New Phytologist

New Phytologist Supporting Information

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

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The following Supporting Information is available for this article:

Figures S1 to S4
Methods S1, S2
Tables S1 to S8 (in separate excel file)

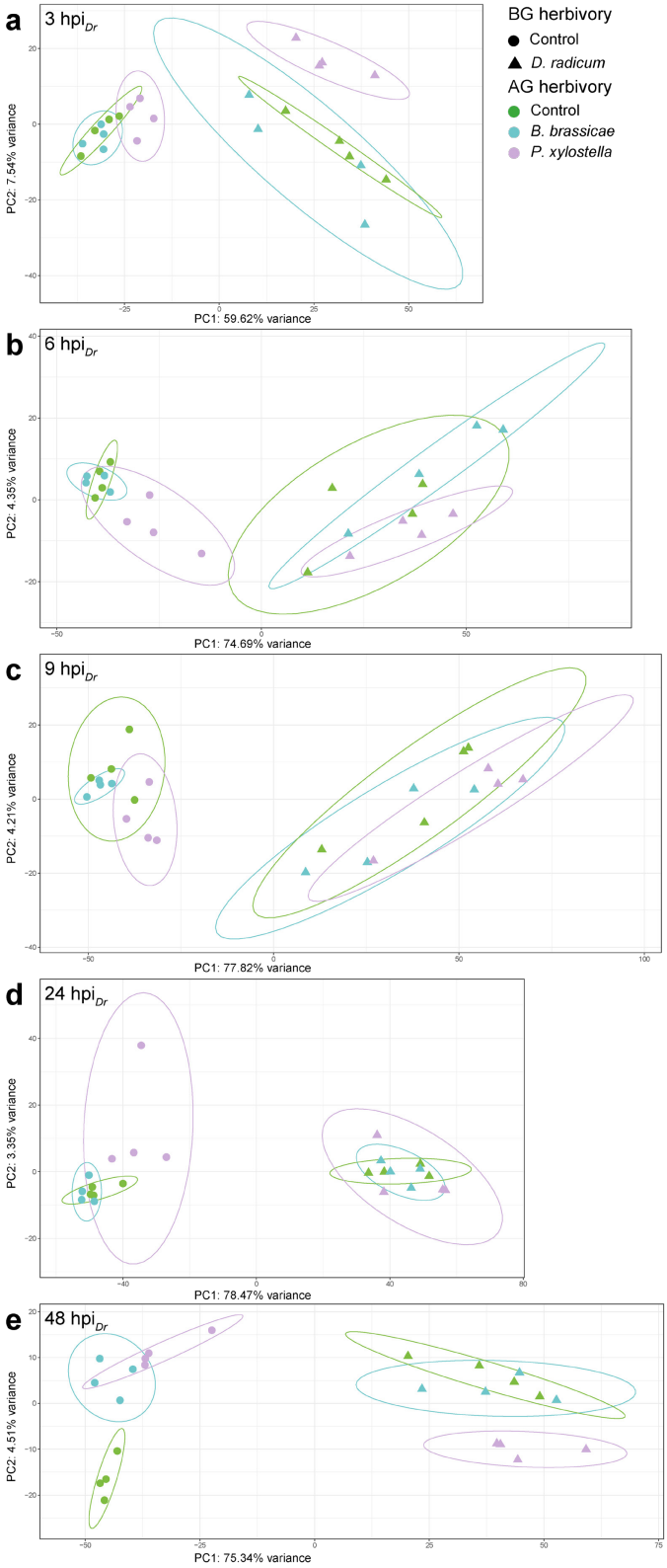

Figure **S1**. Principal component analysis (PCA) of *Brassica oleracea* transcriptomes of the primary root under the influence of aboveground (AG) herbivory by *Brevicoryne brassicae* or *Plutella xylostella* and belowground (BG) herbivory by *Delia radicum* for 3 (**a**), 6 (**b**), 9 (**c**), 24 (**d**), 48 (**e**) hours. Ellipses show 95% confidence intervals. Aboveground herbivores arrived 48 hours prior to infestation by *D. radicum*. hpi$_{Dr}$: hours post infestation by *D. radicum*.

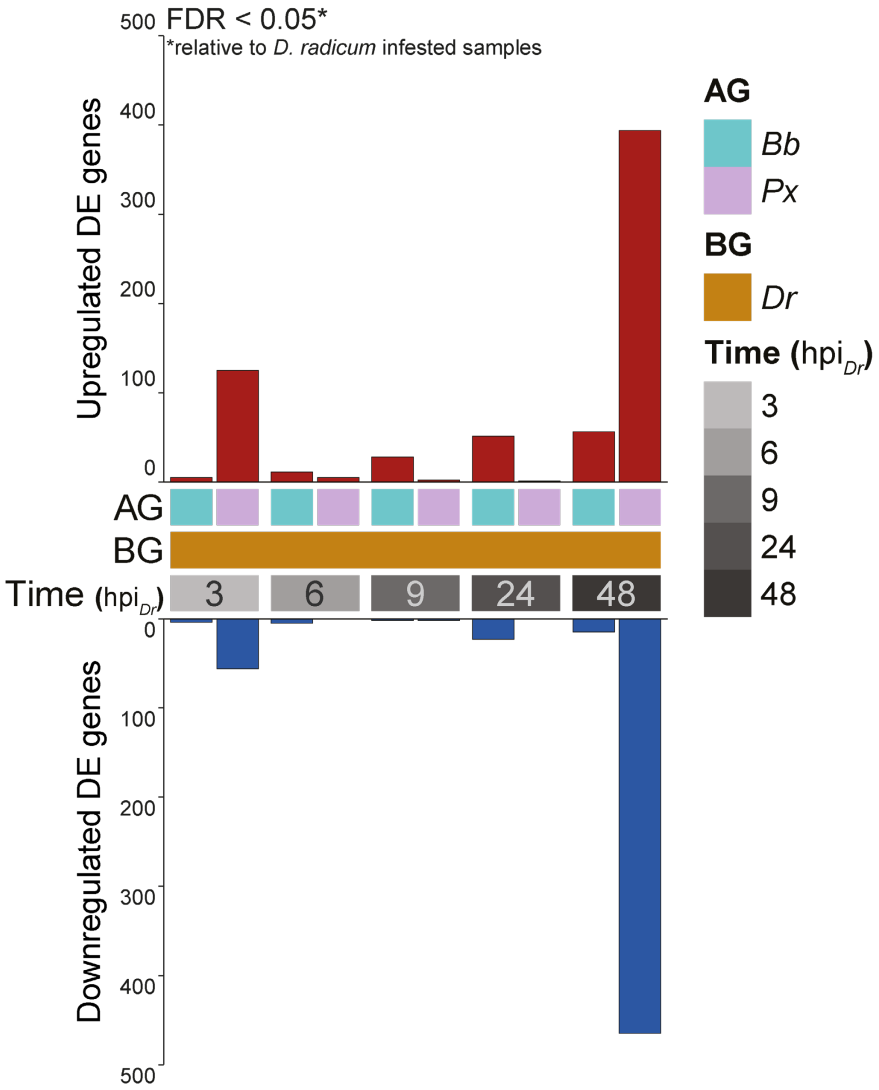

Figure **S2**. Differentially expressed genes (DEGs) relative to root-herbivore (*Delia radicum* (*Dr*)) induced samples in *Brassica oleracea* primary roots in response to aboveground (AG) herbivory by *Brevicoryne brassicae* (*Bb*) or *Plutella xylostella* (*Px*). Herbivores feeding AG were introduced two days prior to infestation by *D. radicum*. FDR: false discovery rate. hpi_{Dr}: hours post infestation by *D. radicum*.

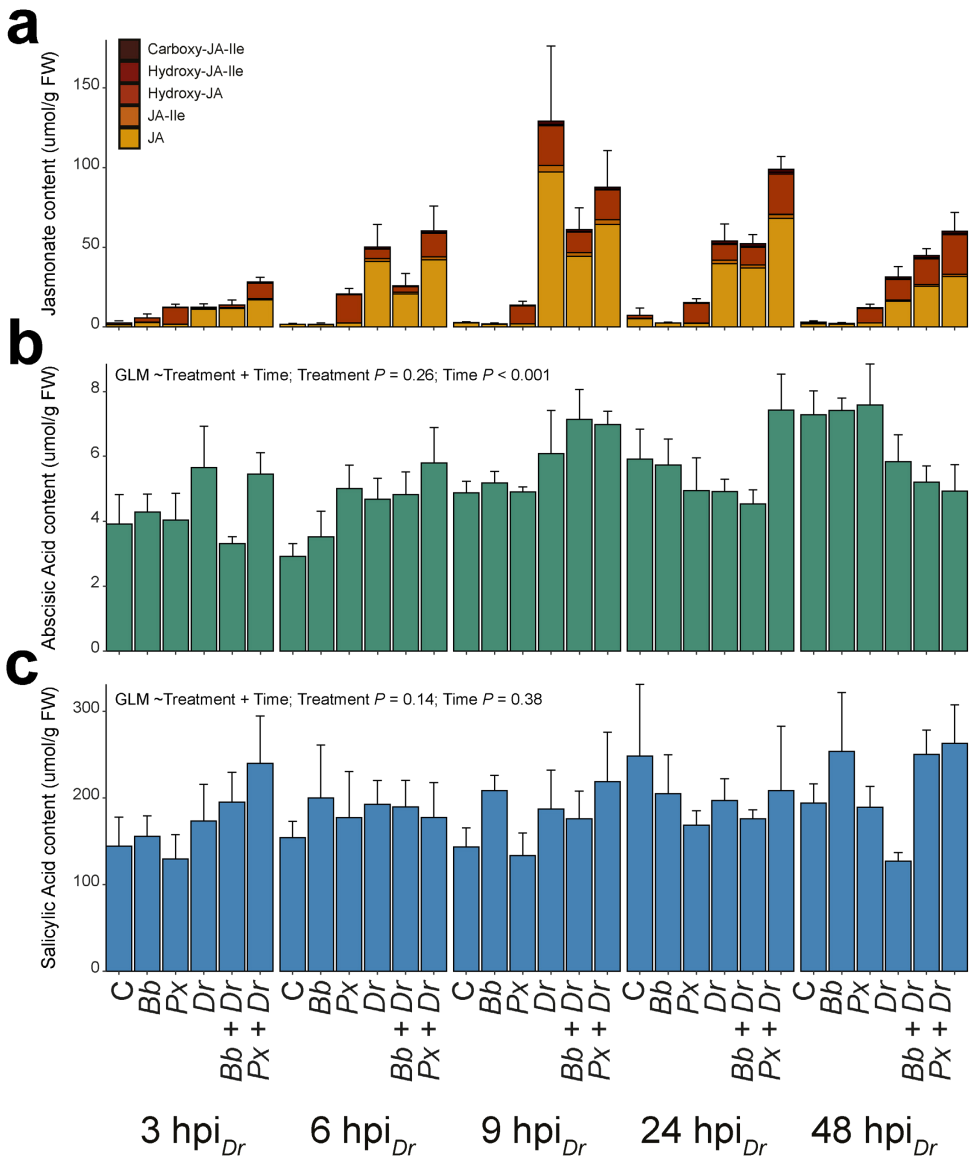

Figure **S3.** Concentrations of jasmonates (**a**) abscisic acid (**b**) and salicylic acid (**c**) in *Brassica oleracea* primary roots in response to aboveground (AG) herbivory by *Brevicoryne brassicae* (*Bb*) or *Plutella xylostella* (*Px*) and belowground (BG) herbivory by *Delia radicum* (*Dr*). Herbivores feeding AG were infested two days prior to root infestation by *D. radicum*. Corresponding statistical information in Table S5. For jasmonates, means are plotted per compound and error bars represent the standard error of the total (N = 3-6). FW: fresh weight. C: control. hpi_{Dr}: hours post infestation by *D. radicum*.

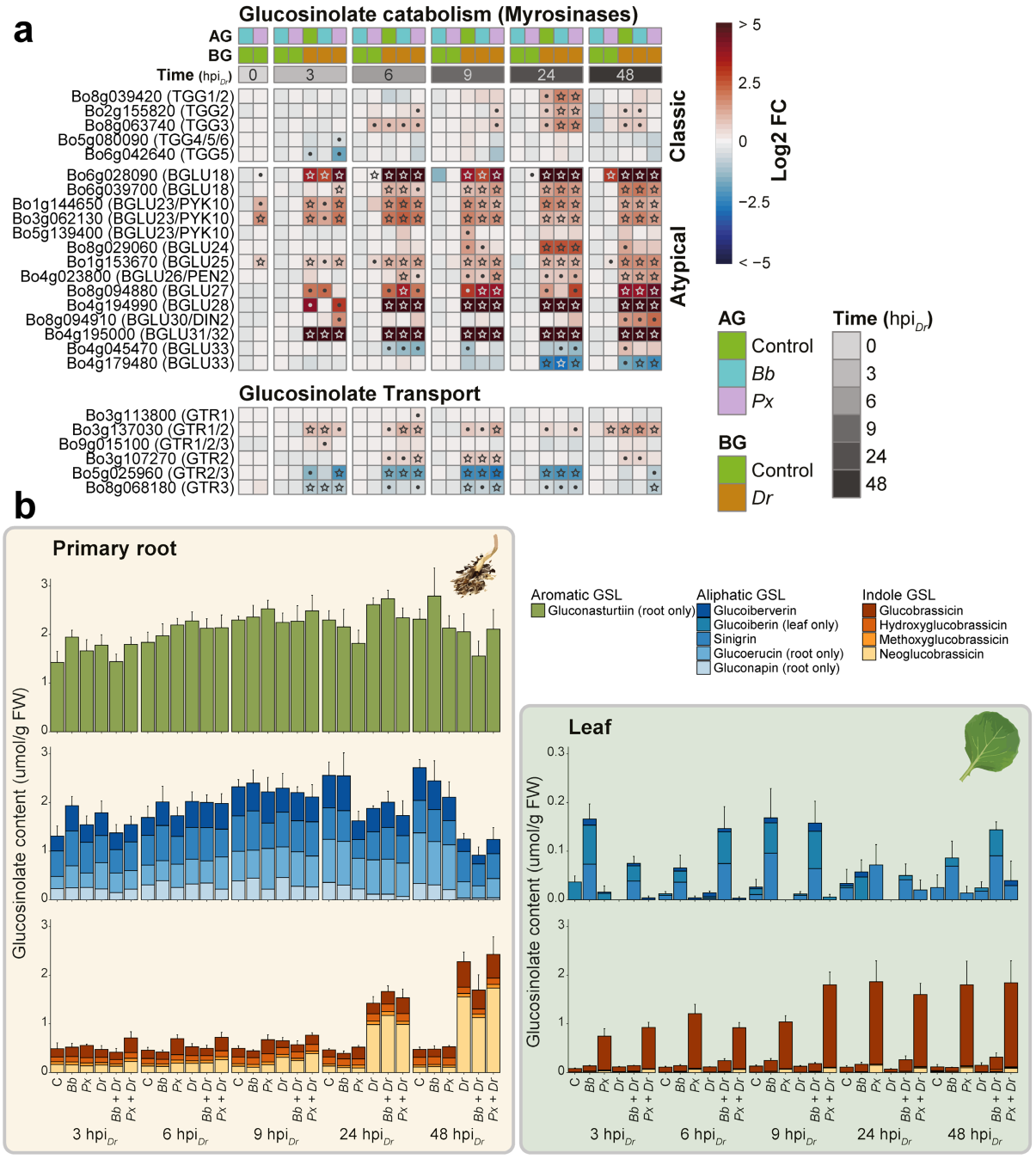

Figure **S4.** Fold changes relative to control for genes involved in glucosinolate catabolism and transport (**a**) in *B. oleracea* primary roots in response to aboveground (AG) herbivory by *Brevicoryne brassicae* aphids (*Bb*) or *Plutella xylostella* (*Px*) caterpillars and belowground (BG) herbivory by *Delia radicum* (Dr). Herbivores feeding aboveground were introduced on plants two days prior to root infestation by *D. radicum*. Concentrations of glucosinolates in *B. oleracea* primary roots and leaves (**b**) of plants from the same experiment used for RNAseq analysis (N = 3-6). 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 (FDR <0.0001) and dots (FDR <0.05). For analyses of GSL concentrations, statistical information can be found in Table S6. Scaling of the y-axis is identical for the three classes of GSLs, with exception of leaf aliphatic GSL. Means are plotted per compound and error bars represent the standard error of the total amount. FW: fresh weight. C: control. hpi$_{Dr}$: hours post infestation by *D. radicum*.

Supporting Methods S1: Genotyping of *myb28* and WT plants.

In the experiments performed to test the effects of aliphatic glucosinolates (GSL) on *Delia radicum*, two cabbage genotypes were used: a wildtype (WT) DH1012 line, and a *myb28* knockout line in the same DH1012 background. In the latter line, two of the three copies of *MYB28* (Bo9175680 and Bo2g161590) have been knocked out using CRISPR-Cas9 (Neequaye *et al.*, 2020). Seeds were kindly provided by Mikhaela Neequaye and Lars Østergaard, who created this mutant line.

Seeds were sown in seedling soil and seedlings were transplanted after 8 days into regular potting soil (Lentse potgrond nr4). We genotyped all *myb28* plants used for the experiments and a subset of WT plants. To this end, a cotyledon was harvested from each plant 26 days after transplanting using a sharp razor to limit wounding. Rapid DNA extraction was performed using the MyTaq™ Extract-PCR Kit (Bioline). A small piece of cotyledon was directly submerged into lysis buffer and crushed using tweezers. Three plants were pooled for each sample. The razor and tweezers were cleaned between samples using a 10% bleach solution, followed by 70% ethanol and finally MQ water. This mixture was incubated at 75 °C for 5 min followed by 95 °C for 10 min. Samples were briefly spun down to reduce debris, and liquid was diluted 10 times prior to PCR. An initial PCR for the Act-2 reference gene was performed to confirm the extraction technique (not shown).

For genotyping, we tested the deletion in the *MYB28* ortholog on chromosome 9 (Bo9175680), as this can easily be visualized on using gel electrophoresis. Mutation in the chromosome 2 ortholog (Bo2g161590) was assumed, as the seeds were harvested from homozygous plants for both mutations. PCR was performed using MyTaq HS Red Mix (Bioline) and primers spanning the deletion (Table M1), with the following protocol: Initial denaturation 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 15 sec, annealing at 60 °C for 30 sec and elongation at 72 °C for 1 min, and final elongation at 72 °C for 5 min. Samples were loaded on a 1 % agarose gel with Midori green. By gel electrophoresis, we confirmed the mutant *myb28* allele in all samples (Figure M1).

Table M1. Genotyping primers, designed by Mikhaela Neequaye, John Innes Centre, UK

Primer name	**Gene code**	**Sequence**	**Product size**	**Comment**
MYB28_C9_F	**Bo9g175680**	**AGAGTTCTCATCAACCGATCT**	**1534bp**	**526bp deletion in mutant**
MYB28_C9_R	**Bo9g175680**	**ACCTTTCTGCTTAGGCACGA**		

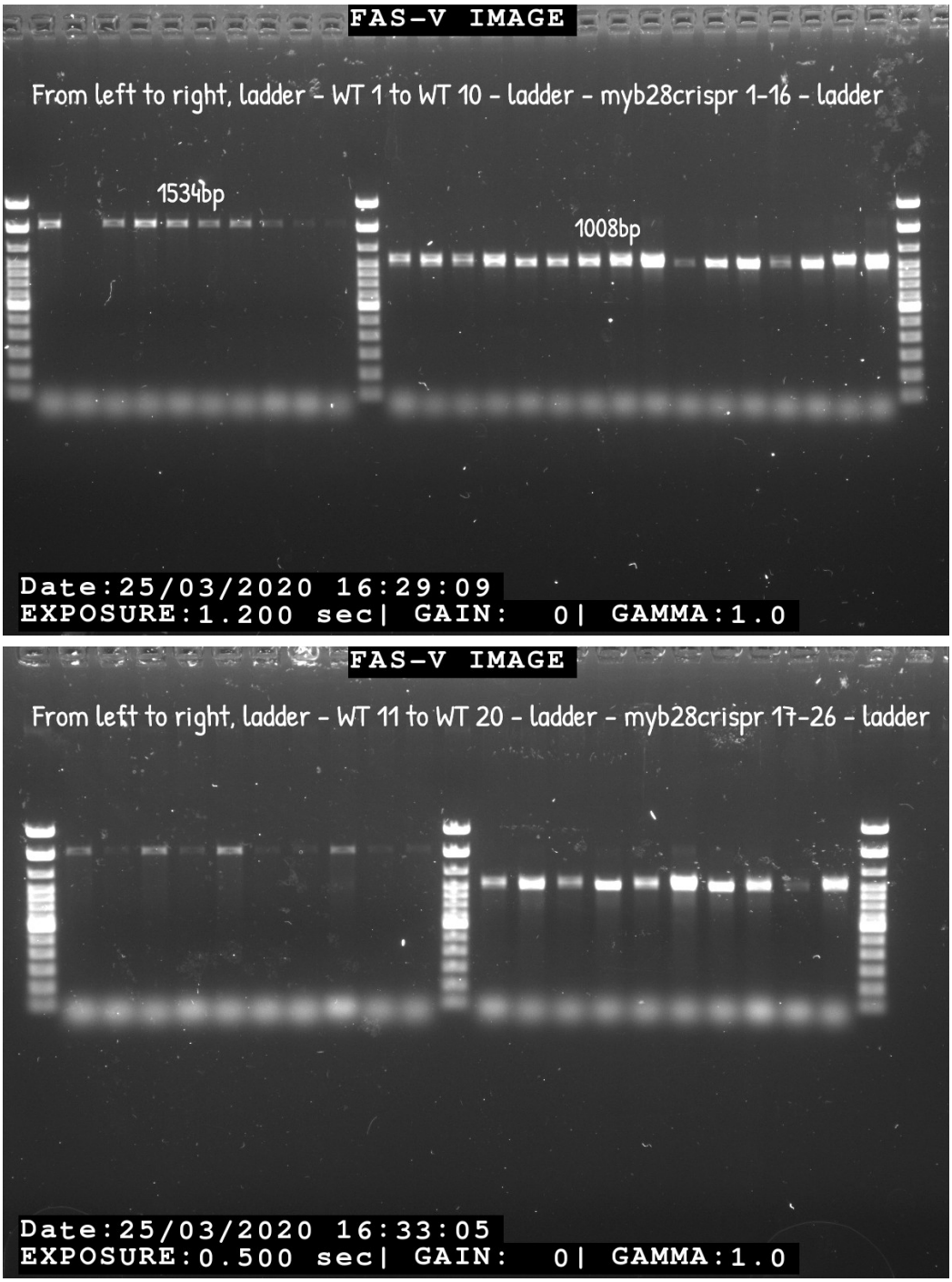

Figure M1. Gel electrophoresis of *MYB28* (Bo9g175680) PCR products in WT and *myb28* mutant plants.

Supporting Methods S2: Chemical analyses.

For phytohormone analysis, 14-96 mg of fresh frozen leaf samples and 8-102 mg of fresh frozen root samples were extracted with 1 ml of 80% methanol (v:v) containing 40 ng D4-SA, 40 ng D6-ABA (Santa Cruz Biotechnology, USA), 40 ng D6-JA, and 8 ng D6-JA-Ile (HPC Standards GmbH, Germany). Phytohormone analysis was performed as in Vadassery *et al.* (2012) on an Agilent 1200 series HPLC system (Agilent Technologies) with the modification that a tandem mass spectrometer QTRAP 6500 (SCIEX, Darmstadt, Germany) was used in multiple reaction monitoring (MRM) mode with parameters listed in Table M2 (below). Concentration of *cis*-OPDA, and OH-JA were determined relative to the quantity of the internal standard D6-JA applying a response factor (RF) of 1.0. OH-JA-Ile and COOH-JA-Ile were quantified relative to D6-JA-Ile: RF 1.0.

Glucosinolates (GSL) were analysed by HPLC-UV as described in Burow *et al.* (2006). In short, 6-25 mg of lyophilized root material was extracted with 1mL 80% methanol solution (v:v) containing 50 nmol of 4-hydroxybenzyl GSL. A 600 µL aliquot of the raw extract was loaded onto DEAE Sephadex A 25 columns and treated with arylsulfatase for desulfation (Sigma-Aldrich) (Brown *et al.*, 2003). The eluted desulfoglucosinolates were separated using high performance liquid chromatography (Agilent 1100 HPLC system, Agilent Technologies) on a reversed phase C-18 column (Nucleodur Sphinx RP, 250 x 4.6 mm, 5µm, Machrey-Nagel, Düren, Germany) with a water (A)-acetonitrile (B) gradient (0-1 min, 1.5% B; 1-6 min, 1.5-5% B; 6-8 min, 5-7% B; 8-18 min, 7-21% B; 18-23 min, 21-29% B; 23-23.1 min, 29-100% B; 23.1-24min 100% B and 24.1-28 min 1.5% B; flow 1.0 mL min-1). Detection was performed with a photodiode array detector and peaks were integrated at 229 nm. Desulfated GSL were identified by comparison of retention time and UV spectra to those of purified standards previously extracted from *Arabidopsis thaliana* (Brown *et al.*, 2003) or by analysis of the desulfoglucosinolate extracts on an LC-ESI-Ion-Trap-mass spectrometer (Esquire6000, Bruker Daltonics). We used the following molar response factors for quantification of individual GSL relative to the internal standard, 4-hydroxybenzyl GSL: aliphatic GSL 2.0, indole GSL 0.5 (83), 2-phenylethyl GSL 2.0. The following GSLs were detected in the samples: 2-propenyl GSL (sinigrin), 3-butenyl GSL (gluconapin), 3-methylsulfinylpropyl GSL (glucoiberin), 4-methylsulfinylbutyl GSL (glucoraphanin), 3-methylthiopropyl GSL (glucoiberverin), 4-hydroxy-indol-3-ylmethyl GSL (hydroxyglucobrassicin), 4-methylthiobutyl GSL (glucoerucin), indol-3-ylmethyl GSL (glucobrassicin), 4-methoxy-indol-3-ylmethyl GSL (methoxyglucobrassicin), 2-phenylethyl GSL (gluconasturtiin), and 1-methoxy-indol-3-ylmethyl GSL (neoglucobrassicin).

Table M2. Details of the analysis of phytohormones by LC-MS/MS using an Agilent HPLC 1260/QTRAP6500 instrument in negative ionisation mode. Abbreviations are: Q1, selected m/z of the first quadrupole; Q3, selected m/z of the third quadrupole; RT, retention time; DP, declustering potential (V); and CE, collision energy (V).

Q1	Q3	RT (min)	compound	Internal standard	DP	CE
136.93	93	3.3	SA	D4-SA	-20	-24
209.07	59	3.6	JA	D6-JA	-20	-24
263	153.2	3.4	ABA	D6-ABA	-20	-22
322.19	130.1	3.9	JA-Ile	D6-JA-Ile	-20	-30
290.9	165.1	4.6	OPDA	D6-JA	-20	-24
225.1	59	2.6	OH-JA	D6-JA	-20	-24
338.1	130.1	3.0	OH-JA-Ile	D6-JA-Ile	-20	-30
352.1	130.1	3.0	COOH-JA-Ile	D6-JA-Ile	-20	-30
140.93	97	3.3	D4-SA		-20	-24
215	59	3.6	D6-JA		-20	-24
214	59	3.6	D5-JA		-20	-24
269	159.2	3.4	D6-ABA		-20	-22
328.19	130.1	3.9	D6-JA-Ile		-20	-30
327.19	130.1	3.9	D5-JA-Ile		-20	-30

SI References

Brown PD, Tokuhisa JG, Reichelt M, Gershenzon J. 2003. Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. *Phytochemistry* **62**(3): 471-481.

Burow M, Müller R, Gershenzon J, Wittstock U. 2006. Altered glucosinolate hydrolysis in genetically engineered *Arabidopsis thaliana* and its influence on the larval development of *Spodoptera littoralis*. *Journal of Chemical Ecology* **32**(11): 2333-2349.

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Vadassery J, Reichelt M, Hause B, Gershenzon J, Boland W, Mithöfer A. 2012. CML42-mediated calcium signaling coordinates responses to *Spodoptera* herbivory and abiotic stresses in *Arabidopsis*. *Plant Physiology* **159**(3): 1159-1175.