New Phytologist

Effect of prior drought and pathogen stress on *Arabidopsis* transcriptome changes to caterpillar herbivory

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Received: *7 October 2015*
Accepted: *6 December 2015*

New Phytologist (2016) **210:** 1344–1356
doi: 10.1111/nph.13847

Key words: abiotic stress, *Botrytis cinerea*, combined stresses, insect herbivory, multiple stresses, *Pieris rapae*, RNAseq, transcriptome.

Summary

- In nature, plants are exposed to biotic and abiotic stresses that often occur simultaneously. Therefore, plant responses to combinations of stresses are most representative of how plants respond to stresses.
- We used RNAseq to assess temporal changes in the transcriptome of *Arabidopsis thaliana* to herbivory by *Pieris rapae* caterpillars, either alone or in combination with prior exposure to drought or infection with the necrotrophic fungus *Botrytis cinerea*.
- Pre-exposure to drought stress or *Botrytis* infection resulted in a significantly different timing of the caterpillar-induced transcriptional changes. Additionally, the combination of drought and *P. rapae* induced an extensive downregulation of *A. thaliana* genes involved in defence against pathogens. Despite a more substantial growth reduction observed for plants exposed to drought plus *P. rapae* feeding compared with *P. rapae* feeding alone, this did not affect weight increase of this specialist caterpillar.
- Plants respond to combined stresses with phenotypic and transcriptional changes that differ from the single stress situation. The effect of a previous exposure to drought or *B. cinerea* infection on transcriptional changes to caterpillars is largely overridden by the stress imposed by caterpillars, indicating that plants shift their response to the most recent stress applied.

Introduction

During their life cycle, plants suffer from a broad range of stresses (Buchanan *et al.*, 2000). These include abiotic stresses (e.g. drought, flooding, heat, cold or nutrient deficiency) (Yamaguchi-Shinozaki & Shinozaki, 2006; Shinozaki & Yamaguchi-Shinozaki, 2007; Roy *et al.*, 2011; Fahad *et al.*, 2015; Mickelbart *et al.*, 2015) and biotic stresses, imposed by, for example, bacteria, fungi, viruses, insects or parasitic plants (Jones & Dangl, 2006; Howe & Jander, 2008; Dicke & Baldwin, 2010; Mithofer & Boland, 2012; Pieterse *et al.*, 2012; Dangl *et al.*, 2013; Pierik *et al.*, 2013; Stam *et al.*, 2014). Under natural conditions, these stresses do not occur in isolation but are commonly present simultaneously (Rizhsky *et al.*, 2004; Mittler & Blumwald, 2010; Vile *et al.*, 2012; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013; Kissoudis *et al.*, 2014; Rivero *et al.*, 2014; Sewelam *et al.*, 2014; Suzuki *et al.*, 2014). Due to their sessile nature, plants have evolved sophisticated mechanisms for tolerating or combatting multiple stresses (Howe & Jander, 2008; Dangl *et al.*, 2013; Mickelbart *et al.*, 2015).

Plants have evolved mechanisms for perceiving microbial pathogens, insect herbivores and abiotic stresses (Yamaguchi-Shinozaki & Shinozaki, 2006; Shinozaki & Yamaguchi-Shinozaki, 2007; Howe & Jander, 2008; Dangl *et al.*, 2013). Upon stress perception, plants can elicit defensive mechanisms in a stressor-specific manner (De Vos *et al.*, 2005; Kilian *et al.*, 2007; Bidart-Bouzat & Kliebenstein, 2011). Plant hormones have emerged as important players underlying the specificity in plant stress responses (Pieterse *et al.*, 2009, 2012; Verhage *et al.*, 2010; Erb *et al.*, 2012). For instance, salicylic acid (SA) especially mediates responses to phloem-feeding insects and biotrophic pathogens (De Vos *et al.*, 2005; Glazebrook, 2005), jasmonic acid (JA) especially mediates responses to chewing insects and necrotrophic pathogens (Reymond *et al.*, 2004; De Vos *et al.*, 2005; Bodenhausen & Reymond, 2007) and abscisic acid (ABA) mediates responses to abiotic stresses such as drought, cold and heat stress (Yamaguchi-Shinozaki & Shinozaki, 2006; Kilian *et al.*, 2007; Huang *et al.*, 2008).

Phytohormonal signalling pathways are known to interact with each other in a phenomenon coined 'crosstalk'. Crosstalk has been hypothesized to allow plants to respond in a fast, specific and cost-effective manner to stresses (Verhage *et al.*, 2010; Pieterse *et al.*, 2012; Vos *et al.*, 2013a). Interactions between phytohormonal signalling pathways can be antagonistic and

synergistic. For example, SA- and JA-mediated defences are known to exert negative effects on each other (Verhage *et al.*, 2010; Sendon *et al.*, 2011; Van der Does *et al.*, 2013; Caarls *et al.*, 2015), whereas the phytohormones ethylene (ET) and ABA have emerged as positive modulators of JA-mediated responses (Van der Ent *et al.*, 2008; Verhage *et al.*, 2010, 2011; Vos *et al.*, 2013b). Responses to necrotrophic pathogens and herbivorous caterpillars are mediated by different branches of the JA signalling cascade, the ERF and MYC branches, respectively (Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007). The ERF branch is activated by JA/ET through the transcription factors ERF1 and ORA59 and results in the expression of genes such as *PDF1.2* (Lorenzo *et al.*, 2003; Pre *et al.*, 2008; Verhage *et al.*, 2010). The MYC branch is activated by JA/ABA through the transcription factor MYC2, resulting in the expression of genes such as *VSP2* (Verhage *et al.*, 2011; Vos *et al.*, 2013b). The ERF and MYC2 transcription factors antagonistically regulate the two branches of the JA signalling pathway (Verhage *et al.*, 2011). Other hormones that mediate the responses of plants to environmental stresses include auxin (IAA), cytokinins, brassinosteroids, strigolactones and gibberellins (Erb *et al.*, 2012; Pieterse *et al.*, 2012; Giron *et al.*, 2013; Song *et al.*, 2014).

The simultaneous occurrence of stresses may modify the overall level of stress imposed on a plant. For instance, abiotic stresses tend to have a negative effect on plant responses to pathogens (Suzuki *et al.*, 2014; Ramegowda & Senthil-Kumar, 2015). Tomato plants exposed to simultaneous stress imposed by salinity and a pathogen were more susceptible to the pathogen than when exposed only to the pathogen (Kissoudis *et al.*, 2015). In *Arabidopsis thaliana,* drought promoted population growth of generalist aphids (Mewis *et al.*, 2012). Therefore, the question emerges of how plants elicit an effective defence response when exposed to multiple stresses. To address this question, several recent studies have conducted whole transcriptome profiling using microarrays, of plants exposed to multiple abiotic and biotic stresses (Atkinson *et al.*, 2013; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013; Sewelam *et al.*, 2014; Ramegowda & Senthil-Kumar, 2015; Sham *et al.*, 2015). These studies have shed light onto plant responses to multiple stresses at the molecular level. A clear pattern that emerged is that responses to combined stresses cannot be predicted from the responses to individual stresses (Atkinson & Urwin, 2012; Atkinson *et al.*, 2013; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013; Sewelam *et al.*, 2014; Suzuki *et al.*, 2014; Ramegowda & Senthil-Kumar, 2015; Sham *et al.*, 2015).

Quantifying the complete set of transcripts under specific ecophysiological conditions is essential to understand the regulatory mechanisms involved in acclimation to those conditions. With the reduction in costs of NGS (Next Generation Sequencing), RNAseq analysis is becoming feasible for large-scale transcriptomic analyses (Wang *et al.*, 2009; Van Verk *et al.*, 2013). RNAseq analysis offers several advantages over microarrays: no restriction to known genes, an unlimited dynamic range in quantitation, a more accurate expression level assessment, less sophisticated normalization needed and no problem with cross-hybridization of similar transcripts (Wang *et al.*, 2009; Ozsolak & Milos, 2011; Van Verk *et al.*, 2013). In addition, RNAseq analysis can extend the studies of transcriptomes to the analysis of splice variants.

Here, we used RNAseq to assess temporal changes in the transcriptomic response of *A. thaliana* to herbivory by *Pieris rapae* caterpillars alone or combined with exposure to drought or the necrotrophic pathogenic fungus *Botrytis cinerea*. These stresses were chosen because the response of *A. thaliana* to these three stresses is highly diverse, yet at the same time all responses are regulated by the plant hormone JA (in combination with ET in response to *P. rapae* and *B. cinerea,* and with ABA in response to drought). The main goals of this study were: to characterize transcriptomic changes of *A. thaliana* in response to herbivory by *P. rapae* caterpillars; to investigate to what extent transcriptome signatures elicited by caterpillar herbivory change when plants had been previously exposed to drought or *B. cinerea* fungal infection; and to specifically identify genes differentially expressed upon exposure to a combination of stresses. To our knowledge, this is the first study using a platform independent from microarrays to address the transcriptional responses of *A. thaliana* to herbivores.

Materials and Methods

Plants, insects and pathogens

Plant growth conditions *Arabidopsis thaliana* (L.) Heynh. Col-0 seeds were sown in containers (10 cm length, 5 cm width, 5 cm height) containing pasteurized (80°C for 4 h) sand that was humidified by adding 50 ml Hoagland solution. Seeds were sown at a density of *c.* 100 seeds per container. In order to keep 100% RH during germination, the containers were enclosed in a tray with a transparent lid. Seeds were vernalized for 2 d at 4°C in a dark room to overcome remaining dormancy and to induce even germination. Thereafter, the tray was moved to a controlled-environment chamber at 23 ± 1°C, 70 ± 10% relative humidity, 100 μmol m^{-2} s^{-1} photosynthetically active radiation and a diurnal cycle of 8 h : 16 h light : dark. After 1 wk, the lid was removed from the tray. Two-week-old seedlings were individually transplanted to 0.08-l pots (5 cm height × 5 cm diameter) containing a 1 : 1 mixture (v/v) of commercial potting soil and sand. Pots were watered at the bottom three times per week. Plants were subsequently grown under similar conditions until they were exposed to the treatments.

Herbivore rearing *Pieris rapae* L. (Lepidoptera: Pieridae; small cabbage white butterfly) were routinely reared on cabbage plants (*Brassica oleracea* var. *gemmifera* cv Cyrus) in a glasshouse as described previously (Van Poecke *et al.*, 2001; De Vos *et al.*, 2005).

Pathogen cultivation *Botrytis cinerea* strain B0510 was grown on 0.5× PDA plates, containing penicillin (100 μg ml^{-1}) and streptomycin (200 μg ml^{-1}), for 2 wk at room temperature. Spores were collected and suspended in 0.5× potato dextrose broth (Difco Laboratories, Sparks, MD, USA) to a final density

of 1.0×10^5 spores ml^{-1}. After a 3 h-incubation period, the spores were used for inoculation (see the Treatments subsection) (Thomma *et al.*, 1998; Pre *et al.*, 2008; Van der Ent *et al.*, 2008).

RNAseq experiment

Treatments Plants were exposed to five treatments: control (C), mock (M), *P. rapae* herbivory (P), drought plus *P. rapae* (DP) and *B. cinerea* plus *P. rapae* (BP). Plants were grown under similar conditions for 32 d after germination (DAG) (see Fig. 1). Plants were exposed to drought by withholding water for 7 d from 33 to 39 DAG. The drought treatment was followed by a 24-h recovery period (40 DAG). *Botrytis cinerea* inoculation was also carried out at 40 DAG. Plants were inoculated with *B. cinerea* by pipetting 5 µl of spores suspended in half-strength PDB at a concentration of 1×10^5 spores ml^{-1} on two leaves of the rosette. Plants were kept at 100% RH for 24 h in order to ensure successful infection by *B. cinerea*. Plants exposed to *P. rapae* as single or combined stress were inoculated with two first-instar (L1) *P. rapae* caterpillars on leaf number 8 (41 DAG). Caterpillars were allowed to move freely on the plant. Two controls were included in this experiment, a group of plants that did not experience any stress (Control) and a group of plants that were inoculated with 5 µl of mock solution on two leaves of the rosette (Mock). Mock solution consisted of the same medium that *B. cinerea* spores were suspended in. Plants treated with mock solution were also kept at 100% RH for 24 h. Only plants exposed to *B. cinerea* or mock solution were kept at 100% RH.

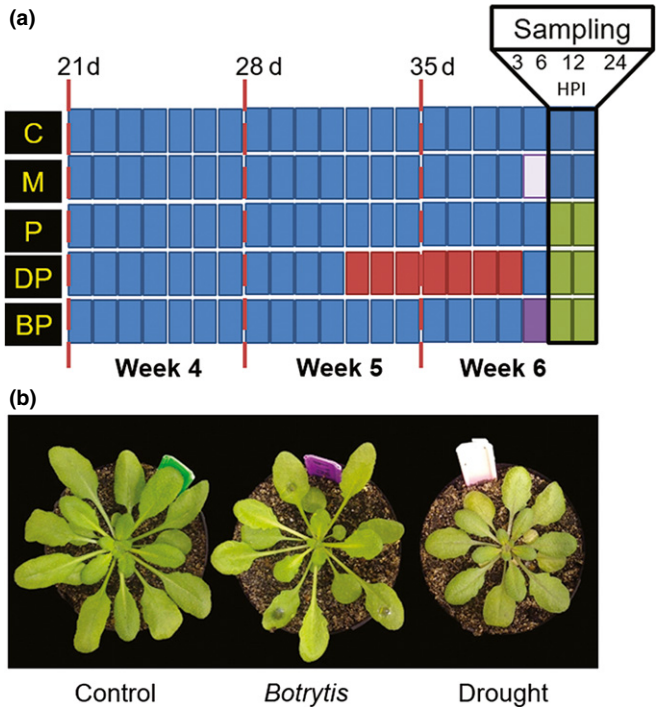

Fig. 1 Experimental Design. (a) Treatment and sampling schedule. *Arabidopsis thaliana* plants were exposed to one of five treatments: Single stress imposed by *Pieris rapae* herbivory (P), combined stresses drought plus *P. rapae* (DP) and *Botrytis cinerea* plus *P. rapae* (BP), Control (C) and Mock (M). Days after germination (DAG) are indicated in red dashed lines. Drought was imposed by not watering the plants for 7 d followed by 24 h recovery after rewatering (red boxes). *Botrytis cinerea* inoculation (dark purple box) was made 24 h before caterpillar inoculation (green boxes). Two types of controls were included: control, that is, plants that were not exposed to stress (blue boxes), and mock, that is, plants that were inoculated with the same medium used to inoculate the plants with *B. cinerea* spores (white box). Following the first stress, plants were inoculated with two neonate *P. rapae* caterpillars (green boxes) and samples for RNAseq analysis were taken for all five treatments at 3, 6, 12 and 24 h after inoculation (HPI). Three biological replicates were included per treatment and time point. Each biological replicate consisted of a pool of four plants. (b) Representative pictures of *A. thaliana* plants that had not been exposed to stress, exposed to *B. cinerea* infection or drought stress.

Experimental design The experiment was carried out in a full factorial design with two factors: time and treatment. Time had four levels (3, 6, 12 and 24 h) and treatment had five levels (control, mock, *P. rapae* herbivory, drought plus *P. rapae* and *B. cinerea* plus *P. rapae*). The experiment was carried out in a growth chamber, following a flat table design with three blocks (Supporting Information Fig. S1). Time was randomized within the three blocks. For every time point, treatments were assigned randomly. Each time and treatment combination consisted of three biological replicates. Each biological replicate consisted of pools of four plants. In total, we collected 60 samples (3 replicates × 5 treatments × 4 time points). RNA extraction was carried out in batches of 20 randomly chosen samples. Samples were sequenced single end (SE) 93 bp, on an Illumina Hi-Seq 2000 sequencer (Illumina Inc., San Diego, CA, USA). Samples were sequenced in three runs. Within each run, samples were randomly assigned to seven lanes of the Illumina flow cells.

Sampling Leaf samples were collected from plants exposed to *P. rapae* herbivory as single and combined stress and its respective controls; this was done at 3, 6, 12 and 24 h post inoculation (HPI) with *P. rapae* (Fig. 1). For plants under control and mock conditions, leaf number 8 was collected. For plants exposed to *P. rapae* as single or combined stress, leaf number 8 was collected when it showed caterpillar feeding damage. Otherwise, the leaf closest to leaf number 8 that displayed feeding damage was collected. Upon collection, leaf samples were immediately frozen in liquid nitrogen and stored at −80°C.

RNA extraction and library preparation RNA was extracted using Qiagen Plant RNeasy Plant Mini Kit (cat. no. 74903). All samples were treated with DNase I on column using the Qiagen RNase-Free DNase Set (cat. no. 79254). Quality and quantity of total RNA were initially measured with a NanoDrop ND-1000 (Nanodrop, Delaware, NC, USA). RNA quality was also checked using the RNA Integrity Number (RIN) with The Agilent 2100 bioanalyzer using RNA Nano chips. For library preparation we used only samples with RIN values ≥ 6. The sample preparation was performed according to the TruSeq Stranded mRNA HT Sample Prep Kit from Illumina. This protocol allows the identification of strand-specific transcripts. First, Poly-A RNA was

isolated from the total RNA using Poly-T oligo attached magnetic beads. Subsequently, RNA was fragmented using divalent cations under elevated temperature. First-strand cDNA was synthesized using random primers. Strand specificity is achieved by replacing dTTP with dUTP in the second Strand Marking Mix (SMM), followed by second strand cDNA synthesis using DNA Polymerase I and RNase H.

Validation of RNAseq by quantitative reverse transcription-PCR RNAseq was validated by reverse transcription RT-PCR as described in Fig. S2 and Table S1.

RNAseq analysis

Gene expression quantification RNAseq reads were mapped to the *A. thaliana* genome version TAIR 10 using TopHat (Trapnell *et al.*, 2009) with standard parameters and the following adjustments: -N 3 --min-intron-length 50 --max-intron-length 5000 -g 1 -M -p 4 -G --read-edit-dist 3 --library-type. The number of reads mapped to each annotated *Arabidopsis* gene (TAIR10) were determined using HTSeq-count (Anders *et al.*, 2014). Finally, raw read counts were subject to a regularized log transformation, implemented in the DESeq2 package (Love *et al.*, 2014), which normalizes counts for differences in sequencing depth across samples and produces expression values that are more suitable for clustering and visualization.

Differential gene expression Differential gene expression analysis was carried out with the DESeq2 Bioconductor package in R (Anders & Huber, 2010; Love *et al.*, 2014). Raw counts, which are the number of read pairs aligned to each TAIR 10 gene model with a maximum of three mismatches were used for this analysis. Differentially expressed genes (DEGs) were computed per time point. For each pair of treatments, we compared the resulting read counts from three biological replicates. Per individual time point, we performed the following comparisons among treatments: control vs *P. rapae*, *P. rapae* vs drought plus *P. rapae*, and *P. rapae* vs *B. cinerea* plus *P. rapae.* DESeq2 fits a generalized linear model (GLM) to the data, where counts per gene and sample are modelled using a negative binomial distribution. *P*-values were computed using a Wald test (Love *et al.*, 2014). Genes were considered to be differentially regulated in a given pair of treatments if they had a *P*-value ≤ 0.01 after Bonferroni correction and a $\log_2$-fold change ≤ -1 or ≥ 1.

Gene ontology analysis We used Bingo, implemented into Cytoscape 3.1.1 platform (Maere *et al.*, 2005), to identify enriched gene ontology (GO) terms in our gene lists. In all cases we used the following parameter settings: hypergeometric test, with Benjamini–Hochberg FDR adjustment, $\alpha = 0.05$. As background, we used only genes that were expressed in at least one sample out of the 60 samples analysed. GO term categories were reduced by semantic similarity using Revigo (Supek *et al.*, 2011). The following settings were used: allowed similarity (small) and semantic similarity measure (Simrel).

Statistical analysis

Principal component analysis We used principal component analysis (PCA) in the DESeq2 package in R (Love *et al.*, 2014). PCA was performed on the regularized $\log_2$-transformed data.

Orthogonal projection to latent structures – discriminant analysis Orthogonal projection to latent structures – discriminant analysis (OPLS-DA) was carried out on the regularized $\log_2$-transformed data with the software Simca P+ v.12 (Umetrics, Umeå, Sweden). The analysis shows the variable importance in the projection (VIP) of each variable (in this case, transcriptional data for the different genes), with variables having VIP values > 2 being most influential in the model (Eriksson *et al.*, 2006). One of the drawbacks of fitting a model with all variables (genes) is overparameterization and poor predictability (Perez-Enciso & Tenenhaus, 2003). Thus, we also fitted a reduced model using only variables with VIP ≥ 2 as described in Perez-Enciso & Tenenhaus (2003) and Burguillo *et al.* (2014). Alternatively, one could use a Student's *t*-test for each variable sequentially (Nguyen & Rocke, 2002). However, VIP is more appropriate because a Student's *t*-test cannot be applied for more than two classes and VIP takes into consideration the effect of a variable (gene) on all categories and OPLS components (Perez-Enciso & Tenenhaus, 2003).

Phenotype expression experiments

Plant bioassay An independent experiment was carried out to quantify the effect of herbivory by *P. rapae* alone or preceded by drought or *B. cinerea* on plant biomass. *A. thaliana* (Col-0) plants were exposed to five treatments: drought (D), herbivory by *P. rapae* (P), combinations of drought and *P. rapae* (DP) and *B. cinerea* and *P. rapae* (BP) and control (C). Pre-treatments before herbivory were applied in the same way as described for sample collection for RNAseq analysis. For treatments involving herbivory, 4-wk-old plants were infested with two L1 *P. rapae* caterpillars. Caterpillars were allowed to feed on the plants for 5 d. At the end of the experiment, shoot FW for the five treatments was quantified. A total of 65 biological replicates were included per treatment. Data were square-root transformed to satisfy the ANOVA assumptions of normality and homogeneity of variances. One-way ANOVA followed by Tukey's Honestly Significant Difference (Tukey HSD) *post hoc* tests for pairwise comparison were executed in R v.3.0.

Insect bioassay An independent experiment was carried out to quantify the effect of drought or *B. cinerea* pre-treatment on insect performance. *A. thaliana* (Col-0) plants were pre-treated with drought, *B. cinerea* or no stress. Pre-treatments before herbivory were applied in the same way as described for sample collection for RNAseq analysis. Subsequently, plants were infested with one L1 *P. rapae* caterpillar. Caterpillars were allowed to feed on the plants for 5 d. At the end of the experiment, caterpillar weight was quantified. A total of 20 biological replicates were included per treatment. Data were square-root transformed to satisfy the ANOVA assumptions of normality and homogeneity of variances. One-way ANOVA was executed in R v.3.0.

Results

Arabidopsis transcriptional changes in response to herbivory by *P. rapae* alone or in combination with previous exposure to drought or *B. cinerea*

Gene expression levels were quantified by RNAseq analysis for noninfested *A. thaliana* plants and for plants infested with *P. rapae* alone and either preceded by drought or infected by *B. cinerea* at four time points (3, 6, 12, and 24 HPI). The dataset was analysed by PCA (Fig. 2). The first two principal components (PCs) explained 42 and 19% of the variation, respectively. The first PC separated caterpillar-infested from noninfested plants and the second component separated the time point 24 HPI from the remaining, earlier, time points (Fig. 2a). When comparing only samples from infested plants either with *P. rapae* infestation alone, with *P. rapae* infestation preceded by exposure to drought, or with *P. rapae* infestation preceded by *B. cinerea* at the four different time points (Fig. 2b), the first two PCs explained 43 and 24% of the variation, respectively. Both PCs contributed to the separation of samples by time points. Separation was also observed for plants infested with *P. rapae* alone or in combination with either drought or *B. cinerea* in a time-dependent manner. For instance, the largest separation was at 3 HPI, whereas at 24 HPI the samples were more similar to each other. Thus, the timing of the transcriptional response to *P. rapae* caterpillars is significantly affected by prior drought or pathogen stress and that the differences diminished when the response developed with time.

Differential expression of *A. thaliana* genes in response to herbivory by *P. rapae* alone

Over the four time points of sample collection, a total of 3548 genes were differentially expressed (2755 up- and 793 downregulated) upon herbivory by *P. rapae* at 1% FDR (P3, P6, P12 and P24 in Fig. 3, Table S2). The number of up- and downregulated genes increased with time. Furthermore, the number of upregulated genes was higher than the number of downregulated genes at all time points (Fig. 3b, S3). A total of 59% and 31% of the differentially expressed genes (DEGs) were up- and downregulated, respectively, at more than one time point (Table 1; Fig. S3). On the one hand, samples collected at 24 HPI had the highest proportion of genes that were differentially expressed at only one time point (hereafter called time-point specific expression): 16% for up- and 31% for downregulated genes, respectively. On the other, samples collected at 3 HPI and 12 HPI displayed the lowest proportion of time-point specific up- (4%) and downregulated (10%) genes, respectively (Table 1). GO-term analysis revealed that up- and downregulated genes were associated with 58 and 16 processes, respectively (Table S3). Upregulated genes were associated with processes involved in secondary metabolism (e.g. production of flavonoids, phenylpropanoids, phytoalexins, and glucosinolates), phytohormone signalling pathways (e.g. JA, ET, ABA, SA and IAA), cell-wall modification and abiotic stresses (e.g. drought and cold responses). Downregulated genes were associated with processes related to plant defences, circadian rhythm and nitrate assimilation (Table S3).

Differential gene expression of *A. thaliana* in response to herbivory by *P. rapae* preceded by exposure to drought

Over the four time points, a total of 1025 (432 up- and 593 downregulated) genes were differentially expressed under combined drought and *P. rapae* stress compared with *P. rapae* alone at 1% FDR (Fig. 3; Table S4). A total of 26% of DEGs were shared with DEGs in plants exposed to *P. rapae* alone and 32% with DEGs in plants exposed to *B. cinerea* plus *P. rapae* (Fig. 3a). The number of up- and downregulated genes increased from 3 HPI to 6 HPI, followed by a lower number at the subsequent time points (Figs 3b, S3, S4). A total of 12% and 15% of all DEGs were up- and downregulated, respectively, at more than

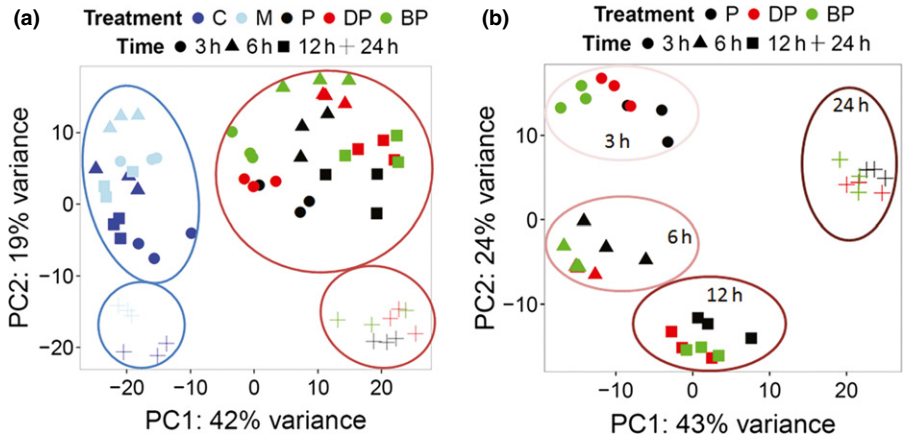

Fig. 2 Principal component analysis (PCA) of gene expression levels in *Arabidopsis thaliana* plants that had been noninfested, infested with *Pieris rapae* or infested by *P. rapae* after previous exposure to either drought or *Botrytis cinerea* infection; samples had been taken at four different time points. PCA was executed with DESeq2 software on the regularized $\log_2$-transformed data. The first two principal components are plotted. (a) PCA on all treatments and (b) PCA on the three treatments that included caterpillar feeding, while excluding the control and mock treatments. Colours indicate different treatments. Shapes indicate different time points. Percentages of variation explained by each PC are indicated along the axes. P, single stress imposed by *P. rapae* herbivory; DP, combined stresses drought plus *P. rapae*; BP, *B. cinerea* plus *P. rapae*; C, control; M, mock.

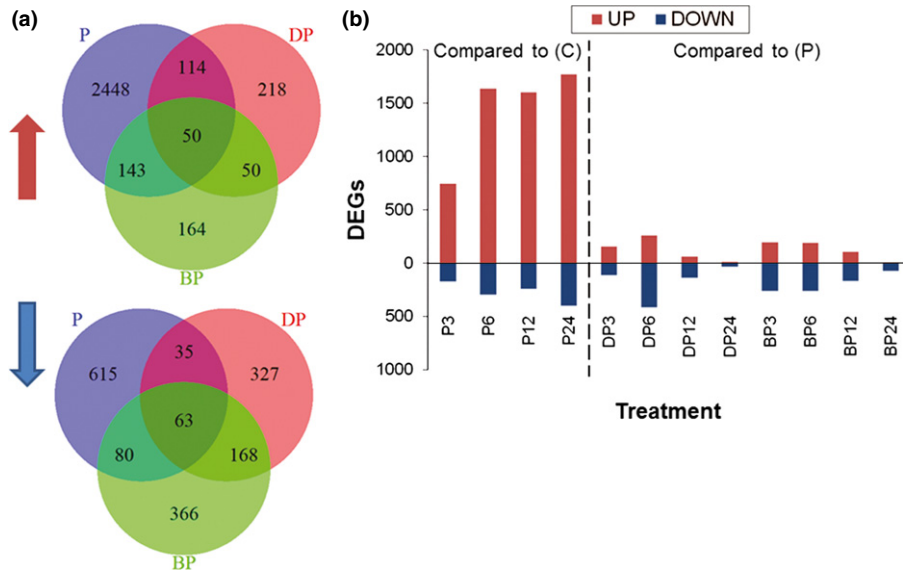

Fig. 3 Differentially expressed genes (DEGs) in *Arabidopsis thaliana* plants exposed to *Pieris rapae* herbivory alone or herbivory preceded by drought or *Botrytis cinerea* over time. (a) Venn diagrams showing DEGs per treatment, P, single stress *P. rapae*; DP, combined stresses drought plus *P. rapae*; BP, combined stresses *B. cinerea* plus *P. rapae*. (b) DEGs per time point and treatment combination. The time indications 3, 6,12 and 24 h refer to the numbers of hours after insect inoculation (HPI; see Fig. 1) at which the samples were collected. Genes were considered to be differentially regulated if they displayed a $\log_2$-fold change ≥1 for upregulated or ≤ −1 for downregulated genes with respect to the reference condition and a *P*-value ≤ 0.01 after Benjamini–Hochberg correction for false discovery rate.Gene expression levels for the single stress *P. rapae* were compared with the untreated control (C). Gene expression levels for the double stresses drought plus *P. rapae,* or *B. cinerea* plus *P. rapae* were compared with the single stress *P. rapae* (P).

Table 1 Proportion of genes up- and downregulated in *Arabidopsis thaliana* plants upon *Pieris rapae* herbivory alone or when preceded by drought or *Botrytis cinerea*, per time point post inoculation.

Time	Up	% Up	Down	% Down
Pieris rapae				
3 h	114	4.1	91	11.5
6 h	324	11.8	129	16.3
12 h	255	9.3	77	9.7
24 h	434	15.8	248	31.3
Common	1628	59.1	248	31.3
Total	2755	100.0	793	100.0
Drought and *P. rapae*				
3 h	132	30.6	81	13.7
6 h	222	51.4	334	56.3
12 h	21	4.9	69	11.6
24 h	5	1.2	19	3.2
Common	52	12.0	90	15.2
Total	432	100.0	593	100.0
Botrytis cinerea and *P. rapae*				
3 h	145	35.6	294	43.4
6 h	121	29.7	121	17.9
12 h	60	14.7	49	7.2
24 h	4	1.0	28	4.1
Common	77	18.9	185	27.3
Total	407	100.0	677	100.0

Number of genes differentially expressed genes specific for a specific time point are indicated. 'Common' reflects the number of genes differentially expressed at more than one time point.

one time point (Table 1; Fig. S3). Samples collected at 6 HPI displayed the highest proportion of time-point specific DEGs (51% up- and 56% for downregulated genes, respectively). On the other hand, samples collected at 24 HPI displayed the lowest proportion of time point-specific DEGs: 1% up- and 3% downregulated genes (Table 1). GO-term analysis revealed that up- and downregulated genes were associated with 22 and 38 processes respectively (Table S5). Upregulated genes were associated with processes involved in cytokinin metabolism and signalling, and flavonoid, phenylpropanoid and pigment biosynthesis. Downregulated genes were associated with processes related to immune responses, response to salicylic acid, photosynthesis and protein phosphorylation (Table S5).

Differential gene expression by *A. thaliana* in response to herbivory by *P. rapae* preceded by *B. cinerea* infection

Over the four time points, a total of 1084 (407 up- and 677 downregulated) genes were differentially expressed after treatment with *B. cinerea* infection followed by *P. rapae* infestation, compared with plants exposed only to *P. rapae,* at 1% FDR (Fig. 3; Table S4). A total of 31% of these 1084 genes were shared with DEGs in response to *P. rapae* alone and 31% with DEGs found in the combined exposure to drought followed by *P. rapae* caterpillars (Fig. 3a). The number of upregulated and downregulated genes decreased steadily over the time points (Fig. 3b). Furthermore, the number of downregulated genes was higher than the number of upregulated genes at all time points (Figs 3b, S3, S4). A total of 19% and 27% were up- and downregulated, respectively, at more than one time point (Table 1; Fig. S3). On the one hand, samples collected at 3 HPI displayed the highest proportion of time-point specific DEGs (36% up- and 43% downregulated genes, respectively). On the other,

samples collected at 24 HPI displayed the lowest proportion of specific up- (1%) and downregulated (4%) genes, respectively (Table 1). Up- and downregulated genes were associated with 24 and 51 processes, respectively (GO-term analysis; Table S5). Upregulated genes were associated with processes involved in lipid metabolism, response to temperature stimulus, wax metabolism, response to insects and regulation of anthocyanin metabolism. Downregulated genes were associated with processes related to immune responses, flavonoid metabolism, protein phosphorylation, defence response by callose deposition in cell wall and indole glucosinolate metabolism (Table S5).

Genes whose expression levels explain most of the observed transcriptomic differences between plants exposed to herbivory by *P. rapae* alone or preceded by exposure to drought or *B. cinerea*

We executed a discriminant analysis using OPLS-DA on the gene expression levels of *A. thaliana* plants that were noninfested, infested with *P. rapae* alone, or with *P. rapae* after exposure to drought or *B. cinerea*, at different time points (Fig. 4). A full model identified two significant components explaining 6% and 5% of the variation, respectively. The first PC separates plants infested with *P. rapae* alone or exposed to *B. cinerea* plus *P. rapae* from plants exposed to drought plus *P. rapae* (Fig. 4a). The second PC separates plants infested with *P. rapae* alone from those pre-infested with *B. cinerea* followed by infestation by *P. rapae* (Fig. 4a). Subsequently, we fitted a reduced model including only genes having VIP values ≥ 2 (see the Materials and Methods section). A total of 420 genes were identified as having VIP values ≥ 2 in the full model (Table S6). The reduced model identified two significant PCs, explaining 33% and 21% of the variance, respectively. The first PC separates plants exposed to drought plus *P. rapae* from plants exposed to *B. cinerea* plus *P. rapae* (Fig. 4b). The second PC separates plants infested with *P. rapae* alone from plants exposed to the two combined stress treatments (Fig. 4b). The expression patterns of the 420 genes with a VIP ≥ 2 are displayed in Fig. 4(c). Four clusters of genes were clearly different across treatments. Cluster 1 consists of 25 genes that were more upregulated at 3 HPI in the *B. cinerea* plus *P. rapae* treatment compared with the other two treatments, that is, drought plus *P. rapae* infestation or *P. rapae* infestation alone. Several genes involved in plant immunity belong to this cluster such as *BAP1* and *ERF104*. Cluster 2 consists of 13 genes that were upregulated at 3 HPI in the drought plus *P. rapae* treatment compared with *B. cinerea* plus *P. rapae* or *P. rapae* alone. Among the genes in cluster 2 are two ABA receptors (*PYL4* and *PYL5*). Cluster 3 consists of 17 genes that were downregulated at all time points in the drought plus *P. rapae* treatment, whereas these genes were induced by *B. cinerea* plus *P. rapae* or *P. rapae* alone. Several receptors were in this group of genes such as two Toll-Interleukin-Resistance (TIR) proteins (*AT1G57630, AT2G20142*). Cluster 4 consists of 52 genes that were downregulated at all time points in the drought plus *P. rapae* treatment compared with *B. cinerea* plus *P. rapae* or *P. rapae* alone. Several genes involved in plant defences against pathogens were present in this cluster, such as receptor-like proteins (*RLP39*, *RLP41*), receptor-like kinases (*CRK1*, *CRK37*, *CRK4*, *CRK6*, *CRK7*, *CRK8*, *WAK3*) and PR proteins (*PR-2*, *PR-5*).

Effect of herbivory by *P. rapae* alone or preceded by drought or *B. cinerea* infection on *A. thaliana* biomass

Because we observed that pretreatment with drought or *B. cinerea* changed the timing of *A. thaliana* responses to *P. rapae*, we investigated if this shift in responses affected plant immunity against *P. rapae*. We observed that *A. thaliana* plants exposed to stress imposed by drought, *P. rapae* alone or *P. rapae* herbivory

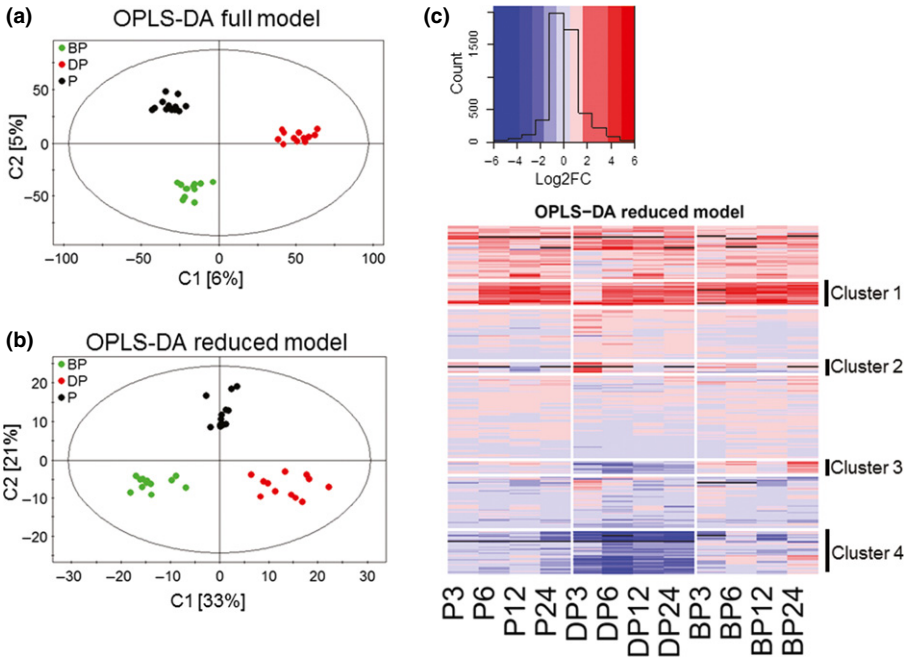

Fig. 4 Discriminant analysis of gene expression levels for *Arabidopsis thaliana* uninfested control plants, plants infested with *Pieris rapae* alone or with *P. rapae* infestation preceded by drought or *Botrytis cinerea* infection at different time points. (a) Orthogonal projection to latent structures – discriminant analysis (OPLS-DA) full model. Treatments are indicated in colours. (b) OPLS-DA reduced model. Treatments are indicated in colours. The reduced model was limited to genes with a VIP value ≥ 2 in the full model. (c) Heatmap showing the $\log_2$-fold changes of genes with VIP values in the reduced model being ≥ 0.8. Blue indicates downregulated genes. Red indicates upregulated genes. Black indicates missing values. P, *P. rapae* as single stress; DP, combination of drought and *P. rapae*; BP, combination of *B. cinerea* and *P. rapae*. The time indications 3, 6, 12 and 24 h refer to the numbers of hours after insect inoculation (HPI) at which the samples were collected.

preceded by drought or *B. cinerea* had a lower shoot FW than control plants not exposed to stress ($P \leq 0.05$) (Fig. 5a). Plants exposed to the combination of drought plus *P. rapae* feeding had a lower shoot FW than plants exposed to *P. rapae* alone or plants exposed to *B. cinerea* plus *P. rapae* ($P < 0.05$). No difference in shoot FW was observed between plants exposed to *P. rapae* alone or *B. cinerea* plus *P. rapae*. Interestingly, pretreatment with drought or *B. cinerea* did not have an effect on caterpillar weight compared with caterpillars fed on plants not previously exposed to stress (Fig. 5b). In conclusion, we recorded that pretreatment with drought or *B. cinerea*. followed by *P. rapae* herbivory elicited transcriptome changes that were different from those elicited by *P. rapae* herbivory alone and that these changes converged over time. Despite these transcriptome differences, pretreatment with drought or *B. cinerea* did not seem to compromise plant immunity against the most damaging stress imposed by the specialist caterpillar *P. rapae*.

Discussion

Transcriptomic responses to herbivory by *P. rapae* caterpillars

Plants have evolved sophisticated mechanisms for detecting and responding to feeding by insect herbivores (Howe & Jander, 2008; Hogenhout & Bos, 2011; Mithofer & Boland, 2012). Herbivore-induced plant defences can be divided in three phases: perception, signal-transduction and response (Heidel-Fischer *et al.*, 2014). Jasmonic acid (JA)-mediated responses have emerged as important components of plant defences against chewing herbivores (Reymond *et al.*, 2000, 2004; De Vos *et al.*, 2005). For instance, Reymond *et al.* (2004) estimated that 67–84% of *Arabidopsis thaliana's* transcriptional responses to *Pieris rapae* were JA-mediated. Furthermore, mutants that are impaired in JA-signalling have been shown to be more susceptible to herbivory by *P. rapae* (Bodenhausen & Reymond, 2007; Verhage *et al.*, 2011; Vos *et al.*, 2013b). In this RNAseq analysis, we observed an extensive transcriptome reprogramming (3548 DEGs) upon *P. rapae* herbivory in *A. thaliana* over a 24-h time period (Fig. 3). This transcriptome reprogramming occurred rapidly as indicated by the large number of DEGs (744 up- and 171 downregulated) identified already at 3 h post inoculation (HPI) (Figs S3, S4). Expression of several genes involved in JA biosynthesis (e.g. *DAD1*, *JMT*, *LOX2*, *LOX3*, *LOX4*, *OPR3*), signal-transduction (e.g. *JAZ1*, *JAZ2*, *JAZ3*, *JAZ5*, *JAZ6*, *JAZ7*, *JAZ8*, *JAZ9*, *JAZ10*) and response (e.g. *NSP4*, *TCH4*, *VSP2*, *TPS4*) were upregulated in response to *P. rapae* herbivory (Table S2). Several of these genes code for proteins involved in anti-insect defences. For example, *VSP2* encodes for a vegetative storage protein. Recombinant AtVSP2 included in diets increased mortality and delayed development in coleopteran and dipteran insects (Liu *et al.*, 2005). Another example is *TPS4* which encodes a geranyllinalool synthase which is induced by JA application and feeding by *P. rapae* and *P. xylostella* larvae in *A. thaliana* and cabbage (*Brassica oleracea*), respectively (Broekgaarden *et al.*, 2007; Huang *et al.*, 2008). Geranyllinalool synthase is involved in the production of terpenes that function in indirect defence of *A. thaliana* (Herde *et al.*, 2008; Markovic *et al.*, 2014; Pangesti *et al.*, 2015).

Several studies have investigated transcriptional responses of *A. thaliana* to herbivory by *P. rapae* using targeted or whole-genome arrays (Reymond *et al.*, 2000, 2004; De Vos *et al.*, 2005; Bodenhausen & Reymond, 2007; Appel *et al.*, 2014). Direct comparison across studies is challenging because research teams used different experimental conditions and protocols. For instance, in the studies mentioned earlier, the time of sample collection ranges from 15 min to 24 h after caterpillar feeding. The number of caterpillars, their developmental instar, plant age and tissue (local vs systemic) also differed between studies. To our knowledge, our study is the first using a gene expression monitoring platform independent from microarrays to address the response of plants to chewing herbivores in *A. thaliana*. Thus, it is interesting to compare our data with results obtained with microarray analyses addressing the response of *A. thaliana* to

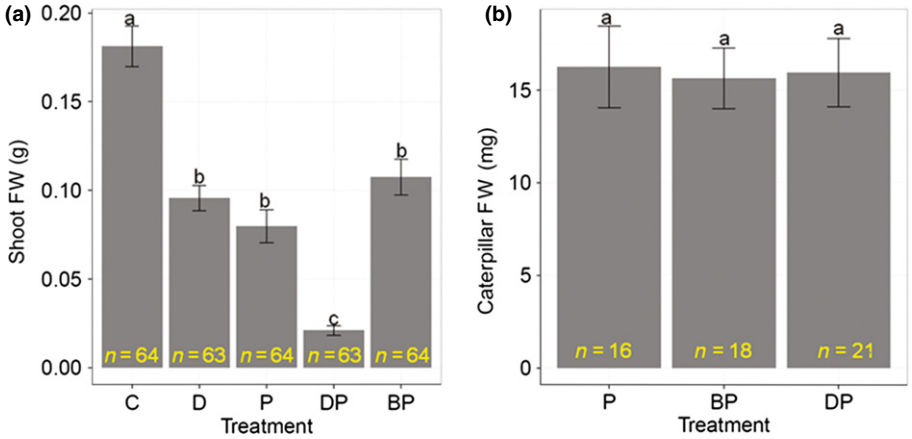

Fig. 5 Phenotypic characterization of *Arabidopsis thaliana* and *Pieris rapae* caterpillars when plants are exposed to *P. rapae* feeding alone or to *P. rapae* herbivory preceded by drought or *Botrytis cinerea* infection. (a) *Arabidopsis thaliana* rosette FW after exposure to different stress treatments. C, control; D, drought; P, *P. rapae*; DP, drought plus *P. rapae*; BP, *B. cinerea* plus *P. rapae*. (b) *Pieris rapae* caterpillar weight after feeding for 5 d on plants that had not been exposed to stress (P), to drought (DP) or to *B. cinerea* infection (BP) before exposure to the caterpillars. Bars show mean values ± SE. Different letters above bars indicate significant differences between treatments (Tukey's HSD test, $P < 0.05$).

chewing herbivores. Despite the limitations mentioned earlier, we made a nonexhaustive cross-experiment comparison. In our RNAseq analysis we found higher numbers for up- and downregulated genes, respectively, in comparison to Reymond *et al.* (2004) (140 up- and three downregulated), De Vos *et al.* (2005) (128 up- and 58 downregulated) and Appel *et al.* (2014) (480 up- and 295 downregulated) (Fig. S5). Although these latter three studies all used microarrays, large differences were observed among them. A total of 68% of the differentially expressed genes (DEGs) identified in the three studies described earlier were also identified in this study; by contrast, only 10% of DEGs identified in this study were also identified in the other studies. Thus, our approach extends beyond what has been recorded with microarrays. On the one hand, the higher number of DEGs identified in the present study in comparison with Reymond *et al.* (2004) is not surprising because Reymond *et al.* (2004) used an array representing only 7200 unique genes of the 33 000 genes annotated in the *A. thaliana* genome. On the other, De Vos *et al.* (2005) and Appel *et al.* (2014) used an array representing almost the whole *A. thaliana* genome (26 000 genes) and we still observed striking differences in the number of DEGs. These differences may be due to inherent differences between RNAseq and microarray analyses. For instance, microarrays have a lower dynamic range for quantitation than RNAseq (Wang *et al.*, 2009; Ozsolak & Milos, 2011; Van Verk *et al.*, 2013). Furthermore, the two platforms seem to correlate well for genes with intermediate expression levels but not for genes with either high or low expression levels, and RNAseq analysis has been proven to outperform microarray analysis in the detection of low abundance transcripts (Wang *et al.*, 2009, 2014). The large number of new DEGs identified with RNAseq analysis underlines the potential of this technology for discovery of genes involved in plant–herbivore interactions. A logical follow-up of this study will be the functional characterization of new candidate genes identified in this study. In the long term these genes could be candidates for development of crops that are better defended against chewing herbivores.

Transcriptomic responses to combined stresses, imposed by drought and *P. rapae* or *B. cinerea* followed by *P. rapae*

In nature, plants are challenged by a diverse range of abiotic and biotic stresses that commonly occur simultaneously (Rizhsky *et al.*, 2004; Mittler & Blumwald, 2010; Vile *et al.*, 2012; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013; Kissoudis *et al.*, 2014; Rivero *et al.*, 2014; Sewelam *et al.*, 2014; Stam *et al.*, 2014; Suzuki *et al.*, 2014). Whole transcriptome profiling using microarrays for plants exposed to multiple abiotic and biotic stresses has shed light onto plant responses to multiple stresses at the molecular level (Atkinson *et al.*, 2013; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013). Here, we recorded a considerable overlap between the transcriptional responses to combined stresses imposed by drought and *P. rapae* or *Botrytis cinerea* and *P. rapae* and the single stress imposed by *P. rapae* (Fig. 3a). Despite this overlap, specific time-dependent transcripts in response to combined stresses were identified, with early time points displaying the biggest difference between single and combined stresses (Figs 2, S4). In one of the first studies addressing transcriptional responses to multiple stress exposure, Voelckel & Baldwin (2004) found that transcriptional changes in *Nicotiana attenuata* plants exposed to sequential or simultaneous attack by the sap-feeding insect *Tupiocorus notatus* and the chewing insect *Manduca sexta* were very similar. Furthermore, these transcriptional changes were different from the changes in response to the single stress situations. In tomato, simultaneous attack by the phloem feeder *Macrosiphum euphorbiae* and the chewing herbivore *Spodoptera exigua* induced a similar set of genes as in the single stress situation. However, the expression patterns were different (Rodriguez-Saona *et al.*, 2010). The specificity observed for the combined stress expression signature varies between studies; for example, Voelckel & Baldwin (2004) observed specificity only at early time points, whereas Rodriguez-Saona *et al.* (2010) observed specificity also 5 d after the treatment had been applied. Here, we identified genes that were specifically differentially expressed in response to a combination of stresses as well as genes with altered expression patterns in the combined stresses compared with the single stress (Figs 3a, 4, S3, S4). For plants exposed to a combined stress imposed by drought and *P. rapae*, we observed a group of genes induced to higher levels at 3 HPI (Cluster 2) than in the single stress scenario (Fig. 4c, Table S6). This cluster 2 contains two abscisic acid (ABA)receptors (*PYL4* and *PYL5*) belonging to a family of 14 members in *A. thaliana*; these receptors are known to be involved in the regulation of *ABI1* and *ABI2*, two genes that encode for negative regulators of the ABA signalling pathway (Ma *et al.*, 2009; Park *et al.*, 2009). Furthermore, PYL4 and PYL5 have been pinpointed as components of the crosstalk between the JA and ABA signalling pathways (Lackman *et al.*, 2011). For instance, expression of *PYL4* is regulated by JA in *Nicotiana tabacum* and *A. thaliana*. On the one hand, loss-of-function mutants in *PYL4* and *PYL5* were hypersensitive to JA treatment, as reflected in reduced growth in comparison to wild-type (WT) Col-0 *A. thaliana* plants. On the other, both mutants *pyl4* and *pyl5* displayed reduced anthocyanin accumulation in response to JA compared with the WT (Col-0) (Lackman *et al.*, 2011). Another group of genes showing altered expression patterns upon the combined stress imposed by drought and *P. rapae* compared with the single stress scenario was Cluster 4 (Fig. 4c; Table S6). Genes in Cluster 4 were downregulated at all time points to a higher degree than in the caterpillar single-stress situation (Fig. 4c). Cluster 4 contains genes involved in plant defences against biotrophic pathogens (e.g. *PR2*, *PR5*, *RLP39*, *RLP41*, *WAK3*). *PR-1* and *PR-2* encode pathogenesis-related proteins that are induced by a broad range of pathogens (Thomma *et al.*, 1998; De Vos *et al.*, 2005). *PR1* is often used as marker for systemic acquired resistance (SAR) (Fu & Dong, 2013). We hypothesize that downregulation of these *PR* genes may be an effect of the drought stress experienced by the plants before caterpillar infestation. In support of this hypothesis, several studies have observed that abiotic stresses have a negative impact on plant defence against pathogens (Suzuki *et al.*, 2014; Ramegowda & Senthil-Kumar, 2015). Tomato plants exposed to simultaneous stress imposed by salinity and a microbial pathogen were more

susceptible to the pathogen than when exposed only to the pathogen (Kissoudis *et al.*, 2015). Application of ABA and drought stress made *A. thaliana* plants more susceptible to the pathogens *Pseudomonas syringae* pv. tomato and *Peronospora parasitica* (Mohr & Cahill, 2003; Goel *et al.*, 2008). For plants exposed to combined stress imposed by *B. cinerea* and *P. rapae*, we observed a group of genes being induced at higher levels at 3 HPI (Cluster 1) than in the single stress scenario; however, no differences were observed at subsequent time points (Fig. 4c; Table S6). Examples of genes in Cluster 1 are *ERF104* and *BAP1*. *ERF104* encodes a transcription factor that is involved in ET-mediated responses through interaction with *MPK6* (Bethke *et al.*, 2009). A homologue of *ERF104* (*ERF 106*) that also interacts with *MPK6* is involved in resistance against *B. cinerea* (Meng *et al.*, 2013), suggesting that *ERF104* may be involved in defence signalling in response to *B. cinerea* infection. *BAP1* encodes a negative regulator of plant defences and is required for growth homeostasis under normal conditions (Yang *et al.*, 2006, 2007). Future efforts will focus on understanding the biological role of genes showing altered gene expression patterns under combined stresses.

Conclusion

Plants in natural and agricultural environments are subjected to multiple stresses. Here, we evaluated the transcriptomic changes to herbivory by *P. rapae* caterpillars alone, and to combinatorial stresses imposed by drought plus *P. rapae,* or *B. cinerea* plus *P. rapae*. The transcriptomic changes elicited by *P. rapae* herbivory alone are fast. Already at 3 HPI, 915 genes are differentially expressed. Moderate differences were observed between the transcriptomic changes in response to the combined stresses compared with the single stress by *P. rapae* herbivory. We identified transcripts that were specifically differentially expressed in the combined stress treatments and transcripts that were expressed in both single and combined stresses but with altered expression pattern in the combined stress. Differences observed in the transcriptomic response to single and combined stresses were larger at early time points and subsequently the responses converged. This indicates that the response to the most recent stress imposed by feeding specialist caterpillars overrides the effects of previous exposure to drought or *B. cinerea*. This study highlights the importance of studying combinations of stresses. How these transcriptomic changes affect the plant phenotype needs further attention. For example, we observed a larger biomass reduction in plants exposed to the combined stress imposed by drought plus *P. rapae* than in the single stresses situation, but how these changes are related to the transcriptome changes remains to be investigated. Future experiments will be directed to mutant analyses of genes differentially expressed under combinatorial stresses compared with single stresses.

Acknowledgements

We are grateful to Hans van Pelt, Kim Vermeer, Michel Arts, Elena Kapsomenou, Benno Augustinus, Robert Veldman and Tom van den Beuken who helped with collecting the samples. Raymond Hulzink and Roy Gorkink (KeyGene) are thanked for their skilled execution of the RNA sequencing work and technical discussions. This work was supported by The Netherlands Organization for Scientific Research (NWO) through the Technology Foundation, Perspective Programme 'Learning from Nature' [STW10988].

Author contributions

N.H.D.O., S.C., P.H., J.J.A.v.L., M.G.M.A., M.P., S.C.M.v.W., C.M.J.P. and M.D. planned and designed the research. N.H.D.O., S.C., P.H., E.S., M.C.v.V, R.H., A.H.J.W. and M.d.V, performed experiments and/or analysed data. N.H.D.O., J.J.A.v.L. and M.D. executed data interpretation. N.H.D.O., J.J.A.v.L., M.G.M.A., M.P., C.M.J.P. and M.D. wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Experimental design.

Fig. S2 Validation of RNAseq data with RT-PCR.

Fig. S3 Dynamic transcriptional responses of *Arabidopsis thaliana* plants exposed to *Pieris rapae* herbivory alone or to *P. rapae* herbivory preceded by drought or *Botrytis cinerea* infection, over time.

Fig. S4 Differences in transcriptional responses of *Arabidopsis thaliana* plants to *Pieris rapae* feeding alone or to *P. rapae* feeding preceded by drought or *Botrytis cinerea* infection over time.

Fig. S5 Venn diagrams comparing differentially expressed genes (DEGs) from this study to those from other relevant studies.

Table S1 Primers for genes used for validation of the RNAseq using RT-PCR

Table S2 Differentially expressed genes in *Arabidopsis thaliana* plants exposed to *Pieris rapae* at 3, 6, 12 and 24 h post inoculation

Table S3 GO-term enrichment for differentially expressed genes in *Arabidopsis thaliana* plants exposed to *Pieris rapae*

Table S4 Differentially expressed genes in *Arabidopsis thaliana* plants exposed to either combinations of drought and *Pieris rapae* or *Botrytis cinerea* and *P. rapae* at 3, 6, 12 and 24 h post *P. rapae* inoculation

Table S5 GO-term enrichment for differentially expressed genes in *Arabidopsis thaliana* plants exposed to either combinations of drought and *Pieris rapae* or *Botrytis cinerea* and *P. rapae*

Table S6 Genes which expression levels explain most of the difference among herbivory by *Pieris rapae* alone or preceded by drought or *Botrytis cinerea*

Methods S1 Preparation of cDNA and validation of RNAseq by quantitative RT-PCR.

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