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

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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 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; Schlaeppi 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; Schlaeppi 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 m^3 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 N_2 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; $\log_2$ transformed) values had to be $\geq$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 Brassicacaeae 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 Arabidopis 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).

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.

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 (Wybouw 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 (SlGSTE1) was up-regulated in midguts of larvae fed on *Brassica juncea* or diet containing allyl-isothiocyanate. *In vitro*, SlGSTE1 was shown to catalyze the conjugation of glutathione and allyl-isothiocyanate, and RNAi-mediated suppression of Slgste1 in the larvae decreased both larval growth and feeding rate (Zou et al., 2016).

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. 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. 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.

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Appendix A. Supplementary data

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

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805

Table 1

Number of differentially regulated contigs

	Total	Induced in Col-0[a]	Induced in *quadGS*[a]
H. virescens	34,867	2,660	1,087
P. brassicae	26,756	118	136

[a]Fold 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	*quadGS* 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	HzCYP321A1	83	0.0	7.40	9.68	4.85	Resistance to xanthotoxin and cypermethrin in *H. zea*[2]
Hvir_C9545	1	HaCYP321B1	90	0.0	3.03	5.66	6.19	Resistance to cypermethrin in *S. litura*[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 *H. armigera*[4,5]
Hvir_C27228	1	BmCYP339A1	61	3E-148	-0.43	1.67	4.31	Induced by ecdysone in *B. mori*[6]
Hvir_C25434	1	SliCYP341A13	79	1E-102	-1.41	1.71	8.69	Expressed in larval antennae *S. littoralis*[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 *H. armigera*[8]
Hvir_C2104	1	HaCYP6AE17	87	2E-149	5.15	6.55	2.66	Induced by gossypol[9] and feeding on bean in *H. armigera*[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 *H. armigera*[9]
Hvir_C798	1	HzCYP6B8	91	0.0	8.17	9.23	2.09	Metabolizes plant toxins and insecticides in *H. zea*[11]
Hvir_C13522	1	HvirCYP9A1v2	100	0.0	2.74	4.69	3.86	Correlated with insecticide resistance in *H. virescens*[12]
Hvir_C2774	4	HaCYP9A17v2	91	2E-169	6.57	8.21	3.10	Induced by deltamethrin and gossypol in *H. armigera*[13]

[1]Petryk et al. (2003); [2]Sasabe et al. (2004); [3]Wang et al. (2016); [4]Wee et al. (2008); [5]Joussen et al. (2012); [6]Gao et al.(2010); [7]Pottier et al. (2012); [8]Chandra et al. (2016); [9]Celorio-Mancera et al. (2011); [10]Celorio-Mancera et al. (2012); [11]Li et al. (2004); [12]Rose et al. (1997); [13]Zhou et al. (2010).
Bm: *Bombyx mori*; Ha: *Helicoverpa armigera*; Hvir: *Heliothis virescens*; Hz: *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	*quadGS* 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 *H. armigera*, associated with resistance[1]
Hvir_C12658	2	HaCCE001c	79	2E-179	-0.34	4.51	28.80	Midgut esterase in *H. armigera*[1]
Hvir_C1953	2	HaCCE001d	91	3E-109	5.63	7.36	3.31	Midgut esterase in *H. armigera*[1]
Hvir_C2486	1	HaCCE001f	87	2E-61	7.19	8.21	2.03	Midgut esterase in *H. armigera*[1]
Hvir_C25103	2	HaCCE001i	85	6E-62	0.43	3.03	6.06	Midgut esterase in *H. armigera*, associated with resistance[1]
Hvir_C8604	1	HaCCE006a	69	0.0	1.35	2.57	2.33	Odorant degrading esterase in *H. armigera*, assoc. with res.[1]
Hvir_C19785	1	HaCCE033a	70	7E-32	-1.05	2.31	10.30	Odorant degrading esterase in *H. armigera*[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 *B. mori*[2]
Hvir_C25474	1	SlGSTe11	94	5E-158	-0.29	1.28	2.98	SlGSTe2/3 induced by insecticides in *S. litura*[3]
UDP-glycosyltransferases								
Hvir_C720	4	HaUGT40M1	93	2E-141	6.83	8.56	3.32	Homologues induced by gossypol in *H. armigera*[4]
Hvir_C2844	1	HaUGT33B5	94	5E-55	5.50	6.67	2.26	Homologues induced by gossypol in *H. armigera*[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 *T. urticae*[5]
Hvir_C6094	1	HsABCC2	100	1E-41	0.46	4.20	13.43	Correlated with *H. virescens* 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 *T. urticae*[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	

[1]Teese et al. (2008); [2]Yamamoto et al. (2011); [3]Deng et al. (2009); [4]Krempl et al. (2016); [5]Dermauw et al. (2013); [6]Gahan 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	*quadGS* RPKM (log_2)	Col-0 RPKM (log_2)	Fold change	Description
Nitrile-specifier proteins								
Pieris brassicae								
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								
Pieris brassicae								
Pbra_C2345	1	PbCAS1	100	0.0	5.89	7.24	2.54	CAS homologue[3]
Glutathione-S transferases								
Pieris brassicae								
Pbra_C1158	1	HaGSTD1	74	9E-97	6.59	7.92	2.52	GS detoxification in *Scaptomyza*[4]
Sulfatases								
Heliothis virescens								
Hvir_C14843	1	PxyCAC86342	69	5E-155	0.00	1.70	3.35	Glucosinolate sulfatase in *P. xylostella*[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

[1]Wittstock et al. (2004); [2]Wheat et al. (2007); [3]van Ohlen et al. (2016); [4]Gloss et al. (2014); [5]Ratzka et al. (2002)
Ha, *Helicoverpa armigera*; Pb, *Pieris brassicae*; Pxy, *Plutella xylostella*

806 **Figure legends**

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

812

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

816

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

821

822

Figure 1

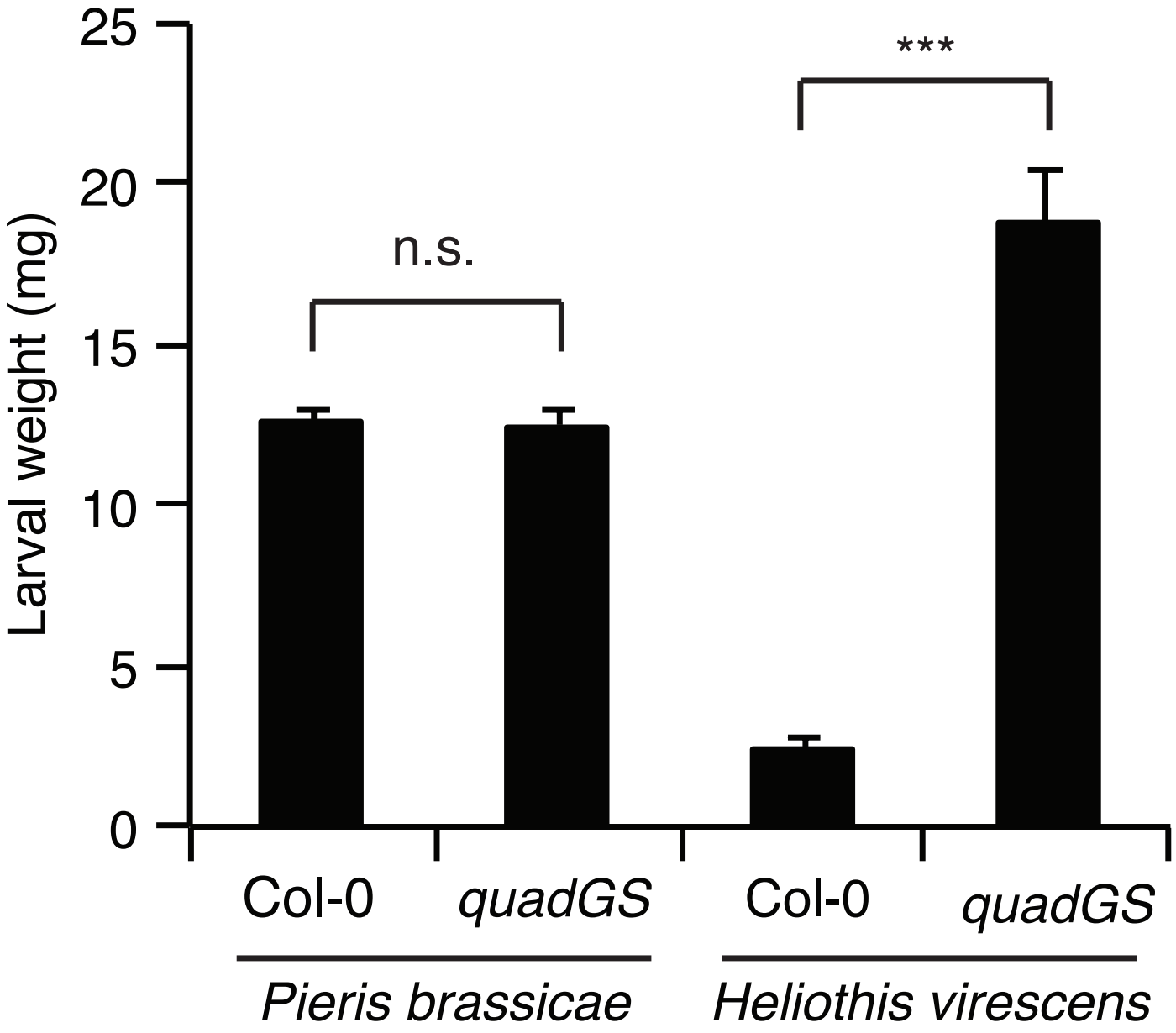
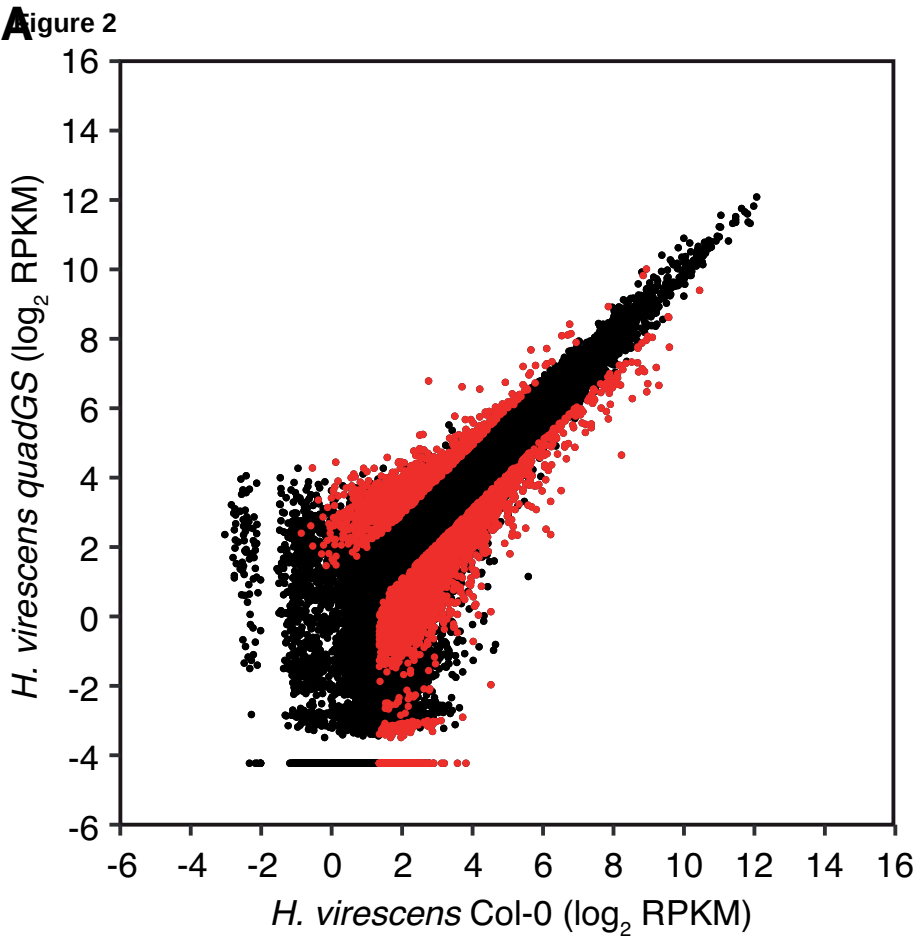


Fig. 1

Figure 2



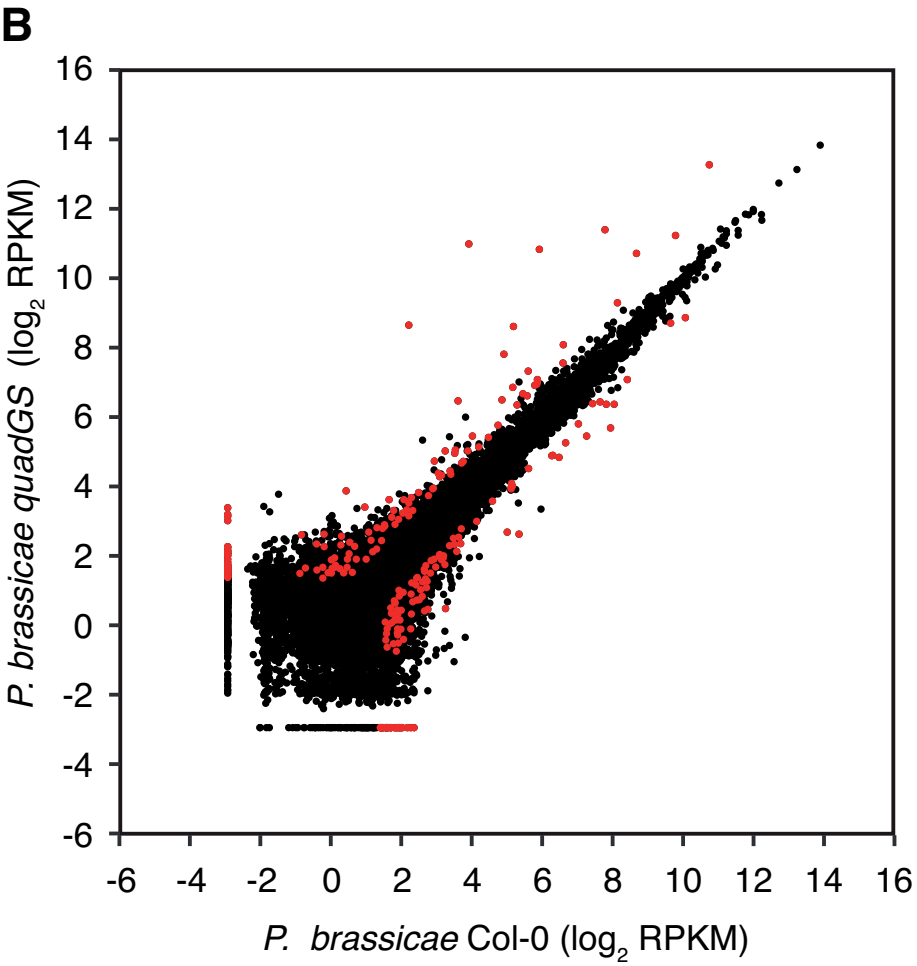


Fig. 2

Figure 3

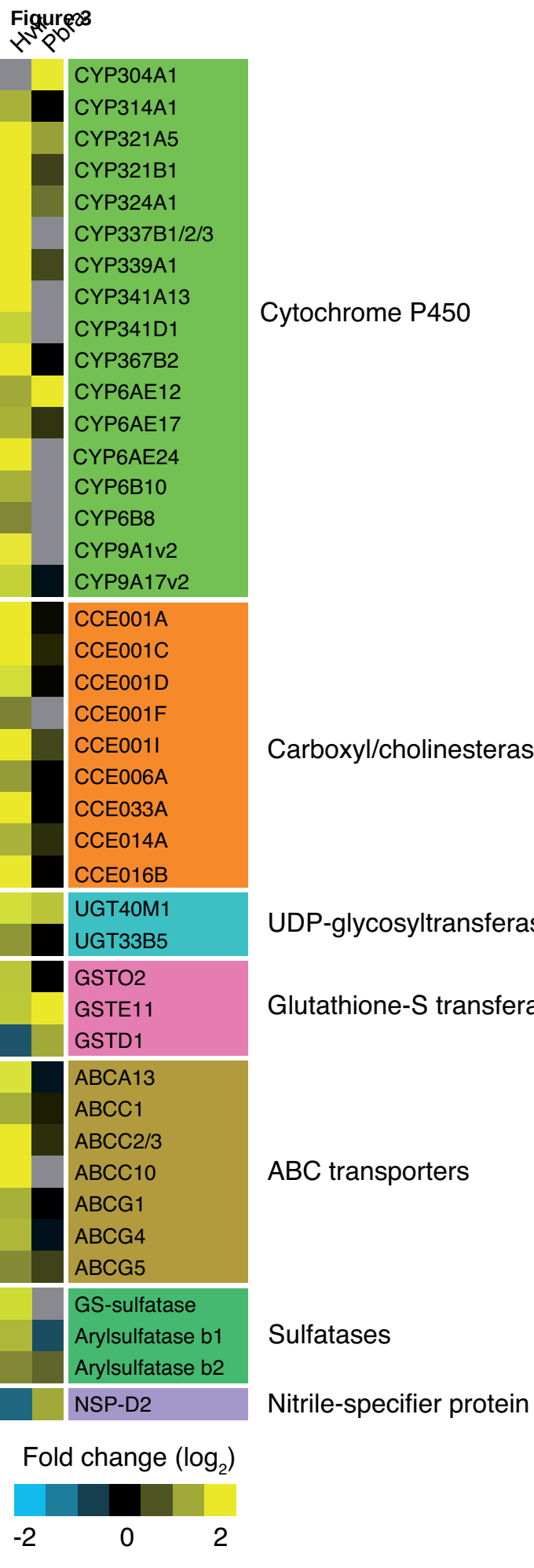


Fig. 3

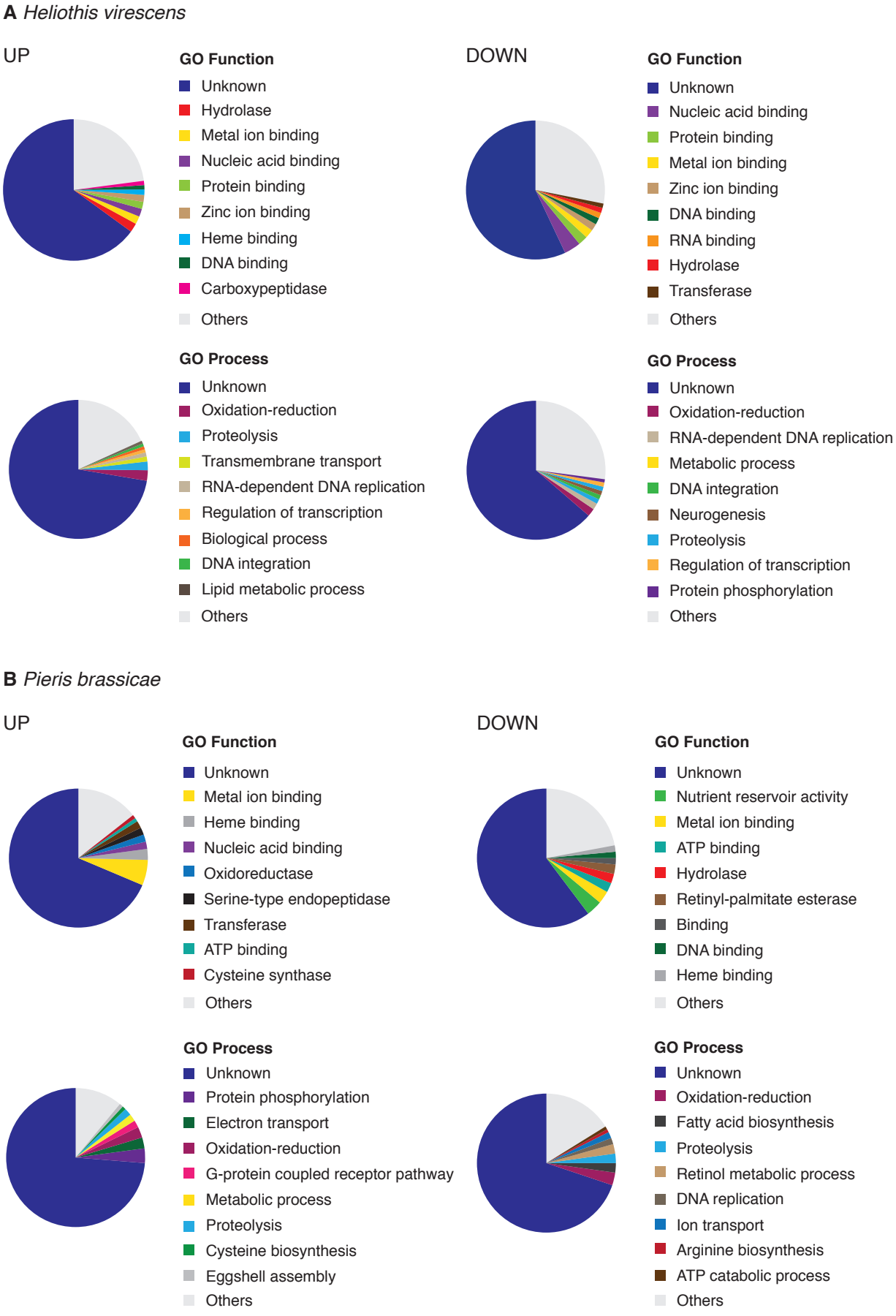


Fig. S1. Gene ontology (GO) analysis of contigs differentially regulated between insect larvae feeding for 48 h on wild-type Arabidopsis Col-0 and quadGS mutant plants. UP, contigs upregulated in larvae feeding on Col-0 plants; DOWN, contigs downregulated in larvae feeding on Col-0 plants.

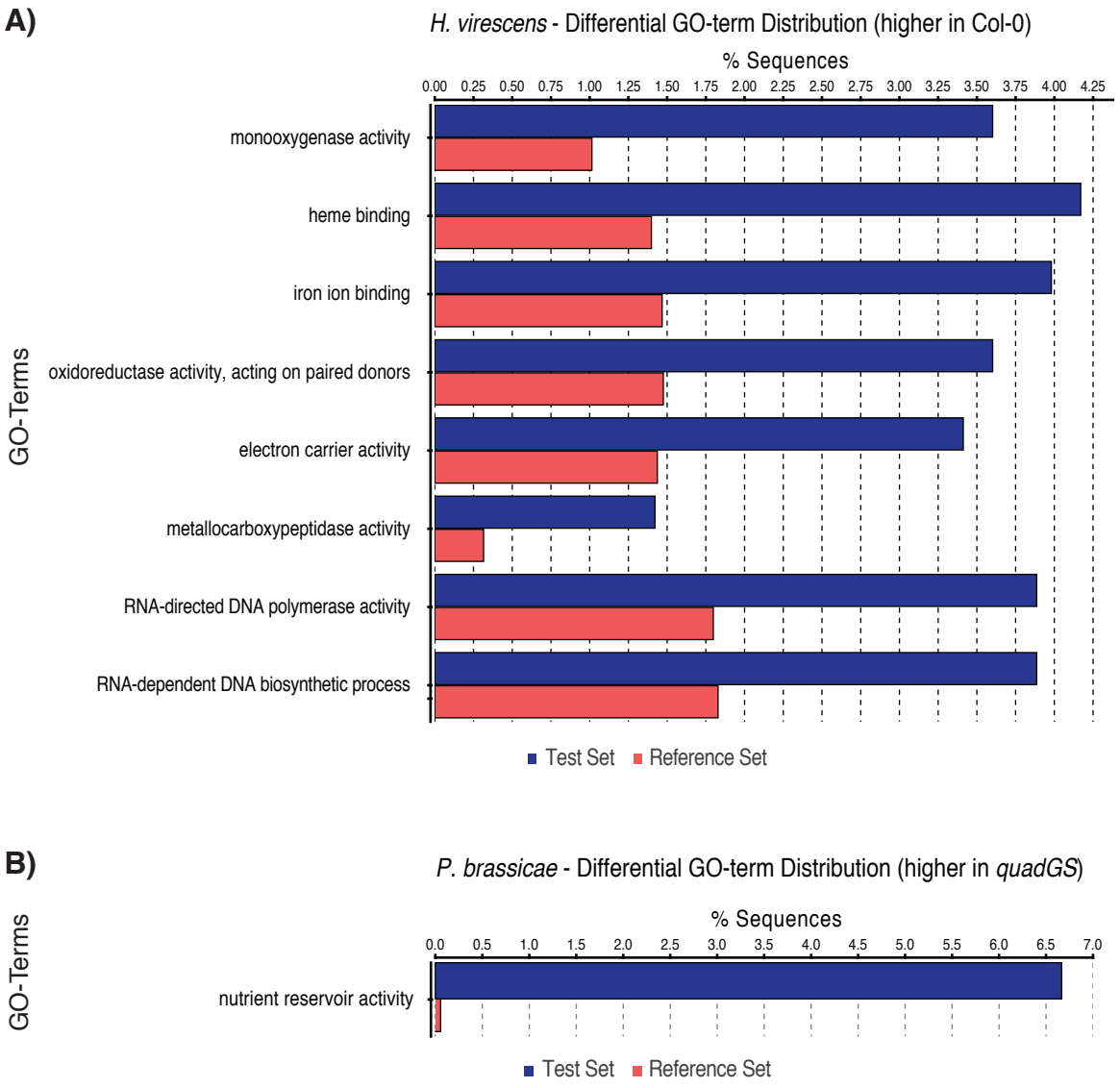


Fig. S2. Relative frequencies of GO term in differentially regulated genes. Differentially expressed contigs were compared to the complete dataset using Fisher's exact test (FDR-adjusted *P*-value <0.01). (A) GO terms over-represented in *H. virescens* larvae exposed to Col-0 (B) GO term over-represented in the *P. brassicae* larval samples exposed to quadGS plants.

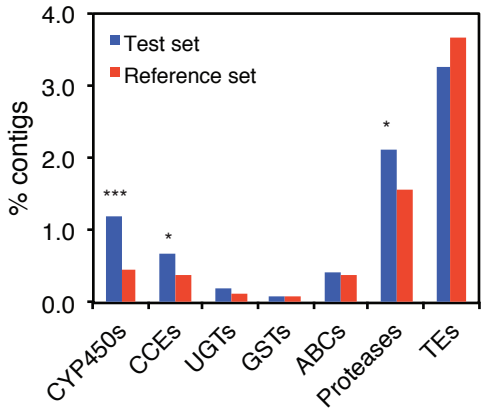


Fig. S3. Proportion of *H. virescens* contigs associated with detoxification, growth and development, digestion and transposition that are differentially regulated by GS exposure (blue bars, test set) compared to the total number of contigs (red bars, reference set). Processes significantly overrepresented in the list of differentially regulated contigs are indicated (Fisher's exact test, *p<0.05, ***p<0.001).

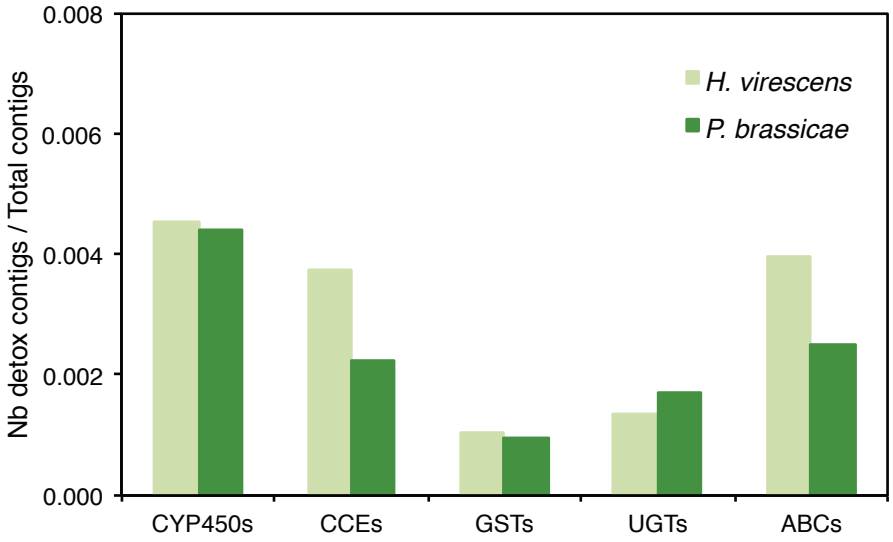


Fig. S4. Proportion of contigs associated with detoxification in *H. virescens* and *P. brassicae* transcriptomes.