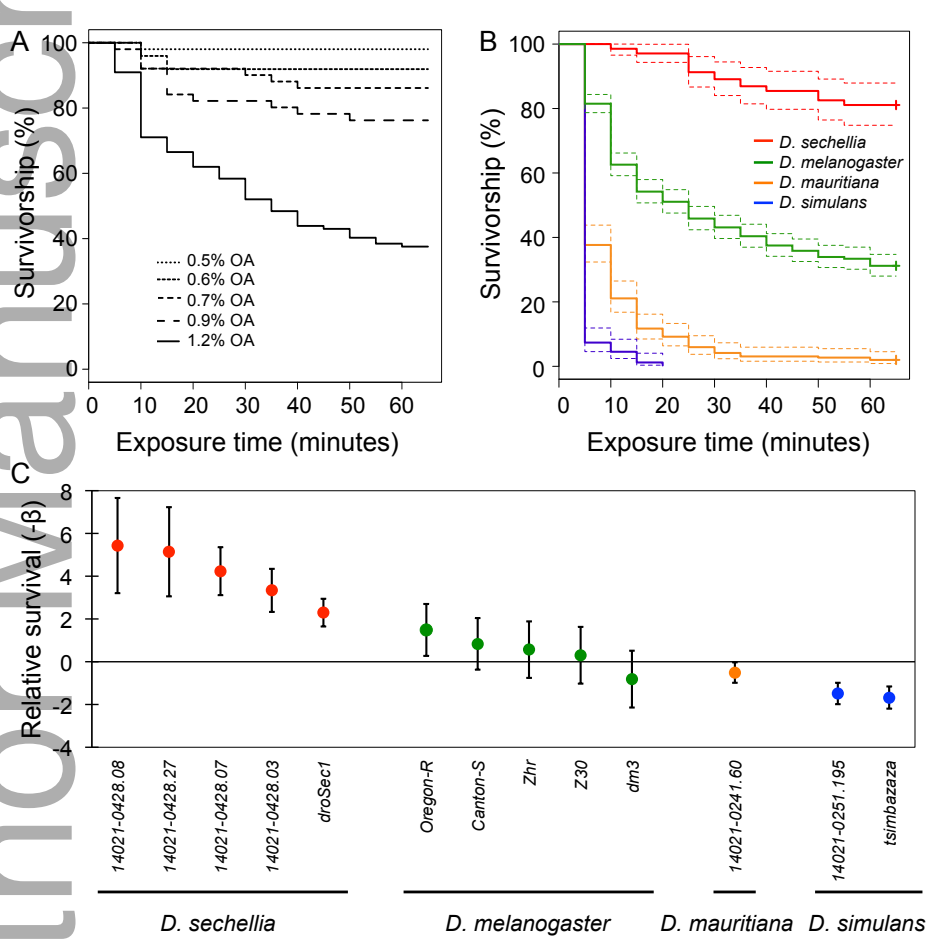

Figure 2

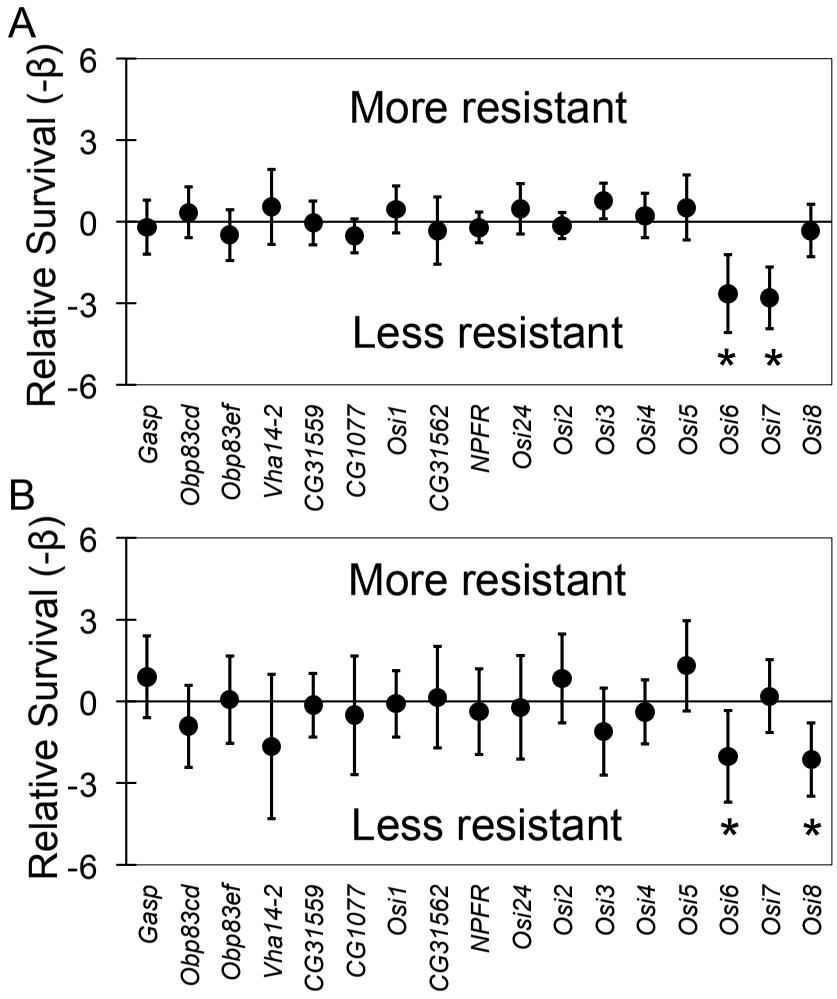

Figure 3

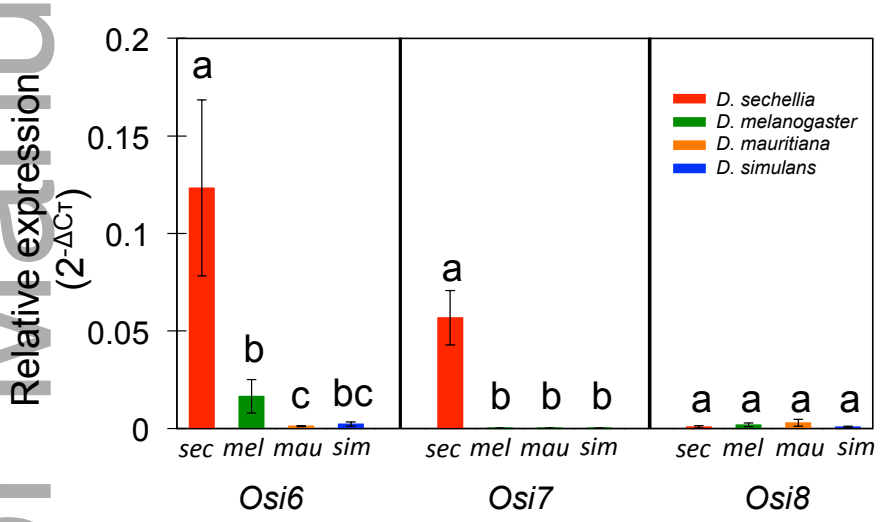

Figure 4

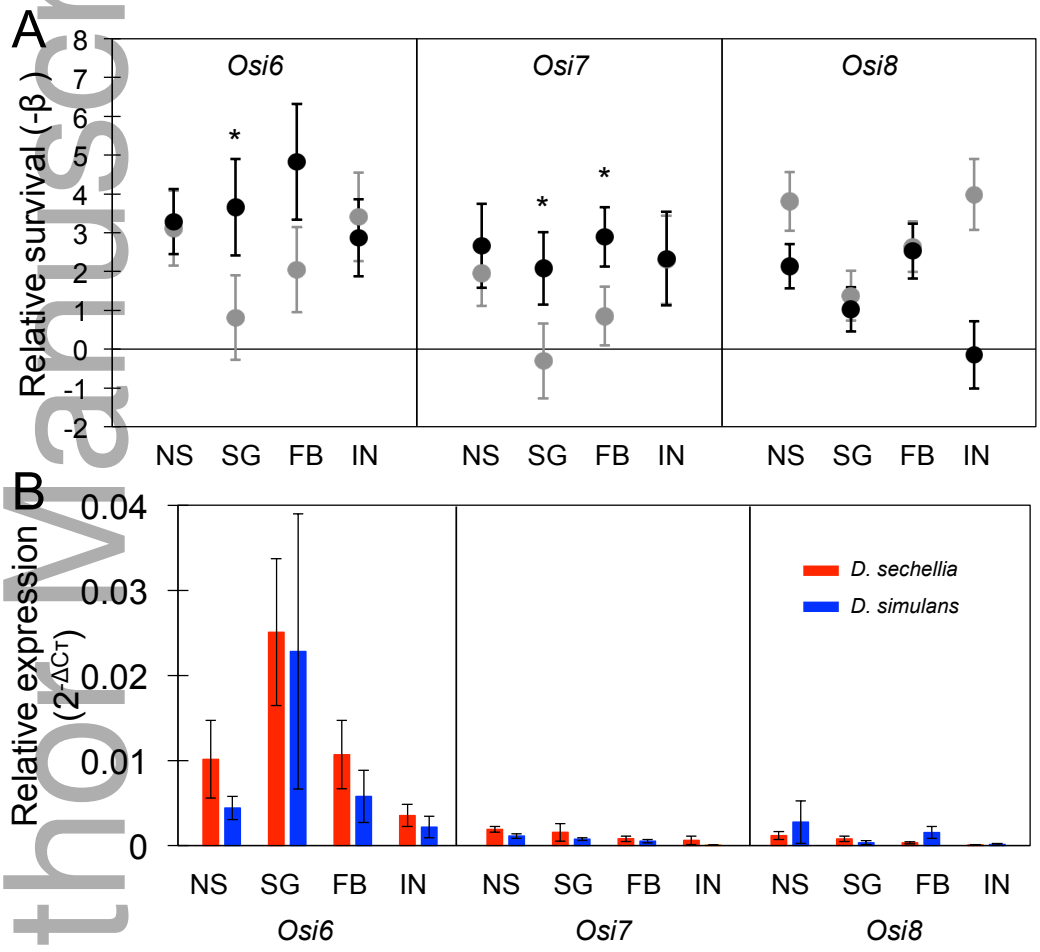

Figure 5