Arabidopsis glucosinolates trigger a contrasting transcriptomic response in a generalist and a specialist herbivore.

Fabian Schweizer^a, Hanna Heidel-Fischer^b, Heiko Vogel^{b,*}, Philippe Reymond^{a,*}

^a*Department of Plant Molecular Biology, University of Lausanne, 1015 Lausanne, Switzerland*

^b*Department of Entomology, Max Planck Institute for Chemical Ecology, 07745 Jena, Germany*

*Corresponding authors:

Department of Entomology, Max Planck Institute for Chemical Ecology, Hans-Knoell-Strasse 8, 07745 Jena, Germany. Tel. +49 (0)36 415 71512

Department of Plant Molecular Biology, Biophore Building, University of Lausanne, 1015 Lausanne, Switzerland. Tel.: +41 (0)21 692 41 90

E-mail addresses: hvogel@ice.mpg.de (H. Vogel), philippe.reymond@unil.ch (P. Reymond).

ABSTRACT

Phytophagous insects have to deal with toxic defense compounds from their host plants. Although it is known that insects have evolved genes and mechanisms to detoxify plant allochemicals, how specialist and generalist precisely respond to specific secondary metabolites at the molecular level is less understood. Here we studied the larval performance and transcriptome of the generalist moth *Heliothis virescens* and the specialist butterfly *Pieris brassicae* feeding on *Arabidopsis thaliana* genotypes with different glucosinolate (GS) levels. *H. virescens* larvae gained significantly more weight on the GS-deficient mutant *quadGS* compared to wild-type (Col-0) plants. On the contrary, *P. brassicae* was unaffected by the presence of GS and performed equally well on both genotypes. Strikingly, there was a considerable differential gene expression in *H. virescens* larvae feeding on Col-0 compared to *quadGS*. In contrast, compared to *H. virescens*, *P. brassicae* displayed a much-reduced transcriptional activation when fed on both plant genotypes. Transcripts coding for putative detoxification enzymes were significantly upregulated in *H. virescens*, along with digestive enzymes ~~hormone-related genes~~ and transposable elements. These data provide an unprecedented view on transcriptional changes that are specifically activated by GS and illustrate differential molecular responses that are linked to adaptation to diet in lepidopteran herbivores.

Keywords: Heliothis virescens, Pieris brassicae, Arabidopsis thaliana, glucosinolates, detoxification, insect transcriptome

1. Introduction

Phytophagous insects are continuously exposed to toxic secondary metabolites from their host plants. Over million years of coevolution, different trajectories have resulted in the specialization of a majority of insect species each to a narrow group of plants whereas other species evolved the ability to feed on a larger host range (Schoonhoven et al., 2005). For specialist insects, specific detoxification or adaptation mechanisms are numerous and generally involve the acquisition/modification of genes to inactivate plant toxins or the evolution of amino-acid substitutions in target site proteins (Després et al., 2007; Heidel-Fischer and Vogel, 2015). In contrast, for generalist insects that face a variety of plant allelochemicals, metabolic enzymes with broad substrate specificity confer some level of protection against the detrimental effect of these molecules. Carboxyl/cholinesterases (CCEs), cytochrome P450 monooxygenases (CYP450s), glutathione *S*-transferases (GSTs), UDP-glycosyltransferases (UGTs), and ABC transporters (ABCs) constitute the main group of detoxification-related gene families and have been associated with resistance to plant allelochemicals but also to xenobiotics, including insecticides (Heidel-Fischer and Vogel, 2015; Li et al., 2007).

Glucosinolates (GS) are nitrogen- and sulfur-containing thioglucosides of the Brassicaceae family. Upon insect feeding, GSs interact with myrosinases that are stored in different compartments, releasing an aglycone that rearranges non-enzymatically into toxic compounds such as isothiocyanates (ITCs) and nitriles (Halkier and Gershenzon, 2006). ITCs are electrophiles that are believed to interact with amino group and cleave disulfide bonds in proteins, whereas nitriles appear to be less toxic (Halkier and Gershenzon, 2006; Lambrix et al., 2001). GSs are always present at basal levels (Wittstock and Gershenzon, 2002) but also accumulate after herbivory (Mewis et al., 2006; Schweizer et al., 2013). The plant model *Arabidopsis thaliana* has been instrumental to identify genes involved in GS biosynthesis (Sønderby et al., 2010) and availability of GS mutants demonstrated the crucial role of GS as feeding deterrents for generalist insects (Beekwilder et al., 2008; Gigolashvili et al., 2007; Kliebenstein et al., 2005; Schlaeppli et al., 2008; Schweizer et al., 2013).

Recently, larvae of lepidopteran generalist herbivores, including *Spodoptera exigua*, *S. littoralis*, *Mamestra brassicae*, *Trichoplusia ni* and *Helicoverpa armigera*, were found to produce glutathione conjugates of ITCs, suggesting some level of GS detoxification (Schramm et al., 2012). However, more efficient ways of dealing with GS evolved in specialist insects. The diamondback moth *Plutella xylostella* contains a sulfatase in the larval gut that prevents formation of hydrolysis products by desulfating intact GS (Ratzka et al., 2002). A similar activity was found in the desert locust *Schistocerca gregaria* (Falk and Gershenzon, 2007). Larvae of the small and large white butterflies, *Pieris rapae* and *P. brassicae*, are equipped with nitrile-specifier proteins (NSPs) that redirect GS hydrolysis to less toxic nitriles instead of ITCs in the caterpillars midgut (Wittstock et al., 2004). As a consequence, these Pierids feed equally well on wild-type *Arabidopsis* plants or on mutants with altered GS contents (Müller et al., 2010; Schlaeppli et al., 2008; Schweizer et al., 2013).

With the advance of genome sequencing projects and availability of large-scale technologies for molecular analyses, insect adaptation to polyphagy and its underlying metabolic processes have recently attracted the attention of scientists. One important question is whether substantial changes in expression of detoxification genes occur when larvae feed on toxin-containing compared to non-toxic plants. To address this, experiments with artificial diets supplemented with specific plant defense metabolite or transfer from one host plant to another were conducted. For example, addition of different concentrations of the cotton toxin gossypol to an artificial diet triggered differential expression of many *CYP450s*, *CCEs*, *UGTs* and *GSTs* in the cotton bollworm, *Helicoverpa armigera* (Celorio-Mancera et al., 2011; Krempl et al., 2016). Similar genes were also regulated when *H. armigera* larvae were transferred from artificial diet to different cotton tissues (Celorio-Mancera et al., 2012). When larvae of the Swedish comma butterfly, *Polygonia c-album*, were shifted from *Urtica dioica*, their usual plant host, to the recently colonized *Ribes uva-crispa*, there was a general upregulation of genes coding for peptidases, membrane proteins, transporters, and proteins involved in cuticle structure (Celorio-Mancera et al., 2013). Midgut transcriptome of *Spodoptera littoralis* larvae transferred from artificial diet to maize showed upregulation of genes encoding digestive and detoxifying enzymes, transporters, and immunity-related proteins (Roy et al., 2016).

Whether or not specialist insects display a reduced transcriptional activity compared to generalist insects when feeding on the same host is an important question raised in this context. Only few studies have tested this hypothesis but in two experiments comparing lepidopteran generalist and specialist species, the generalist regulated more transcripts globally. In fact, larvae of the polyphagous *Heliothis virescens* regulated between 17 to 38-times more genes than larvae of the nicotine-adapted *Manduca sexta*, when feeding on wild-type *Nicotiana attenuata* plants or on various mutants defective in defense metabolite production (Govind et al., 2010). Differentially regulated transcripts in response to maize feeding were 1.7 to 3-times more abundant in *S. littoralis* than in grass-adapted *Spodoptera frugiperda* strains (Roy et al., 2016). These results suggest that plant host specialization is accompanied by a decreased transcriptional regulation of detoxification- and metabolism-related genes. However, more studies with different plants and insects are necessary to test the generality of this observation.

The effect of GS on arthropod transcriptomes has recently been investigated. First, a transfer of the spider mite *Tetranychus urticae* from bean to Arabidopsis revealed 483 differentially expressed transcripts, including genes coding for CYP450s, CCE2s, ABCs transporters, GSTs, and peptidases, although the specific contribution of GS from Arabidopsis diet was not evaluated (Grbic et al., 2011). In a more targeted approach, a comparison of *T. urticae* genes differentially regulated when feeding on bean or on Arabidopsis mutants with varying GS levels identified only a few transcripts that responded specifically to the presence of GS in the food, including CYP450s and UGTs (Zhurov et al., 2014). A study of the fly *Scaptomyza flava*, a Drosophila related species that has adapted to Brassicaceae, reported 341 transcripts differentially regulated between larvae reared on wild-type

Arabidopsis Col-0 and on the GS deficient mutant *quadGS*. Surprisingly, very few genes were associated with detoxification (Whiteman et al., 2012). Finally, only one experiment was done with a lepidopteran herbivore. The generalist cabbage looper *Trichoplusia ni* was reared on Col-0 or on *tgg1tgg2*, a mutant that lack myrosinases. A midgut transcriptome analysis identified 86 genes upregulated in Col-0 compared to *tgg1tgg2*, including *CYP450s*, *UGTs* and *CCEs* (Herde and Howe, 2014). However, this number may be underestimated since *tgg1tgg2* plants can still produce some levels of GS breakdown products non-enzymatically (Barth and Jander, 2006).

Here, we performed a larval transcriptome analysis of the generalist *H. virescens* and the specialist *P. brassicae* feeding on wild-type *Arabidopsis* (Col-0) and on a GS-deficient quadruple mutant (*quadGS*) (Schweizer et al., 2013). The aim of the study was to obtain a specific and comprehensive view on the effect of GS exposure on transcriptional activity. We found considerable changes in gene expression when the generalist fed on GS-containing Col-0 compared to *quadGS*. In contrast, the specialist showed a much reduced transcriptional response between the two genotypes. The pattern of expression in the generalist herbivore includes known detoxification genes but unveil additional metabolic responses that may reflect stress and the detrimental effect of GS on *H. virescens* larval performance. Although the overall total number of differentially expressed genes was low in the specialist *P. brassicae* when feeding on the *quadGS* mutant, several genes related to host plant GS detoxification were downregulated and genes encoding insect storage proteins were drastically induced.

2. Materials and methods

2.1. Plants and insects

Growth conditions of *Arabidopsis thaliana* wild-type (Col-0) and the quadruple mutant *cyp79b2cyp79b3myb28myb29* (*quadGS*) were reported previously (Schweizer et al., 2013). *Heliothis virescens* (tobacco budworm) eggs were obtained from Syngenta (Stein, Switzerland). A *Pieris brassicae* (large white butterfly) colony was reared on *Brassica oleracea* var. *gemmifera* in 1 m³ cages in a greenhouse (25°C, 60 % RH).

2.2. Insect performance assays

No-choice insect bioassays with *H. virescens* and *P. brassicae* larvae were described previously (Bodenhausen and Reymond, 2007). Experiments were performed with five-week-old plants in transparent plastic boxes. Just after hatching, forty neonate larvae were placed on each genotype for 8 days of feeding and thus only consumed *Arabidopsis* leaves. Larvae were then weighed on a precision

balance Mettler-Toledo MT5 (Mettler-Toledo). Experiments were repeated once (*H. virescens*) and twice (*P. brassicae*) with similar results.

2.3. Insect feeding experiment and RNA isolation

Before the treatment, neonate larvae were fed on artificial diet (*H. virescens*) or on Arabidopsis Col-0 plants (*P. brassicae*). To identify transcriptional changes that do not depend on insect developmental stage (larval instar), we combined RNA isolated from second- and fourth-instar larvae. For each experiment, 15 second-instar and 15 fourth-instar larvae were placed for 48 h on five-week-old wild-type or *quadGS* plants and then stored in -80°C. Whole larvae were ground in liquid N₂ with mortar and pestle and total RNA extracted using RNeasy® plant mini kit (Qiagen, <http://www.qiagen.com>). This experiment was replicated three times independently.

For RNA sequencing (RNA-Seq), equal RNA amounts from each replicate experiment with second- and fourth-instar larvae were pooled to minimize expression changes due to different developmental stages. This resulted in 3 RNA pools per plant genotype per insect species, giving a total of 12 RNA-Seq samples.

2.4. Illumina sequencing, transcriptome assembly and annotation

Transcriptome sequencing was carried out by GATC Biotech on an Illumina HiSeq2500 Genome Analyzer platform, using paired-end (2 x 100bp) read technology for the 12 whole-larvae diet samples. This yielded approximately 15 million reads for each of the 12 samples. Quality control measures and *de novo* transcriptome assemblies, combining all 6 RNA-Seq samples per species, were carried out using CLC Genomics Workbench v8.1 (<http://www.clcbio.com>). Selecting the presumed optimal consensus transcriptome as well as subsequent transcriptome annotation using BLAST, Gene Ontology and InterProScan were done as described previously (Vogel et al., 2014; Jacobs et al., 2016). In brief, three assemblies were generated for each species, with standard settings and two additional CLC-based assemblies with the following parameters: word size = 64 (automatic); bubble size = 150 (automatic); scaffolding option selected; reads were mapped back to contigs with the following alternative options: nucleotide mismatch cost = 1(2); insertion = deletion costs = 2(3); length fraction = 0.5(0.7); similarity = 0.9(0.85). Conflicts among individual bases were resolved in all assemblies by voting for the base with the highest frequency. Contigs shorter than 250 bp were removed from the final analysis. The three assemblies were compared according to quality criteria such as N50 contig size, total number of contigs and the number of sequence reads not included in the contig assembly. For each assembly, the 100 largest contigs were manually inspected for chimeric sequences. The *de novo* reference transcriptome assembly contig sequences were used to search the NCBI nr nucleotide database with the blastall program. Homology searches (BLASTx and BLASTn), and functional

annotation according to GO terms (<http://www.geneontology.org>), InterPro terms (InterProScan, EBI), enzyme classification (EC) codes, and metabolic pathways (Kyoto Encyclopedia of Genes and Genomes, KEGG) were carried out using BLAST2GO v2.3.1 (<http://www.blast2go.de>) as previously described (Vogel et al., 2014). Homology searches were conducted remotely on the NCBI server by QBLAST using a sequential strategy. First, sequences were searched against the NCBI nr protein database using an E value cutoff of 10^{-3} , with predicted polypeptides of a minimum length of 15 amino acids. Enzyme classification codes and KEGG metabolic pathway annotations were generated from the direct mapping of GO terms to their enzyme code equivalents. Finally, InterPro searches were carried out remotely using BLAST2GO via the InterProEBI web server. To identify homologues of detoxification genes (Table 2-4), contigs were translated (translate tool, www.expasy.org) and blasted against NCBI non-redundant protein database (blastp, <https://blast.ncbi.nlm.nih.gov>). Homologous proteins from insect species and with similarity >60% were selected. Then, homologues associated with responses to plants chemicals or insecticides in the literature were included in the Tables. Contigs with top BLAST hits to plant sequences were discarded since they probably originate from ingested Arabidopsis food.

All the sequence data have been deposited in the European Nucleotide Archive (ENA) with study accession number PRJEB19607. The study is also accessible directly at the following URL: <http://www.ebi.ac.uk/ena/data/view/PRJEB19607>. The *H. virescens* RNAseq data sets can be found with sample accession numbers ERS1568621- ERS1568626. The *P. brassicae* RNAseq data sets can be found with sample accession numbers ERS1568627- ERS1568632.

2.5. Gene expression analysis

Digital gene expression analysis was carried out using CLC Genomics workbench to generate BAM mapping files, to remap the Illumina reads from all 12 samples onto the two respective reference transcriptomes, and finally by counting the sequences to estimate expression levels, using previously described parameters for read mapping and normalization (Vogel et al., 2014; Jacobs et al., 2016). To control for the effect of global normalization using the RPKM method, we analyzed a number of highly-conserved housekeeping genes, including GAPDH, ribosomal proteins (e.g. Rps4e), elongation factor 1 alpha and eukaryotic translation initiation factors 4 and 5a. The overall variation of expression levels for these housekeeping genes across samples and treatments was lower than 1.2-fold (based on log₂ transformed RPKM values), indicating they were not differentially expressed. To identify contigs differentially expressed between larvae feeding on wild-type or *quadGS* plants, a threshold of 2-fold change and an FDR-adjusted *p*-value of 0.05 were selected. In addition, average RPKM (reads per kilo base of transcript per million mapped reads; log₂ transformed) values had to be ≥ 1.0 in at least one treatment. To avoid infinite expression ratios, a RPKM value for contigs with 0 reads in each replicate

was set to -5.15. RPKM values, expression ratio and DNA sequences for all contigs can be found in Table S1.

3. Results and Discussion

3.1. Larval performance on wild-type and *GS*-deficient *Arabidopsis* plants

To evaluate the effect of *GS* on insect performance, neonate larvae were placed on *Arabidopsis* plants for 8 days and their weight was measured at the end of the experiment. The performance of *P. brassicae* was not statistically different when feeding on wild type Col-0 or *quadGS*, confirming that this species is adapted to the detrimental effect of *GS* breakdown products (Fig. 1). In sharp contrast, *H. virescens* larvae reached an 8-fold larger mass when feeding on *quadGS*, illustrating the defensive function of *GS* against generalist herbivores.

3.2. RNA-Seq and transcriptome assembly

We prepared cDNA libraries from *H. virescens* and *P. brassicae* whole larvae fed on wild type Col-0 or *quadGS* *Arabidopsis* plants and carried out Illumina HiSeq2500 sequencing to generate approximately 15 million 100-bp paired-end reads for each of the three replicate samples per species per treatment. A total of 90 million paired-end reads were pooled for each of the respective Lepidopteran transcriptome assemblies. The *de novo* reference transcriptome assembly (backbone) of *H. virescens* contained 34,887 contigs (minimum contig size = 250 bp) with an N50 contig size of 1,129 bp and a maximum contig length of 17,202 bp while the *P. brassicae* transcriptome assembly contained 26,802 contigs with an N50 contig size of 1,659 bp and a maximum contig length of 22,155 bp. The transcriptome contig sequences were translated using BLASTx and functionally annotated by assigning Gene Ontology (GO) terms, enzyme classification (EC) codes, InterPro terms, and metabolic pathway classifications using Blast2GO PRO.

3.3. Comparative gene expression profiles between *H. virescens* and *P. brassicae*

RNA-Seq was conducted on whole caterpillars to assess the impact of *GS* on insect transcriptomic response. The overall pattern of expression varied considerably between the generalist and the specialist insect. Using a threshold of ≥ 2 fold change, an FDR-adjusted *p*-value < 0.05 and $\log_2$ RPKM ≥ 1 , there were 3,747 contigs (10.1%) differentially regulated between *H. virescens* larvae feeding on Col-0 and *quadGS*, whereas only 254 contigs (0.9%) varied in *P. brassicae* (Table 1, Fig. 2). *H. virescens*-upregulated contigs were twice as many as downregulated ones, while an equal number of up- and downregulated contigs were observed in *P. brassicae* (Table 1, Fig. 2).

A global gene ontology (GO) analysis of differentially regulated contigs revealed different sets of functions and processes, with no dominant class. A large fraction of regulated contigs in both insect species (60-70%) were not associated with any GO term (Fig. S1). Among functions with highest number of contigs in both up- and downregulated categories were "hydrolases", "metal ion binding", "nucleic acid and DNA binding", "zinc ion binding", "protein binding" and "heme binding". For processes, top terms included "oxidation-reduction", "proteolysis", "RNA-dependent DNA replication", "DNA integration", "protein phosphorylation", and "regulation of transcription" (Fig. S1).

We then compared the relative frequencies of GO terms between the replicated RNA-Seq datasets from larvae exposed to wild type Col-0 (control) and *quadGS*, using GO information from all contigs with above described criteria for differential gene expression. The differentially expressed contigs were compared to the complete dataset using Fisher's exact test implemented in Blast2GO, with an FDR-adjusted p -value <0.01 . After filtering for specificity, we identified several GO terms that were over-represented in the *H. virescens* larvae exposed to the wild type Col-0 compared to *quadGS* plants, including "monooxygenase activity", "oxidoreductase activity", "metallocarboxypeptidase activity" and "RNA-directed DNA polymerase activity" (Fig. S2A). In contrast, the only GO term over-represented in the *P. brassicae* larval samples exposed to Arabidopsis *quadGS* plants was "nutrient reservoir activity" (Fig. S2B).

The finding that GS differentially regulated ten times more contigs in *H. virescens* than in *P. brassicae* is intriguing. This is similar to expression differences reported between generalist and specialist insects responding to nicotine or to maize defense compounds (Govind et al., 2010; Roy et al., 2016). Thus, the ability to specifically detoxify major plant toxins may prevent activation of a large transcriptional program. One reason for such reduced response may be that specialist larvae apparently do not suffer to the same extent from eating toxins they are adapted to, compared to generalist larvae exposed to the same toxins. They therefore do not have to cope with detrimental effects on growth and development by altering the expression of hormone-related and nutrition-related genes. The absence of induced detoxification genes in larvae feeding on wild-type plants supports the ability of *P. brassicae* to cope with GS present in Arabidopsis. Likewise, the induced response in the generalist herbivore may not be fine-tuned. A toxic stress signal may be triggered by a diverse array of chemicals, leading to a broad transcriptomic response with only a subset of the induced enzymes actually acting on any given substrate. In the case of a specialist insect, a rapid and efficient disarming of specific plant toxins would prevent the generation of the stress signal, hence abolish any global response.

Although *H. virescens* larvae responded to feeding on Arabidopsis by expressing detoxification genes, whether this allows them to complete their life-cycle on this host was not tested in our experiment. However, previous data of larval performance, pupation and adult eclosion on Brassicaceae host plants has shown the ability of *H. virescens* larvae to indeed complete their life cycle on members of this plant family (data not shown). Interestingly, a study on the spider mite *T.*

urticae showed that a transfer from bean to tomato for 30 generations evolved mite populations that better performed on the new host and exhibited an enhanced expression of detoxification genes compared to non-adapted mites (Wybouw et al., 2015). In addition, our experimental design did not take into account tissue-specific gene expression and temporal accumulation of transcripts in response to feeding on GS. If *H. virescens* transcriptomic responses would evolve after successful generations on GS-containing Arabidopsis plants is another important aspect. More work will thus be necessary to address these interesting questions.

The large number of differentially regulated contigs identified in our study contrasts with the relatively low number of genes activated by GS-exposure in the spider mite *T. urticae* (Zhurov et al., 2014) or *Scaptomyza* (Whiteman et al., 2012). In the latter case, enhanced activity of GSTD1 towards ITCs (GS breakdown products) may indicate that this fly has specialized to feed on GS-containing plants, lowering the need to induce generic detoxification genes (Gloss et al., 2014). In the case of *T. urticae*, the difference is more difficult to explain since mites were clearly performing better on *quadGS* plants, indicating that GS are also detrimental to chelicerates (Zhurov et al., 2014). A likely mechanism could be that they rely on a smaller number of highly efficient detoxification enzymes. More transcriptomes of insects and arthropods from different feeding guilds are clearly needed to obtain a clearer picture of molecular changes induced by plant allelochemicals.

3.4. Expression of storage and cuticle protein genes in *P. brassicae*

A more detailed analysis of the differentially-expressed genes revealed that, in contrast to the large number of differentially regulated contigs from *H. virescens* that included gene families with known function in detoxification and insect development (see below), a much smaller number of genes were differentially regulated in *P. brassicae* when fed on the *quadGS* compared to wild-type plants. Most prominently, storage proteins were significantly upregulated in the *P. brassicae* larvae when feeding on the *quadGS* plants, with the highest fold-change (219 fold) of a contig encoding a methionine rich storage protein, and 137-fold upregulation of a contig encoding a hexamerin protein. Three more storage protein sequences were upregulated in the caterpillars with values ranging from 42- to 6.8-fold increase on the *quadGS* mutant (Table S1).

~~*P. brassicae* also displayed a drastic increase in the expression of genes encoding for insect storage proteins of the hexamerin gene family (which includes arylphorins and methionine rich storage proteins) when feeding on *quadGS*.~~

Hexamerins are synthesized and secreted by the fat body and reach very high concentration in the hemolymph prior to metamorphosis. Before pupation they are taken up by the fat body and are primarily incorporated into new tissues and proteins. In addition, they are also incorporated into cuticle as intact proteins, bind and thus regulate ecdysteroid hormones, support foraging activities in honey bees, but are also involved in insect immune defense and were shown to respond to dietary

changes (Martins et al., 2010; Ryan et al., 1985; Banville et al., 2012; Afshar et al., 2013). The upregulation of these proteins in the absence of GS could be interpreted in two different ways. Either the lack of GS might free resources otherwise used for detoxification and excretion processes in caterpillars. As a result, storage protein production might accumulate for further use. Alternatively, the lack of GS in the diet could also result in low sulphur levels, an element present in GS. The upregulation of storage proteins might therefore be a mechanism to compensate for sulphur deficiency. ~~Increased levels of storage proteins could thus improve the distribution of existent sulphur to tissues with the highest demand.~~

Among the upregulated contigs in *P. brassicae* larvae feeding on Col-0 wild-type plants are three encoding cuticle-related proteins. This is similar to the situation in *Manduca sexta*, where larvae grown on different plants compared to artificial diet exhibited an increase in GO terms linked to “structural composition of cuticle” (König et al., 2015). Modifications of the cuticle protein composition can lead to thicker, more robust and less permeable cuticle to prevent water loss and promote survival (Hegedus et al., 2009; Li & Denlinger 2009; Stuckas et al., 2014). Similarly, cuticle protein components of the gut peritrophic matrix are responsible for the strength, elasticity and permeability of this structure, which is an important physical and biochemical barrier (Agrawal et al., 2014; Kelkenberg et al., 2014).

3.5. Expression of detoxification genes

Since detoxification of defense metabolites in polyphagous insects occurs via a common set of enzymes families, we looked specifically at CYP450s, CCEs, GSTs, UGTs, and ABCs in the list of differentially regulated contigs. Strikingly, a considerable number of such genes were upregulated when *H. virescens* fed on Col-0, indicating that the presence of GS in leaves triggered a strong (2 to 30-fold) induction of those genes (Fig. 3, Tables 2 and 3). The most abundant upregulated contigs were CYP450s (17 genes), CCEs (9 genes) and ABCs (7 genes), whereas only 2 GSTs and 2 UGTs were upregulated. Relative to the total number of detoxification-related genes in *H. virescens* transcriptome, CYP450s and CCEs were significantly enriched in the set of differentially regulated contigs (Fig. S3). In contrast, homologues from *P. brassicae* showed almost no differential regulation (Fig. 3).

Although there are conserved roles for specific P450 clades, such as the CYP4 clade associated with pheromone metabolism (Maibeche-Coisne et al., 2004), the CYP2 clade and mitochondrial P450s contributing to hormone, sterol and fatty acid metabolism (Feyereisen, 1999; 2006), it can be problematic to determine the function of a P450 solely based on sequence homology (Feyereisen, 1999). However, the CYP3 clade is known to facilitate the detoxification of synthetic insecticides, and CYP450s from this as well as other clades have frequently been shown to be inducible when insects are exposed to plant metabolites (Celorio-Mancera et al., 2011; Feyereisen, 1999, 2006; Hung, 1997;

König et al., 2015; Snyder and Glendinning, 1996; Yamamoto et al., 2010). We thus performed a literature search on the role and regulation of detoxification genes homologous to *H. virescens* contigs identified in this study. Remarkably, a majority of regulated contigs have insect counterparts that have been associated with responses to plant allelochemicals or insecticide resistance (Table 2 and 3). For CYP450s, CYP321A1 metabolizes xanthotoxin, a plant furanocoumarin, and the pyrethroid insecticide cypermethrin in *Helicoverpa zea* (Sasabe et al., 2004); CYP321B1 confers resistance to cypermethrin in *Spodoptera litura* (Wang et al., 2016); resistance of *Helicoverpa armigera* to the pyrethroid fenvalerate is due to a chimeric CYP337B3 (Joußen et al., 2012); CYP6AE12, CYP6AE17, CYP6B10, and CYP9A17 are induced by the cotton toxin gossypol in *H. armigera* (Celorio-Mancera et al., 2011; Chandra et al., 2016; Zhou et al., 2010); CYP6B proteins metabolize furanocoumarins in swallowtail caterpillars (Li et al., 2003); CYP6B8 metabolizes six diverse plant toxins (xanthotoxin, quercetin, flavone, chlorogenic acid, indole-3-carbinol, and rutin) and three classes of insecticides (the organophosphate diazinon, the chlorinated aldrin, and cypermethrin) in *H. zea* (Li et al., 2004); CYP9A1 is associated with resistance to the carbamate insecticide thiodicarb in *H. virescens* (Rose et al., 1997). For CCEs, CCE001a, CCE001i, CCE006a, CCE014a, and CCE016b are associated with resistance to various insecticides in *H. armigera* (Teese et al., 2010). For GSTs, GSTo2 is induced by the insecticides diazinon, permethrin and the neonicotinoid imidacloprid in the silkworm *Bombyx mori* (Yamamoto et al., 2011) and GSTe2 and GSTe3, which are in the same clade as GSTe11, are induced by various insecticides in *S. litura* (Deng et al., 2009). For UGTs, UGT40M1 and UGT33B5 belong to families that have expanded in Lepidoptera and may accept a larger range of compounds detoxified or regulated by glycosylation (Ahn et al., 2012). Close homologues of these two UGTs are induced by gossypol in *H. armigera* and *H. virescens*, and a UGT40D1 was shown to glycosylate the toxin *in vitro* (Krempl et al., 2016). Finally for ABCs, ABCC1 and ABCG1 are induced in *T. urticae* shifted from bean to Arabidopsis (Dermauw et al., 2013); ABCC2 is associated with resistance to *Bacillus thuringiensis* (Bt) Cry1Ac toxin in *H. virescens* (Gahan et al., 2010) and ABCA2 confers resistance to Cry2Ab in *H. armigera* (Tay et al., 2015).

We thus identified many upregulated CYP450s, CCEs, and ABCs homologous to genes that are also upregulated in other phytophagous species in response to a variety of insecticides or plant secondary metabolites. This induction of known families of detoxification genes previously associated with plant host shift or treatment with plant allelochemicals and insecticides in various insects (Heidel-Fischer and Vogel, 2015; Li et al., 2007) illustrates a conserved and generic mechanism to respond to highly different chemical structures. Similarly, larvae feeding on nicotine-containing *N. attenuata* plants exhibited induced expression of genes coding for detoxification enzymes and peptidases (Govind et al., 2010). Beyond the lepidopteran lineage, studies with aphids and spider mites have also revealed that plant host shift is associated with the upregulation of detoxification genes (Wybouw et al., 2015; Mathers et al., 2017). With increased knowledge on the role of plant defense metabolites against herbivores and availability of knock-out techniques for non-model species,

future experiments should attempt to define whether conserved expression signatures are found in generalist herbivores and whether these are closely associated with the presence of a particular toxin.

3.6. Expression of specialization genes

H. virescens is a polyphagous species but, to our knowledge, has not been reported to be specifically adapted to GS-containing plants. However, in the field *H. virescens* larvae are reported to readily attack cabbage plants, especially in cases where the preferred host plants such as soybean, alfalfa or cotton are less abundant (Martin et al., 1976; Mitter et al., 1993; Cho et al., 2008). We noticed that three sulfatase genes were induced when *H. virescens* larvae were feeding on Col-0 plants (Fig. 3). Intriguingly, one contig is homologous to a *P. xylostella* gene that is responsible for GS detoxification by removing a sulfate group from intact GS (Ratzka et al., 2002). This finding suggests that generalist insects may use similar mechanisms than specialists, although less efficiently. This hypothesis however awaits the biochemical characterization of *H. virescens* sulfatases.

Nitrile-specifier proteins (NSPs) are known to confer GS resistance to Pierids by diverting breakdown products towards less toxic nitriles (Wittstock et al., 2004). Accordingly, a contig encoding one member of the small NSP gene family (consisting of genes named NSP and MA) was induced in *P. brassicae* when larvae fed on Col-0 (Fig. 3). In addition, we observed that a non-regulated NSP contig from *P. brassicae* had a similar high expression level than the regulated MA (Table 4). The very high expression levels of two NSPs in *P. brassicae*, comparable to housekeeping genes, are noticeable and support the importance of these proteins for GS detoxification. Although NSPs can modify the outcome of most glucosinolates, cyanide is released during metabolism of phenylacetonitrile, a product of benzylglucosinolate breakdown (Stauber et al., 2012). In *Pieris rapae*, this highly toxic metabolite is converted to non-toxic β -cyanoalanine by a small gene family encoding β -cyanoalanine synthase (CAS, also named cysteine synthase) enzymes (Wybough et al., 2014; van Ohlen et al., 2016). One of the three CAS gene orthologs identified in our *P. brassicae* transcriptome was upregulated in larvae fed on Col-0 wild-type plants. For *P. rapae* it was shown that the breakdown products of aromatic glucosinolates can undergo further metabolism, including the generation of sulfated compounds (Agerbirk et al., 2010), indicative of a sulfotransferase activity in *Pieris* larval guts. Sulfotransferases are Phase II detoxifying enzymes that mediate the sulfate conjugation of numerous xenobiotic molecules (Weinshilboum et al., 1997), and in *P. brassicae* larvae one of the identified sulfotransferase is more highly expressed (3 fold) in larvae fed on Col-0 wild-type plants compared to the *quadGS* mutant. More studies on expression of these genes are required to identify the regulatory factors involved in their differential expression, e.g. in the absence or presence of individual GS classes.

Interestingly, a *GSTD1* homologue was upregulated in *P. brassicae* but downregulated in *H. virescens* when larvae were fed on Col-0 plants (Fig. 3). *GSTD1* can efficiently metabolize ITCs in

Scaptomyza flava and *Scaptomyza nigrita*, two fly species that feed on Brassicaceae. Importantly, this gene was duplicated in *S. nigrita* and is induced when feeding on GS-containing *Arabidopsis* (Gloss et al., 2014; Whiteman et al., 2011). Although it is tempting to speculate that *P. brassicae* uses GSTD1 in addition to NSPs to resist GS, there is no biochemical data indicating that *Pieris* larvae generate glutathione conjugates of GS breakdown products (Agerbirk et al., 2010; Vergara et al., 2006; Wittstock et al., 2004). Two other GSTs are upregulated in *H. virescens* larvae on Col-0 wild-type plants, including one GSTE homologue. In the generalist herbivore *Spodoptera litura*, a GSTE gene (SIGSTE1) was up-regulated in midguts of larvae fed on *Brassica juncea* or diet containing allyl-isothiocyanate. *In vitro*, SIGSTE1 was shown to catalyze the conjugation of glutathione and allyl-isothiocyanate, and RNAi-mediated suppression of *Slgst1* in the larvae decreased both larval growth and feeding rate (Zou et al., 2015).

The intimate association between Pierid species and Brassicales is estimated to have coevolved for the last 70 Myr, and is explained by the acquisition of NSPs (Edger et al., 2015; Wheat et al., 2007). Such long-lasting specialization may have led to the loss of generic detoxification genes that are used by generalist herbivores. To the contrary, we found that there is an equal proportion of CYP450s, GSTs, and UGTs expressed in *H. virescens* and *P. brassicae* transcriptomes, and more than 50% of CCEs and ABCs (Fig. S4). Although these genes are not induced in response to GS in *P. brassicae*, an open question is whether they are functionally active and whether they can still play a role when larvae are exposed to alternative host plants. There might be conditions in nature where *P. brassicae* is obliged to feed on other host species, in which case having an existing, flexible detoxification machinery would be crucial. Thus, toxic secondary metabolites other than GS in Brassicales host plants could provoke a much more global response, including differential regulation of detoxification-related genes. More studies on expression of these genes and on *P. brassicae* feeding behavior are needed to test this hypothesis. However, from an evolutionary perspective, the finding that homologues of detoxification genes are conserved in *P. brassicae* genome and expressed strongly suggests that they have kept their enzymatic function.

~~3.7. Analysis of hormone-related genes~~

~~Given the drastic effect of GS on *H. virescens* larval growth (Fig. 1), we looked at hormone-related genes in the list of differentially regulated contigs. *CYP314A1*, which mediates the last biosynthesis step of the molting steroid hormone 20-hydroxyecdysone (Petryk et al., 2003), was upregulated in the presence of GS (Fig. 4). The same was true for the ecdysone receptor. In contrast, a delta-24 sterol reductase that is involved in cholesterol synthesis, a precursor of ecdysone, was downregulated. Contigs homologous to five ecdysteroid 22-kinases were all upregulated. Phosphorylation of ecdysone generates a physiologically inactive hormone (Makka et al., 2002). Ecdysone is also inactivated through the sequential activity of ecdysone oxidase and 3-~~

~~dehydroecdysone 3 α -reductase (Gilbert et al., 2002). Curiously, three contigs homologous to ecdysone oxidases were downregulated whereas two contigs homologous to 3-dehydroecdysone 3 α -reductase were upregulated. In addition, a 3-dehydroecdysone 3 β -reductase, which can revert 3-dehydroecdysone to ecdysone was not regulated in *H. virescens* but downregulated in *P. brassicae* (Fig. 4).~~

~~—— Juvenile hormone (JH) is a key larval factor that prevents metamorphosis (Jindra et al., 2013). JH is synthesized from the mevalonate pathway and secreted in hemolymph where it interacts with JH binding proteins that transport or protect it from degradation (Gilbert et al., 2000). Interestingly, a putative JH-methyltransferase, which participates in one of the last step of JH biosynthesis (Bellés et al., 2005), was induced in *H. virescens* feeding on Col-0 (Fig. 4). Of five identified JH-binding homologues, three were upregulated and two were downregulated. Finally, one contig annotated as JH-inducible was upregulated (Fig. 4).~~

~~—— Insulin signaling links nutrition and growth (Nijhout and Callier, 2015). Two insulin receptors were upregulated in *H. virescens*, whereas an insulin-degrading enzyme was downregulated. Key downstream targets of the insulin pathway were also upregulated, namely the forkhead transcription factor (FOXO) and the target of rapamycin 1 complex (TORC1). Noticeably, none of these factors were regulated in *P. brassicae* (Fig. 4).~~

~~—— Collectively, this significant enrichment of hormone-related genes (Fig. S3) illustrates the physiological adaptation of *H. virescens* to the detrimental nature of GS.~~

3.7. Proteases and transposable elements

Induction of digestive enzymes in response to plant toxins is known (Herde and Howe, 2014; Roy et al., 2016). They may be required for directly disarming proteinaceous toxins but may also be needed to enhance nutrient acquisition as part of a compensatory feeding behavior. Many contigs encoding lipases and glucosidases were upregulated in *H. virescens* feeding on GS (Table S1). There was also a significant enrichment of proteases in upregulated contigs (Fig. S3). These 56 contigs include a majority of carboxypeptidases, chymotrypsins, and serine proteases (Table S1).

Transposable elements (TEs) constitute a large fraction of insect genomes. TE activity can be induced by stress (Capy et al., 2000; Maumus et al., 2015) and their mutagenic potential can provide selective advantage to organisms (Chénais et al., 2012). We found 87 TE-related contigs upregulated in *H. virescens*, including RNA-based LTR and non-LTR retrotransposons as well as DNA transposons (Table S1). Although there was not a significant enrichment of TE genes in upregulated contigs compared to the total number of TE-related genes in the transcriptome (Fig. S3), this number was considerably larger than the only TE contig upregulated in *P. brassicae* (Table S1).

Upregulation of TEs in response to GS feeding in *H. virescens* is quite interesting. In some striking examples of adaptation, resistance to insecticides has been associated with insertion of TEs

near detoxification or target genes (Aminetzach et al., 2005; Daborn et al., 2002; Mateo et al., 2014; Schmidt and Robin, 2011). Increased expression and transposition of TEs in response to GS might be sufficient to generate heritable genetic diversity in lepidopteran larvae and confer further selective advantages. Analyses of resistance in natural populations of generalist herbivores associated with Brassicaceae coupled to genome-wide association studies may in the future unveil the importance of TEs for adaptation to GS and to plant defense compounds in general.

3.8. Detection of plant allelochemicals

One intriguing yet unsolved question is how insects detect plant allelochemicals and xenobiotics and how they activate the expression of hundreds of genes. Given the apparent conserved and generic transcriptional response towards molecules of great chemical diversity, a high-affinity ligand-binding process seems unlikely. Genomes of polyphagous insects would need to harbor a large number of specific receptors to accommodate the wide variety of plant toxins they encounter. In addition, the observation that chemically synthesized insecticides induce similar detoxification genes as natural products suggests that a rather non-specific mechanism is responsible for the detection of these molecules. Overexpression of *CYP6G1* in *D. melanogaster* increased survival on different classes of insecticides (Daborn et al., 2007). A population genetic study on GSTD1 showed that amino acid changes associated with the DDT-degrading activity of the protein predate the use of DDT, suggesting that they evolved in response to another toxin (Low et al., 2007). These examples underline the versatility of detoxification enzymes towards different substrates. However, how this is correlated with "sensing" of plant allelochemicals and activation of the full set of detoxification genes is still unclear.

4. Discussion

~~—— Arabidopsis *quadGS* mutant devoid of GS provides an excellent tool to test the specific effect of a well studied class of plant defense metabolites on insect responses. Exposure to GS drastically lowered larval performance and triggered a massive transcriptome reprogramming in the generalist *H. virescens*.~~

~~—— The long lasting specialization of *P. brassicae* to plants containing GS also explains the observed similar growth rate of *P. brassicae* larvae on both Arabidopsis lines, as NSP allows caterpillars to feed on glucosinolate-containing plants with impunity. The complete lack of GS in the Arabidopsis *quadGS* mutant, however, results in the downregulation of a specific member of each of the NSP and CAS gene family, both of which are central components of GS detoxification or GS metabolites, respectively.~~

~~—The drastic *H. virescens* larval mass reduction triggered by GS consumption was accompanied by differential regulation of growth and molting related genes. Induction of insulin receptors and downstream targets of the insulin pathway, as well as downregulation of insulin degrading enzyme indicate that larvae may cope with a reduced growth by stimulating this pathway. Although the nutritional value of Col-0 and *quadGS* is likely the same, the stress caused by GS breakdown products seems to inhibit a proper nutrient acquisition/perception and may generate a signal that leads to the induction of insulin related genes. Interestingly, TORC, an important downstream target of the insulin pathway upregulated in *H. virescens* feeding on wild-type *Arabidopsis* plants, is activated by starvation in *Drosophila* and enhances stress resistance (Wang et al., 2008).~~

~~—The regulation of ecdysone related genes is however more difficult to interpret. Depending on the concentration, ecdysone stimulates growth or molting (Nijhout and Callier, 2015). Upregulation of ecdysteroid 22 kinases and 3-dehydroecdysone 3 α reductases in *H. virescens* exposed to GS suggests that ecdysone is modified to inactive forms. In addition, downregulation of a delta 24-sterol reductase and upregulation of a JH synthesizing gene support the hypothesis that *H. virescens* larvae produce less ecdysone and more JH to inhibit molting. However, upregulation of ecdysone receptor and *CYP314A1*, along with a downregulation of ecdysone oxidases, may indicate that larvae activate the ecdysone pathway. Whether this is a feedback loop or a compensatory mechanism should be tested. Further analyses of hormone levels in larvae feeding on plants with different GS levels will be important to correlate gene expression, hormonal status and larval development. Interestingly, genes coding for 3-dehydroecdysone 3 α reductases and ecdysteroid 22 kinases were also induced in *H. virescens* feeding on nicotine containing *N. attenuata* plants (Govind et al., 2010) and a 3-dehydroecdysone 3 α reductase gene was induced in *T. ni* feeding on *Arabidopsis* Col-0 (Herde and Howe, 2014), illustrating a conserved response to different plant allelochemicals in different insects.~~

4. Conclusion

Our analysis reveals the profound effect of GS exposure on performance and transcriptional signature of a non-adapted herbivore. It also provides evidence that acquisition of a specific detoxification mechanism in a specialist avoids metabolic costs associated with activation of a general stress response. However, an exciting finding is the downregulation of GS detoxification genes when GS are absent from the diet. Genome-wide changes in *H. virescens* larval gene expression reflect the physiological remodeling induced by GS but also illustrate the potential to overcome detrimental effects of these molecules. Future investigations should aim at understanding how herbivores detect plant allelochemicals and may help to develop strategies to target general detoxification processes in insect pests. In addition, as pointed out by Ali and Agrawal (2012), a rigorous comparison of different insect species with different diet specialization, within the same phylogenetic lineage and/or feeding

guild, will be necessary to appreciate the generality of the contrasting transcriptomic response to plant allelochemicals between specialist and generalist herbivores.

Author's contribution

F.S. and P. R. planned the experiments. F. S. performed larval bioassay and RNA extraction. H. H.-F. and H. V. performed RNA sequencing and contig assembly. H.V. performed initial bioinformatics, data analysis and plotting, database management and contributed to manuscript writing. P. R. analyzed the data and wrote the manuscript. All authors have read the final draft of the manuscript.

Acknowledgements

We thank Roland Reist and Oliver Kindler for providing *H. virescens*. We thank Charles Robin (University of Melbourne) for useful insights and unpublished information. The Swiss National Science Foundation (grant 31003A_149286 to P.R.) and Fondation Herbette (UNIL) supported this work. H.V. thanks the Max-Planck-Gesellschaft for funding.

Appendix A. Supplementary data

Supplementary data related to this article can be found at ...

References

- Afshar, K., Dube, F.F., Najafabadi, H.S., Bonneil, E., Thibault, P., Salavati, R., Bede, J.C., 2013. Insights into the insect salivary gland proteome: Diet-associated changes in caterpillar labial salivary proteins. *J. Insect Physiol.* 59, 351-366.
- Agerbirk, N., Olsen, C.E., Poulsen, E., Jacobsen, N., Hansen, P.R., 2010. Complex metabolism of aromatic glucosinolates in *Pieris rapae* caterpillars involving nitrile formation, hydroxylation, demethylation, sulfation, and host plant dependent carboxylic acid formation. *Insect Biochem. Mol. Biol.* 40, 126–137.
- Agrawal, S., Kelkenberg, M., Begum, K., Steinfeld, L., Williams, C.E., Kramer, K.J., Beeman, R.W., Park, Y., Muthukrishnan, S., Merzendorfer, H., 2014. Two essential peritrophic matrix proteins mediate matrix barrier functions in the insect midgut. *Insect Biochem. Mol. Biol.* 49, 24e34.
- Ahn, S.-J., Vogel, H., Heckel, D.G., 2012. Comparative analysis of the UDP-glycosyltransferase multigene family in insects. *Insect Biochem. Mol. Biol.* 42, 133–147.
- Ali J.G., Agrawal, A.A., 2012. Specialist versus generalist insect herbivores and plant defense. *Trends Plant Sci.* 17, 293-302.
- Aminetzach, Y.T., Macpherson, J.M., Petrov, D.A., 2005. Pesticide resistance via transposition-mediated adaptive gene truncation in *Drosophila*. *Science* 309, 764–767.
- Banville, N., Browne, N., Kavanagh, K., 2012. Effect of nutrient deprivation on the susceptibility of *Galleria mellonella* larvae to infection. *Virulence* 3, 497-503.
- Barth, C., Jander, G., 2006. Arabidopsis myrosinases TGG1 and TGG2 have redundant function in glucosinolate breakdown and insect defense. *Plant J.* 46, 549–562.

- Beekwilder, J., van Leeuwen, W., van Dam, N.M., Bertossi, M., Grandi, V., Mizzi, L., Soloviev, M., Szabados, L., Molthoff, J.W., Schipper, B., Verbocht, H., de Vos, R.C.H., Morandini, P., Aarts, M.G.M., Bovy, A., 2008. The impact of the absence of aliphatic glucosinolates on insect herbivory in *Arabidopsis*. PLoS ONE 3, e2068.
- ~~Bellés, X., Martín, D., Piulachs, M. D., 2005. The mevalonate pathway and the synthesis of juvenile hormone in insects. Annu. Rev. Entomol. 50, 181–199.~~
- Bodenhause, N., Reymond, P., 2007. Signaling pathways controlling induced resistance to insect herbivores in *Arabidopsis*. Mol. Plant-Microbe Interact. 20, 1406–1420.
- Capy, P., Gasperi, G., Biémont, C., Bazin, C., 2000. Stress and transposable elements: co-evolution or useful parasites? Heredity 85, 101–106.
- Celorio-Mancera, M. de L.P., Ahn, S.-J., Vogel, H., Heckel, D.G., 2011. Transcriptional responses underlying the hormetic and detrimental effects of the plant secondary metabolite gossypol on the generalist herbivore *Helicoverpa armigera*. BMC Genomics 12, 575.
- Celorio-Mancera, M. de L.P., Heckel, D.G., Vogel, H., 2012. Transcriptional analysis of physiological pathways in a generalist herbivore: responses to different host plants and plant structures by the cotton bollworm, *Helicoverpa armigera*. Entomol. Exp. Appl. 144, 123–133.
- Celorio-Mancera, de, M., Wheat, C.W., Vogel, H., Söderlind, L., Janz, N., Nylin, S., 2013. Mechanisms of macroevolution: polyphagous plasticity in butterfly larvae revealed by RNA-Seq. Mol. Ecol. 22, 4884–4895.
- Chandra, G.S., Asokan, R., Manamohan, M., 2016. Cytochrome P450 isoforms transcriptional, larval growth and development responses to host allelochemicals in the generalist herbivore, *Helicoverpa armigera*. Curr. Sci. 5, 901–906.
- Chénais, B., Caruso, A., Hiard, S., Casse, N., 2012. The impact of transposable elements on eukaryotic genomes: from genome size increase to genetic adaptation to stressful environments. Gene 509, 7–15.
- Cho, S., Mitchell, A., Mitter, C., Regier, J., Matthews, M., Robertson, R., 2008. Molecular phylogenetics of heliothine moths (Lepidoptera: Noctuidae: Heliothinae), with comments on the evolution of host range and pest status. Syst. Entomol. 33, 581–594.
- Daborn, P.J., Lumb, C., Boey, A., Wong, W., ffrench-Constant, R.H., Batterham, P., 2007. Evaluating the insecticide resistance potential of eight *Drosophila melanogaster* cytochrome P450 genes by transgenic over-expression. Insect Biochem. Mol. Biol. 37, 512–519.
- Daborn, P.J., Yen, J.L., Bogwitz, M.R., Le Goff, G., Feil, E., Jeffers, S., Tijet, N., Perry, T., Heckel, D., Batterham, P., Feyereisen, R., Wilson, T.G., ffrench-Constant, R.H., 2002. A single p450 allele associated with insecticide resistance in *Drosophila*. Science 297, 2253–2256.
- Deng, H., Huang, Y., Feng, Q., Zheng, S., 2009. Two epsilon glutathione S-transferase cDNAs from the common cutworm, *Spodoptera litura*: characterization and developmental and induced expression by insecticides. J. Insect Physiol. 55, 1174–1183.
- Dermauw, W., Osborne, E.J., Clark, R.M., Grbic, M., Tirry, L., Van Leeuwen, T., 2013. A burst of ABC genes in the genome of the polyphagous spider mite *Tetranychus urticae*. BMC Genomics 14, 317.
- Després, L., David, J.-P., Gallet, C., 2007. The evolutionary ecology of insect resistance to plant chemicals. Trends Ecol. Evol. 22, 298–307.
- Edger, P.P., Heide-Fischer, H.M., Bekaert, M., Rota, J., Glöckner, G., Platts, A.E., Heckel, D.G., Der, J.P., Wafula, E.K., Tang, M., Hofberger, J.A., Smithson, A., Hall, J.C., Blanchette, M., Bureau, T.E., Wright, S.I., dePamphilis, C.W., Eric Schranz, M., Barker, M.S., Conant, G.C., Wahlberg, N., Vogel, H., Pires, J.C., Wheat, C.W., 2015. The butterfly plant arms-race escalated by gene and genome duplications. Proc. Natl. Acad. Sci. USA 112, 8362–8366.
- Falk, K.L., Gershenzon, J., 2007. The desert locust, *Schistocerca gregaria*, detoxifies the glucosinolates of *Schouwia purpurea* by desulfation. J. Chem. Ecol. 33, 1542–1555.
- Feyereisen, R., 2006. Evolution of insect P450. Biochem. Soc. Trans. 34, 1252–1255.
- Feyereisen, R., 1999. Insect P450 enzymes. Annu. Rev. Entomol. 44, 507–33.
- Gahan, L.J., Pauchet, Y., Vogel, H., Heckel, D.G., 2010. An ABC transporter mutation is correlated with insect resistance to *Bacillus thuringiensis* Cry1Ac toxin. PLoS Genet. 6, e1001248.
- Gao, R.-N., Wei, Z.-G., Zhang, T., Wang, R.-X., Zhao, G.-D., Li, B., Shen, W.-D., 2010. Changes in the expression of CYP3 family genes under the induction of ecdysone in *Bombyx mori*. Acta

- Entomol. Sinica 53, 943–948.
- Gigolashvili, T., Yatusovich, R., Berger, B., Müller, C., Flügge, U.-I., 2007. The R2R3-MYB transcription factor HAG1/MYB28 is a regulator of methionine-derived glucosinolate biosynthesis in *Arabidopsis thaliana*. Plant J. 51, 247–261.
- ~~Gilbert, L.I., Granger, N.A., Roe, R.M., 2000. The juvenile hormones: historical facts and speculations on future research directions. Insect Biochem. Mol. Biol. 30, 617–644.~~
- ~~Gilbert, L.I., Rybczynski, R., Warren, J.T., 2002. Control and biochemical nature of the ecdysteroidogenic pathway. Annu. Rev. Entomol. 47, 883–916.~~
- Gloss, A.D., Vassão, D.G., Hailey, A.L., Nelson Dittrich, A.C., Schramm, K., Reichelt, M., Rast, T.J., Weichsel, A., Cravens, M.G., Gershenzon, J., Montfort, W.R., Whiteman, N.K., 2014. Evolution in an ancient detoxification pathway is coupled with a transition to herbivory in the drosophilidae. Mol. Biol. Evol. 31, 2441–2456.
- Govind, G., Mittapalli, O., Griebel, T., Allmann, S., Böcker, S., Baldwin, I.T., 2010. Unbiased transcriptional comparisons of generalist and specialist herbivores feeding on progressively defenseless *Nicotiana attenuata* plants. PLoS ONE 5, e8735.
- Grbic, M., Van Leeuwen, T., Clark, R.M., Rombauts, S., Rouzé, P., Grbic, V., Osborne, E.J., Dermauw, W., Ngoc, P.C.T., Ortego, F., Hernandez-Crespo, P., Diaz, I., Martinez, M., Navajas, M., Sucena, E., Magalhaes, S., Nagy, L., Pace, R.M., Djuranovic, S., Smagghe, G., Iga, M., Christiaens, O., Veenstra, J.A., Ewer, J., Mancilla Villalobos, R., Hutter, J.L., Hudson, S.D., Velez, M., Yi, S.V., Zeng, J., Pires-daSilva, A., Roch, F., Cazaux, M., Navarro, M., Zhurov, V., Acevedo, G., Bjelica, A., Fawcett, J.A., Bonnet, E., Martens, C., Baele, G., Wissler, L., Sanchez-Rodriguez, A., Tirry, L., Blais, C., Demeestere, K., Henz, S.R., Gregory, T.R., Mathieu, J., Verdon, L., Farinelli, L., Schmutz, J., Lindquist, E., Feyereisen, R., Van de Peer, Y., 2011. The genome of *Tetranychus urticae* reveals herbivorous pest adaptations. Nature 479, 487–492.
- Halkier, B.A., Gershenzon, J., 2006. Biology and biochemistry of glucosinolates. Annu. Rev. Plant Biol. 57, 303–333.
- Hegedus, D., Erlandson, M., Gillott, C., Toprak, U., 2009. New insights into peritrophic matrix synthesis, architecture, and function. Annu. Rev. Entomol. 54, 285–302.
- Heidel-Fischer, H.M., Vogel, H., 2015. Molecular mechanisms of insect adaptation to plant secondary compounds. Curr. Opin. Insect Sci. 8, 8–14.
- Herde, M., Howe, G.A., 2014. Host plant-specific remodeling of midgut physiology in the generalist insect herbivore *Trichoplusia ni*. Insect Biochem. Mol. Biol. 50, 58–67.
- Jacobs, C.G., Steiger, S., Heckel, D.G., Wielsch, N., Vilcinskis, A., Vogel, H., 2016. Sex, offspring and carcass determine antimicrobial peptide expression in the burying beetle. Scientific Reports 6, 25409.
- ~~Jindra, M., Palli, S.R., Riddiford, L.M., 2013. The juvenile hormone signaling pathway in insect development. Annu. Rev. Entomol. 58, 181–204.~~
- Joußen, N., Agnolet, S., Lorenz, S., Schöne, S.E., Ellinger, R., Schneider, B., Heckel, D.G., 2012. Resistance of Australian *Helicoverpa armigera* to fenvalerate is due to the chimeric P450 enzyme CYP337B3. Proc. Natl. Acad. Sci. USA 109, 15206–15211.
- Kelkenberg, M., Odman-Naresh, J., Muthukrishnan, S., Merzendorfer, H., 2014. Chitin is a necessary component to maintain the barrier function of the peritrophic matrix in the insect midgut. Insect Biochem. Mol. Biol. 56, 21–28.
- Kliebenstein, D.J., Kroymann, J., Mitchell-Olds, T., 2005. The glucosinolate-myrosinase system in an ecological and evolutionary context. Curr. Opin. Plant Biol. 8, 264–271.
- König, C., Bretschneider, A., Heckel, D. G., Grosse-Wilde, E., Hansson, B. S., Vogel, H., 2015. The plastic response of *Manduca sexta* to host and non-host plants. Insect Biochem. Mol. Biol. 63, 72–85.
- Krempl, C., Sporer, T., Reichelt, M., Ahn, S.-J., Heidel-Fischer, H., Vogel, H., Heckel, D. G., Joußen, N., 2016. Potential detoxification of gossypol by UDP-glycosyltransferases in the two Heliothine moth species *Helicoverpa armigera* and *Heliothis virescens*. Insect Biochem. Mol. Biol. 71, 49–57.
- Lambrix, V.M., Reichelt, M., Mitchell-Olds, T., Kliebenstein, D.J., Gershenzon, J., 2001. The *Arabidopsis* epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences *Trichoplusia ni* herbivory. Plant Cell 13, 2793–2807.

- Li, X., Schuler, M.A., Berenbaum, M.R., 2003. Diversification of furanocoumarin-metabolizing cytochrome P450 monooxygenases in two papilionids: Specificity and substrate encounter rate. *Proc. Natl. Acad. Sci. USA* 100, 14593-14598.
- Li, X., Baudry, J., Berenbaum, M.R., Schuler, M.A., 2004. Structural and functional divergence of insect CYP6B proteins: From specialist to generalist cytochrome P450. *Proc. Natl. Acad. Sci. USA* 101, 2939-2944.
- Li, X., Schuler, M.A., Berenbaum, M.R., 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu. Rev. Entomol.* 52, 231-253.
- Li, A., Denlinger, D., 2009. Pupal cuticle protein is abundant during early adult diapause in the mosquito *Culex pipiens*. *J. Med. Entomol.*, 46, 1382-1386.
- Low, W.Y., Ng, H.L., Morton, C.J., Parker, M.W., Batterham, P., Robin, C., 2007. Molecular evolution of glutathione S-transferases in the genus *Drosophila*. *Genetics* 177, 1363-1375.
- Maïbèche-Coisne, M., Nikonov, A.A., Ishida, Y., Jacquín-Joly, E. and Leal, W.S., 2004. Pheromone anosmia in a scarab beetle induced by *in vivo* inhibition of a pheromone-degrading enzyme. *Proc. Natl. Acad. Sci. USA* 101, 11459-11464.
- ~~Makka, T., Seino, A., Tomita, S., Fujiwara, H., Sonobe, H., 2002. A possible role of 20-hydroxyecdysone in embryonic development of the silkworm *Bombyx mori*. *Arch. Insect. Biochem. Physiol.* 51, 111-120.~~
- Martin, P.B., Lingren, P.D., Greene, G.L., 1976. Relative abundance and host preferences of cabbage looper, soybean looper, tobacco budworm, and corn earworm on crops grown in northern Florida. *Environ. Entomol.* 5, 878-882
- Martins, J.R., Nunes, F.M., Cristino, A.S., Simões, Z.L., Bitondi, M.M., 2010. The four hexamerin genes in the honey bee: structure, molecular evolution and function deduced from expression patterns in queens, workers and drones. *BMC Mol. Biol.* 11:23.
- Mathers, T.C., Chen, Y., Kaithakottil, G., Legeai, F., Mugford, S.T., Baa-Puyoulet, P., Bretaudeau, A., Clavijo, B., Colella, S., Collin, O., Dalmay, T., Derrien, T., Feng, H., Gabaldón, T., Jordan, A., Julca, I., Kettles, G.J., Kowitwanich, K., Lavenier, D., Lenzi, P., Lopez-Gomollon, S., Loska, D., Mapleson, D., Maumus, F., Moxon, S., Price, D.R., Sugio, A., van Munster, M., Uzest, M., Waite, D., Jander, G., Tagu, D., Wilson, A.C., van Oosterhout, C., Swarbreck, D., Hogenhout, S.A., 2017. Rapid transcriptional plasticity of duplicated gene clusters enables a clonally reproducing aphid to colonise diverse plant species. *BMC Biol.* 18, 27.
- Mateo, L., Ullastres, A., González, J., 2014. A transposable element insertion confers xenobiotic resistance in *Drosophila*. *PLoS Genet.* 10, e1004560.
- Maumus, F., Fiston-Lavier, A.-S., Quesneville, H., 2015. Impact of transposable elements on insect genomes and biology. *Curr. Opin. Insect Sci.* 7, 30-36.
- Mewis, I., Tokuhisa, J.G., Schultz, J.C., Appel, H.M., Ulrichs, C., Gershenzon, J., 2006. Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. *Phytochemistry* 67, 2450-2462.
- Mitter, C., Poole, R. W., Matthews, M. 1993. Biosystematics of the Heliethinae (Lepidoptera: Noctuidae). *Annu. Rev. Entomol.* 38, 207-225.
- Müller, R., de Vos, M., Sun, J.Y., Sønderby, I.E., Halkier, B.A., Wittstock, U., Jander, G., 2010. Differential effects of indole and aliphatic glucosinolates on lepidopteran herbivores. *J. Chem. Ecol.* 36, 905-913.
- ~~Nijhout, H.F., Callier, V., 2015. Developmental mechanisms of body size and wing-body scaling in insects. *Annu. Rev. Entomol.* 60, 141-156.~~
- ~~Petryk, A., Warren, J.T., Marqués, G., Jarcho, M.P., Gilbert, L.I., Kahler, J., Parvy, J. P., Li, Y., Dauphin-Villemant, C., O'Connor, M.B., 2003. Shade is the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl. Acad. Sci. USA* 100, 13773-13778.~~
- Pottier, M.-A., Bozzolan, F., Chertemps, T., Jacquín-Joly, E., Lalouette, L., Siaussat, D., Maïbèche-Coisne, M., 2012. Cytochrome P450s and cytochrome P450 reductase in the olfactory organ of the cotton leafworm *Spodoptera littoralis*. *Insect Mol. Biol.* 21, 568-580.
- Ratzka, A., Vogel, H., Kliebenstein, D.J., Mitchell-Olds, T., Kroymann, J., 2002. Disarming the mustard oil bomb. *Proc. Natl. Acad. Sci. USA* 99, 11223-11228.

- Rose, R.L., Goh, D., Thompson, D.M., Verma, K.D., Heckel, D.G., Gahan, L.J., Roe, R.M., Hodgson, E., 1997. Cytochrome P450 (CYP)9A1 in *Heliothis virescens*: the first member of a new CYP family. *Insect Biochem. Mol. Biol.* 27, 605–615.
- Roy, A., Walker, W.B., Vogel, H., Chattington, S., Larsson, M.C., Anderson, P., Heckel, D.G., Schlyter, F., 2016. Diet dependent metabolic responses in three generalist insect herbivores *Spodoptera* spp. *Insect Biochem. Mol. Biol.* 71, 91–105.
- Ryan, R.O., Anderson, D.R., Grimes, W.J., Law, J.H., 1985. Arylphorin from *Manduca sexta*: carbohydrate structure and immunological studies. *Arch. Biochem. Biophys.* 243, 115–124.
- Sasabe, M., Wen, Z., Berenbaum, M.R., Schuler, M.A., 2004. Molecular analysis of CYP321A1, a novel cytochrome P450 involved in metabolism of plant allelochemicals (furanocoumarins) and insecticides (cypermethrin) in *Helicoverpa zea*. *Gene* 338, 163–175.
- Schlaeppli, K., Bodenhausen, N., Buchala, A., Mauch, F., Reymond, P., 2008. The glutathione-deficient mutant *pad2-1* accumulates lower amounts of glucosinolates and is more susceptible to the insect herbivore *Spodoptera littoralis*. *Plant J.* 55, 774–786.
- Schmidt, J.M., Robin, C., 2011. An adaptive allelic series featuring complex gene rearrangements. *PLoS Genet.* 7, e1002347.
- Schoonhoven, L.M., van Loon, J.J.A., Dicke, M., 2005. *Insect-plant biology*. Oxford University Press, USA.
- Schramm, K., Vassão, D.G., Reichelt, M., Gershenzon, J., Wittstock, U., 2012. Metabolism of glucosinolate-derived isothiocyanates to glutathione conjugates in generalist lepidopteran herbivores. *Insect Biochem. Mol. Biol.* 42, 174–182.
- Schweizer, F., Fernández-Calvo, P., Zander, M., Diez-Diaz, M., Fonseca, S., Glauser, G., Lewsey, M.G., Ecker, J.R., Solano, R., Reymond, P., 2013. *Arabidopsis* basic helix-loop-helix transcription factors MYC2, MYC3, and MYC4 regulate glucosinolate biosynthesis, insect performance, and feeding behavior. *Plant Cell* 25, 3117–3132.
- Snyder, M.J., Glendinning, J.I., 1996. Causal connection between detoxification enzyme activity and consumption of a toxic plant compound. *J. Comp. Physiol. A* 179, 255–261.
- Sønderby, I.E., Geu-Flores, F., Halkier, B.A., 2010. Biosynthesis of glucosinolates--gene discovery and beyond. *Trends Plant Sci.* 15, 283–290.
- Stauber, E.J., Kuczka, P., van Ohlen, M., Vogt, B., Janowitz, T., Piotrowski, M., Beuerle, T., Wittstock, U., 2012. Turning the ‘Mustard Oil Bomb’ into a ‘Cyanide Bomb’: Aromatic glucosinolate metabolism in a specialist insect herbivore. *PLoS ONE* 7: e35545.
- Stuckas, H., Mende, M.B., Hundsdoerfer, A.K., 2014. Response to cold acclimation in diapause pupae of *Hyles euphorbiae* (Lepidoptera: Sphingidae): candidate biomarker identification using proteomics. *Insect Mol. Biol.* 23, 444–456.
- Tay, W.T., Mahon, R.J., Heckel, D.G., Walsh, T.K., Downes, S., James, W.J., Lee, S.-F., Reineke, A., Williams, A.K., Gordon, K.H.J., 2015. Insect resistance to *Bacillus thuringiensis* toxin Cry2Ab is conferred by mutations in an ABC transporter subfamily A protein. *PLoS Genet.* 11:e1005534.
- Teese, M.G., Campbell, P.M., Scott, C., Gordon, K.H.J., Southon, A., Hovan, D., Robin, C., Russell, R.J., Oakeshott, J.G., 2010. Gene identification and proteomic analysis of the esterases of the cotton bollworm, *Helicoverpa armigera*. *Insect Biochem. Mol. Biol.* 40, 1–16.
- van Ohlen, M., Herfurth, A.-M., Kerbstadt, H., Wittstock, U., 2016. Cyanide detoxification in an insect herbivore: Molecular identification of β -cyanoalanine synthases from *Pieris rapae*. *Insect Biochem. Mol. Biol.* 70, 99–110.
- Vergara, F., Svatoš, A., Schneider, B., Reichelt, M., Gershenzon, J., Wittstock, U., 2006. Glycine conjugates in a lepidopteran insect herbivore - the metabolism of benzylglucosinolate in the cabbage white butterfly, *Pieris rapae*. *ChemBiolChem*, 7, 1982–1989.
- Vogel, H., Badapanda, C., Knorr, E., Vilcinskis, A., 2014. RNA-sequencing analysis reveals abundant developmental stage-specific and immunity-related genes in the pollen beetle *Meligethes aeneus*. *Insect Mol. Biol.* 23, 98–112.
- ~~Wang, B., Goode, J., Best, J., Meltzer, J., Schilman, P.E., Chen, J., Garza, D., Thomas, J.B., Montminy, M., 2008. The insulin-regulated CREB coactivator TORC promotes stress resistance in *Drosophila*. *Cell Metab.* 7, 434–444.~~
- Wang, R.L., Zhu-Salzman, K., Baerson, S.R., Xin, X.-W., Li, J., Su, Y.J., Zeng, R.S., 2016. Identification of a novel cytochrome P450 CYP321B1 gene from tobacco cutworm (*Spodoptera*

- litura*) and RNA interference to evaluate its role in commonly used insecticides. Insect Science.
- Wee, C.W., Lee, S.F., Robin, C., Heckel, D.G., 2008. Identification of candidate genes for fenvalerate resistance in *Helicoverpa armigera* using cDNA-AFLP. Insect Mol. Biol. 17, 351–360.
- Weinshilboum, R.M., Otterness, D.M., Aksoy, I.A., Wood, T.C., Her, C., and Raftogianis, R.B., 1997. Sulfation and sulfotransferases 1: sulfotransferase molecular biology: cDNAs and genes. FASEB J. 11, 3–14.
- Wheat, C.W., Vogel, H., Wittstock, U., Braby, M.F., Underwood, D., Mitchell-Olds, T., 2007. The genetic basis of a plant-insect coevolutionary key innovation. Proc. Natl. Acad. Sci. USA 104, 20427–20431.
- Whiteman, N.K., Gloss, A.D., Sackton, T.B., Groen, S.C., Humphrey, P.T., Lapoint, R.T., Sønderby, I.E., Halkier, B.A., Kocks, C., Ausubel, F.M., Pierce, N.E., 2012. Genes involved in the evolution of herbivory by a leaf-mining, Drosophilid fly. Genome Biol. Evol. 4, 900–916.
- Whiteman, N.K., Groen, S.C., Chevasco, D., Bear, A., Beckwith, N., Gregory, T.R., Denoux, C., Mammarella, N., Ausubel, F.M., Pierce, N.E., 2011. Mining the plant-herbivore interface with a leafmining *Drosophila* of *Arabidopsis*. Mol. Ecol. 20, 995–1014.
- Wittstock, U., Agerbirk, N., Stauber, E.J., Olsen, C.E., Hippler, M., Mitchell-Olds, T., Gershenzon, J., Vogel, H., 2004. Successful herbivore attack due to metabolic diversion of a plant chemical defense. Proc. Natl. Acad. Sci. USA 101, 4859–4864.
- Wittstock, U., Gershenzon, J., 2002. Constitutive plant toxins and their role in defense against herbivores and pathogens. Curr. Opin. Plant Biol. 5, 300–307.
- Wybouw, N., Dermauw, W., Tirry, L., Christian Stevens, C., Grbić, M., Feyereisen, R., Van Leeuwen, T., 2014. A gene horizontally transferred from bacteria protects arthropods from host plant cyanide poisoning. eLife 3, e02365.
- Wybouw, N., Zhurov, V., Martel, C., Bruinsma, K. A., Hendrickx, F., Grbic, V., Van Leeuwen, T., 2015. Adaptation of a polyphagous herbivore to a novel host plant extensively shapes the transcriptome of herbivore and host. Mol. Ecol. 24, 4647–4663.
- Yamamoto, K., Teshiba, S., Shigeoka, Y., Aso, Y., Banno, Y., Fujiki, T., Katakura, Y., 2011. Characterization of an omega-class glutathione S-transferase in the stress response of the silkworm. Insect Mol. Biol. 20, 379–386.
- Zhou, X., Ma, C., Li, M., Sheng, C., Liu, H., Qiu, X., 2010. CYP9A12 and CYP9A17 in the cotton bollworm, *Helicoverpa armigera*: sequence similarity, expression profile and xenobiotic response. Pest Manag. Sci. 66, 65–73.
- Zou, X., Xu, Z., Zou, H., Liu, J., Chen, S., Feng, Q., Zheng, S., 2016. Glutathione S-transferase SIGSTE1 in *Spodoptera litura* may be associated with feeding adaptation of host plants. Insect Biochem. Mol. Biol. 70, 32–43.
- Zhurov, V., Navarro, M., Bruinsma, K.A., Arbona, V., Santamaria, M.E., Cazaux, M., Wybouw, N., Osborne, E.J., Ens, C., Rioja, C., Vermeirssen, V., Rubio-Somoza, I., Krishna, P., Diaz, I., Schmid, M., Gómez-Cadenas, A., Van de Peer, Y., Grbic, M., Clark, R.M., Van Leeuwen, T., Grbic, V., 2014. Reciprocal responses in the interaction between *Arabidopsis* and the cell-content-feeding chelicerate herbivore spider mite. Plant Physiol. 164, 384–399.

Figure legends

Figure 1. Effect of glucosinolates (GS) on performance of a specialist and a generalist herbivore. Freshly hatched larvae were placed on five-week-old Arabidopsis wild-type (Col-0) plants or on the GS-deficient quadruple mutant *quadGS* and larval weight (mean $\pm$ SE) was measured after 8 days of feeding. Asterisks indicate statistically significant differences between the tested genotypes (Student's t test; *** $p < 0.001$).

Figure 2. Scatter plots of differentially regulated contigs. Contig gene expression in *H. virescens* (A) or *P. brassicae* (B) larvae feeding on Col-0 or *quadGS* plants for 48 h. Contigs expression ratios that are larger than 2 (adjusted p -value < 0.05) are indicated by red dots.

Figure 3. Heat map of detoxification genes significantly regulated between *H. virescens* (Hvir) and *P. brassicae* (Pbra) larvae feeding on Col-0 or *quadGS* plants. Contigs upregulated in the presence of GS (Col-0) have value > 1 whereas contigs downregulated have value < -1 . Grey boxes indicate that no *H. virescens* or *P. brassicae* homologue was identified.

~~**Figure 4.** Heat map of hormone related genes significantly regulated between *H. virescens* (Hvir) and *P. brassicae* (Pbra) larvae feeding on Col-0 or *quadGS* plants. Contigs upregulated in the presence of GS (Col-0) have value > 1 whereas contigs downregulated have value < -1 . Grey boxes indicate that no *P. brassicae* homologue was identified.~~

Table 1

Number of differentially regulated contigs

	Total	Induced in Col-0 ^a	Induced in <i>quadGS</i> ^a
<i>H. virescens</i>	34,867	2,660	1,087
<i>P. brassicae</i>	26,756	118	136

^aFold change ≥ 2 , $\log_2$ RKPM ≥ 1 , $P < 0.05$

Table 2

Heliothis virescens CYP450 genes upregulated by Arabidopsis glucosinolates. Each contig sequence was translated and blasted against NCBI non-redundant protein database. Insect homologous proteins with high BLAST scores were retrieved. Homologous proteins with known biological information are listed, otherwise homologous proteins with the highest similarity are included. Contig DNA sequences can be found in Table S1.

Contig	Nb	Homologue	Similarity (%)	E-value	<i>quadGS</i> RPKM (log ₂)	Col-0 RPKM (log ₂)	Fold change	Description
Hvir_C2993	2	HaCYP314A1	96	0.0	2.35	3.76	2.65	20-Ecdysone synthesis ¹
Hvir_C289	4	HxCYP321A1	83	0.0	7.40	9.68	4.85	Resistance to xanthotoxin and cypermethrin in <i>H. zea</i> ²
Hvir_C9545	1	HaCYP321B1	90	0.0	3.03	5.66	6.19	Resistance to cypermethrin in <i>S. litura</i> ³
Hvir_C7033	7	HaCYP324A1	86	0.0	3.84	6.21	5.21	
Hvir_C20757	2	HaCYP337B1/2	85	5E-89	-0.72	1.29	4.00	Resistance to fenvalerate in <i>H. armigera</i> ^{4,5}
Hvir_C27228	1	BmCYP339A1	61	3E-148	-0.43	1.67	4.31	Induced by ecdysone in <i>B. mori</i> ⁶
Hvir_C25434	1	SliCYP341A13	79	1E-102	-1.41	1.71	8.69	Expressed in larval antennae <i>S. littoralis</i> ⁷
Hvir_C13818	1	HaCYP341D1	90	0.0	1.55	3.19	3.10	
Hvir_C17579	1	HaCYP367B2	95	3E-158	-1.48	1.64	8.73	
Hvir_C135	4	HaCYP6AE12	88	3E-144	6.60	7.94	2.53	Induced by gossypol in <i>H. armigera</i> ⁸
Hvir_C2104	1	HaCYP6AE17	87	2E-149	5.15	6.55	2.66	Induced by gossypol ⁹ and feeding on bean in <i>H. armigera</i> ¹⁰
Hvir_C3213	1	HaCYP6AE24	90	0.0	3.49	6.70	9.27	
Hvir_C871	1	HvirCYP6B10	100	0.0	5.99	7.37	2.60	Induced by gossypol, tomatine, xanthotoxin in <i>H. armigera</i> ⁹
Hvir_C798	1	HxCYP6B8	91	0.0	8.17	9.23	2.09	Metabolizes plant toxins and insecticides in <i>H. zea</i> ¹¹
Hvir_C13522	1	HvirCYP9A1v2	100	0.0	2.74	4.69	3.86	Correlated with insecticide resistance in <i>H. virescens</i> ¹²
Hvir_C2774	4	HaCYP9A17v2	91	2E-169	6.57	8.21	3.10	Induced by deltamethrin and gossypol in <i>H. armigera</i> ¹³

¹Petryk et al. (2003); ²Sasabe et al. (2004); ³Wang et al. (2016); ⁴Wee et al. (2008); ⁵Joussen et al. (2012); ⁶Gao et al.(2010); ⁷Pottier et al. (2012); ⁸Chandra et al. (2016); ⁹Celorio-Mancera et al. (2011); ¹⁰Celorio-Mancera et al. (2012); ¹¹Li et al. (2004); ¹²Rose et al. (1997); ¹³Zhou et al. (2010).

Bm: *Bombyx mori*; Ha: *Helicoverpa armigera*; Hvir: *Heliothis virescens*; Hx: *Helicoverpa zea*; Sli: *Spodoptera littoralis*

Table 3

Heliothis virescens detoxification genes upregulated by Arabidopsis glucosinolates. Each contig sequence was translated and blasted against NCBI non-redundant protein database. Insect homologous proteins with high BLAST scores were retrieved. Homologous proteins with known biological information are listed, otherwise homologous proteins with the highest similarity are included. Contig DNA sequences can be found in Table S1.

Contig	Nb	Homologue	Similarity (%)	E-value	<i>quadGS</i> RPKM (log ₂)	Col-0 RPKM (log ₂)	Fold change	Description
Carboxyl/cholinesterases								
Hvir_C1895	6	HaCCE001a	96	4E-97	6.86	8.44	4.00	Midgut esterase in <i>H. armigera</i> , associated with resistance ¹
Hvir_C12658	2	HaCCE001c	79	2E-179	-0.34	4.51	28.80	Midgut esterase in <i>H. armigera</i> ¹
Hvir_C1953	2	HaCCE001d	91	3E-109	5.63	7.36	3.31	Midgut esterase in <i>H. armigera</i> ¹
Hvir_C2486	1	HaCCE001f	87	2E-61	7.19	8.21	2.03	Midgut esterase in <i>H. armigera</i> ¹
Hvir_C25103	2	HaCCE001i	85	6E-62	0.43	3.03	6.06	Midgut esterase in <i>H. armigera</i> , associated with resistance ¹
Hvir_C8604	1	HaCCE006a	69	0.0	1.35	2.57	2.33	Odorant degrading esterase in <i>H. armigera</i> , assoc. with res. ¹
Hvir_C19785	1	HaCCE033a	70	7E-32	-1.05	2.31	10.30	Odorant degrading esterase in <i>H. armigera</i> ¹
Hvir_C16628	1	HaCCE014a	97	1E-48	2.35	3.76	2.65	Associated with resistance in dipteran and hymenopteran ¹
Hvir_C1996	2	HaCCE016b	60	6E-155	2.59	4.58	3.95	Associated with resistance in dipteran and hymenopteran ¹
Glutathione-S transferases								
Hvir_C728	1	BmGSTo2	76	2E-122	6.75	8.30	2.93	Induced by insecticides in <i>B. mori</i> ²
Hvir_C25474	1	SlGSTe11	94	5E-158	-0.29	1.28	2.98	SIGSTe2/3 induced by insecticides in <i>S. litura</i> ³
UDP-glycosyltransferases								
Hvir_C720	4	HaUGT40M1	93	2E-141	6.83	8.56	3.32	Homologues induced by gossypol in <i>H. armigera</i> ⁴
Hvir_C2844	1	HaUGT33B5	94	5E-55	5.50	6.67	2.26	Homologues induced by gossypol in <i>H. armigera</i> ⁴
ABC transporters								
Hvir_C34071	3	PxABCA13	61	5E-22	-0.55	1.27	3.53	
Hvir_C15652	2	SlABCC1	99	9E-100	3.14	4.50	2.57	Induced by diet shift bean->Arabidopsis in <i>T. urticae</i> ⁵
Hvir_C6094	1	HsABCC2	100	1E-41	0.46	4.20	13.43	Correlated with <i>H. virescens</i> resistance to Bt ⁶
Hvir_C29951	1	SlABCC10	98	6E-70	-1.26	2.02	9.76	
Hvir_C13258	2	BmABCG1	91	0.0	1.11	2.50	2.63	Induced by diet shift bean->Arabidopsis in <i>T. urticae</i> ⁵
Hvir_C6673	1	PxABCG4	85	0.0	1.84	3.29	2.71	
Hvir_C17474	1	BmABCG5	97	2E-159	2.12	3.21	2.12	

¹Teese et al. (2008); ²Yamamoto et al. (2011); ³Deng et al. (2009); ⁴Krempl et al. (2016); ⁵Dermauw et al. (2013); ⁶Gahan et al. (2010)

Bm: *Bombyx mori*; Ha: *Helicoverpa armigera*; Hs, *Heliothis subflexa*; Px, *Papilio xuthus*; Sl: *Spodoptera litura*

Table 4

Glucosinolate detoxification genes. Each contig sequence was translated and blasted against NCBI non-redundant protein database. Insect homologous proteins with high BLAST scores were retrieved. Homologous proteins with known biological information are listed, otherwise homologous proteins with the highest similarity are included. Contig DNA sequences can be found in Table S1.

Contig	Nb	Homologue	Similarity (%)	E-value	<i>quadGS</i> RPKM (log ₂)	Col-0 RPKM (log ₂)	Fold change	Description
Nitrile-specifier proteins								
<i>Pieris brassicae</i>								
Pbra_C162	1	PbNSP-D2	100	0.0	9.26	10.59	2.52	GS detoxification in Pierids ^{1,2}
Pbra_C85	1	PbNSP-D3	100	0.0	10.54	10.69	1.10	GS detoxification in Pierids ^{1,2}
Cyanoalanine synthase								
<i>Pieris brassicae</i>								
Pbra_C2345	1	PbCAS1	100	0.0	5.89	7.24	2.54	CAS homologue ³
Glutathione-S transferases								
<i>Pieris brassicae</i>								
Pbra_C1158	1	HaGSTD1	74	9E-97	6.59	7.92	2.52	GS detoxification in <i>Scaptomyza</i> ⁴
Sulfatases								
<i>Heliothis virescens</i>								
Hvir_C14843	1	PxyCAC86342	69	5E-155	0.00	1.70	3.35	Glucosinolate sulfatase in <i>P. xylostella</i> ⁵
Hvir_C20233	1	PxyXP_011550435	67	6E-154	-0.30	1.14	2.71	Arylsulfatase b
Hvir_C2274	2	PxyXP_011554395	72	5E-109	2.66	3.73	2.10	Arylsulfatase b

¹Wittstock et al. (2004); ²Wheat et al. (2007); ³van Ohlen et al. (2016); ⁴Gloss et al. (2014); ⁵Ratzka et al. (2002)

Ha, *Helicoverpa armigera*; Pb, *Pieris brassicae*; Pxy, *Plutella xylostella*

Figure 1

Fig. 1

Fig. 2

Fig. 3

● Differentially
expressed
genes