Plant defence responses in oilseed rape MINELESS plants after attack by the cabbage moth Mamestra brassicae

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Abstract

The Brassicaceae family is characterized by a unique defence mechanism known as the ‘glucosinolate–myrosinase’ system. When insect herbivores attack plant tissues, glucosinolates are hydrolysed by the enzyme myrosinase (EC 3.2.1.147) into a variety of degradation products, which can deter further herbivory. This process has been described as ‘the mustard oil bomb’. Additionally, insect damage induces the production of glucosinolates, myrosinase, and other defences. Brassica napus seeds have been genetically modified to remove myrosinase-containing myrosin cells. These plants are termed MINELESS because they lack myrosin cells, the so-called toxic mustard oil mines. Here, we examined the interaction between B. napus wild-type and MINELESS plants and the larvae of the cabbage moth Mamestra brassicae. No-choice feeding experiments showed that M. brassicae larvae gained less weight and showed stunted growth when feeding on MINELESS plants compared to feeding on wild-type plants. M. brassicae feeding didn’t affect myrosinase activity in MINELESS plants, but did reduce it in wild-type seedlings. M. brassicae feeding increased the levels of indol-3-yl-methyl, 1-methoxy-indol-3-yl-methyl, and total glucosinolates in both wild-type and MINELESS seedlings. M. brassicae feeding affected the levels of glucosinolate hydrolysis products in both wild-type and MINELESS plants. Transcriptional analysis showed that 494 and 159 genes were differentially regulated after M. brassicae feeding on wild-type and MINELESS seedlings, respectively. Taken together, the outcomes are very interesting in terms of analysing the role of myrosin cells and the glucosinolate–myrosinase defence system in response to a generalist cabbage moth, suggesting that similar studies with other generalist or specialist insect herbivores, including above- and below-ground herbivores, would be useful.

Key words: Brassica napus (oilseed rape), defence cells, generalist, glucosinolates, jasmonates, myrosinase, plant–insect interaction, transcriptional profiling.

Introduction

The presence of the dual ‘glucosinolate–myrosinase’ system is a distinctive characteristic of the Brassicaceae family. Glucosinolates (β-thioglucoside–N-hydroxysulfates) are well known plant secondary metabolites that comprise a diverse group of sulfur-rich compounds and occur mainly in the order Brassicales (Bones and Rossiter, 1996; Kliebenstein et al., 2005; Hopkins et al., 2009; Sønderby et al., 2010). Though the intact glucosinolates are shown to provide resistance to insect herbivores, their defensive potential is increased upon hydrolysis by the enzyme myrosinase (β-thioglucosidases; E.C. 3.2.1.147) (Kim and Jander, 2007; Ahuja et al., 2010; Björkman et al., 2011). Glucosinolate hydrolysis by myrosinases produces a range of compounds such as isothiocyanates, nitriles, epithionitriles, and oxazolidine-thiones, depending
upon the glucosinolate structure, specifier proteins, and reaction conditions (Rask et al., 2000; Rohloff and Bones, 2005; Bones and Rossiter, 2006; Wittstock and Burow, 2010). The protein cofactors that affect glucosinolate hydrolysis are epi-thiospecifiers (ESPs), nitrile-specifiers (NSPs), and thiocyanate-forming proteins (TFPs) (MacLeod and Rossiter, 1985; Lambrich et al., 2001; Bones and Rossiter, 2006; Burow et al., 2009; Kissen and Bones, 2009; Kong et al., 2012).

Myrosinases are present in specialized ‘myrosin cells’ (Bones and Iversen, 1985; Bones et al., 1991; Kissen et al., 2009), which are dispersed throughout plant tissues. Immunocytochemical and in situ hybridization studies carried out on seeds of Brassicaceae species have shown that myrosinases are exclusively present in the myrosin cells of embryonic cotyledons and the radicle periphery (Bones et al., 1991). The roles of myrosin cells and myrosinase have been well documented in several studies (Rask et al., 2000; Kissen et al., 2009; Textor and Gershenzon, 2009; Ahuja et al., 2010).

The programmed cell death of myrosin cells was achieved during the seed development phase by directing the expression of an RNase (barnase) from a cell-specific myrosinase promoter (Borgen et al., 2010). These plants have been named MINELESS because their myrosin cells (toxic mines) have been removed, leading to a dramatic reduction in myrosinase activity in seeds (Ahuja et al., 2011). Additionally, the MINELESS plants have reduced amount of glucosinolate–myrosinase hydrolysis products, but higher amount of glucosinolates (Borgen et al., 2010; Ahuja et al., 2011).

The Lepidopteran insect Mamestra brassicae L. (Noctuidae) is a generalist that feeds on plants from at least 70 species and 22 families, of which members of the Brassicaceae and Chenopodiaceae are among the most preferred (McKinlay, 1992; Rojas et al., 2000; Ulland et al., 2008). It is common in Europe and Asia. Feeding by the caterpillars causes severe damage to the plants, and it is an economically devastating pest in agriculture (Ulland et al., 2008). M. brassicae preference and performance are both affected by glucosinolate content and composition. M. brassicae larvae prefer to feed and perform best on glucosinasturtiin-type rather than glucobrassicin-type Barbarea vulgaris plants (van Leur et al., 2008). The larvae are also reported to benefit from a reduction in glucoraphanin, a predominant glucosinolate in Arabidopsis (Beckwilder et al., 2008); and showed reduced performance on B. oleracea plants with high concentrations of the glucosinolate glucobrassicin (Poelman et al., 2009). Furthermore, silencing of the foliar myrosinase genes TGG1/TGG2 enhanced growth of M. brassicae larvae after feeding on Arabidopsis plants (Zheng et al., 2011).

Here, we took an exclusive opportunity to study the responses of B. napus wild-type cv. Westar and MINELESS plants after they had been attacked by the larvae of M. brassicae. The aim was to find out what happens in plants that lack defence cells, such as myrosin cells; or in other words what kind of defence responses are regulated in plants with a modified glucosinolate–myrosinase defence system upon attack by the generalist insect herbivore M. brassicae. Since glucosinolate levels were higher and glucosinolate–myrosinase hydrolysis products were lower in MINELESS than the wild-type, we also evaluated how M. brassicae larvae develop on wild-type and MINELESS plants. The results are presented as a comparative study of wild-type vs MINELESS plants in response to insect herbivory by larvae of M. brassicae.

### Materials and methods

#### Plant and insect rearing

The B. napus wild-type cv. Westar and MINELESS seeds were germinated in soil (Borgen et al., 2010; Ahuja et al., 2011), and the plants were grown under greenhouse conditions (S3 security class), with a 16 h photoperiod. The day and night temperatures were 21 and 18°C, respectively, at a light intensity of 70–80 μmol m⁻² s⁻¹. The eggs of M. brassicae were kept at 21°C/16°C, light 16 h/12 h.

#### Insect no-choice feeding experiments

Seven-to-nine-day-old wild-type and MINELESS seedlings were infested with neonate M. brassicae larvae (Cabbage moth; Laboratory of Entomology, Wageningen University) (Supplementary Table S1). Neonate larvae were weighed to assess their average starting weight and then distributed over pots (four seedlings per pot) of each of the wild-type and MINELESS seedlings. Each seedling was infested with one neonate larva by placing it on one of the two cotyledons. The larvae were trapped in cages made of opaque plastic sheets. The seedlings were completely enclosed by placing one side of the cage on a pot and covering the other side with a muslin cloth. The experiments were repeated twice. In a first experiment, the insect no-choice feeding assay was performed for 12 days by taking insect weights at four time points (day 3, 8, 10, and 12) (Supplementary Table S1). In a second experiment, the same was performed for 12 days by taking insect weights at two time points (day 7 and 12) (Supplementary Table S1). Larvae were given new seedlings at each time point. The above-ground tissue (cotyledons + hypocotyl) from each seedling was scanned and comprised one biological observation. The area of scanned tissue was calculated with software Compu Eye, Leaf & Symptom Area (Bakr, 2005). To account for the damage to tissue during the experiment, the amount of consumed area was calculated by comparison with the control tissue.

#### Induction experiment

The effects of larval attack on wild-type and MINELESS seedlings were measured in the following way: 10-day-old M. brassicae larvae were kept on 6–7-day-old seedlings of wild-type and MINELESS, respectively (Supplementary Table S1). In order to retain the larvae, the pots (four seedlings per pot) were enclosed in cages. Controls were treated in the same way, without insects. Pots with control (non-infested) and M. brassicae-infested seedlings were kept under the same conditions. The control and M. brassicae-challenged seedlings were harvested after 24 h of infestation. Cages and larvae were removed and the above-ground tissue from seedlings was harvested and flash frozen in liquid nitrogen. These samples were homogenized to a fine powder and used for myrosinase activity assays, glucosinolate analysis, microarrays, and qRT-PCR experiments. Fresh tissue was used for extraction of glucosinolate–myrosinase hydrolysis products.

#### Myrosinase activity and protein assays

The extraction of myrosinase and specific myrosinase activity were measured from control and insect-challenged samples following previous methodology (Borgen et al., 2010; Ahuja et al., 2011). The wild-type and MINELESS seedlings were crushed and the proteins were extracted in 100 μl of imidazole-HCl buffer (10 mM, pH 6.0). The myrosinase activity was measured using the GOD-Perid assay, as described previously (Bones and Slupphaug, 1989; Borgen et al., 2010). In order to calculate the specific myrosinase activity, the total protein content of samples was measured using Bradford reagent
Glucosinolate analysis

Glucosinolate analysis was performed as described previously (van Dam et al., 2004). Lyophilized, finely ground above-ground tissue was dissolved in 1 ml 70% MeOH in water (v/v) in a 2 ml Eppendorf tube, vortexed, and immediately boiled for 5 min to inactivate any remaining myrosinase. The tubes were placed in an ultrasonic bath for 15 min and centrifuged (10 min, 10 000 rpm). The extraction was repeated for the pellet, but with the boiling step omitted. Both supernatants were combined per sample and applied to a DEAE-Sephadex A 25 column, desulphated with aroylsulphatase (Sigma, St. Louis, IL, USA), and separated on a reversed phase C-18 column on HPLC with a CH3CN–H2O gradient. Glucosinolate detection was performed with a Photo Diode Array (PDA) detector (200–350 nm) with 229 nm as the integration wavelength. Sinigrin (2-propenylglucosinolate) was used as an external standard. The response factors at 229 nm from three sources (Buchner, 1987; EC, 1990; Brown et al., 2003) were used to calculate the concentrations of the glucosinolates. Desulphoglucosinolate peaks were identified by comparing HPLC retention times and UV spectra with standards kindly provided by M. Reichelt (MPI Chemical Ecology, Jena, Germany) and a certified rape seed standard (Community Bureau of Reference, Brussels, code BCR-367R).

Analysis of glucosinolate–myrosinase hydrolysis products

Glucosinolate hydrolysis products were analysed as described previously (Ahuja et al., 2011). The seedlings were crushed with a glass rod in MQ H2O in a 2 ml screw-top vial with a PTFE/silicone septum. The mixture was left for 10 min at ambient temperature for hydrolysis. A mixture of 0.5 ml hexane:dichloromethane (3:2) with an internal standard (12 μg 3-butyl-isothiocyanate) was injected through the septum into the vial, and the sample was vortexed for 50 s. After centrifugation at 3 1000 rpm for 2 min, the solvent phase was pipetted into a 2 ml screw-top vial with a PTFE/silicone septum, and concentrated under nitrogen flow to a volume of 50 μl. Agilent 6890/5975 GC–MS (Agilent Technologies Inc., Palo Alto, CA) was used for all analyses. Mass spectra were acquired in EI mode, and a mass range of m/z 39–250 was recorded. The compounds were quantified as described previously (Ahuja et al., 2011).

RNA isolation, cDNA synthesis, microarray experiments, and data analysis

Microarray experiments were performed to compare the expression profiles of wild-type controls against M. brassicae-challenged wild-type seedlings, and the MINELESS controls against M. brassicae-challenged MINELESS seedlings by taking four biological replicas of each. All procedures were followed as described previously (Kusnierczyk et al., 2008). Statistical analysis was done using R (version 2.10.1), and the Linear Models for Microarray Data (LIMMA) package (Smyth, 2004). The data sets were log-transformed and normalized using the print tip-locss approach. Within-array replicated measurements for the same gene were merged by taking the average between replicates. No background subtraction was performed. Probe-specific dye effects were identified and genes with a strong dye bias were removed. Adjusted P-values were calculated using the Benjamini-Hochberg step-up procedure, controlling the false discovery rate. Genes with an adjusted P-value < 0.05 were considered to be significantly differentially expressed. To evaluate the difference in responses between M. brassicae-challenged wild-type seedlings and M. brassicae-challenged MINELESS seedlings, a LIMMA contrast analysis was performed using control (non-infested) plants as a common reference. The genes were grouped depending on the biological process with which they are mainly associated using the Arabidopsis Information Resource (TAIR) database (http://arabidopsis.org/index.jsp) (Poole, 2008), GenomeNet (http://www.genome.jp), and cited literature. The microarray data were deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE40932, and are compliant with the MIAME guidelines.

Quantitative PCR

Quantitative PCR was performed as described previously (Kusnierczyk et al., 2011), with minor modifications. The cDNA was synthesized from 1 μg total RNA using the Quantitect® Reverse Transcription Kit (Qiagen). The qRT-PCR was carried out using the LightCycler 480 SYBR Green I Master mix (Roche Applied Science, Mannheim, Germany), following the manufacturer’s instructions. PCR was performed in a LightCycler 480 as follows: (i) Pre-incubation for 5 min at 95°C; (ii) 45 amplification cycles consisting of 95°C for 10 s, 55°C for 15 s, and 72°C for 10 s; (iii) 95°C for 5 s, 65°C for 1 min, followed by a melting curve analysis by heating from 65°C to 97°C with a ramp rate of 2.2°C s−1. Each 20 μl reaction contained 0.5 μM of each of forward and reverse primer and a cDNA quantity corresponding to 0.05 μg of total RNA. Cp values and melting curves were calculated by the LightCycler 480 analysis programs using the 2nd derivative maximum function and exported in text format. PCR efficiencies were determined employing the Limma-PCR (Ramakers et al., 2003). Relative expression ratios were calculated using the Relative Expression Software Tool (REST 2008) (Pfaffl et al., 2002). All biological replicas were tested with each primer set. The primer sequences for Brassica genes representing Arabidopsis homologues (which were regulated in the microarrays) are described and pooled (Supplementary Tables S2 and S3).

Statistical analysis of feeding experiments

Statistical analyses were performed using R language (R Development Core Team, 2011), and IBM SPSS Statistics version 20. Nonparametric tests were used, since data in general could not be assumed to be normally distributed (based on Anderson-Darling normality tests and quantile-quantile plots). For the no-choice experiments the differences between the insect weight gain of larvae retrieved from the wild-type and MINELESS plants on days 3, 7, 8, 10, and 12 were analysed using Wilcoxon Mann-Whitney tests, where P < 0.05 was considered significant. For the induction experiments, the levels of myrosinase activity, glucosinolates, and glucosinolate hydrolysis products among four different groups (wild-type control, wild-type M. brassicae, MINELESS control, and MINELESS M. brassicae) were analysed by a non-parametric version of one-way analysis of variance, the Kruskal-Wallis test (where P < 0.05 was considered significant); after being considered significant, they were followed by the pairwise Wilcoxon Mann-Whitney tests using Bonferroni post hoc correction (where P < 0.0083 is considered significant for six tests). For the myrosinase activity and glucosinolates, only four observations were available in each group, and using a Wilcoxon Mann-Whitney test with two groups of four observations each (without ties), the smallest P-value that can be obtained is 0.02857, and thus in our data no pairwise comparisons could be found to be significant after Bonferroni correction (which requires P < 0.0083 when six comparisons are made). For the glucosinolate–myrosinase products, six observations were available in each group and the multicomView R-package (version 0.1–5) was used to categorize the four groups based on significance, as presented in Fig. 6 (http://cran.r-project.org/ and choose package multcompView).

Results

M. brassicae larvae show stunted growth on MINELESS plants

The feeding experiments with M. brassicae showed that larvae gained significantly less weight on MINELESS seedlings compared to the wild-type seedlings at all time points: (Experiment I) day 3, 8, 10, and 12, P < 0.001; (Experiment II)
day 7 and 12, \( P < 0.001 \) (Fig. 1). The average weights of larvae feeding on MINELESS plants were 1.5, 2.2, 4.1, and 3.6 times lower than the average weights of larvae that had been feeding on the wild-type for day 3, 8, 10, and 12, respectively (Experiment I) (Fig. 1A). Similarly, in feeding Experiment II, the average larvae weights were observed to be 2.2 and 2.6 times lower when feeding MINELESS seedlings compared to the wild-type for day 7 and 12, respectively (Fig. 1B). Both experiments showed similar reduction (2.2 times) in larvae weights for day 7 (Experiment II) and day 8 (Experiment I). How larvae feed on wild-type and MINELESS plants, and how they appeared after 12 days of feeding, can be seen in Supplementary Videos V1 and V2.

Due to lower weight gain and reduced development, most larvae that had been feeding on MINELESS plants appeared smaller than the larvae from wild-type plants (Fig. 2A–D) (Supplementary Videos V1 and V2). Larvae that had been feeding on MINELESS plants also showed a greater reduction in number compared to the wild-type (Fig. 1). The larvae also attacked MINELESS seedlings less than wild-type seedlings as the MINELESS seedlings were consumed less in comparison (Fig. 2E, F) (Fig. 3). This is consistent with the lower weight of larvae feeding on MINELESS plants (Fig. 1). The first experiment showed that larvae consumed 41.6%, 54.3%, 61.2%, and 31.9% less MINELESS tissue than the wild-type for day 3, 8, 10, and 12, respectively (day 3, 8, 10 and 12; \( P < 0.001 \)) (Fig. 3A) (Supplementary Fig. S1). Similarly, the second experiment showed this consumption to be 26% and 60.6% less for MINELESS compared to the wild-type for day 7 (\( P < 0.0077 \)) and day 12 (\( P < 0.001 \)) (Fig. 3B).

MINELESS plants show no difference for myrosinase activity after M. brassicae feeding

The Kruskal-Wallis test showed significant differences among the four groups wild-type control, wild-type M. brassicae, MINELESS control, and MINELESS M. brassicae (\( P < 0.05 \)) (Fig. 4). The myrosinase activity was nearly comparable in MINELESS control seedlings and MINELESS M. brassicae seedlings. The myrosinase activity was very low in MINELESS control seedlings (1.85 nmol glucose min \(^{-1} \) mg \(^{-1} \) protein) compared to the wild-type control seedlings (54.4 nmol glucose min \(^{-1} \) mg \(^{-1} \) protein). The feeding by larvae of M. brassicae caused a 41% reduction in myrosinase activity in wild-type seedlings. The pair-wise comparisons of wild-type control vs MINELESS control, wild-type control vs wild-type M. brassicae, and wild-type M. brassicae vs MINELESS M. brassicae showed differences with a \( P \) value of 0.02857, except MINELESS control vs MINELESS M. brassicae (Fig. 4).

Glucosinolate concentration changes after M. brassicae feeding

Five different glucosinolates were detected in wild-type control as well as MINELESS control seedlings (Fig. 5). These five glucosinolates were (2S)-2-hydroxy-3-butenyl (epiprogoitrin); and the indole glucosinolates indol-3-yl-methyl (13M) (glucobrassicin), 4-hydroxy-indol-3-yl-methyl (4OH-13M) (4-hydroxyglucobrassicin), 4-methoxy-indol-3-yl-methyl (4MO-13M) (4-methoxyglucobrassicin), and 1-methoxy-indol-3-yl-methyl (1MO-13M) (neoglucobrassicin). The Kruskal-Wallis test (\( P < 0.05 \)) showed significant differences among the four groups wild-type control, wild-type M. brassicae, MINELESS control, and MINELESS M. brassicae for 4-hydroxy-13M, 13M, 1-methoxy-13M, and total glucosinolates, with levels higher in MINELESS control seedlings compared to wild-type control seedlings (Fig. 5).

M. brassicae feeding increased 13M, 10M-13M, and total glucosinolate levels in both wild-type and MINELESS seedlings (\( P = 0.02857 \)); however, 4OH-13M was observed to be enhanced only in MINELESS seedlings (\( P = 0.02857 \)) (Fig. 5). The glucosinolate 4OH-13M was higher in MINELESS M. brassicae-challenged seedlings than wild-type M. brassicae-challenged seedlings (\( P = 0.02857 \)). In contrast, 1MO-13M was nearly significantly different (\( P = 0.057 \)) in wild-type

\[ \text{Fig. 1. Growth of } M. \text{ brassicae on wild-type and MINELESS plants in no-choice feeding experiments. (A, B) The weights of larvae feeding on wild-type and MINELESS seedlings differed significantly for day 3, 8, 10, and 12 (A); and day 7 and 12 (B). } n, \text{ number of larvae. Values represent mean ± SE; ***, } P < 0.001 \text{ (Wilcoxon Mann-Whitney test).} \]
M. brassicae-challenged seedlings and MINELESS M. brassicae challenged seedlings.

Glucosinolate–myrosinase hydrolysis products vary between wild-type and MINELESS with and without M. brassicae feeding

In total, seven glucosinolate-degradation products could be detected by the solvent extraction of wild-type and MINELESS seedlings (Fig. 6). These compounds were 3-butenyl isothiocyanate (3BITC), 1-methyl thiopentane (1MTPe), 1-methyl thiohexane (1MTHx), benzyl nitrile (BNIT), 1-methoxy indole-3-yl-methylnitrile (1MI3M NIT), indole-3-yl-methylnitrile (I3M NIT), and 4-methoxy indole-3-yl-methylnitrile (4MI3M NIT). The Kruskal-Wallis test \((P < 0.05)\) showed significant differences among the four groups wild-type control, wild-type M. brassicae, MINELESS control, and MINELESS M. brassicae for all compounds except 3BITC. The wild-type control and MINELESS control seedlings differed significantly for 1MTPe, 1MTHx, I3M NIT, 1MI3M NIT, 4MI3M NIT, and for the total glucosinolate–myrosinase hydrolysis products \((P < 0.05)\). I3M NIT, 4MI3M NIT, and total glucosinolate–myrosinase hydrolysis products were low in MINELESS control seedlings compared to their wild-type control, while 1MTPe and 1MTHx showed higher levels in MINELESS control seedlings (Fig. 6).

M. brassicae feeding significantly increased levels of 1MI3M NIT \((P < 0.002)\) in both wild-type and MINELESS seedlings (Fig. 6E). The wild-type M. brassicae-challenged seedlings differed from MINELESS M. brassicae-challenged seedlings for 1MTPe, 1MTHx \((P < 0.002)\), and 4MI3M NIT \((P < 0.004)\) (Fig. 6).
Gene regulation in wild-type and MINELESS plants upon M. brassicae attack

Oligonucleotide microarrays from Arabidopsis were used and the microarray data were validated by qRT-PCR using Brassica-specific sequences corresponding to Arabidopsis genes. B. napus and Arabidopsis are both members of the Brassicaceae family and shared a common ancestor about 13–17 million years ago. This means that orthologous/paralogous gene pairs from these plants have retained high homology to each other even at the DNA level. Previous analyses have shown that 87% of sequences are conserved between B. napus and Arabidopsis (Cavell et al., 1998). The close evolutionary relationship between Arabidopsis and other Brassica spp. means that Arabidopsis microarrays can be used for gene expression studies of Brassica spp. Arabidopsis microarrays have been successfully applied and compared for gene expression studies in Brassica spp. Examples include studies of insect feeding by Pieris rapae in B. oleracea (Broekgaarden et al., 2007), infestation by Brevicoryne brassicae (Broekgaarden et al., 2008), and the identification of plant defence genes in canola (B. napus) (Schenk et al., 2008).

Transcriptional analysis showed 494 genes to be differentially regulated in wild-type seedlings after M. brassicae feeding. Out of these 494 genes, 393 were upregulated (log2 ratio > 0.5; adjusted P ≤ 0.05), and 101 downregulated (log2 ratio < –0.5; adjusted P ≤ 0.05) (Fig. 7A). In contrast, in MINELESS seedlings, after M. brassicae feeding, only 159 genes were found to be significantly regulated. Out of these 159 genes, 129 showed induction (log2 ratio > 0.5; adjusted P ≤ 0.05), while 30 genes showed suppression (log2 ratio < –0.5; adjusted P ≤ 0.05) (Fig. 7B). Comparison of gene expression data of wild-type M. brassicae-challenged seedlings to MINELESS M. brassicae-challenged seedlings showed 326 genes to be differentially regulated. Of these, 209 genes showed suppression (log2 ratio < –0.4; adjusted P ≤ 0.05), while the remaining 117 genes showed induction in MINELESS seedlings (log2 ratio > 0.4; adjusted P ≤ 0.05) (Fig. 7C).

Expression patterns of genes representing jasmonic acid biosynthesis and signalling pathway

Several genes involved in the jasmonic acid (JA) biosynthesis and signalling pathway were upregulated in wild-type seedlings after M. brassicae attack (Fig. 8) (Supplementary Table S4). B. napus genes encoding homologues of Arabidopsis LOX2, LOX3, LOX4, AOS, AOC2, and AOC3, all involved in the initiation of JA synthesis in plastids, were upregulated. The gene OPCLI, encoding enzyme OPC-8:CoA ligase 1, that esterifies a CoA to the acyl group of OPC-8:0 (Acosta and Farmer, 2010), also showed upregulation in wild-type. Two acyl-coenzyme A oxidase genes,
Oilseed rape MINELESS plants after *Mamestra brassicae* attack

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**Fig. 5.** Glucosinolate levels in control and *M. brassicae* challenged seedlings (n = 4). WC, wild-type control; MC, MINELESS control; WM, wild-type *M. brassicae*; MM, MINELESS *M. brassicae*. Error bars represent SE. No significant differences were found after post hoc Bonferroni corrections (P < 0.00833).

*ACX1* and *ACX5*, and *KAT2*, involved in (the three cycles of) β-oxidation leading to the production of JA, were also induced. JA insensitive 1 (*JIN1/MYC2*), which regulates the transcription of JA-responsive genes, was also upregulated. Three JA-responsive genes (*JR1*, *JR2*, and *JR3*) and *VSP2* were strongly upregulated. Some of the genes involved in the JA biosynthesis and signaling pathways (*LOX2*, *AOS*, and *OPCL1*) were also upregulated in MINELESS seedlings after *M. brassicae* attack, although their regulation was relatively low compared to gene regulation in the wild-type (Fig. 8) (Supplementary Table S5). After *M. brassicae* attack on MINELESS seedlings, the genes that were most induced were homologues of *AOS*, *LOX2*, *OPCL1*, *ACX1*, *ACX5*, *KAT2*, *JAZ1*, *JAZ2*, *JAZ3*, *JAZ9*, *JR2*, *JR3*, and *VSP2* showed lower induction or downregulation in MINELESS seedlings compared to the wild-type seedlings. (Fig. 8) (Table S6).

Expression patterns of genes representing pathways of tryptophan biosynthesis and indole glucosinolate biosynthesis

The attack by *M. brassicae* on wild-type plants resulted in upregulation of *B. napus* genes homologous to *Arabidopsis ASA1*, *TRP1*, *IGPS*, *TSA1*, *TSB1*, *TSB2*, and *TSB3*, which are involved in tryptophan biosynthesis from chorismate (Fig. 9) (Supplementary Table S4). In contrast, MINELESS seedlings only showed induction of *ASA1*, *TRP1*, *IGPS*,

A comparison of *M. brassicae*-challenged MINELESS seedlings to *M. brassicae*-challenged wild-type seedlings showed that the JA responses were stronger in wild-type seedlings compared to MINELESS seedlings. *B. napus* genes homologous to *LOX4*, *AOS*, *AOC2*, *AOC3*, *OPCL1*, *ACX1*, *ACX5*, *KAT2*, *JAZ1*, *JAZ2*, *JAZ3*, *JAZ9*, *JR2*, *JR3*, and *VSP2* showed lower induction or downregulation in MINELESS seedlings compared to the wild-type seedlings. (Fig. 8) (Table S6).
cytochrome P450 proteins, and GSTF9, GGP1, and SUR1, involved in the biosynthesis of I3M glucosinolate from tryptophan (Fig. 9) (Supplementary Table S6). CYP81F4 was strongly upregulated in wild-type seedlings after M. brassicae attack. CYP81F4 has been reported to be responsible for the production of 1MOI3M glucosinolate (Pfalz et al., 2011), which is produced through a hydroxylation reaction of the glucosinolate indole ring leading from I3M to the 1-hydroxy-indol-3-yl-methyl (1-OH-I3M) glucosinolate intermediate. The 1-OH-I3M glucosinolate intermediate is converted to 1MOI3M by either indole glucosinolate methyltransferase 1 (IGMT1) or IGMT2. In wild-type seedlings, IGMT5 was induced after M. brassicae feeding. IGMT5 shares 70% of its sequence identity with IGMT1 and IGMT2 (Pfalz et al., 2011). The B. napus homologues of CYP79B2, CYP79B3, CYP83B1, GSTF9, and SUR1, all key genes in glucosinolate biosynthesis, showed moderate to strong upregulation in MINELESS seedlings upon M. brassicae feeding (Fig. 9) (Supplementary Table S5). The comparison of wild-type M. brassicae-challenged seedlings to MINELESS M. brassicae-challenged seedlings showed that B. napus homologues of CYP79B2, CYP79B3, CYP83B1, GSTF9, SUR1, CYP81F4, and IGMT5 were downregulated in MINELESS seedlings compared to wild-type seedlings (Fig. 9) (Supplementary Table S6).

Validation of genes belonging to JA biosynthesis and indole glucosinolate pathways using qPCR

A subset of genes belonging to JA and glucosinolate pathways that were differentially regulated in wild-type and MINELESS seedlings after M. brassicae feeding were selected for qPCR analysis. Based on the Arabidopsis gene information, locus gene IDs, gene sequences, and microarray probe sequences, the corresponding B. napus gene homologues were identified, and these DNA sequences were used to design B. napus-specific primers (Supplementary Tables S2 and S3). For wild-type M. brassicae-challenged seedlings, the expression patterns of seven genes, LOX3, AOS, and VSP1 (JA-biosynthesis and signalling), CYP79B2, SUR1, CYP83B1, and CYP81F4 (indole glucosinolate biosynthesis), corresponded well to the profiles obtained from microarray data (Fig. 10A). Similarly, for MINELESS M. brassicae-challenged seedlings, the expression patterns of five genes, AOS, VSP1, CYP79B2, SUR1, and CYP83B1 matched well with the patterns obtained from microarray data (Fig. 10B). The qRT-PCR validation of three genes from wild-type M. brassicae-challenged seedlings compared to MINELESS M. brassicae-challenged seedlings also showed correspondence with gene expression profiles obtained from microarray data (Fig. 10C).

Discussion

Based on insect no-choice feeding experiments, we found that M. brassicae (a generalist) larvae show reduced growth and less preference for MINELESS seedlings compared to the wild-type seedlings (Fig. 1) (Supplementary videos V1 and V2). Accordingly, we observed that M. brassicae larvae showed upregulation in wild-type seedlings upon M. brassicae feeding: CYP79B2, CYP79B3, and CYP83B1, encoding and TSA1 after M. brassicae attack (Fig. 9) (Supplementary Table S5). The comparison of wild-type M. brassicae-challenged seedlings to MINELESS M. brassicae-challenged seedlings showed that genes corresponding to ASA1, IGP5, TSA1, TSB1, and TSB2 were downregulated or less affected in MINELESS seedlings (Fig. 9) (Supplementary Table S6).
consumed more of wild-type cotyledons than MINELESS seedlings (Figs 2–3, Supplementary Figure S1). Additionally, the data showed a reduction in survival rate of larvae on MINELESS compared to the wild-type during their development period from 3 to 12 days (Fig. 1). The MINELESS plants seemed to be more resistant to M. brassicaceae herbivory compared to the wild-type as larvae either developed more slowly or died on MINELESS plants. This could be due to high glucosinolate levels (Fig. 5), or to the presence of new glucosinolate–myrosinase hydrolysis products, 1MTPe and 1MTHx (Fig. 6), which were 36.5- and 38.8-fold higher in the MINELESS control compared to the wild-type control. The detection of high amounts of these compounds particularly in MINELESS seedlings is interesting as we have not found these compounds before in MINELESS seeds or seedlings (Borgen et al., 2010; Ahuja et al., 2011; Borgen et al., 2012). 1 MT Pe has been detected in B. napus and B. oleracea (Romanesco cauliflower) (Tollsten and Bergström, 1988; Valette et al., 2003), and 1MTHx in seeds of Prunus domestica (Ahmed et al., 2007).

Plants show a diverse range of defences that may vary in effectiveness against generalist and specialist insect herbivores (Ahuja et al., 2010; Ali and Agrawal, 2012; Poelman et al., 2008). The results from previous insect feeding experiments with Arabidopsis myrosinase mutants seem to vary for generalist and specialist herbivores. In that study, the weight of Trichoplusia ni larvae was significantly increased on tgg1 tgg2 mutants, while the specialist Pieris rapae performed better on the wild-type than on tgg1 tgg2 mutants, which has been considered to be due to reduced feeding stimulants (glucosinolate–myrosinase hydrolysis products) in tgg1 tgg2 mutants. Our results of challenging myrosinase-mutated MINELESS plants to M. brassicaceae can be seen as somewhat similar to the response of P. rapae to tgg1 tgg2 mutants (Barth and Jander, 2006), as myrosinase-mutated MINELESS plants also have reduced feeding stimulants (glucosinolate–myrosinase hydrolysis products) (Ahuja et al., 2011). The authors speculated about the possibility that the Arabidopsis double mutant tgg1 tgg2 could produce another deterrent to compensate for the loss of glucosinolate–myrosinase hydrolysis products (Barth and Jander, 2006). Additionally, it is worth mentioning that even if M. brassicaceae is not a specialist, it has some association or preference for cruciferous crops (Bretherton et al., 1979; Rojas et al., 2000; Beekwilder et al., 2008; Ploomi et al., 2009), further highlighting that M. brassicaceae may be a specialist for Brassica plants and not a generalist. Furthermore, an approximate 4-fold difference in myrosinase activity among lines of B. juncea decreased feeding by Plutella xylostella (a crucifer specialist) on the lines with highest activity relative to the lowest, but there was no difference in feeding by Spodoptera eridania (a generalist) (Li et al., 2000). In another similar observation, in B. rapa populations that were artificially selected for divergent myrosinase levels (~2.5-fold higher), the high myrosinase population was more resistant to the flea beetle Phyllotreta cruciferae than the low myrosinase population (Siemens and Mitchell-Olds, 1998). On the other hand, variation in myrosinase levels had no effect on the feeding behaviour of Brevicoryne brassicae on Arabidopsis (Barth and Jander, 2006) or Athalia rosae on Sinapis alba (Müller and Sieling, 2006; Travers-Martín and Müller, 2007).

The myrosinase activity in wild-type seedlings was almost in the same range as it has been reported previously for B. napus hypocotyls (Bones, 1990). In recent years, some non-traditional myrosinases (glucosidases) have been reported to degrade indole glucosinolates (Bednarek et al., 2009; Clay et al., 2009; Bednarek et al., 2011). This indicates that there might also be such glucosidases in B. napus. If present, where these are potentially acting is still unknown. The standard myrosinase assay revealed low myrosinase activity in MINELESS, and analysis of glucosinolate hydrolys products clearly shows low concentrations, which could be taken as a measure that if such non-traditional myrosinases are present, they are less active than the traditional thioglucosidases.

Myrosinase activity was reduced by M. brassicaceae feeding in wild-type seedlings, but not in MINELESS seedlings. The latter was not surprising as the myrosinase activity in non-challenged MINELESS seedlings is extremely low. Previous studies on myrosinase activity in response to herbivores have also reported a net decline in activity, for example after A. rosae feeding on B. juncea (Müller and Sieling, 2006) and P. xylostella feeding on B. napus (Pontoppidan et al., 2005). These studies showed decreases in soluble activity, which were also observed for the wild-type after M. brassicaceae feeding. On the other hand, some studies revealed induction or increases in myrosinase activity after herbivore attack by B. brassicaceae on wild-type and MINELESS seedlings (Borgen et al., 2012) and, for example, by P. xylostella on B. rapa and by A. rosae on S. alba (Martin and Müller, 2007; Travers-Martín and Müller, 2007). Moreover, Textor and Gershenson mentioned in a review that there is no general induction of myrosinase activity after herbivory, but that it instead triggers the appearance of various associated proteins, the functions of which remain to be explained (Textor and Gershenson, 2009).

In this study, the total glucosinolate levels were observed to be higher in MINELESS control seedlings relative to wild-type
control seedlings. Moreover, in our previous studies, we have shown the total glucosinolate levels to be higher in MINELESS control (non-infested) seeds than wild-type control seeds and seedlings (Borgen et al., 2010; Ahuja et al., 2011; Borgen et al., 2012). Glucosinolates form a constitutive defence (Wittstock and Gershenzon, 2002), but it is also evident that these compounds accumulate in response to wounding or herbivory (Doughty et al., 1995; Bartlet et al., 1999; Brader et al., 2001; Mikkelsen et al., 2003). A variety of glucosinolates are required to limit the growth of various insect herbivores (Gols et al., 2008; Müller et al., 2010). Gols and colleagues found a negative relationship between the total levels of glucosinolates and survival of M. brassicae larvae, proposing that M. brassicae responds to high levels of glucosinolates rather than to specific glucosinolates (Gols et al., 2008). The levels of three glucosinolates (4OH-I3M, I3M, and 4MO-I3M), although non-significant, showed more elevation in MINELESS control seedlings compared to the wild-type seedlings. At the same time, MINELESS showed low myrosinase activity, which meant that more glucosinolates were intact, giving the seedlings higher glucosinolate levels (Borgen et al., 2010; Ahuja et al., 2011; Borgen et al., 2012), which probably also affects the growth of M. brassicae larvae. The levels of most of the indole glucosinolates were increased in both

**Fig. 8.** Regulation of genes involved in the jasmonic acid (JA) pathway and JA responsiveness after M. brassicae feeding. The colour scale represents log₂-transformed gene expression ratios. The grey boxes represent non-regulated genes. More detailed information about genes is given in Supplementary Tables S4, S5, and S6. Abbreviations: MM-MC, MINELESS M. brassicae challenged vs MINELESS control; WM-WC, wild-type M. brassicae challenged vs wild-type control; MM-WM, MINELESS M. brassicae challenged vs wild-type M. brassicae challenged.

**Fig. 9.** Regulation of genes involved in tryptophan and glucosinolate (GSL) biosynthesis pathways after M. brassicae feeding. The colour scale represents log₂-transformed gene expression ratios. The grey boxes represent non-regulated genes. More detailed information about genes is given in Supplementary Tables S4, S5, and S6. Abbreviations: MM-MC, MINELESS M. brassicae challenged vs MINELESS control; WM-WC, wild-type M. brassicae challenged vs wild-type control; MM-WM, MINELESS M. brassicae challenged vs wild-type M. brassicae challenged.
wild-type and **MINELESS** *M. brassicae*-challenged seedlings. Similarly, indole glucosinolate biosynthesis genes (*CYP79B2, CYP79B3, SUR2, SUR1*, and *GSTF9*), also showed upregulation in both wild-type and **MINELESS** after *M. brassicae* feeding. The genes *CYP79B2* and *SUR2*, catalysing the conversion of tryptophan to indole-3-acetaldoxime (IAOx), also showed induction in *B. oleracea* and *Arabidopsis* after *P. rapae* feeding (Reymond et al., 2004; Broekgaarden et al., 2008). Similar responses for these genes have also been observed in *Arabidopsis* ecotypes upon feeding by aphids *Brevicoryne brassicae* and *Myzus persicae* (Kusnierczyk et al., 2007). An induction of genes of the indole glucosinolate pathway, and of indole glucosinolates levels, has frequently been reported as a herbivore response in *Brassicaceae* and the model plant *Arabidopsis* (Bodnaryk, 1992; Hopkins et al., 1998; Bartlet et al., 1999; Kusnierczyk et al., 2007; Kusnierczyk et al., 2008; Poelman et al., 2008; Textor and Gershenzon, 2009). *CYP81F4* (strongly induced in wild-type seedlings) has been shown to be mainly responsible for the production of 1MO-13M (Pfalz et al., 2011) (Figs. 9 and 10A). Our results showed a corresponding increase in glucosinolate 1MO-13M in wild-type seedlings after *M. brassicae* feeding (Fig. 5), thereby supporting the role of *CYP81F4* in production of glucosinolate 1MO-13M. A very similar trend was also observed for its hydrolysis product 1MI3M NIT (Fig. 6E).

The induction of JA synthesis and signalling pathways, and JA responsive genes, is a well known response to insect attack (Acosta Iván and Farmer Edward, 2010; Ballaré, 2011; Verhage et al., 2011). Genes involved in the biosynthesis of JA are upregulated after feeding by insect herbivores such as *P. rapae* and *P. xylostella* (Reymond et al., 2004; Broekgaarden et al., 2007; Ehlting et al., 2008; Kusnierczyk et al., 2011), and JA has been shown to be responsible for *Arabidopsis* resistance to cabbage looper (*T. ni*) (Chehab et al., 2011). It is therefore not surprising that key genes involved in JA synthesis and signalling, such as *LOX2, AOS, AOC2, OPCL1, OPR1, ACX1, KAT1, MYC2*, and several JAZs, were upregulated in wild-type seedlings after *M. brassicae* feeding. However, in **MINELESS** seedlings after *M. brassicae* feeding, only a few genes (*LOX2, AOS, OPCL1, OPR1, ACX1, KAT1, MYC2*, and several JAZs) showed upregulation with relatively low induction levels compared to the wild-type (Fig. 8). The regulation of fewer genes of the JA pathway, and their low expression levels in **MINELESS** compared to the wild-type, could be due to the reduced feeding by *M. brassicae* larvae on **MINELESS** plants. However, we cannot rule out the possibility that it could also be due to lower levels of myrosinase and glucosinolate–myrosinase hydrolysis products, leading to reduced induction of genes in the JA-signalling pathway in **MINELESS** plants after attack by *M. brassicae* larvae.

**Conclusions and perspectives**

Plant–insect interactions have been studied using the insect herbivore cabbage moth (*M. brassicae*), and wild-type and **MINELESS** *B. napus* plants that lack plant defence cells
called myrosin cells, also known as the toxic mustard oil mines. The results showed that *M. brassicae* larvae chewed more and performed better on wild-type *B. napus* plants than on *MINELESS B. napus* plants. The reduced performance of *M. brassicae* larvae on *MINELESS* seedlings is possibly due to the higher levels of indole- and total glucosinolates in *MINELESS* control (non-infested) seedlings. Due to the reduction in myrosinase levels, *MINELESS* plants have reduced amounts of glucosinolate hydrolysis products. However, the glucosinolate–myrosinase hydrolysis products, 1MTPe and 1MTHx, were observed in very high amounts in *MINELESS* compared to the wild-type seedlings, which might be affecting preference or feeding behaviour. The results also highlight that *M. brassicae*, which is a generalist herbivore but has some preference for Brassicaceae plants, can be a specialist for brassicains and not a generalist. As expected, the transcriptional responses showed JA as the key mediator of the defence response towards insect herbivory as several genes involved in the JA biosynthesis pathway, signalling, and JA responsiveness were upregulated in both wild-type and *MINELESS* seedlings. The genes belonging to tryptophan biosynthesis and indole glucosinolate pathway genes were induced, and the indole glucosinolate levels were elevated by *M. brassicae* feeding in both types of plants. A much higher induction of 1MO-I3M glucosinolate was observed for *M. brassicae* in wild-type plants compared with *MINELESS* plants. The comparison of wild-type *M. brassicae* and *MINELESS M. brassicae*-challenged seedlings showed a number of genes for JA biosynthesis, signalling, and JA-responsiveness, and tryptophan and glucosinolate biosynthesis, to be downregulated in *MINELESS* seedlings. The downregulation of genes *ASA1, IGPS, TSA1, TSB1, TSB2, CYP79B2, CYP79B3, CYP83B1, GSTD9*, and *SURI* in *MINELESS brassicae*-challenged seedlings compared to the wild-type *M. brassicae*-challenged seedlings probably leads to lower accumulation of the glucosinolates indolyl-3-yl-methyl, 1-methoxy-indol-3-yl-methyl, and 4-methoxy-indol-3-yl-methyl with respect to their control (non-infested) seedlings. Moreover, the induction of fewer genes and lower expression levels in *MINELESS* after *M. brassicae* feeding compared to the wild-type could be due to less feeding by *M. brassicae* larvae in comparison to the wild-type, which needs to be explored further. Currently, we are using *MINELESS* plants as a representative model for studying defence responses against other insect herbivores, including both generalists and specialists, and above- and below-ground herbivores, to get an overview about the role of myrosin cells in plant–insect interactions. We think that performing such studies will provide more information about the importance of plant defence cells in Brassicaceae plants.

**Supplementary material**

Supplementary data can be found at *JXB* online.

**Supplementary Table S1.** Time-line of insect no-choice and induction experiments.

**Supplementary Table S2.** Selected *B. napus* genes confirmed by qRT-PCR based on differential regulation in microarray results.

**Supplementary Table S3.** Primer sequences.

**Supplementary Table S4.** Regulation of JA biosynthesis, signalling, and JA-responsive genes; and tryptophan and glucosinolate biosynthesis pathway genes. This is in wild-type *M. brassicae*-challenged seedlings, after comparison of these to wild-type control seedlings.

**Supplementary Table S5.** Regulation of JA biosynthesis, signalling, and JA-responsive genes; and tryptophan and glucosinolate biosynthesis pathway genes. This is in *MINELESS M. brassicae*-challenged seedlings, after comparison of these to *MINELESS* control seedlings.

**Supplementary Table S6.** Regulation of JA biosynthesis, signalling, and JA-responsive genes; and tryptophan and glucosinolate biosynthesis pathway genes. This is in *MINELESS M. brassicae*-challenged seedlings, after comparison of wild-type *M. brassicae*-challenged seedlings to *MINELESS M. brassicae*-challenged seedlings.

**Supplementary Figure S1.** Control (non-infested) and *M. brassicae*-damaged cotyledons of wild-type and *MINELESS* seedlings from no-choice feeding experiments. (A, E, I, M) Wild-type control cotyledons from day 3, 8, 10, and 12, respectively. (B, F, J, N) Wild-type damaged cotyledons from day 3, 8, 10, and 12, respectively. (C, G, K, O) *MINELESS* control cotyledons from day 3, 8, 10, and 12, respectively. (D, H, L, and P) *MINELESS* damaged cotyledons from day 3, 8, 10, and 12, respectively.

**Supplementary Video S1.** *M. brassicae* larvae feeding on wild-type plants.

**Supplementary Video S2.** *M. brassicae* larvae feeding on *MINELESS* plants.

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**References**


