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Specialist root herbivore modulates plant transcriptome and downregulates defensive secondary metabolites in a brassicaceous plant

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Summary

- Plants face attackers aboveground and belowground. Insect root herbivores can lead to severe crop losses, yet the underlying transcriptomic responses have rarely been studied.
- We studied the dynamics of the transcriptomic response of Brussels sprouts (*Brassica oleracea* var. *gemmifera*) primary roots to feeding damage by cabbage root fly larvae (*Delia radicum*), alone or in combination with aboveground herbivory by cabbage aphids (*Brevicoryne brassicae*) or diamondback moth caterpillars (*Plutella xylostella*). This was supplemented with analyses of phytohormones and the main classes of secondary metabolites; aromatic, indole and aliphatic glucosinolates.
- Root herbivory leads to major transcriptomic rearrangement that is modulated by aboveground feeding caterpillars, but not aphids, through priming soon after root feeding starts. The root herbivore downregulates aliphatic glucosinolates. Knocking out aliphatic glucosinolate biosynthesis with CRISPR-Cas9 results in enhanced performance of the specialist root herbivore, indicating that the herbivore downregulates an effective defence.
- This study advances our understanding of how plants cope with root herbivory and highlights several novel aspects of insect–plant interactions for future research. Further, our findings may help breeders develop a sustainable solution to a devastating root pest.

Introduction

Crop losses due to insect herbivory are a drain on food resources and finding sustainable solutions for crop protection is imperative to reach the United Nations (UNs) zero hunger goal by 2030. Therefore, insight in the molecular interactions between plants and insect herbivores is important, as plant breeders can exploit this when selecting future-proof crops.

Plants are engaged in an evolutionary arms race with insect herbivores that feed on their leaf and root tissues. When under attack, plants attempt to defend themselves by producing toxic secondary metabolites or anti-nutritional proteins such as proteinase inhibitors (Erb & Reymond, 2019). These defence responses are orchestrated by an intricate network of phytohormones. Jasmonic acid (JA) plays a central role in plant defence against insects, together with salicylic acid (SA), abscisic acid (ABA) and ethylene (ET) (Erb *et al.*, 2012; Pieterse *et al.*, 2012; Erb & Reymond, 2019). Plant defences can hamper herbivore growth and development, or lead to behavioural avoidance by the herbivores. However, insect herbivores might overcome plant defence by detoxifying plant toxins (Welte *et al.*, 2016), or by tricking plants into inducing a suboptimal defence response (Chung *et al.*, 2013). Most studies on plant defence focus on leaves, but roots are also threatened by insect herbivores and receive more attention in recent years (Johnson & Rasmann, 2015; Johnson *et al.*, 2016). Despite many similarities, defence in plant roots differs from that in leaves. For instance, profiles of glucosinolates (GSLs), the main group of secondary plant metabolites in brassicaceous plants, differ substantially between roots and shoots (Tsunoda *et al.*, 2017). In terms of defence signalling, JA plays a central role in the defence against root herbivores like in foliar tissues, but the functions of SA, ABA and ET are less clear.

In the past decade, sequencing technology has broadened our understanding of defence signalling in plants. This led to extensive studies on how *Arabidopsis thaliana* (Arabidopsis hereafter) plants respond to exogenous application of JA (Hickman *et al.*, 2017; Zander *et al.*, 2020), SA (Hickman *et al.*, 2019), or combinations of these hormones (Hickman *et al.*, 2019). Coolen *et al.* (2016) studied how the Arabidopsis leaf transcriptome changes after stress by drought, infection by the necrotrophic pathogen *Botrytis cinerea*, chewing insect herbivory by *Pieris rapae*, or combinations of these stresses. This study revealed that the last stress plants were exposed to dominated the

transcriptomic response, but also that earlier stresses left a legacy with consequences for the effectiveness of the defence response (Coolen *et al.*, 2016). Plant responses to insect root herbivores have rarely been the subject of transcriptome analyses (Barr *et al.*, 2010), presenting a sizeable knowledge gap.

Plants are seldom attacked by a single herbivore in natural settings but must cope with different insect herbivores throughout the growing season (Stam *et al.*, 2014). By activating plant defences, herbivores feeding on the same plant can affect each other. Such plant-mediated interactions can have long-lasting effects on the insect community associated with plants under field conditions (Poelman *et al.*, 2008). The identity and sequence of arrival of herbivores can be determining factors in the outcome of plant-mediated interactions (Erb *et al.*, 2011; Johnson *et al.*, 2012; Stam *et al.*, 2014). Additionally, plants appear to be more adapted to respond to commonly occurring combinations of insect herbivores (Mertens *et al.*, 2021). Plant-mediated interactions between insect herbivores can occur across plant compartments, even though the herbivores are not in direct contact (Johnson *et al.*, 2012; Biere & Goverse, 2016; Papadopoulou & van Dam, 2017). In most published studies, foliar herbivory by chewing herbivores negatively affects belowground chewers, and *vice versa* (Johnson *et al.*, 2012; Papadopoulou & van Dam, 2017). However, feeding by leaf chewers can induce susceptibility to root-feeding nematodes (Biere & Goverse, 2016). Such interactions suggest that induction of plant defence occurs not only locally, but also in distal systemic tissues.

Indeed, defence signalling can cross the root–shoot interface (Ankala *et al.*, 2013; Gulati *et al.*, 2014; Wang *et al.*, 2019). For instance, herbivory on tobacco leaves triggers a systemic signal in roots to produce nicotine, a secondary metabolite only produced in root tissue and transported to foliar tissues for defence (Gulati *et al.*, 2014). Experiments with mutant tomato plants revealed that intact JA biosynthesis is more important in shoots than roots when defending against root-knot nematodes. In this case, following infestation of the roots, an electrical signal moves up the stem to trigger JA biosynthesis in leaves, which then activates defence in roots (Wang *et al.*, 2019). Another well-studied example of plant defence signalling that crosses the root–shoot interface occurs when beneficial microbes in the rhizosphere trigger induced systemic resistance in leaves (Berendsen *et al.*, 2012). This is an example of defence priming, in which the plant is prepared for future attack, leading to a faster and/or stronger response upon attack (Conrath *et al.*, 2015; Hilker *et al.*, 2016). Moreover, insect eggs may induce defence priming against caterpillars that hatch from those eggs (Hilker & Fatouros, 2015; Valsamakis *et al.*, 2020). Defence priming is a potential mechanism underlying interactions between different herbivores feeding on the same plant (Hilker *et al.*, 2016).

Here, we investigate transcriptomics in primary roots exposed to herbivory, alone or in combination with aphids or caterpillars that were placed on the leaves 2 d earlier. The study system consists of Brussels sprouts (*Brassica oleracea* var. *gemmifera*) and three of its pest species, cabbage aphids (*Brevicoryne brassicae*), diamondback moth caterpillars (*Plutella xylostella*) and cabbage root fly larvae (*Delia radicum*). These three insect species are important pests of cabbage and often occur together. In this study system, we previously discovered that diamondback moth caterpillars negatively affect root-feeding cabbage root fly larvae, but cabbage aphids do not (Karssemeijer *et al.*, 2020). Based on the transcriptome analysis in the present study, two hypothesis-driven follow-up experiments were carried out. In the first, we tested whether aliphatic GSLs confer defence against *Delia radicum* using *myb28* knockout *Brassica oleracea* plants. In the second, we investigated whether *P. xylostella* primes a faster plant defence response against *Delia radicum*.

Materials and Methods

Study system

Brassica oleracea L. plants were used throughout the experiments, grown in a glasshouse compartment at 22 ± 2°C, 50–70% relative humidity (RH), with a 16 h : 8 h, light : dark (L : D) cycle.

Delia radicum L. (Diptera: Anthomyiidae) was reared in a climate cabinet at 20 ± 1°C, with 50–70% RH and a 16 h : 8 h, L : D cycle, larvae were reared on rutabaga (*Brassica napus* L. var. *napobrassica*) and adult flies were fed honey and a mixture of yeast, sugar and milk powder (1 : 1 : 1). *Brevicoryne brassicae* L. (Hemiptera: Aphididae) and *P. xylostella* L. (Lepidoptera: Plutellidae) were reared on Brussels sprouts (*Brassica oleracea* var. *gemmifera* cv. Cyrus) plants at 22 ± 2°C, 50–70% RH, with a 16 h: 8 h, L : D cycle.

Transcriptomics of the herbivore-induced primary root

Plant treatments Three-week-old Brussels sprouts plants were induced by placing either 10 *Brevicoryne brassicae* apterous adults or 10 *P. xylostella* L1–L2 larvae on a leaf. The induced leaf was always the same, i.e. the third leaf counted from the bottom. Two days later, half of the plants received 10 *Delia radicum* neonate larvae at the base of the plant's stem. This resulted in six treatments: Control (C), *Brevicoryne brassicae* (*Bb*), *P. xylostella* (*Px*), *Delia radicum* (*Dr*), *Brevicoryne brassicae* followed by *Delia radicum* (*Bb*+*Dr*), and *P. xylostella* followed by *Delia radicum* (*Px*+*Dr*). Plants were harvested just before adding *Delia radicum* larvae (0 h), and 3, 6, 9, 24, and 48 h after adding the *Delia radicum* larvae. Per treatment at each time point, six biological replicates were harvested, of which four were selected for sequencing and chemical analysis. When harvesting, three leaf disks were taken from the induced leaf, plants were uprooted, the secondary roots were cut off using scissors, and the primary roots were separated by cutting the stem at the position where the soil surface had been. Samples were immediately frozen in liquid nitrogen. All equipment was cleaned using RNaseZap (ThermoFisher Scientific, Waltham, MA, USA) between samples.

RNA-seq and read processing Total RNA was extracted using Maxwell 16 LEV Plant RNA kit (Promega, Madison, WI, USA), subjected to poly-A isolation, digestion, and complementary DNA (cDNA) synthesis, followed by end repair and ligation of a universal adapter. Sequencing was performed to an average depth

of 39 M reads, 150-bp paired end (Illumina HiSeq X; Genewiz, South Plainfield, NJ, USA). Quality of reads was assessed using FastQC (Andrews, 2010) and multiQC (Ewels *et al.*, 2016). Reads were processed with Trimmomatic (Bolger *et al.*, 2014) and aligned to the cabbage TO1000 genome (Parkin *et al.*, 2014), using Star (Dobin *et al.*, 2013). On average, 92.7% of the reads were aligned to the genome (Supporting Information Table S1). Raw sequencing data is available from the European Nucleotide Archive (https://www.ebi.ac.uk/ena, study accession no. PRJEB49273).

Differential gene expression Read counts were processed in R using the DESeq2 package (Love *et al.*, 2014). Genes with < 10 counts on average across all samples were omitted, resulting in a total of 30 908 genes. To calculate differentially expressed genes (DEGs), a model with a combined factor for the different time points and treatments was run. The model was releveled to the control treatment for each time point, and genes were classified as DEG if they were different from the control of the relevant time point with a false discovery rate (FDR) lower than 0.0001 and $\log_2$-fold change (LFC) higher than two using the apeGLM shrinkage estimator (Zhu *et al.*, 2018). A separate analysis was performed in DESeq2 to calculate DEGs in multiple herbivore treatments relative to *Delia radicum* alone; here, an FDR lower than 0.05 was used as threshold.

PCA analysis Principal component analysis (PCA) was performed with PCAexplorer (Marini & Binder, 2019). Variance-stabilized counts were used as input, and the top 10 000 most variant genes were included. Association between covariates and the first two principal component (PC) axes was assessed using Kruskal–Wallis tests. Genes in the top and bottom loadings of the first PC were extracted and functionally characterized. The closest *Arabidopsis* homologue was identified using Plaza v.4.5 (Van Bel *et al.*, 2017), and the function of these genes was manually assigned based on descriptions in the Tair (Berardini *et al.*, 2015) and UniProt (The UniProt Consortium, 2021) databases.

Clustering Normalized counts of the DEGs were clustered in R using the dynamictreecut function in WGCNA (Langfelder *et al.*, 2008; Langfelder & Horvath, 2008). Clusters were subjected to gene ontology (GO) enrichment analysis, relative to all 30 908 expressed genes, using Plaza v.4.5 (Van Bel *et al.*, 2017).

Analysis of defence pathways *Arabidopsis* genes involved in plant defence pathways were selected based on recent literature on JA (Wasternack & Feussner, 2018), SA (Rekhter *et al.*, 2019; Zhang & Li, 2019), ABA (Cutler *et al.*, 2010; Yoshida *et al.*, 2015; Hauser *et al.*, 2017), ET (Chang *et al.*, 2013; Pattyn *et al.*, 2021), and GSL biosynthesis (Gigolashvili *et al.*, 2009; Sønderby *et al.*, 2010; Pfalz *et al.*, 2016), catabolism (Barth & Jander, 2006; Sugiyama & Hirai, 2019), and transport (Jørgensen *et al.*, 2017). Many *Arabidopsis* genes have multiple homologs in cabbage due to a whole genome duplication event. Therefore, we used the PLAZA integrative orthology viewer to extract multiple homologs per gene based on four evidence types: synteny, Blast, orthologous gene family, and/or hierarchical trees (Van Bel *et al.*, 2017). Cabbage homologs with at least two evidence types were selected. In some cases, multiple *Arabidopsis* genes matched a cabbage homolog with equal evidence types; if the other *Arabidopsis* gene was also in the query, the cabbage gene was retained and the name adjusted (e.g. *LOX3/4*), if not, the gene was discarded. Finally, the *MAM* and *AOP* genes were selected based on earlier studies on *Brassica oleracea* (Liu *et al.*, 2014; Yi *et al.*, 2015; Abrahams *et al.*, 2020).

Effect of aliphatic glucosinolates on *Delia radicum*

To assess the effects of aliphatic GSLs on *Delia radicum* performance, we used a *myb28* cabbage mutant in which aliphatic GSL biosynthesis is strongly knocked down. In the *myb28* line, two copies of the *MYB28* gene (Bo2g161590 and Bo9g014610) were knocked out using CRISPR-Cas9 technology (Neequaye *et al.*, 2021). The genetic background of these plants is *Brassica oleracea* DH1012.

Seeds were sown in seedling soil and seedlings were transplanted after 8 d into regular potting soil. Starting 12 d after transplanting, plants were fertilized thrice weekly. One cotyledon was harvested from each plant 26 d after transplanting for genotyping (Methods S1). Five weeks after transplanting, 10 *Delia radicum* neonate larvae were placed on the primary root just below the soil surface of each induced plant. Control plants remained uninfested. To assess GSL contents during the larval feeding stage, a subset of plants was harvested 5 d post infestation (dpi). All other plants were harvested 18 dpi, after *Delia radicum* pupated in the soil. Primary root samples were collected as earlier, ground while frozen in liquid nitrogen, lyophilized (Martin Christ, Osterode am Harz, Germany), and subjected to chemical analysis. After harvesting, pots were covered in mesh nets to catch flies emerging from their pupae. Nets were checked daily for emergence, flies were caught, dried and weighed to the nearest 0.001 mg (Sartorius CP2P; Göttingen, Germany).

Priming of plant defence against *Delia radicum* by *P. xylostella*

Plant treatments Early transcriptional responses of *Brassica oleracea* plants to *Delia radicum* were studied to assess whether defence was primed by *P. xylostella*. In a first experiment, 3-wk-old Brussels sprouts plants were induced by 10 *P. xylostella* L1–L2 larvae on a leaf as described earlier. Two days later, half of the plants received 10 *Delia radicum* neonate larvae directly on the primary root, resulting in four treatments: control (C), *P. xylostella* (*Px*), *Delia radicum* (*Dr*) and *P. xylostella* followed by *Delia radicum* (*Px*+ *Dr*). After 15, 30, 60 and 120 min of *Delia radicum* feeding, primary roots were sampled as described earlier.

In a second experiment, we studied whether priming by *P. xylostella* would occur if there was a noninfested period between the two herbivores. Three-week-old Brussels sprouts plants were induced by 10 *P. xylostella* L1–L2 larvae on a leaf for 2 d, after which they were removed. After a 1- or 7-d noninfested period, 10 *Delia radicum*

neonate larvae were introduced directly on the primary root and samples were taken 30 and 60 min later. In this experiment, plants were divided over three treatments: control (C), *Delia radicum* (*Dr*) and *P. xylostella* followed by *Delia radicum* (*Px+ Dr*).

Gene expression analysis Primary root samples were ground while frozen in liquid nitrogen, followed by RNA extraction (Isolate II Plant RNA kit; GCBiotech, Waddinxveen, the Netherlands) and cDNA synthesis (SensiFAST; Meridian Bioscience, Cincinnati, OH, USA). Gene expression was quantified by quantitative polymerase chain reaction (qPCR) analysis (SensiFAST SYBR; Bioline; CFX96™ Real-Time System; Bio-Rad, Hercules, CA, USA). The optimal combination of reference genes was determined using GeNorm (Vandesompele *et al.*, 2002) in qbase+ (Biogazelle, Gent, Belgium), these were *Btub* and *SAR1a* for the first experiment and *PER4* and *SAR1a* for the second. We measured transcript levels of *AOS*, *MYC2*, *CYP81F4*, and *MYB28* (Table S2). Relative expression was calculated in qbase+.

Chemical analyses

We analysed root and leaf samples for phytohormone and GSL concentrations (Methods S2) following established methods (Brown *et al.*, 2003; Burow *et al.*, 2006; Vadassery *et al.*, 2012).

Statistical analysis

Statistical analyses were performed in R v.3.6.3 (R Core Development Team, 2017). For analysis of phytohormones and GSLs, a small fraction (1.23×10^{-7}) was added to circumvent measurements below the detection threshold. We used (generalized) linear models ((G)LM) for data analysis using the LME4 package, with a Gamma (log or inverse link) or Gaussian distribution. Based on Akaike information criterion (AIC), we selected the best model that included all fixed factors. *Delia radicum* emergence was analysed by a generalized linear mixed model (GLMM) with a binomial distribution and plant as a random factor to avoid pseudoreplication. Significance was assessed with likelihood ratio tests using the LMTEST package. *Post hoc* analysis by Tukey's honestly significant difference (HSD) pairwise comparisons were analysed using the EMMEANS package.

Results

Delia radicum feeding causes a major transcriptomic shift in the primary roots

Multivariate analysis revealed distinct patterns in the transcriptome of the primary root following root herbivory. The first PC clearly separates the root transcriptomes based on the presence or absence of *Delia radicum* (Fig. 1a). The second PC separates samples by the different time points at which the roots were sampled following root infestation (Fig. 1b). There is a distinction between infested root tissue sampled very early (3 h), early (6 and 9 h) and later (24 and 48 h). In roots that are not infested with *Delia radicum*, the transcriptome of the primary root was affected by *P. xylostella* infestation compared to uninfested control plants or plants with an aboveground infestation by *Brevicoryne brassicae*

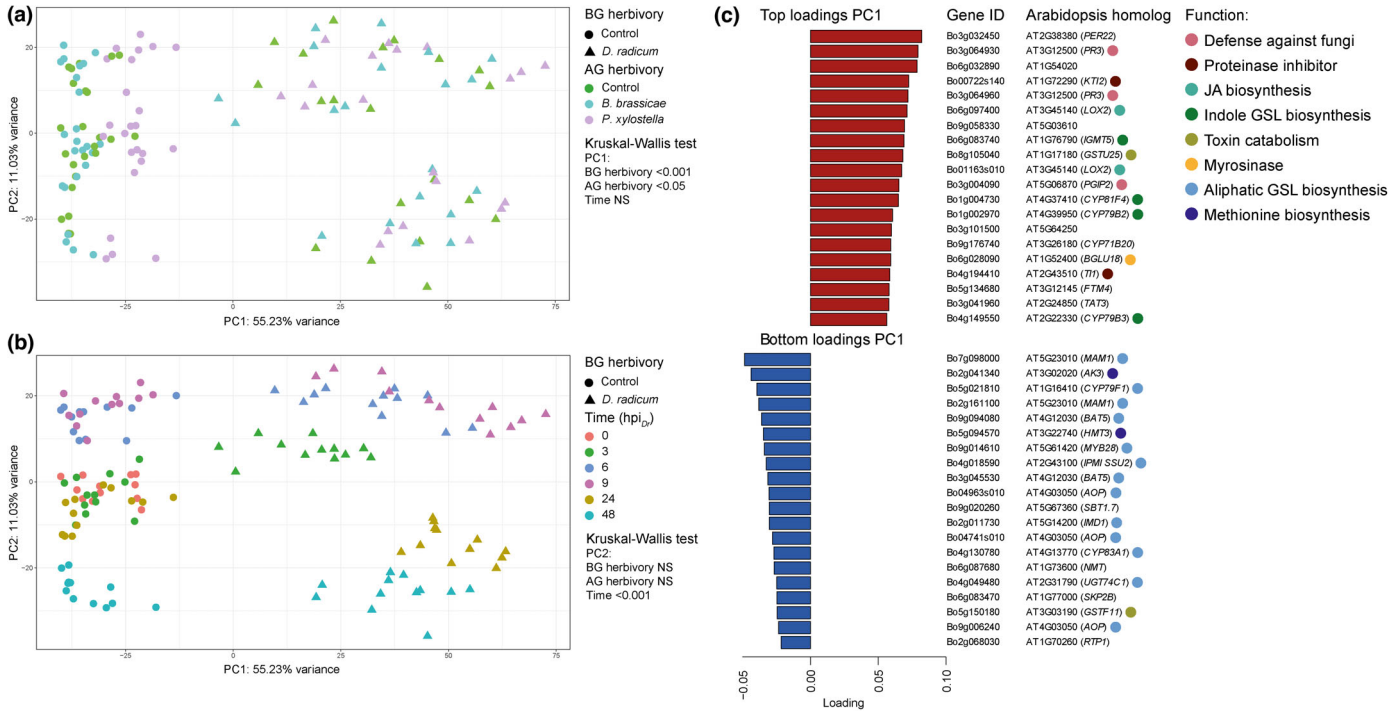

Fig. 1 Principal component analysis of *Brassica oleracea* transcriptomes of the primary root when subjected to aboveground (AG) herbivory by *Brevicoryne brassicae* or *Plutella xylostella* and belowground (BG) herbivory by *Delia radicum*. Aboveground herbivores were introduced 48 h prior to infestation by *Delia radicum*. Samples are coloured by herbivore treatments (a) or time points (b). Top and bottom loadings of the first principal component (PC1) and the function of their *Arabidopsis* homologs (c). hpi_{Dr}, hours post infestation by *Delia radicum*.

aphids. In addition, we ran a separate PCA for each time point (Fig. S1), showing that after 3 and 48 h of feeding by *Delia radicum*, plants pre-treated with *P. xylostella* exhibited a transcriptomic profile separate from the other samples with root-infestation by *Delia radicum*. This effect was not evident at other time points.

We functionally categorized genes that contributed most to the separation on the first PC (PC1) (Fig. 1c; Table S3). Top loadings of PC1 (i.e. genes associated with the positive values on PC1, corresponding with *Delia radicum*-infested roots) include genes involved in well-known defence processes, such as JA biosynthesis, proteinase inhibitors, indole GSL biosynthesis, GSL catabolism, and a peroxidase gene (*PER22*) which strongly responded to root infestation. Bottom loadings of PC1 (i.e. genes associated with negative values on PC1, corresponding with uninfested roots) consist almost exclusively of genes involved in the biosynthesis of aliphatic GSLs and their amino-acid precursor methionine.

Transcriptome of the primary root in response to *Delia radicum*

In total, 8469 genes were differentially expressed between control and any of the treatments over the course of the experiment, 4702 were upregulated and 3868 were downregulated (Fig. 2a); this corresponds to roughly 14% of the genome. Most of these genes responded to infestation by the root herbivore *Delia radicum*. As soon as 3 h after infestation by the root herbivore, over a thousand genes were upregulated in the roots. Feeding by shoot herbivores alone did not lead to many DEGs in the roots; their

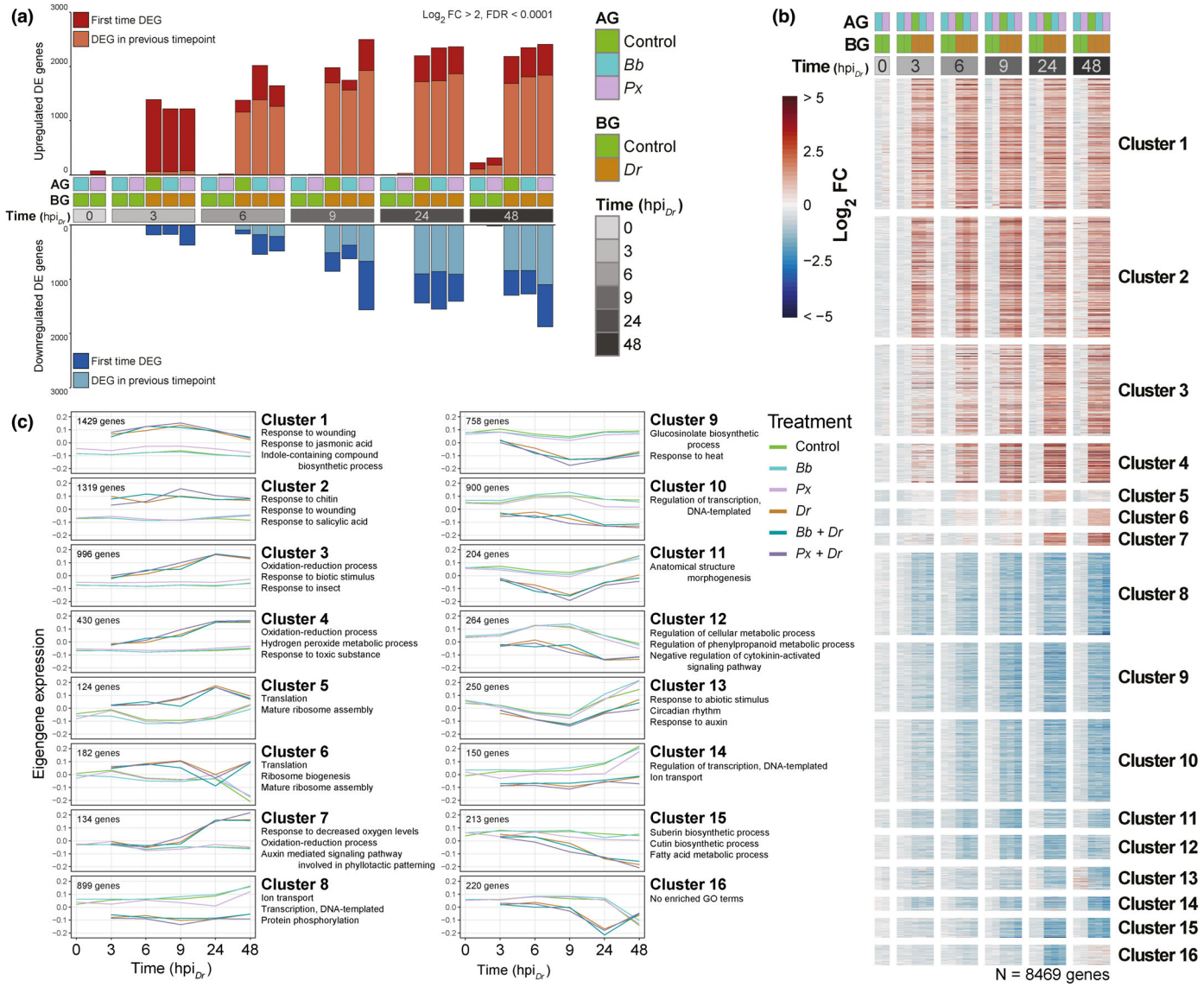

Fig. 2 (a) Differentially expressed genes (DEGs) in *Brassica oleracea* primary roots in response to aboveground (AG) herbivory by *Brevicoryne brassicae* (*Bb*) or *Plutella xylostella* (*Px*) and belowground (BG) herbivory by *Delia radicum* (*Dr*). Herbivores feeding AG arrived 2 d prior to infestation by *Delia radicum*. (b) Cluster analysis of DEGs. (c) Eigengene expression and enriched gene ontology (GO) terms of each cluster. FC, fold change relative to control for each time point; FDR, false discovery rate; hpi_{Dr}, hours post infestation by *D. radicum*.

largest effect was seen at the latest time point, 96 h after shoot herbivory had started. At several time points, more genes were differentially expressed when plants were facing multiple herbivory: for instance, after 3, 6, 9 and 48 h, more genes were downregulated in plants infested with both *P. xylostella* and *Delia radicum* compared to plants only infested by *Delia radicum*. To further assess the effects of shoot herbivores on the root transcriptomic response to *Delia radicum*, we analysed DEGs of dual-infested plants relative to plants only infested with *Delia radicum* (Fig. S2). This analysis revealed that *P. xylostella* mainly caused changes at the first and last time points. Infestation by *Brevicoryne brassicae* had little effect compared to infestation with *Delia radicum* only.

A cluster analysis to gain more insight in the functions of the DEGs resulted in seven clusters of upregulated genes and nine clusters of downregulated genes in response to *Delia radicum* feeding (Fig. 2b). For each cluster, we performed GO enrichment analysis (Fig. 2c; Table S4). Clusters 1 and 2 include genes that are upregulated rapidly upon infestation by *Delia radicum*, and are involved in responses to wounding, chitin, JA, SA, and in the biosynthesis of indole GSLs. Clusters 3, 4 and 7 encompass genes that respond to root herbivory at later time points, and include genes involved in oxidation–reduction processes, which may be involved in reactive oxygen species production and detoxification. Further, we found that processes involved in the production of proteins, i.e. translation, ribosome biogenesis, were upregulated by *Delia radicum*, peaking at 9 (cluster 4) and 24 h (cluster 5) after infestation. In clusters of downregulated genes, we found genes involved in GSL biosynthesis (cluster 9), upon closer inspection these are genes involved in aliphatic GSL biosynthesis. Other processes downregulated upon infestation by *Delia radicum* include ion transport and protein phosphorylation (cluster 8), regulation of circadian rhythm and responses to auxin (cluster 13), and biosynthesis of cutin and suberin (cluster 15). No clusters specifically correspond with changes in the roots induced by *Brevicoryne brassicae* or *P. xylostella* feeding on aboveground tissues.

Jasmonic acid and ethylene are involved in the plant response to *Delia radicum*

We studied the expression of genes involved in biosynthesis and signalling of defence-related phytohormones (Fig. 3). JA signalling plays a central role in plant response to *Delia radicum*, as biosynthesis, regulation and signalling in this pathway are rapidly upregulated upon infestation (Fig. 3a). This upregulation of genes is reflected in jasmonate concentrations (Fig. 3b; Table S5). Inactivated jasmonates, such as hydroxy-JA, were found in higher concentrations in roots of plants exposed to *P. xylostella* feeding on the leaves compared to control plants (Fig. S3; Table S5). Genes involved in biosynthesis of ethylene were induced upon *Delia radicum* infestation, especially *ACS2*, *ACO2* and *ACO4* were upregulated strongly and in early stages of the defence response (Fig. 3c). Conversely, *ACS6* (Bo9g091320), was downregulated by *Delia radicum* after 24 and 48 h. Further, expression of transcription factors *ERF1* and *ERF2* and ethylene response gene *PR3* were strongly upregulated by *Delia radicum* (Fig. 3c). There seems to be no clear role of ABA in the defence response against *Delia radicum*. Some genes, such as *NCED9* and *RAB18*, are upregulated, whereas others, such as *ABA1*, *ABF3* and *RD29B*, are slightly downregulated (Fig. 3d). Furthermore, none of the treatments affected ABA hormone levels in primary roots (Fig. S3; Table S5). Infestation by *Delia radicum* did not have a uniform effect on SA biosynthesis and signalling, *ICS* genes are downregulated while other biosynthesis genes are upregulated (Fig. 3e). Concentrations of SA were not affected by infestation (Fig. S3; Table S5). In conclusion, jasmonates, together with ethylene, appear to be involved in the plant response against *Delia radicum*.

Infestation by *Delia radicum* leads to contrasting responses in glucosinolate biosynthesis

PCA and cluster analyses revealed that the plant response to feeding by *Delia radicum* involves regulation of GSL biosynthesis. We analysed expression of genes in the indole and aliphatic GSL pathways and concentrations of these secondary metabolites (Fig. 4). Ten GSLs were detected, four indole GSLs, five aliphatic GSLs and the aromatic GSL gluconasturtiin (Fig. S4).

Indole GSL biosynthesis was rapidly upregulated upon *Delia radicum* infestation (Fig. 4a). Four out of six transcription factors (Bo7g098110, Bo9g014380, Bo8g067910 and Bo6g118350) involved transcriptional regulation of indole GSL biosynthesis genes were already upregulated 3 h after infestation. In the core GSL biosynthesis pathway, genes encoding enzymes specific for indole GSL are upregulated by *Delia radicum*. Several genes are involved in biosynthesis of the core structure for both aliphatic and indole GSL. Of these genes, the *Brassica oleracea* homolog of *GGP1* is upregulated by *Delia radicum* whereas *SUR1* and *UGT74B1* are downregulated. In the indole GSL secondary modification steps, *CYP81F4* and *IGMT5* are most strongly upregulated by *Delia radicum*, and indeed, the GSLs glucobrassicin and neoglucobrassicin are especially abundant in response to *Delia radicum* feeding (Fig. 4d). Notably, in plants experiencing dual herbivory by *P. xylostella* and *Delia radicum*, these two compounds are produced faster compared to plants only exposed to *Delia radicum* (Fig. 4d; Table S6). Several genes in the indole GSL pathway (e.g. *MYB34*, *CYP79B2|3*, *CYP81F4*) are also upregulated in roots of *P. xylostella* induced plants (Fig. 4a).

Conversely, aliphatic GSL biosynthesis was downregulated in plants induced by *Delia radicum* (Fig. 4c). The three cabbage homologs of *MYB28*, in particular, were rapidly downregulated in response to *Delia radicum*. Interestingly, *MYB29* (Bo9g15680) was upregulated 3 h after *Delia radicum* infestation but downregulated at later time points. Genes encoding proteins involved in chain elongation and core aliphatic GSL biosynthesis, such as *BAT5* and *CYP79F2*, were downregulated in plants infested by both *P. xylostella* on the leaves and *Delia radicum* on the roots after 3 h, whereas this downregulation is seen after 6 h in plants only infested with *Delia radicum*. Concentrations of aliphatic GSLs in primary roots showed a reduction of glucoiberverin from 24 h and gluconapin at 48 h after *Delia radicum*

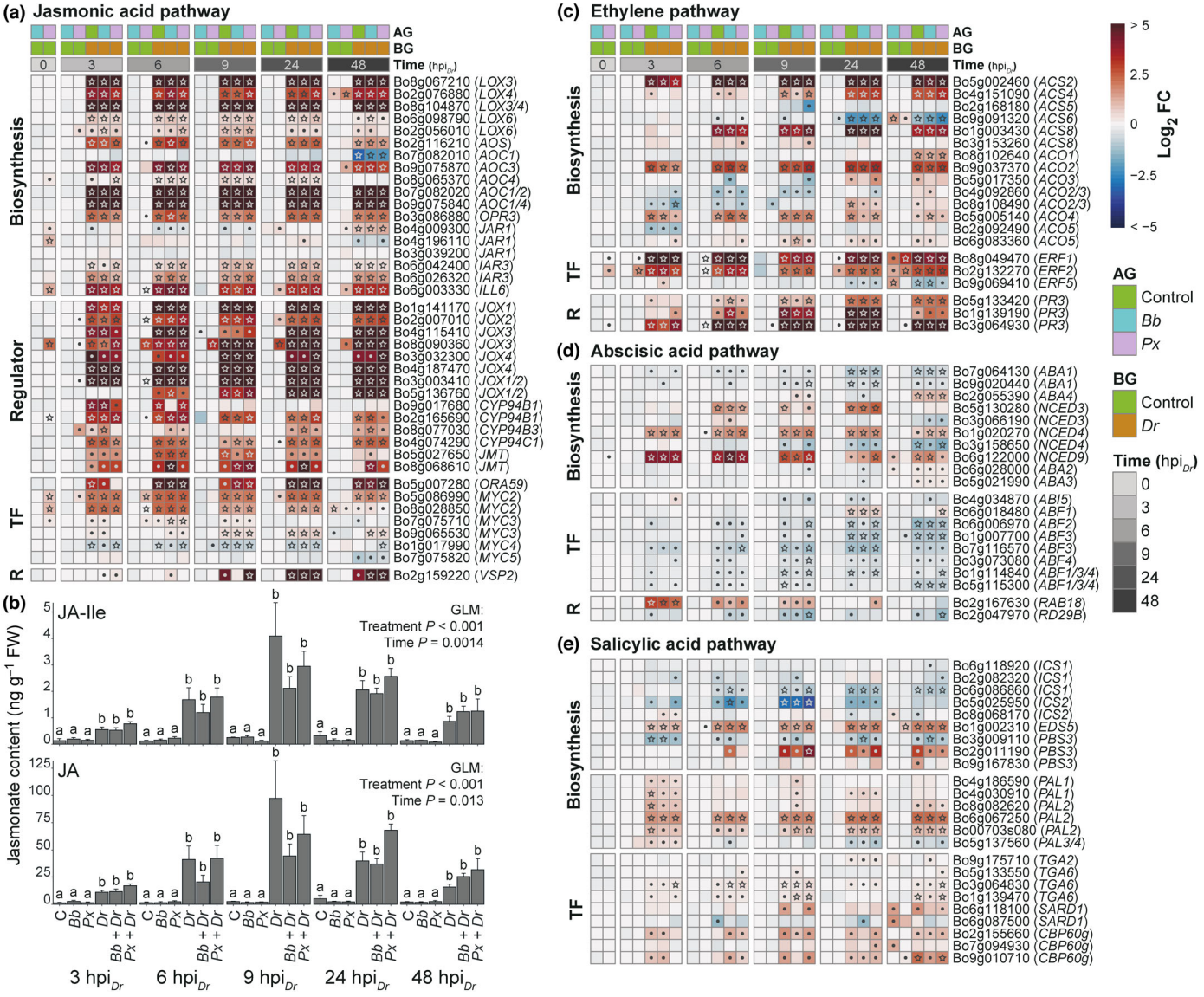

Fig. 3 Phytohormonal response of *Brassica oleracea* primary roots to aboveground (AG) herbivory by *Brevicoryne brassicae* (*Bb*) or *Plutella xylostella* (*Px*) and belowground (BG) herbivory by *Delia radicum* (*Dr*). Herbivores feeding AG were introduced 2 d prior to root infestation by *Delia radicum*. Fold changes of genes involved in the jasmonic acid (JA) pathway relative to control (a), concentrations of JA-Ile and JA; error bars represent standard error of the mean ($n = 3$–6) (b), fold changes of genes involved in ethylene (c), abscisic acid (d) and salicylic acid (e) signalling relative to control. Selection of genes and names of homologs are based on *Arabidopsis*. Genes differentially expressed in the uninfested control of that time point are indicated by stars (false discovery rate (FDR) < 0.0001) and dots (FDR < 0.05). In (b), different letters indicate statistical differences between treatments for each time point (Tukey's HSD, $P < 0.05$). C, control; FC, fold change relative to control for each time point; hpi$_{Dr}$, hours post infestation by *D. radicum*; TF, transcription factor. For gene expression, $n = 4$ for each treatment × timepoint combination. GLM, generalized linear model.

infestation (Fig. 4e). In plants dually infested with *B. brassicae* plus *Delia radicum*, glucoerucin was also reduced 48 h after *Delia radicum* started feeding, compared to control plants (Fig. 4e).

We assessed expression of genes involved in GSL catabolism and transport. Expression of genes encoding several myrosinases, enzymes that hydrolyse GSL into toxic products, was affected by *Delia radicum* infestation (Fig. S4). While transcription of genes encoding classic myrosinase homologs (*TGG1-6* in *Arabidopsis*) was not strongly affected by *Delia radicum* infestation, genes encoding several atypical myrosinases (*BGLU18-33* in *Arabidopsis*) were upregulated. Especially, expression of *Brassica* homologs of *BGLU18*, *PYK10*, *BGLU25* and *BGLU31/32* were strongly increased upon *Delia radicum* herbivory. Moreover, we assessed the expression of five *Brassica oleracea GTR* homologs (Fig. S4), which encode proteins responsible for GSL transport throughout the plant (Andersen *et al.*, 2013). Differences in expression upon *Delia radicum* infestation occurred mainly for two of these genes: a *GTR1/2* homolog (*Bo3137030*) was slightly upregulated, and a *GTR2/3* homolog (*Bo5025960*) was downregulated. The functions of these genes have not been studied in *Brassica oleracea*; thus, assumptions on how changes in *GTR* expression affect GSL transport are premature. Nevertheless, concentrations of GSLs in roots and leaves did not show clear evidence for GSL transport (Fig. S4). For instance, the reduction of aliphatic GSLs in *Delia*

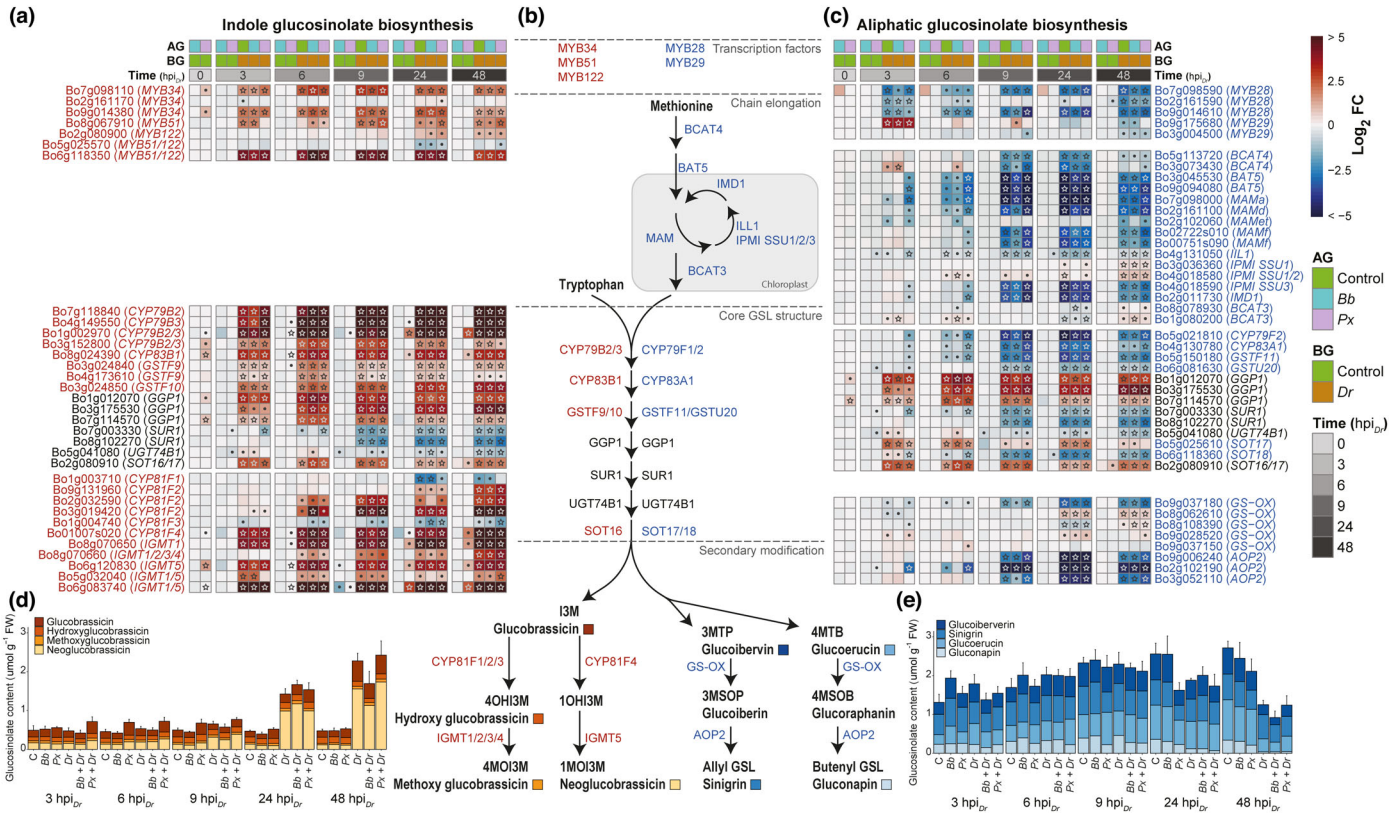

Fig. 4 Fold changes relative to control for genes involved in indole (a) and aliphatic (c) glucosinolate (GSL) biosynthetic pathways in *Brassica oleracea* primary roots in response to aboveground (AG) herbivory by *Brevicoryne brassicae* (*Bb*) or *Plutella xylostella* (*Px*) and belowground (BG) herbivory by *Delia radicum* (*Dr*). Herbivores feeding aboveground were introduced on plants 2 d prior to root infestation by *Delia radicum*. Overview of GSL biosynthesis pathways derived from the amino acids tryptophan (indole) and methionine (aliphatic) (b). Selection of genes and names of homologs are based on *Arabidopsis*. Concentrations of indole (d) and aliphatic (e) GSLs, means are plotted per compound and error bars represent the standard error of the cumulative mean ($n = 3$–6). Genes presented in red are involved in indole GSL biosynthesis, those presented in blue are involved in aliphatic GSL biosynthesis, and genes presented in black are shared between the two pathways. Gene expression levels different from the uninfested control of that time point are indicated by stars (false discovery rate (FDR) < 0.0001) and dots (FDR < 0.05). For analyses of GSL concentrations, statistical information can be found in Supporting Information Table S6. hpi_{Dr}, hours post infestation by *Delia radicum*; FC, fold change relative to control for each time point; C, control; FW, fresh weight. For gene expression, $n = 4$ for each treatment × timepoint combination.

radicum infested roots did not lead to higher aliphatic GSL concentrations in leaves. Likewise, the increase in indole GSLs in roots did not coincide with a reduction of leaf indole GSL concentrations (Fig. S4).

Aliphatic glucosinolates provide defence against *Delia radicum*

We hypothesized that downregulation of aliphatic GSL biosynthesis (Fig. 4) would reduce plant defence and favour *Delia radicum* performance. To address this hypothesis, we studied *Delia radicum* performance using a *myb28* knockout cabbage line (Neequaye *et al.*, 2021). Successful development of *Delia radicum*, quantified as adult fly emergence, increased from 60% on wild-type plants to 82% on *myb28* mutants (Fig. 5a), while adult fly weight was not affected (Fig. 5b). GSL content in primary roots of these plants was measured at 5 (during larval feeding) and 18 (after pupation) dpi, and revealed that indeed, aliphatic GSL production is effectively knocked down in *myb28* plants (Fig. 5c; Table S7). Further, in accordance with our prior results (Fig. 4), in both wild-type and *myb28* plants, indole GSLs are present in higher concentrations in plants induced by *Delia radicum* (Fig. 5c; Table S7).

Plutella xylostella primes the defence response against *Delia radicum*

Transcriptome analysis revealed that *P. xylostella* induces changes in the early (3 h post infestation) plant response to *Delia radicum* (Figs S1a, S2). We therefore hypothesized that the plant response to *Delia radicum* may be primed by *P. xylostella*, leading to a faster or stronger response. To study this, we sampled primary roots at very early time points after induction by *Delia radicum* on plants with or without prior leaf feeding by *P. xylostella* and quantified transcripts of genes involved in JA, indole GSLs, and aliphatic GSLs (Fig. 6a).

Expression levels of *AOS*, *MYC2*, and *CYP81F4* were higher 15 min after the start of *Delia radicum* feeding when there had been a prior infestation by *P. xylostella* compared to the other treatments, whereas plants exposed to *Delia radicum* alone

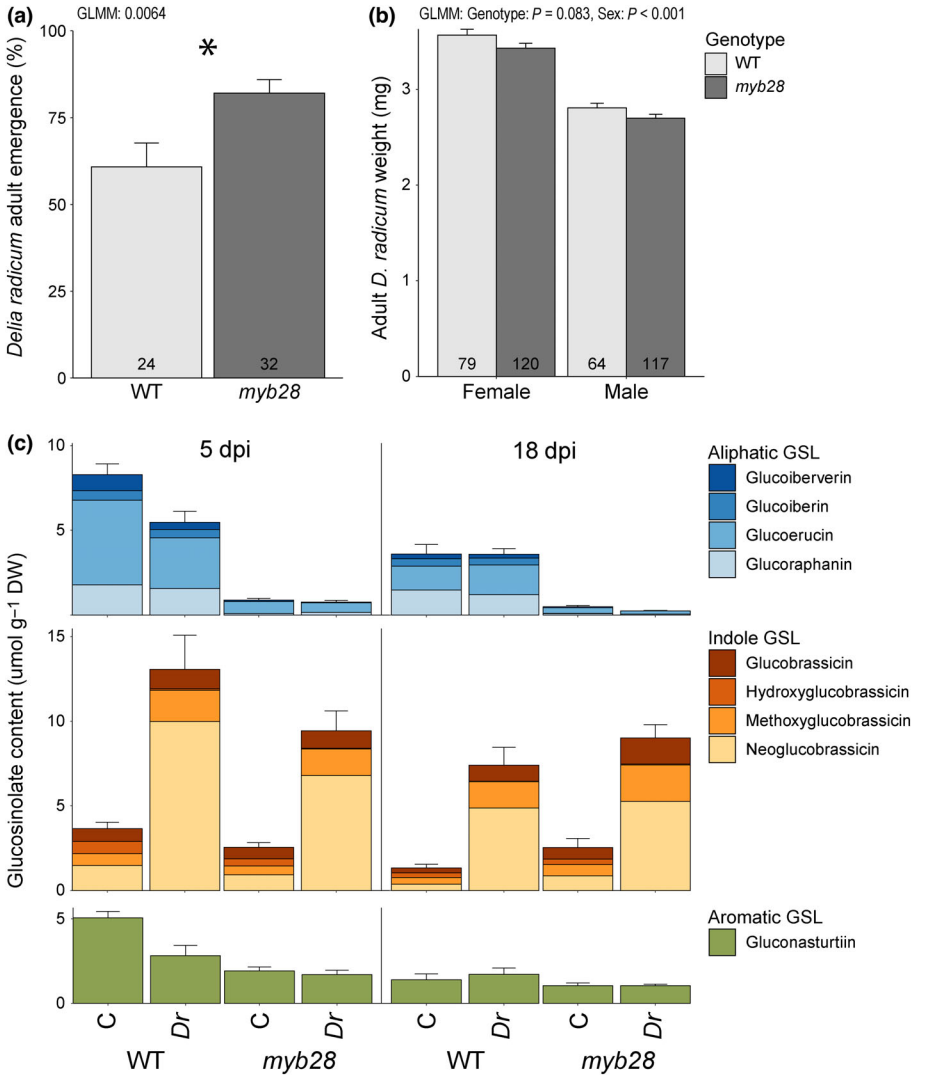

Fig. 5 Emergence (a) and adult weight (b) of *Delia radicum* feeding on wild-type (WT) or *myb28* knockout *Brassica oleracea* plants. Numbers at the bottom of bars represent the number of plants or flies. (c) Glucosinolate (GSL) concentrations in primary roots at 5- and 18-d-post-infestation by *Delia radicum* (dpi). Means are plotted per compound and error bars represent the standard error of the cumulative mean; $n = 10$ plants for all groups, except for *D. radicum*-infested plants at 18 dpi, in which case all plants used for *D. radicum* survival assay were measured ($n = 24$–32). The asterisk indicates a statistically significant difference in root fly emergence (GLMM: $P < 0.05$). For analyses of GSL concentrations, statistical information can be found in Supporting Information Table S7. GLMM, generalized linear mixed model; DW, dry weight; C, control; *Dr*, *Delia radicum*.

responded after 30 or 60 min (Fig. 6b–d; Table S8). *MYB28* expression was downregulated after 60 min of *Delia radicum* feeding in plants induced with the root herbivore alone or in combination with *P. xylostella*, but the downregulation was stronger in dual-infested plants (Fig. 6e). After 2 h, expression levels of all four genes were the same in plants induced by *Delia radicum* alone or in combination with *P. xylostella* (Fig. 6b–e).

We then studied whether priming of defence against *Delia radicum* by *P. xylostella* would be retained over time. To this end, we introduced a noninfested period of 24 h or 7 d between removal of *P. xylostella* and exposure to *Delia radicum*. When such a noninfested period was introduced, a faster response in terms of gene expression was no longer observed in terms of expression of *AOS, MYC2* and *MYB28* (Fig. 6f–k,m; Table S8). Expression of *CYP81F4* was higher in plants that had been exposed to *P. xylostella* prior to *Delia radicum* with a noninfested period of 7 d, but not with a noninfested period of 24 h (Fig. 6h, l). Thus, prior infestation by *P. xylostella* leads to a faster response to *Delia radicum*, but the effect of *P. xylostella* infestation diminishes after 24 or more hours since their feeding had stopped.

Discussion

Plant transcriptomic responses to root herbivory are rarely studied. Our in-depth transcriptomic analyses of plant responses to insect root herbivores show that plants invest heavily in responding to root herbivory, as demonstrated by major transcriptome reconfiguration in primary root tissue. Many of these genes are involved in processes commonly observed in response to herbivore attack, such as responses to wounding and JA. We found contrasting regulation of indole and aliphatic GSL in the response to *Delia radicum*, and we provide evidence indicating that aliphatic GSL are toxic to this specialist herbivore. Moreover, untargeted analysis of the primary root transcriptome revealed several processes that may be important in plant defence against *Delia radicum*. Prior herbivory by *B. brassicae* aphids or *P. xylostella* caterpillars did not lead to a large shift in the plant response to *Delia radicum*. However, prior caterpillar attack on the leaves primes for an earlier defence response in the roots.

The phytohormones JA and ET are involved in the plant defence response against *Delia radicum*. In leaves, JA and ET

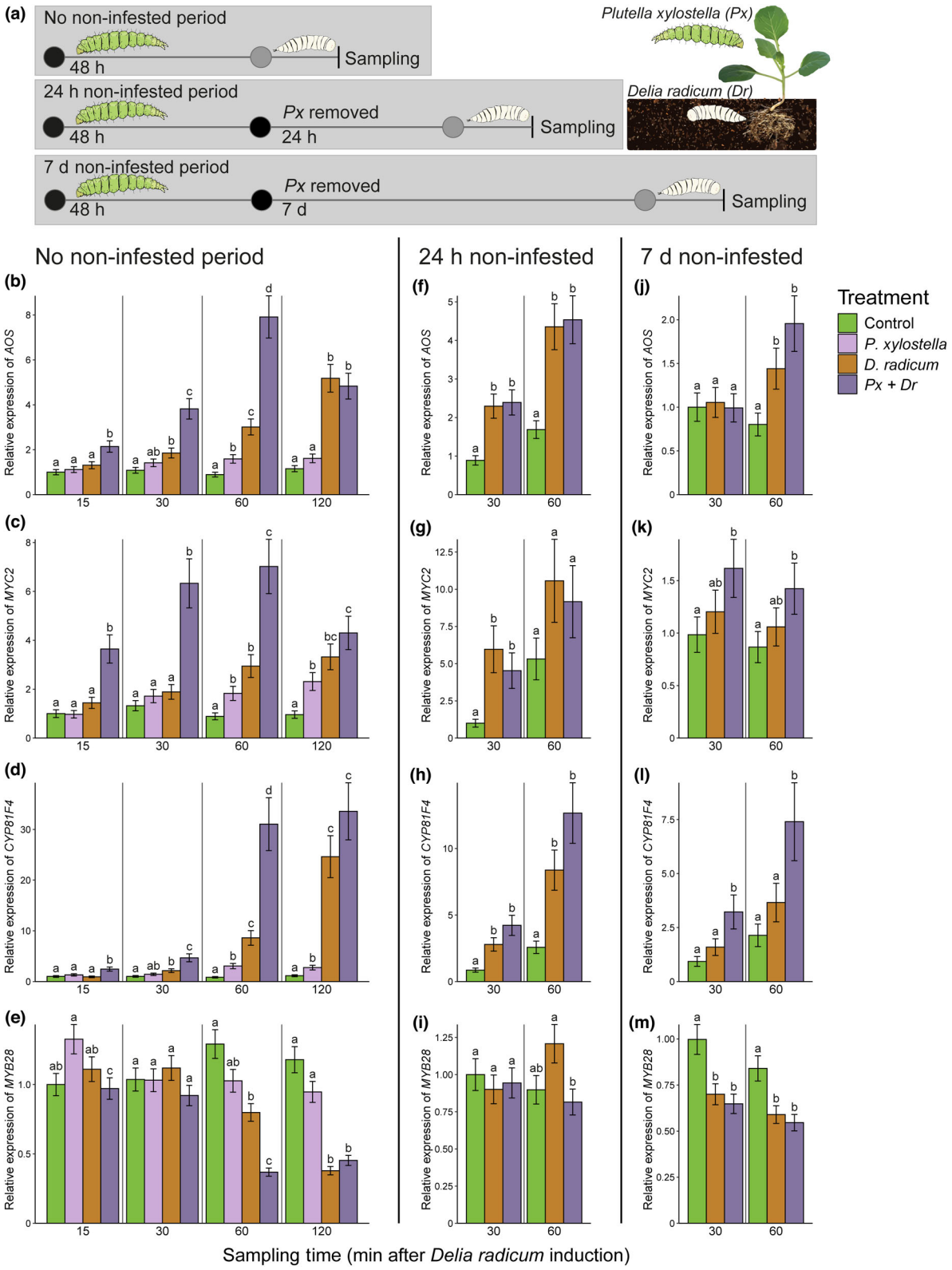

Fig. 6 (a) Experimental setup to study the effects of *Plutella xylostella* (*Px*) leaf herbivory on the gene expression response of *Brassica oleracea* primary roots to the root herbivore *Delia radicum* (*Dr*). *Plutella xylostella* fed for 2 d prior to *Delia radicum* arrival directly afterwards or with a noninfested period of 24 h or 7 d. Relative expression of *AOS* (b, f, j), *MYC2* (c, g, k), *CYP81F4* (d, h, l), and *MYB28* (e, i, m). Statistical information of main effects can be found in Supporting Information Table S8. Different letters indicate differences (Tukey's HSD: $P < 0.05$) in pairwise comparisons between treatments within sampling times. Error bars represent the standard error of the mean. *n* = four biological replicates consisting of three plants.

coordinate responses to necrotrophic pathogens, whereas JA and ABA regulate responses to chewing herbivores (Pieterse *et al.*, 2012). We previously reported that *Delia radicum* downregulated ET biosynthesis in primary roots based on expression of *ACS6* (Karssemeijer *et al.*, 2020). While our current data support that *ACS6* is downregulated, other *ACS* genes and ET response genes are strongly induced upon *Delia radicum* infestation, underlining that conclusions based on marker gene expression should be made with care. Biosynthesis and signalling in the ABA pathway were not upregulated by root herbivory. Thus, while JA regulates responses to insect herbivores in both leaves and roots, finetuning of the response by ET and ABA appears to differ between the two compartments. Several explanations may be given for the activation of JA and ET rather than ABA in response to root herbivory. First, each of these three hormones have ancillary functions in root tissue, for instance in regulation of root development (Saini *et al.*, 2013; Johnson *et al.*, 2016). This may cause differences in defence signalling between shoots and roots, as disrupting hormone homeostasis could affect normal root growth. Second, responses to pathogens may be beneficial when responding to root herbivores, as their feeding sites can be used for infection. Finally, many herbivores manipulate their host–plant defences (Acevedo *et al.*, 2015; Favery *et al.*, 2020). For instance, Colorado potato beetles carry bacteria in their saliva that induce SA instead of JA when administered to tomato plants (Chung *et al.*, 2013). A similar mechanism is not yet known for *Delia radicum*.

Herbivory by *Delia radicum* leads to strong induction of indole GSLs, whereas aliphatic GSLs are downregulated. Combined transcriptomic and chemical analyses clearly show the close connection between GSL-biosynthesis gene expression and the accumulation of different GSLs. Comparison of GSL concentrations in leaves and roots, as well as expression patterns of GSL transporter (*GTR*) genes, suggests that local production drives root GSL accumulation. In *Brassica rapa*, *GTR* genes were strongly induced by *Delia radicum*, but this did not coincide with changes in GSL concentrations in distal tissues, suggesting that *GTR* genes may be involved in GSL retention rather than distal transport (Touw *et al.*, 2020). Previous studies found similar GSL regulation in response to *Delia radicum* and other herbivores, where indole GSLs are highly inducible while aliphatic GSLs are mostly nonresponsive or downregulated (van Dam & Raaijmakers, 2006; Textor & Gershenzon, 2009; Pierre *et al.*, 2012; Touw *et al.*, 2020). Some indole GSLs are toxic to nematodes and pathogens in *Arabidopsis* roots (Iven *et al.*, 2012; Pfalz *et al.*, 2016). However, it is generally believed that isothiocyanates that are formed upon aliphatic GSL hydrolysis are more toxic to herbivorous insects than indole GSL breakdown products (Jeschke *et al.*, 2016). Using mutant *Arabidopsis* plants, Müller *et al.* (2010) found that generalist herbivores were negatively affected by both indole and aliphatic GSL, whereas specialist herbivores were not affected. Many specialist herbivores of brassicaceous plants have evolved mechanisms to detoxify or sequester GSLs (Textor & Gershenzon, 2009; Jeschke *et al.*, 2016; Yang *et al.*, 2021). *Delia radicum* harbours gut microbes that can detoxify breakdown products of the aromatic GSL gluconasturtiin (Welte *et al.*, 2016). Several studies found no link between GSL content and *Delia radicum* performance in wild or cultivated *Brassica oleracea*, but the relationship was not directly studied (Pierre *et al.*, 2012; van Geem *et al.*, 2015). Here, we made use of transgenic *Brassica oleracea* plants to show that *Delia radicum* survival increases when feeding on plants with knocked down aliphatic GSL concentrations. This finding implies that downregulation of aliphatic GSL biosynthesis is adaptive for the root herbivore, and plants that do not respond in this manner are expected to be more resistant to *Delia radicum*.

Hydrolysis of GSLs catalysed by myrosinases is a crucial step in the production of toxic breakdown products, such as nitriles, isothiocyanates and oxazolidine-2-thiones. Brassicaceous plants are equipped with two types of myrosinase enzymes, differing in amino acids at the active site (Sugiyama & Hirai, 2019). In response to *Delia radicum*, expression of classic myrosinases did not change much whereas atypical myrosinases were upregulated. Classic myrosinases localize in specific cells throughout the plant and hydrolyse both indole and aliphatic GSLs, while atypical myrosinases accumulate in endoplasmic reticulum (ER) bodies and show activity towards indole GSLs (Kissen *et al.*, 2009; Zhao *et al.*, 2015; Nakano *et al.*, 2017). Interestingly, ER bodies are constitutively present in roots of *Arabidopsis*, while they are inducible upon wounding in leaves, presenting another difference between shoot and root defences (Ogasawara *et al.*, 2009; Nakano *et al.*, 2014). Different myrosinases may yield different hydrolysis products, with consequences for defensive effectiveness (Zhao *et al.*, 2015). Like many components of plant defence pathways, atypical myrosinases have other functions besides hydrolysing indole GSLs. BGLU18 and BGLU33 can hydrolyse inactive ABA-*O*-glucoside, resulting in bioactive ABA (Han *et al.*, 2019), and PYK10 can hydrolyse scopolin into scopoletin *in vitro* (Ahn *et al.*, 2009). Scopoletin, in turn, plays an important role in communication between roots and the rhizosphere microbiome as well as iron uptake (Stringlis *et al.*, 2019). Downstream of myrosinases, other factors including epithiospecifier proteins determine which biologically active compounds result from GSL hydrolysis, providing potential for specific regulation of toxins (Zhang *et al.*, 2006; Mumm *et al.*, 2008). It will be interesting to elucidate which biologically active compounds are produced from the accumulated neoglucobrassicin in *Delia radicum*-infested roots.

We discovered several processes of interest that may be involved in plant defence against *Delia radicum*. First, transcription of genes involved in responses to hydrogen peroxide and redox reactions were increased upon *Delia radicum* feeding. Moreover, the gene associated most strongly with *Delia radicum* herbivory was a homolog of the *Arabidopsis PER22* gene, a class III peroxidase that confers resistance to cold stress in *Arabidopsis* (Kim *et al.*, 2012). Reactive oxygen species such as hydrogen peroxide are produced upon wounding and during the early onset of plant defence (Erb & Reymond, 2019). Interestingly, Class III peroxidases and hydrogen peroxide production are involved in wheat genotypes resistant against *Mayetiola destructor* (Liu *et al.*, 2009). Larvae of this pest feed within galls in wheat stems, and whereas *Delia radicum* does not induce galls, resistance may be achieved in a similar manner as

young larvae feed within the primary root. Second, genes involved in responses to chitin were induced in primary roots by *Delia radicum*, including homologs of the *Arabidopsis PR3* gene, which encodes a JA/ET inducible chitinase (van Loon *et al.*, 2006). Chitinases have been studied extensively for their antifungal activity (van Loon *et al.*, 2006; Grover, 2012). Since the rhizosphere is a microbial hotspot, it makes sense for plants to prepare for defence against opportunistic pathogens upon root herbivory (Johnson *et al.*, 2016). Maize roots infested with *Diabrotica virgifera virgifera* also exhibited increased expression of chitinases (Barr *et al.*, 2010). Moreover, maize chitinases may play a role in defence against *Spodoptera exigua* caterpillars by disrupting the peritrophic matrix in the midgut, thereby enhancing entomopathogen infection (Mason *et al.*, 2019; Han *et al.*, 2021). Thus, responses to chitin may be targeted directly at *Delia radicum*, and/or at secondary infection by fungal pathogens. Finally, we found that root herbivory led to upregulation of many transcripts encoding proteins involved in translation and ribosome biogenesis. Ribosomes are comprised of ribosomal RNA (rRNA) and ribosomal proteins, and by changing the composition of these ribosomal proteins, of which over 250 are known in *Arabidopsis*, plants may regulate translation (Martinez-Seidel *et al.*, 2020). Indeed, in *Arabidopsis* roots, deficiency in phosphate and iron leads to changes in ribosomal composition (Wang *et al.*, 2013). Furthermore, using TRAP-sequencing, a sequencing technique which specifically targets messenger RNA (mRNA) bound to ribosomes, Kimberlin *et al.* (2021) found that wounding leads to changes in transcripts associated to ribosomes. This level of regulation in plant defence against herbivorous insects presents an exciting avenue for future research.

We compared responses to single and dual infestation in primary roots to uncover mechanisms underlying plant-mediated interactions. Aboveground herbivory by *Brevicoryne brassicae* aphids or *P. xylostella* caterpillars did not lead to major differences in the overall transcriptomic response to *Delia radicum*. This corresponds with earlier findings that the latest stressor has a dominant effect over earlier induction (Coolen *et al.*, 2016). Shoot infestation by the caterpillars had more effect than shoot infestation by the aphids, and we found most differences between single and dual infested plants in the first and last time point studied. When we specifically studied responses in the first hour following root herbivory, a clearly faster response was seen in *P. xylostella*-induced plants, and thus priming by the caterpillar infestation, which may be responsible for the plant-mediated antagonism we previously recorded (Karssemeijer *et al.*, 2020). Interestingly, after 2 h, the genes we studied were expressed at the same level regardless of priming, indicating that especially the onset of the response was altered. Another element of priming is its retention over time, or 'memory' (Hilker *et al.*, 2016), which can be transferred through seeds to the next generation (Rasmann *et al.*, 2012). When we introduced a noninfested time interval, we no longer observed a faster response to root herbivory, indicating that continuous feeding by *P. xylostella* is required for priming to be sustained.

Here, we present an extensive analysis of primary root responses to a specialist root herbivore. We provide evidence that aliphatic GSL can interfere with the performance of specialist insect herbivores. Our study opens new avenues of research in insect–plant interactions. While much remains to be discovered to fully grasp the nature and evolution of these interactions, this study advances our understanding of how plants cope with root herbivores.

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Author contributions

PNK, JJAL and MD designed and planned the project. PNK, KAK and RG performed the experiments. MR and JG performed chemical analyses. MN developed the *myb28* Crispr-Cas9 line. PNK processed and analysed the data. PNK wrote the manuscript together with JJAL and MD, with input from all authors.

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Data availability

Raw sequencing data is available from the European Nucleotide Archive (https://www.ebi.ac.uk/ena/), under study accession no. PRJEB49273.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Principal component analysis (PCA) of *Brassica oleracea* transcriptomes of the primary root under the influence of aboveground and belowground herbivory by *Delia radicum* for 3, 6, 9, 24, or 48 h.

Fig. S2 Differentially expressed genes (DEGs) relative to root-herbivore induced samples in *Brassica oleracea* primary roots in response to aboveground herbivory.

Fig. S3 Concentrations of jasmonates, abscisic acid, and salicylic acid in *Brassica oleracea* primary roots in response to aboveground and belowground herbivory.

Fig. S4 Concentrations of glucosinolates in *Brassica oleracea* primary roots in response to aboveground and belowground herbivory.

Methods S1 Genotyping of the *myb28* mutants.

Methods S2 Chemical analyses of phytohormones and glucosinolates.

Table S1 RNA-sequencing sample metadata (Table S1.1) and information about their processing (Table S1.2).

Table S2 Primers used for quantitative polymerase chain reaction (qPCR).

Table S3 Gene descriptions and functional analysis of top and bottom loadings of first principal component (PC1) (Fig. 1).

Table S4 Gene ontology (GO) enrichment of gene clusters (Fig. 2b,c) and selected GO terms for visualization.

Table S5 Statistical information of phytohormone analyses (Fig. 3b).

Table S6 Statistical information of glucosinolate analysis (Fig. 4d,e).

Table S7 Statistical information of *myb28* mutant experiment, including *Delia radicum* measurements and glucosinolates (Fig. 5).

Table S8 Statistical information of gene expression analyses in the priming experiments (Fig. 6).

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