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RESOURCE

Transcriptome dynamics of Arabidopsis during sequential biotic and abiotic stresses

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SUMMARY

In nature, plants have to cope with a wide range of stress conditions that often occur simultaneously or in sequence. To investigate how plants cope with multi-stress conditions, we analyzed the dynamics of whole-transcriptome profiles of *Arabidopsis thaliana* exposed to six sequential double stresses inflicted by combinations of: (i) infection by the necrotrophic fungus *Botrytis cinerea*, (ii) herbivory by chewing larvae of *Pieris rapae*, and (iii) drought stress. Each of these stresses induced specific expression profiles over time, in which one-third of all differentially expressed genes was shared by at least two single stresses. Of these, 394 genes were differentially expressed during all three stress conditions, albeit often in opposite directions. When two stresses were applied in sequence, plants displayed transcriptome profiles that were very similar to the second stress, irrespective of the nature of the first stress. Nevertheless, significant first-stress signatures could be identified in the sequential stress profiles. Bioinformatic analysis of the dynamics of co-expressed gene clusters highlighted specific clusters and biological processes of which the timing of activation or repression was altered by a prior stress. The first-stress signatures in second stress transcriptional profiles were remarkably often related to responses to phytohormones, strengthening the notion that hormones are global modulators of interactions between different types of stress. Because prior stresses can affect the level of tolerance against a subsequent stress (e.g. prior herbivory strongly affected resistance to *B. cinerea*), the first-stress signatures can provide important leads for the identification of molecular players that are decisive in the interactions between stress response pathways.

Keywords: combinatorial plant stress, transcript profiling, *Botrytis cinerea*, *Pieris rapae*, drought stress, gene regulatory network, plant hormones, RNA-Seq, *Arabidopsis thaliana*.

INTRODUCTION

Plants are continuously threatened by a wide range of harmful microbial pathogens and insect herbivores. Besides these biotic stresses, plants are also exposed to extreme abiotic environmental conditions such as drought, heat, cold, water logging, high salinity or toxicity. Adaptive plant responses to single biotic and abiotic stresses have been extensively studied. Both biotic and abiotic stress responses are associated with the action of the phytohormones jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), and salicylic acid (SA), and to a lesser extent with cytokinin, brassinosteroids and auxin (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012; Giron *et al.*, 2013; O'Brien and Benková, 2013; Kazan and Lyons, 2014; Broekgaarden *et al.*, 2015). JA and ET are generally involved in defense against pathogens with a necrotrophic lifestyle, whereas defenses against biotrophs are commonly

controlled by SA (Glazebrook, 2005). ABA is associated with plant development and abiotic stresses (Yamaguchi-Shinozaki and Shinozaki, 2006), such as drought, but its role in modulating JA-dependent defenses against insect herbivores and SA-dependent defenses against pathogens is becoming increasingly evident (Yasuda *et al.*, 2008; Verhage *et al.*, 2011; Vos *et al.*, 2013b). Antagonistic and synergistic interactions between hormonal signal-transduction pathways are thought to provide the plant with a regulatory potential to adapt to its complex biotic and abiotic environment while utilizing its resources in a cost-efficient manner (Reymond and Farmer, 1998; Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012; Vos *et al.*, 2013a, 2015).

In natural and agricultural settings, plants often have to cope with multiple stress conditions simultaneously. In the context of climate change, it is highly likely that the frequency and complexity of these multi-stress conditions will increase and further threaten crop yield. Abiotic stresses can significantly affect plant responses to biotic stresses and vice versa, depending on the timing, nature, and severity of the stresses (Atkinson and Urwin, 2012; Appel *et al.*, 2014; Rejeb *et al.*, 2014). How plants regulate and prioritize their adaptive response when exposed to multiple stresses is largely unknown. Several studies have investigated plant responses to different stress factors occurring simultaneously or sequentially (Mohr and Cahill, 2003; De Vos *et al.*, 2006; Van Oosten *et al.*, 2008; Atkinson *et al.*, 2013; Prasch and Sonnewald, 2013; Rasmussen *et al.*, 2013; Santino *et al.*, 2013; Kissoudis *et al.*, 2014; Rivero *et al.*, 2014; Sewelam *et al.*, 2014; Stam *et al.*, 2014; Suzuki *et al.*, 2014; Ramegowda and Senthil-Kumar, 2015; Sham *et al.*, 2015). From these studies, the picture emerged that different stress signaling pathways are interconnected in a network that is under control of key regulators such as MAP kinases, transcription factors and the above-mentioned stress-related hormones (Fujita *et al.*, 2006; Pieterse *et al.*, 2009; Robert-Seilaniantz *et al.*, 2011; Rejeb *et al.*, 2014; Caarls *et al.*, 2015). In order to gain insight in the complexity of the plant response to combinatorial stresses, several recent studies investigated changes in the transcriptome of *Arabidopsis thaliana* (hereafter called Arabidopsis) in response to simultaneous exposure to abiotic and biotic stresses (Atkinson *et al.*, 2013; Prasch and Sonnewald, 2013; Rasmussen *et al.*, 2013; Suzuki *et al.*, 2014; Ramegowda and Senthil-Kumar, 2015; Sham *et al.*, 2015). Generally, the responses to the single stresses were different from those to the double stresses. However, these studies often focused on a single time point, representing only a snapshot of the transcriptional changes that are induced by a single or combinatorial stress. The influence of one stress on the other may primarily have an effect on the timing of the response to the second stress, causing the detection of large transcriptional differences in combinatorial stresses at one time point, while over time these differences may be much smaller or are the result of a shift in the phasing of the expression profiles.

In order to gain detailed insight into how plants cope with multiple stresses, we here investigated how a first stress influences the nature and dynamics of the transcriptional response that is induced by a second stress. We chose to study the response of the model plant species Arabidopsis to two biotic stresses (infection by the necrotrophic fungus *Botrytis cinerea* and herbivory by larvae of *Pieris rapae*) and to one abiotic stress (drought stress by water withhold). These stresses were chosen because in previous studies it was demonstrated that the plant hormones JA, ABA, and/or ET are involved in adaptive plant responses to these respective stresses. We hypothesized that combining these stresses may lead to hormonal signal interactions that potentially affect the outcome of the response to the second stress. Several previous studies have identified thousands of Arabidopsis genes that change in expression in response to the selected single stresses (Reymond *et al.*, 2000, 2004; De Vos *et al.*, 2005; Ferrari *et al.*, 2007; Huang *et al.*, 2008; Rowe *et al.*, 2010; Birkenbihl *et al.*, 2012; Windram *et al.*, 2012; Rehrig *et al.*, 2014; Clauw *et al.*, 2015), but their dynamic behavior during multi-stress conditions is largely unknown.

Botrytis cinerea is considered to be the second most important plant pathogen (Dean *et al.*, 2012), infecting over 200 cultivated plant species and causing significant economic damage to crops worldwide. Moreover, *B. cinerea* has become an important model for studying interactions between plants and necrotrophic pathogens (Van Kan, 2006; Laluk and Mengiste, 2010). As a necrotroph, *B. cinerea* kills plant tissue prior to feeding by using different mechanisms that cause plant decay, e.g. enzymatic degradation of the cell walls, generation of toxic reactive oxygen compounds, or secretion of host non-selective toxins. JA and ET participate in the defense response of Arabidopsis against *B. cinerea* (Thomma *et al.*, 1998, 1999; Diaz *et al.*, 2002; Geraats *et al.*, 2002; Rowe *et al.*, 2010; El Oirdi *et al.*, 2011), while ABA and SA can have a negative effect on *B. cinerea* resistance (El Oirdi *et al.*, 2011; Liu *et al.*, 2015; Vos *et al.*, 2015).

Insect herbivores consume over 15% of the plant biomass produced annually in temperate and tropical ecosystems making insect herbivory a major conduit by which energy flows through food webs (Cyr and Pace, 1993; Agrawal, 2011; Johnson, 2011). The Small Cabbage White butterfly *P. rapae* is one of the most destructive pests of cruciferous plants because it has adapted to the glycoside toxins known as glucosinolates that are produced by crucifers as chemical defenses (Hopkins *et al.*, 2009). Arabidopsis and other plants activate additional defense responses that reduce the performance of leaf-chewing *P. rapae* caterpillars on pre-infested plants (De Vos *et al.*, 2006). It has been shown that this herbivore- or wound-

induced resistance also extends systemically to undamaged plant parts (Howe and Jander, 2008; Vos *et al.*, 2013b). JA is an important primary signal in herbivore-induced local and systemic defenses in various plant–herbivore interactions, while ABA has a modulating role in the JA responsiveness (Bodenhausen and Reymond, 2007; Howe and Jander, 2008; Soler *et al.*, 2013; Vos *et al.*, 2013b). SA is reported to inhibit the JA-dependent defense pathway that is induced by *P. rapae* feeding (Koornneef *et al.*, 2008).

Drought is one of the most frequently experienced abiotic environmental stresses in plants. Low water availability in the rhizosphere leads to a reduction in leaf stomatal conductance and growth (Schachtman and Goodger, 2008). Adaptive responses to drought also involve metabolic, osmotic, and structural adjustment, as well as the production of proteins with DNA damage control and repair functions (Ingram and Bartels, 1996). ABA accumulation is essential for the adaptation to drought, but also ABA-independent regulatory systems are involved in drought stress-responsive gene expression. In the latter, JA and ET have been implicated as important regulators (Bray, 1997; Shinozaki *et al.*, 2003; Riera *et al.*, 2005; Huang *et al.*, 2008).

In this study, we used RNA-Seq to analyze the dynamics of the transcriptome changes that occurred in Arabidopsis over four time points in response to *B. cinerea* infection, *P. rapae* feeding, drought stress, and all six combinations of sequential double stresses. Our results show that irrespective of the first stress, Arabidopsis is capable of swiftly adapting its transcriptome to respond to the second stress. Over time, this second stress-induced transcriptome is highly similar to that of plants that did not receive a first stress, but contains clear first-stress signatures, which may play a role in the phenotypic interaction between consecutive stresses.

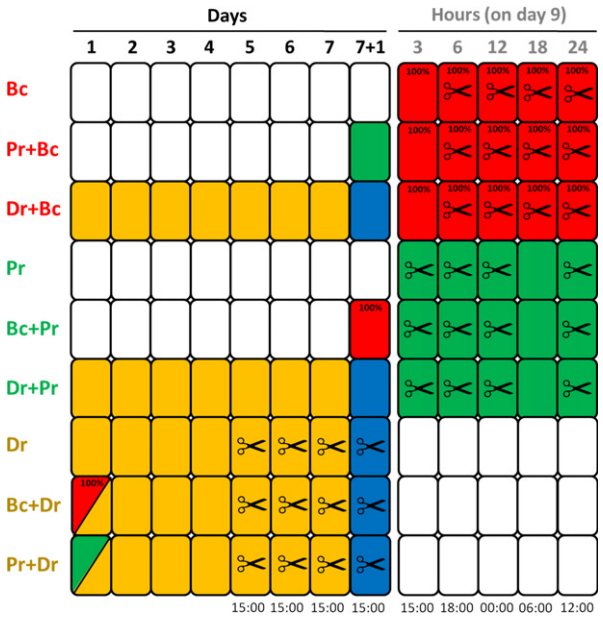

Figure 1. Experimental schedule of treatments and harvests for RNA-Seq time series of single and sequential double stresses.
The schedule shows the timing of treatments and time points of harvest for the three main treatments, *B. cinerea* (Bc, red), *P. rapae* (Pr, green) and drought (Dr, yellow), and the respective pre-treatments. Each single and sequential double stress sample had a mock/control (not visualized) that was harvested at the same time point as the stress treatment. Mock-treated plants were cultivated under the same conditions as their respective *B. cinerea*-treated plants (same periods of 100% RH). Untreated control plants were cultivated under the same conditions as their respective *P. rapae*- and/or drought-treated plants. First stresses were stopped by either lowering RH from 100 to 70% (after 1 day in case of Bc pre-treatment), removing caterpillars from plants (after 1 day), or re-watering after a 7-day period of drought (7 + 1; blue). In case the second stress was drought, the pre-treatments with *B. cinerea* and *P. rapae* were performed right after the last moment of watering. 100%; period of 100% RH instead of standard 70% RH; time indications at the bottom indicate time of the day at which plants were harvested.

RESULTS

Experimental approach for RNA-Seq analysis of single and sequential stress time series

In order to capture a maximal dynamic range of the stress responses, the response to each of the three main stresses was monitored in a different time frame of four time points, depending on how quickly the stress response developed (Figure 1). The transcriptional response to each single and sequential stress was compared at each time point to a non-treated control (for treatments not involving *B. cinerea*) or a mock-treated control (same 100% relative humidity conditions as *B. cinerea* treatments) that was harvested at the same time as the stress treatment. For the study of *B. cinerea* stress, a time span between 6 and 24 h after inoculation with a 5-μl droplet of 5×10^5 spores ml^{-1} was chosen, because previous studies showed that the earliest transcriptional changes can be observed around 6 h after application of the inoculum, while at 24 h after inoculation massive changes in gene expression can be detected (Windram *et al.*, 2012; Vos *et al.*, 2015). For the study of *P. rapae* stress, we chose a time span between 3 and 24 h after infestation by larvae of stage L1 because previous studies demonstrated that this would yield a maximal dynamic range of transcriptional responses (Reymond *et al.*, 2000, 2004; De Vos *et al.*, 2005; Verhage *et al.*, 2011). For the induction of drought stress, 4-week-old Arabidopsis plants that had previously been watered with equal amounts of water were subsequently withheld from water for 7 days. At day 5 of water withhold, drought-stressed plants were clearly smaller and darker colored than the watered control plants, a phenotype that progressed further on day 6 and 7 when they were at the point of wilting. The transcriptome time series were chosen at 5, 6 and 7 days after water withhold, and at day 8 (7 + 1 day), which was 1 day after re-watering. The recovery response

at day 8 was chosen as the fourth time point of the drought time series because this recovery response after drought stress is interesting by itself, and at this time point the sequential treatment with *B. cinerea* and *P. rapae* was executed and thus could function as a reference treatment. Prior to applying the second stress, further development of the first stress was stopped by changing the 100% relative humidity condition to 70% (first stress *B. cinerea*), by removing the caterpillar (first stress *P. rapae*), or by re-watering the plants (first stress drought). Developmental leaf number 8 was used for applying *B. cinerea* or *P. rapae* as second stress. For all treatments, leaf number 8 was harvested for RNA-Seq analysis. When leaf number 8 was not damaged by *P. rapae*, the next-closest *P. rapae*-damaged leaf was harvested. Three biological replicates per treatment and time point were subjected to RNA-Seq. Each of the three biological replicates consisted of four 'number 8' leaves that were pooled to form one sample. After harvest, leaves were processed and subjected to RNA-Illumina sequencing. On average, 14.6 million reads (range 8.5–29.8 million) were generated per sample with >90% of sequences aligning to the Arabidopsis genome after quality filtering (Van Verk *et al.*, 2013).

Time series transcriptome profiling following single and sequential stresses

In this study, our aim was to analyze the dynamic transcriptome changes that are triggered by the single stresses and investigate how the nature and dynamics of these transcriptome profiles were affected by pre-exposure to each of the other two stresses. First, a set of differentially expressed genes (DEGs) derived from each single stress time series was selected according to their significance in fold-change expression (false discovery rate (FDR) <0.05) and an additional threshold level of at least two-fold change ($-1 > \log_2 > 1$) in comparison to the respective control (Table S1). The first observation that can be made from the RNA-Seq results is that over time there are clear differences in the number of genes that are significantly activated or repressed during the different single stress conditions (Figure 2). For responses to *B. cinerea* (total 2076 unique DEGs) and *P. rapae* (total 3952 unique DEGs), a strong increase in the number of activated genes is observed over time, while relatively few genes are repressed. Upon exposure to drought stress (total 4032 unique DEGs for the first three time points, plus 482 additional unique DEGs for the 1 day after re-watering time point), relatively more genes become repressed than activated. A prior stress did not dramatically change the number of DEGs relative to the single stresses (Figure 2). Clustering the union of DEGs of the single stress sets (total 7355 unique DEGs), and subsequent gene ontology (GO) analysis (Boyle *et al.*, 2004) of overrepresented biological processes in each cluster highlights the differentially regulated biological processes during the plant response to the single stresses and uncovers similarities and contrasts between the different stress responses (Figure 3 and Table S2).

Core DEGs that are shared between the single stress responses

To investigate to what extent genes and biological processes are shared between the three single stress responses, we compared their DEGs. Figure 4(a) shows

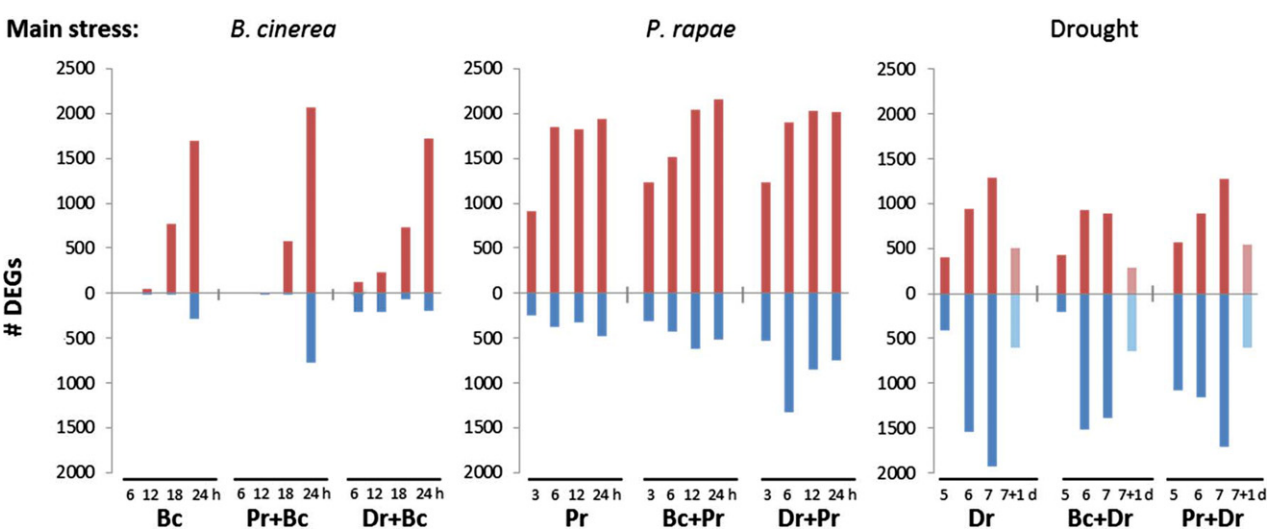

Figure 2. Numbers of DEGs at different time points in single and sequential stress responses.
Graphs show the number of activated (red bars) and repressed (blue bars) differentially expressed genes (DEGs) for all single stresses and their corresponding sequential double stresses at different time points after treatment (FDR <0.05; >2-fold). The one day after re-watering time point of the drought treatments is indicated as '7 + 1 day'. Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought; Pr + Bc, Dr + Bc, Bc + Pr, Dr + Pr, Bc + Dr, and Pr + Dr, respective sequential double stresses.

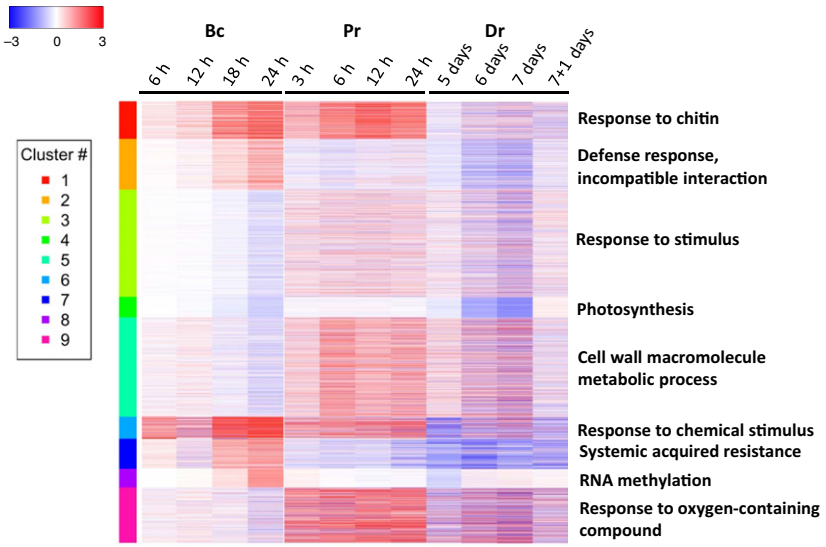

Figure 3. Clustering of the single stress DEGs. Heatmap showing the expression patterns of the union of differentially expressed genes (DEGs) in the three single stresses at different time points after induction (total 7173 unique genes). DEGs were clustered using mclust yielding nine gene clusters (colored bars on the left). On the right side, the most significant GO term for each cluster (full data set in Table S2). Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought. For drought stress, the time point 1 day after re-watering (7 + 1 day) was included in the cluster analysis. Blue–red color key for change in gene expression level: $-3 > \log_2$ fold change >3.

that there is a large overlap between the DEGs of the single stress responses, ranging from 1716 genes shared between the drought and *P. rapae* sets, to 788 genes between the drought and *B. cinerea* sets, and 777 genes between the *P. rapae* and *B. cinerea* sets. Of all 7173 DEGs (excluding the 1 day after re-watering time point), 2493 DEGs (35%) are shared with one or both of the other stresses. A core set of 394 DEGs (5%) was differentially expressed in response to all three single stresses, and clustered into 12 co-expressed gene clusters (Figure 4b). Among this core set of shared DEGs are several well-characterized hormone-responsive marker genes, including *LOX2* (At3g45140), *JAZ7* (At2g34600), and *JAZ8* (At1g30135) (JA responsive), *PDF1.2* (At5g44420) and *ORA59* (At1g06160) (JA/ET responsive), *PR4* (At3g04720), *ERF5* (At5g47230), *ERF6* (At4g17490), and *ACS2* (At1g01480) (ET responsive), *Rap2.6L* (At5g13330), and *HAI1* (At5g59220) (ABA responsive), and *PR-1* (At2g14610), *PR-5* (At1g75040), and *FRK1* (At2g19190) (SA responsive) (Table S1 for details on their expression patterns). In Figure 4(c) the expression patterns of well characterized marker genes of the response to *B. cinerea* (*PDF1.2*; At5g44420), *P. rapae* (*LOX2*; At3g45140), and drought stress (*RAD18*; At5g66400) are depicted, confirming that the different stress treatments resulted in the expected response. When looking at the co-expressed gene clusters, only the genes of cluster 1 (top GO terms related to 'response to oxygen-containing compound', 'response to JA' and 'response to wounding'; Table S3) are regulated in the same direction (activated) during all three individual stress conditions (Figure 4b). All other gene clusters behave clearly different in response to the three single stresses and are often regulated in opposite directions (Figure 4b). For example cluster 8 (top GO terms related to 'response to other organism', 'defense response' and 'immune system process') is activated by *B. cinerea*, but repressed by *P. rapae* and drought. Conversely, cluster 10 (top GO terms 'multidimensional cell growth', 'response to light stimulus', and 'cell wall organization') is activated by *P. rapae*, but repressed by *B. cinerea* and drought. The fact that there is an overlap in the expression of genes under all three single stresses, whether in the same or in opposite directions, suggests that these genes or their regulators may act as a point of convergence if plants were to experience these stresses in combination.

Botrytis cinerea data set: effect of herbivory and drought stress on dynamics of *B. cinerea*-induced gene expression

To investigate the effect of *P. rapae* infestation and drought stress on the dynamics of the transcriptome changes that are induced by *B. cinerea* infection, we analyzed the expression patterns over time of all 2076 *B. cinerea*-responsive DEGs. Clustering of this group of genes yielded 10 clusters of co-expressed genes across the *B. cinerea* single and sequential stress data sets. Gene clusters that are activated in response to *B. cinerea* infection are enriched for GO terms such as 'response to chitin' (Figure 5 (cluster 3) and Table S4), reflecting recognition of fungal chitin by the plant immune system (Pel and Pieterse, 2013), and 'response to ET stimulus', reflecting the high level of ET emission that is related to plant responses to *B. cinerea* infection (Broekgaarden *et al.*, 2015). In addition, gene clusters that are repressed in response to *B. cinerea* infection are associated with GO terms such as 'multidimensional cell growth' (Figure 5 (cluster 9) and Table S4), highlighting the antagonistic relationship between plant growth and defense (Wang and Wang, 2014).

Interestingly, the expression patterns over time in the sequential double stress treatments appear in general very similar to the ones of the *B. cinerea* treatment alone. This

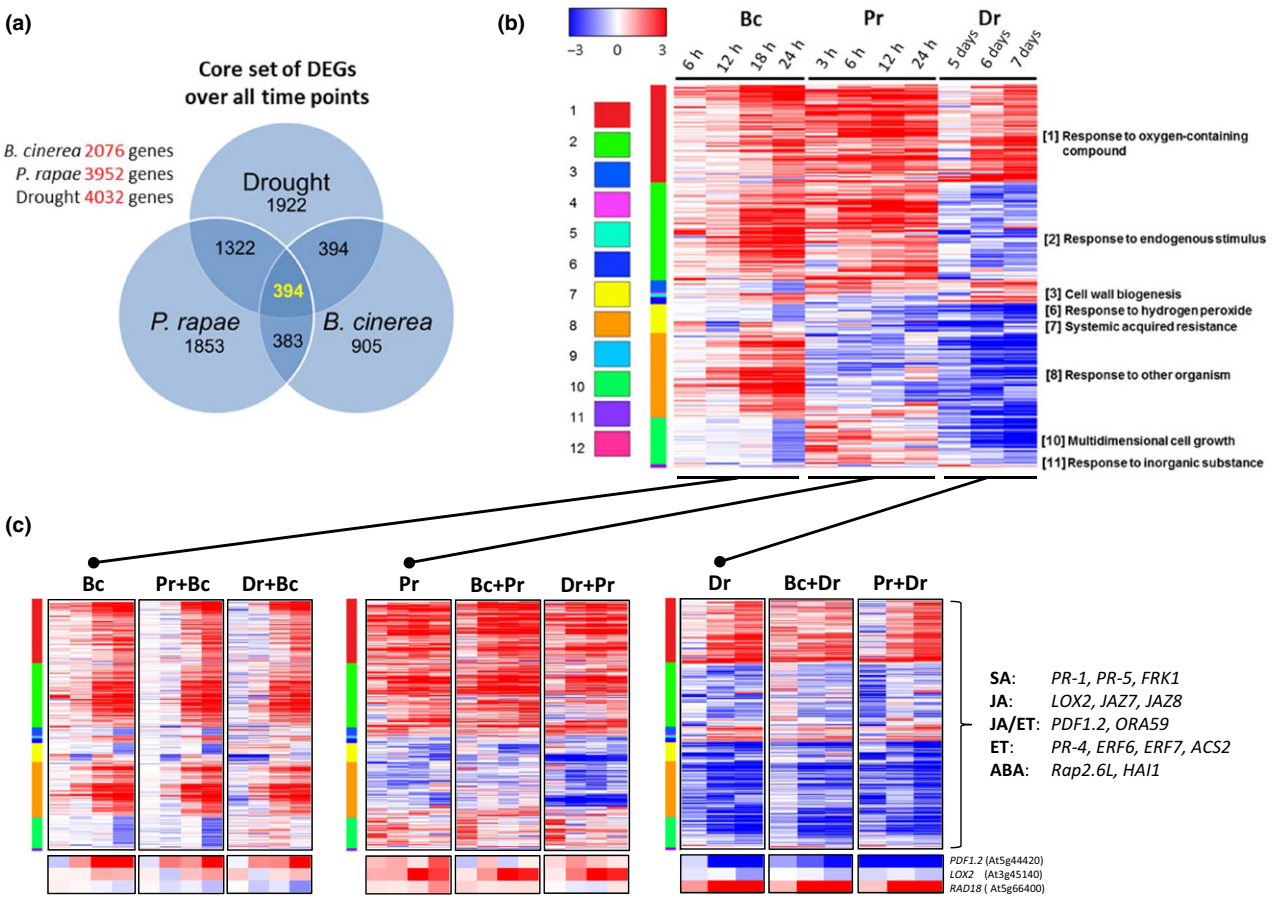

Figure 4. Shared DEGs between the single stress responses.
(a) Venn diagram showing the overlap between the DEGs of each of the single stress responses. The total number of unique DEGs per single stress over all time points is shown in red (full data set in Table S1).
(b) Hierarchical clustering of the 394 core DEGs that are shared between the three single stresses (Cosine similarity metric; 12 clusters are color coded in the square boxes on the left). On the right side, the most significant GO term for the largest clusters (full data set in Table S3).
(c) Comparison of the expression patterns of the 394 core DEGs in response to the single and respective sequential double stresses. Different lanes in (c) reflect the transcription profiles at the time points after treatment as indicated above the lanes in (b). Gene names in the lower right corner represent marker genes of the SA, JA, JA/ET, ET and ABA response pathways that are among the 394 core DEGs. *PDF1.2*, *LOX2*, and *RAD18* represent known marker genes for the response to *B. cinerea*, *P. rapae*, and drought, respectively. Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought; Pr + Bc, Dr + Bc, Bc + Pr, Dr + Pr, Bc + Dr, and Pr + Dr, respective sequential double stresses. Blue-red color key for change in gene expression level: $-3 > \log_2$ fold change >3.

suggests that Arabidopsis swiftly reprogrammes its transcriptome to the response that is induced by *B. cinerea* infection, thereby overruling effects of the prior stresses herbivory and drought on *B. cinerea*-responsive gene expression. For example, at the moment drought pre-treated plants were inoculated with *B. cinerea* (one day after re-watering of drought-treated plants), more than 1000 genes were still differentially expressed in the analyzed leaf (number 8) due to the prior drought treatment (508 DEGs activated and 610 DEGs repressed at time point 7 + 1 day; Table S1; Figure 3 last lane). At 6 h after *B. cinerea* inoculation this drought-induced effect is still clearly visible in the *B. cinerea*-responsive gene set (compare the 6 h lane in Bc versus Dr + Bc treatment in Figure 5), but at the later time points this effect quickly dampens off and the *B. cinerea*-responsive genes start to follow the same pattern as in the *B. cinerea* single treatment. This effect is also visible in the left panel of Figure 4(c) where the expression patterns of the core set of 394 DEGs are plotted. Also prior exposure to *P. rapae* inflicted clear differences in the expression patterns of the *B. cinerea*-responsive genes during the first two time points (compare the first two time points in the Pr + Bc treatments with those in the Bc treatment), but at later time points the gene expression patterns of the core DEGs become very similar to that of the *B. cinerea* single stress treatment. Nevertheless, during the sequential stress responses some clusters show a first-stress signature, e.g. the genes in clusters 2, 3 and 5 in Figure 5 show a delayed activation when plants experienced herbivory or drought stress prior to *B. cinerea* infection.

In order to identify in greater detail co-regulated genes of which the expression pattern in response to *B. cinerea*

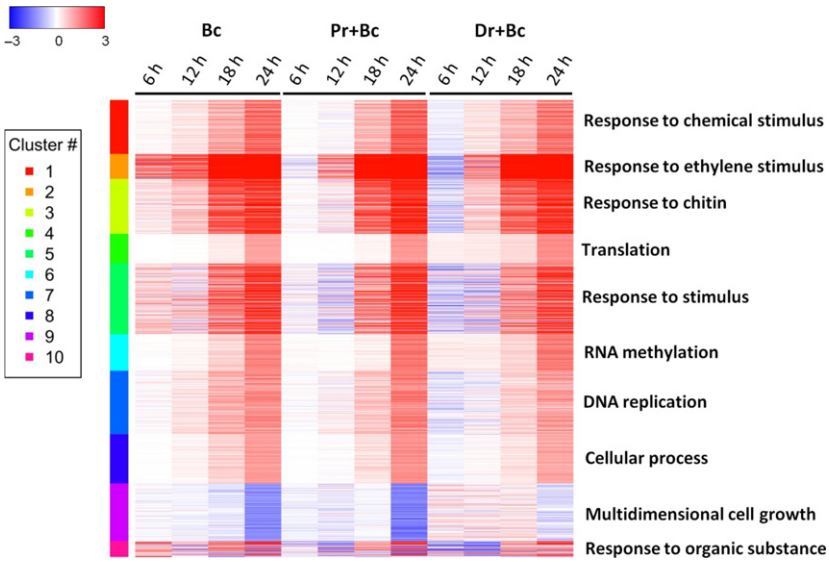

Figure 5. Dynamics of the expression of the *B. cinerea* set of DEGs during single and sequential double stresses.
Heatmap showing the expression patterns of the 2076 *B. cinerea*-responsive DEGs during *B. cinerea* infection on mock pre-treated (Bc), *P. rapae* pre-infested (Pr + Bc) or drought pre-treated (Dr + Bc) Arabidopsis plants. The *B. cinerea*-responsive DEGs were clustered using mclust yielding 10 clusters (colored bars on the left). On the right side, the most significant GO term for each cluster (full data set in Table S4). Blue–red color key for change in gene expression level: $-3 > \log_2$ fold change >3.

infection was affected by either herbivory or drought stress, we used the bioinformatics tool Wigwams (Polanski *et al.*, 2014). The Wigwams algorithm identifies gene modules showing evidence for co-regulation in multiple gene expression time series and identifies signatures of condition-dependent regulatory mechanisms in co-regulated gene sets. Wigwams identified 35 modules of co-regulated genes in the *B. cinerea* data sets (Figure S1). Analysis of these clusters for co-expression revealed gene modules of which the expression patters were clearly affected in one or both of the sequential stress treatments in comparison to the *B. cinerea* treatment alone (examples shown in Figure 6). These gene modules represent signatures of a previous stress in the *B. cinerea*-induced transcriptome profile, and may thus be functionally related to the effect of the first stress on the outcome of the plant response to *B. cinerea* infection. The genes in these Wigwams modules are given in Table S5 along with their GO term analysis.

Among the *B. cinerea*-responsive Wigwams modules of which the co-expression pattern is different when plants were previously exposed to herbivory or drought stress, are gene modules with GO term enrichments for rather general plant processes such as nucleoside biosynthesis and metabolism (modules 6 and 9), and cell growth (module 25), but also modules related to more specific plant processes, such as response to chitin and nitrogen (module 15). Functional analysis of underlying candidate genes should reveal their importance for effects on the outcome of the second stress response.

Further knowledge about the biological processes that are affected when *B. cinerea* infection is preceded by either drought stress or herbivory can be gained by analyzing the phasing of gene expression under the different single and sequential stress conditions. We did this by analyzing at which time point a GO term becomes significantly overrepresented in the *B. cinerea*-related DEG set. For this, we clustered all *B. cinerea*-responsive DEGs according to their time point of first differential expression, divided them over activated and repressed genes, and performed GO term analysis on them. Figure 7 shows the timing and strength of the onset of significant GO term enrichment in the single and sequential double stress conditions. In the *B. cinerea* single stress data set, GO terms related to responses to ET, fungus, chitin, SA, and oxygen-containing compound, or to processes such as systemic acquired resistance, respiratory burst, and defense appear early in the activated gene set, reflecting the importance of these processes in the plant response to this necrotrophic pathogen. Prior infestation with *P. rapae* clearly delayed the appearance of these GO terms (become visible at 18 hai in Figure 7), while pre-treatment with drought stress did not have a dramatic effect on the phasing of the activated genes. For the repressed genes in the *B. cinerea* set of DEGs, pre-infestation with *P. rapae* has clearly only minor effects on the GO term phasing. By contrast, pre-treatment with drought stress noticeably affected the phasing of GO terms related to responses to fungus, JA, SA, ABA, chitin, and oxygen-containing compound, and to auxin metabolic process, defense, systemic acquired resistance, and glucosinolate biosynthetic process. Remarkably, biological processes related to hormone action prevail in the *B. cinerea*-responsive processes that are sensitive to modulation by prior exposure to one of the other stresses.

Effect of herbivory or drought stress on resistance to *B. cinerea*

Both herbivory and drought stress imposed a first-stress-signature in the dynamics of the *B. cinerea*-induced

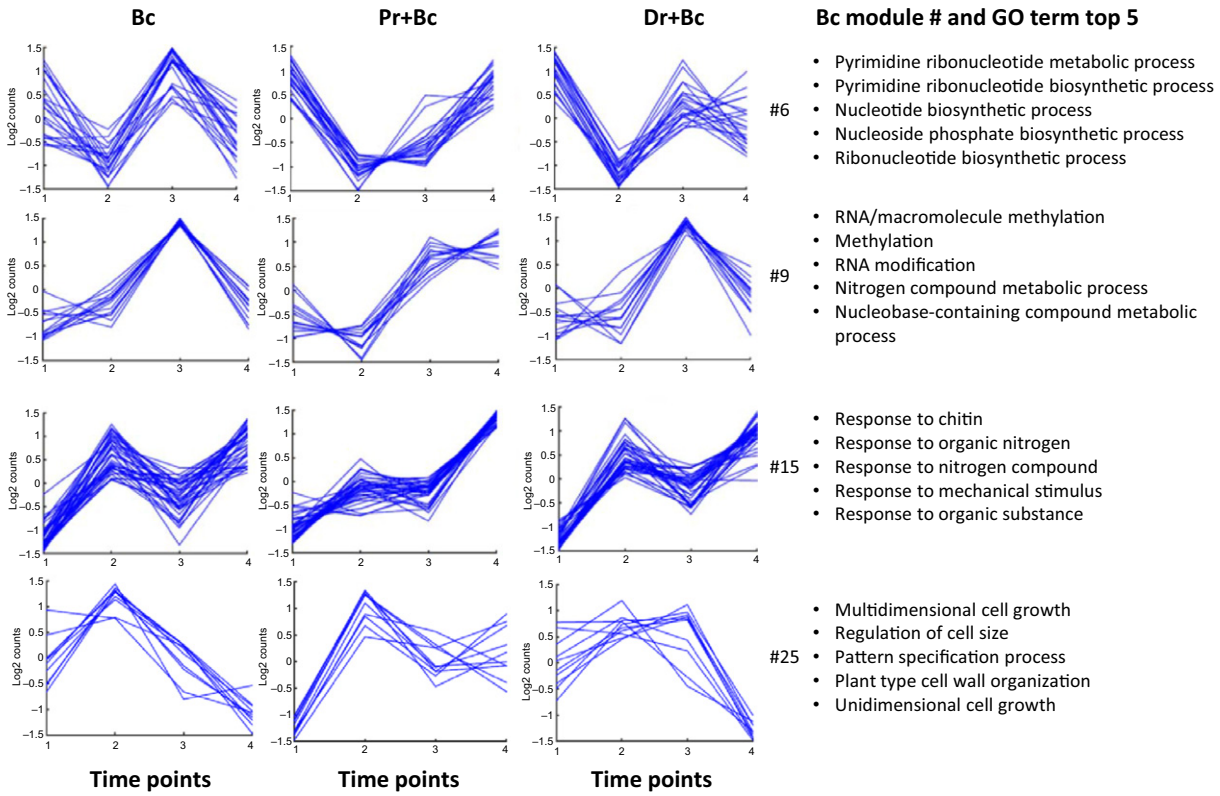

Figure 6. Expression patterns of selected Wigwams modules from the *B. cinerea* set of DEGs during single and sequential stress conditions.
A selection of Wigwams modules of co-expressed gene clusters is depicted that show a different pattern in one or both of the sequential stresses *P. rapae-B. cinerea* (Pr + Bc) and drought-*B. cinerea* (Dr + Bc) in comparison to the single stress *B. cinerea* (Bc). The modules represent standardized patterns of differential gene expression over time ($\log_2$ counts). Blue-colored graphs indicate modules of which the genes are significantly co-expressed over time in the given stress condition. Time points 1, 2, 3 and 4 represent 6, 12, 18 and 24 h after *B. cinerea* inoculation. The top five GO terms with highest significance in the respective modules are given (full data set for all Wigwams modules is presented in Table S5).

transcriptome profiles. Wigwams analysis gained insight into the identity of candidate genes related to these first-stress signatures (Figure S1 and Table S5), whereas analysis of GO term enrichment provided global insight into the biological processes that were affected by the stress pre-treatments (Figure 7). To investigate whether the two prior stresses affected the resistance level to *B. cinerea* infection we performed disease resistance bioassays. Inoculation of 5-week-old Arabidopsis Col-0 plants with *B. cinerea* resulted in the development of spreading lesions in about 60% of the inoculated leaves (Figure 8). Plants that were exposed to drought stress prior to *B. cinerea* inoculation showed a similar percentage of leaves with spreading lesions (approximately 70%). Interestingly, plants that were exposed to herbivory prior to *B. cinerea* inoculation showed a significantly enhanced level of resistance against *B. cinerea* infection (average approximately 35% spreading lesions). Together these results indicate that a first stress can have strong effects on the outcome of the adaptive stress response to a second stress, depending on the nature of the first stress.

Pieris rapae data set: effect of *B. cinerea* infection and drought stress on dynamics of *P. rapae*-induced gene expression

The same approach as described above for the *B. cinerea* transcriptome data was taken to investigate the effect of prior *B. cinerea* infection and drought stress on the transcriptional dynamics that are induced by *P. rapae* feeding. Analysis of the global expression profiles of the 3952 *P. rapae*-responsive DEGs yielded nine clusters of co-expressed genes during single and sequential *P. rapae* stress (Figure 9). As expected, *P. rapae* feeding induced many genes related to the GO term 'response to JA stimulus' (Figure 9 (cluster 7) and Table S6), reflecting induced defenses that are triggered by herbivory-inflicted wounding (Wasternack, 2015). In addition, *P. rapae* feeding repressed SA-related genes associated with GO terms 'defense response' and 'systemic acquired resistance' (Figure 9 (cluster 3) and Table S6), reflecting the antagonistic relationship between JA- and SA-dependent defenses (Pieterse *et al.*, 2012). In analogy with what we observed in the *B. cinerea* data sets, the general gene expression patterns

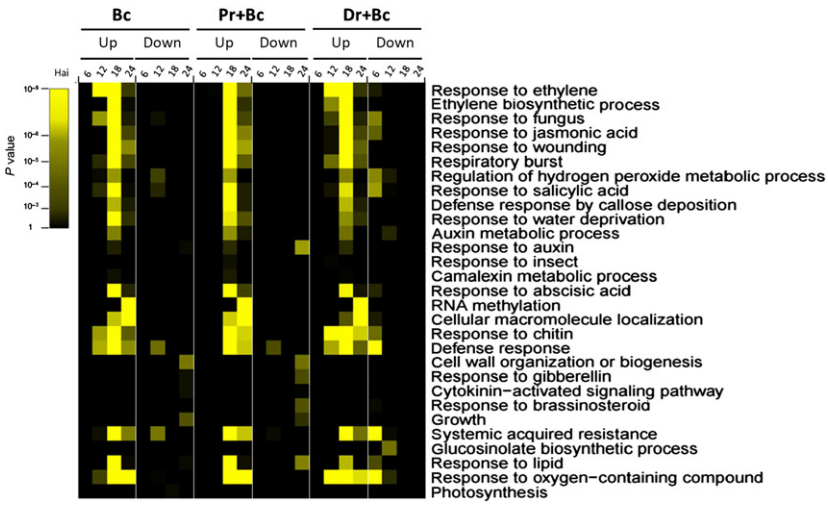

Figure 7. Timing of GO term overrepresentation patterns in *B. cinerea* single and sequential stress data sets.
Heatmap represents the strengths of the *P* values of GO term overrepresentation in the *B. cinerea*-responsive DEG sets (corresponding to the gene lists in Table S1) that become significantly activated (up) or repressed (down) for the first time at the given stress conditions and time points. Color index represents level of significance (*P* values). On the right, overrepresented GO terms. Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought; hai, h after *B. cinerea* infection.

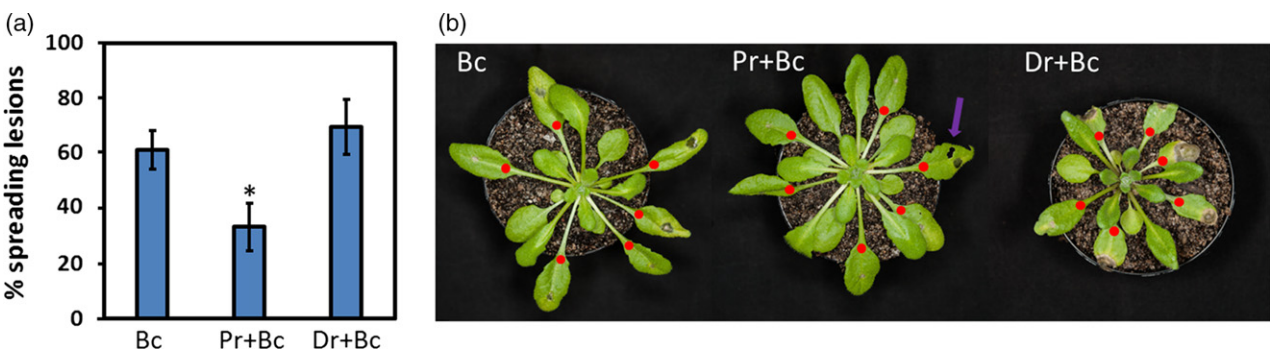

Figure 8. Effect of herbivory and drought stress on resistance of Arabidopsis to *B. cinerea.*
(a) Quantification of *B. cinerea* disease symptoms on Arabidopsis accession Col-0 plants (% spreading lesions per plant). On each plant, six leaves were inoculated with one droplet of *B. cinerea* spores. Three days later, the average number of leaves with spreading lesions was determined per plant. Asterisk indicates statistically significant difference from single stress (Bc) treatment ($n = 6$ plants; Student's *t*-test; $P < 0.05$).
(b) Photographs of *B. cinerea* disease symptoms 3 days after inoculation. Bc, *B. cinerea*-inoculated plants; Pr + Bc, *B. cinerea*-inoculated plants that prior to inoculation were exposed to herbivory by *P. rapae* larvae for 24 h; Dr + Bc, *B. cinerea*-inoculated plants that prior to inoculation received a drought treatment for 7 days, followed by a re-watering phase of one day. Red dots, *B. cinerea*-inoculated leaves; purple arrow, damage caused by *P. rapae* feeding.

over time overlapped greatly between the responses to *P. rapae* single and sequential double stress treatments, again suggesting that Arabidopsis is capable of reprogramming its transcriptome to the last stress encountered, thereby overruling the effects of the prior stresses. For instance, while prior drought stress impacted the expression of over 1000 genes in the leaf tissue just before the start of the *P. rapae* treatment (Table S1 and Figure 3 last lane), already from the first time point (3 h) after herbivory this effect was mostly vanished in the *P. rapae*-induced profiles, which readily followed a similar expression pattern as in the *P. rapae* single treatment (Figure 9). A similar pattern is visible in the core set of 394 DEGs (Figure 4c, middle panel). Nevertheless, during the sequential stresses first-stress signatures can be detected, e.g. genes in cluster 3 of Figure 9 and clusters 7 and 8 of Figure 4(c) (middle panel) show a weaker repression in the *B. cinerea* pre-treatment and a stronger repression in the drought pre-treatment. In general, these *P. rapae*-related results confirm previous findings (Davila Olivas *et al.*, 2016).

To pinpoint co-regulated genes whose expression pattern in response to herbivory is affected by prior *B. cinerea* infection or drought stress, the set of *P. rapae*-responsive DEGs was analyzed with the Wigwams algorithm. Wigwams identified 93 modules of co-regulated genes in the *P. rapae* set of DEGs. Analysis of these clusters for co-expression under the single and sequential double stress conditions revealed gene modules of which the expression patterns were clearly affected by one or both of the sequential double stress treatments in comparison to the *P. rapae* treatment alone (examples shown in Figure 10; full set in Figure S2). The identities of the genes in the *P. rapae*-related Wigwams gene modules are given in Table S5 along with their GO term analysis. It is beyond the scope of this paper to discuss the identity of the genes in detail. However, among the *P. rapae*-responsive

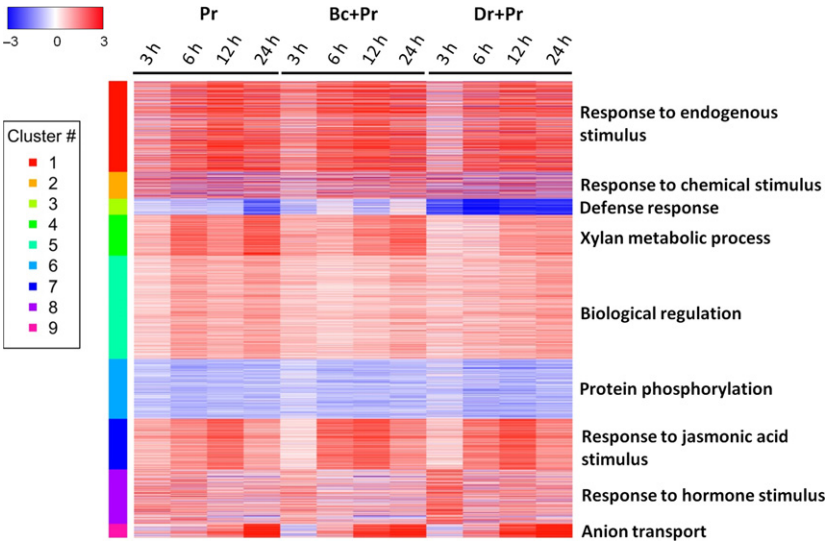

Figure 9. Dynamics of the expression of the *P. rapae* set of DEGs during single and sequential double stresses.
Heatmap showing the expression patterns over time of the 3952 *P. rapae*-responsive DEGs during feeding of *P. rapae* on control (Pr), *B. cinerea* pre-infected (Bc + Pr), or drought pre-treated (Dr + Pi) Arabidopsis plants. The *P. rapae*-responsive DEGs were clustered using mclust yielding nine clusters (colored bars on the left). On the right side, the most significant GO term for each cluster (full data set in Table S6). Blue–red color key for change in gene expression level: $-3 > \log_2$ fold change >3.

Wigwams modules of which the co-expression pattern is clearly different when plants were pre-exposed to *B. cinerea* infection or drought stress, are gene modules with GO term enrichments for xylem, phloem and tissue development (module 3), cell wall biosynthesis processes (module 45), and responses to JA and wounding (module 69).

Also for the *P. rapae* data set, phasing of the regulation of biological processes that are associated with herbivory and influenced by prior *B. cinerea* infection or drought stress was determined. Therefore, the level of significance of overrepresentation of the GO terms for the *P. rapae* set of DEGs at their first time of differential expression was assessed. In the *P. rapae* single stress data set, GO terms related to responses to chitin, wounding, JA, ET, SA, auxin, ABA, water deprivation, osmotic stress, and oxygen-containing compound are already highly enriched at 3 h after infestation in the activated set of DEGs (Figure 11), reflecting the importance of these processes in the response of Arabidopsis to herbivory. In many cases, pre-infection with *B. cinerea* strengthened the level of overrepresentation of these GO terms at different time points after *P. rapae* infestation, while pre-treatment with drought stress often weakened them. In the repressed set of DEGs, drought stress clearly enhanced the overrepresentation of GO terms related to responses to chitin, JA, fungus, ABA, SA, bacterium, and oxygen-containing compound, and to processes such as systemic acquired resistance, defense response to fungus, and negative regulation of programmed cell death, while *B. cinerea* infection had no major effect on the phasing of these GO terms. Overall, these data indicate that *B. cinerea* infection and drought treatment prior to *P. rapae* infestation affects the timing of several defense-related processes, in particular responses to JA, ABA, SA and ET, corroborating the notion that different stresses interact via the hormone-regulated signaling network.

Drought data set: effect of *B. cinerea* infection and herbivory on dynamics of drought stress-induced gene expression

Also for drought stress we investigated the effect of the other two stresses on the dynamics of the transcriptome changes that are induced by this abiotic stress. We analyzed the dynamics of the global expression patterns of the 4032 drought-responsive DEGs during single and sequential stress with drought as the second stress, which yielded 10 clusters of co-expressed genes (Figure 12). GO term analysis of overrepresented biological processes in each cluster highlights the main differentially regulated biological processes. As expected, drought stress induced a relatively large number of genes related to GO term 'response to water deprivation' (Figure 12 (cluster 6) and Table S7) and GO terms related to 'response to oxygen-containing compound' (clusters 3 and 10, and cell wall-related processes (cluster 7). Another feature that stands out is the association of drought stress with massive repression of genes, many of which are associated with biological processes such as 'photosynthesis' and 'defense response' (clusters 1, 2, 5 and 8), reflecting the fact that drought-stressed plants shift their strategy from energy-demanding processes related to growth and immunity to adaptation to the abiotic stress condition. Interestingly, after 1 day of re-watering (7 + 1 day columns in Figure 12), the drought-induced transcriptional changes that intensified over the 7-day period of water withhold, were for 77% (3106 of the 4032 DEGs; Table S1) reset towards basal levels within 24 h, demonstrating the plant's ability to swiftly redirect transcriptional programming when drought stress is relieved. Similar to what we observed for the *B. cinerea*

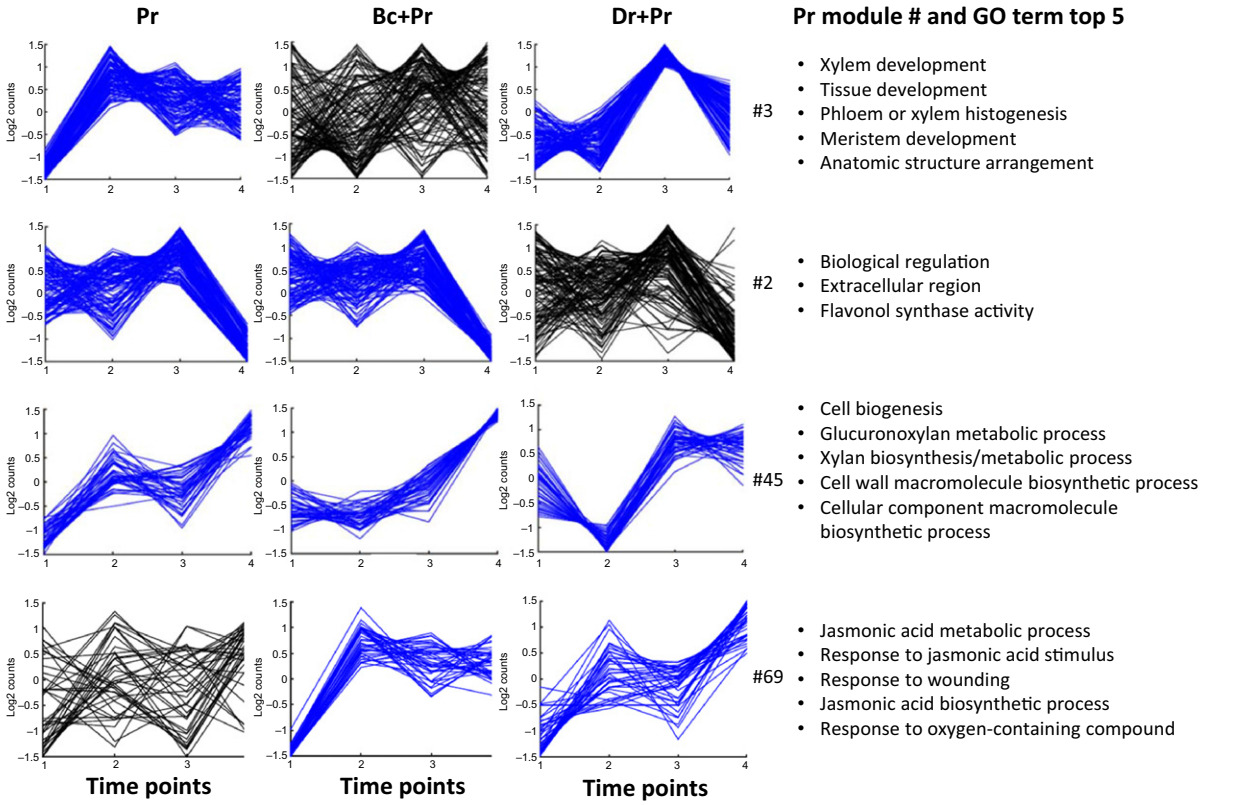

Figure 10. Expression patterns of selected Wigwams modules from the *P. rapae* set of DEGs during single and sequential stress conditions.
A selection of Wigwams modules of co-expressed gene clusters is depicted that show a different pattern in one or both of the sequential stresses *B. cinerea*-*P. rapae* (Bc + Pr) and drought-*P. rapae* (Dr + Pr) in comparison to the single stress *P. rapae* (Pr). The modules represent standardized patterns of differential gene expression over time ($\log_2$ counts). Blue-colored graphs indicate modules of which the genes are significantly co-expressed over time in the given stress condition. In the black-colored graphs, the genes in the module are not significantly co-expressed. Time points 1, 2, 3 and 4 represent 3, 6, 12 and 24 h after *P. rapae* infestation. The top 5 GO terms with highest significance in the respective modules are given (full data set for all Wigwams modules is presented in Table S5).

and *P. rapae* sequential double stress responses, the gene expression patterns over time in the sequential drought double stress treatments were to a large extent similar to those inflicted by the single drought treatment. In the core set of 394 DEGs it is clear that on the first time point after the start of the drought treatment (5 days) *B. cinerea* and *P. rapae* pre-treatment still had noticeable effects on the drought-induced gene expression profiles (Figure 4c, right panel; compare the left lanes of Dr, Bc + Dr, and Pr + Dr). However, at the later time points (6 and 7 days) these effects dampened off and the expression patterns became more similar to that of the drought single treatment. Nevertheless, prior stress caused by *B. cinerea* infection or *P. rapae* infestation left first-stress signatures in the drought-induced transcriptome.

Wigwams analysis of co-regulated genes in the drought data sets identified 72 co-expressed gene modules under the single and sequential double drought stress conditions (examples in Figure 13; full set in Figure S3). The identities of the genes in these Wigwams gene modules are given in Table S5 along with their GO term analysis. Wigwams modules with clear changes in expression pattern when drought-stressed plants were pre-treated with either *B. cinerea* or *P. rapae* represent genes related to the biological processes such as SA and defense signaling (module 10 and 55), and nucleosome organisation (module 11). Future analysis of candidate genes in these modules should reveal their importance for the outcome of the combinatorial stress responses.

Also for the drought DEGs, we analyzed the timing and level of significance of overrepresentation of all the GO terms in the single and sequential double stress time series (Figure 14). In the drought single stress data set, GO terms related to responses to oxidative stress, water deprivation, osmotic stress, ABA, and oxygen-containing compound, and to processes such as phenylpropanoid biosynthesis, cell wall biogenesis, and lignin metabolism are enriched in the activated gene set at the first day of sampling, while responses to wounding and JA follow somewhat later. In the repressed gene set, GO terms related to responses to JA, SA, chitin, fungus, insect and oxygen-containing compound and to processes such as

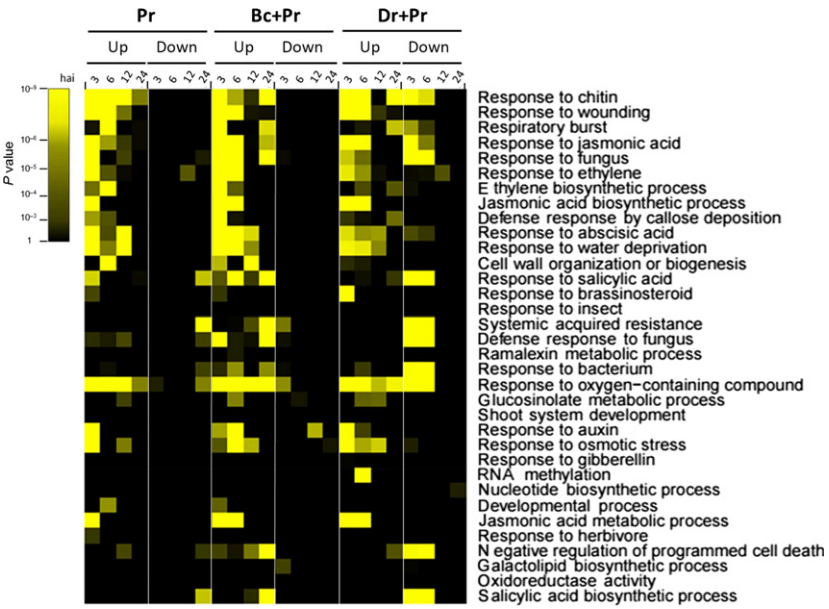

Figure 11. Timing of GO term overrepresentation patterns in *P. rapae* single and sequential double stress data sets.
Heatmap represents the strengths of the *P* values of GO term overrepresentation of *P. rapae*-responsive DEG sets (corresponding to the gene lists in Table S1) that become significantly activated (up) or repressed (down) for the first time at the given stress conditions and time points. Color index represents level of significance (*P* values). On the right, overrepresented GO terms. Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought; hai, h after *P. rapae* infestation.

photosynthesis, shoot system development, systemic acquired resistance, glucosinolate metabolic process, nitrogen compound transport, and respiratory burst are enriched already at the first sampling point. This highlights the biological processes that are engaged or affected during drought stress. Interestingly, pre-infection with *B. cinerea* accelerated the phasing of activated genes associated with biological processes such as responses to chitin, wounding, osmotic stress, ABA, and JA. In the repressed gene set, GO terms related to responses to chitin, fungus, and SA, and to systemic acquired resistance became later enriched than in the single stress data set. When plants were pre-infested with *P. rapae*, the phasing of the drought-responsive genes is also clearly affected. Many GO terms in the drought activated gene set become more prominently enriched at later time points. Moreover, in the repressed gene sets GO terms related to responses to water deprivation, osmotic stress, wounding, JA, ABA and ET are highly overrepresented at the first time point of sampling, while this is not the case in the single stress treatment. Like in the *B. cinerea* and *P. rapae* data sets, biological processes related to hormone action become relatively often differentially enriched in the sequential double treatments in comparison to the single stress treatment.

Effect of stress interactions on plant resistance

For all three main stresses tested, prior treatment with one of the other stresses imposed a first-stress-signature in the dynamics of their transcriptome profiles. Wigwams analysis provided insight into the identity of the co-expressed genes related to these first-stress signatures (Figures 6, 10 and 13), whereas analysis of GO term enrichment at the onset of gene induction provided global insight into the biological processes that were affected during the time course by the prior stress treatment (Figures 7, 11 and 14). As an example for the effect of prior stress on the level of plant resistance to a second stress, we showed that the level of infection by *B. cinerea* can be significantly altered when plants were pre-disposed to herbivory (Figure 8). The bioassays with the other sequential stress treatments showed no strong effects of prior stress treatment on the performance of the specialist herbivore *P. rapae* (Davila Olivas *et al.*, 2016). In future research, we will functionally analyze candidate genes from the first-stress signatures in the second stress profiles to investigate their putative role in a diverse range of multi-stress interactions.

DISCUSSION

Plants are often exposed to different abiotic and biotic stresses, which can occur simultaneously or sequentially. How plants selectively adapt their response to this complexity of stresses is largely unknown. In this study, we aimed to gain insight into how plants respond to a biotic or abiotic stress when previously exposed to another stress, using a necrotrophic pathogen, an insect herbivore, and drought as main stress factors. By analyzing the dynamics of the Arabidopsis transcriptome over four consecutive time points we were able to show that: (i) on average 35% of the DEGs in a given single stress is also differentially regulated in one or both of the other two single stresses, albeit often in different directions; (ii) irrespective of the nature of the first and second stress applied, genes responsive to the second stress rapidly follow a similar pattern as that induced by the second stress alone; (iii) the Wigwams algorithm identified first-stress signatures of co-expressed genes that behave

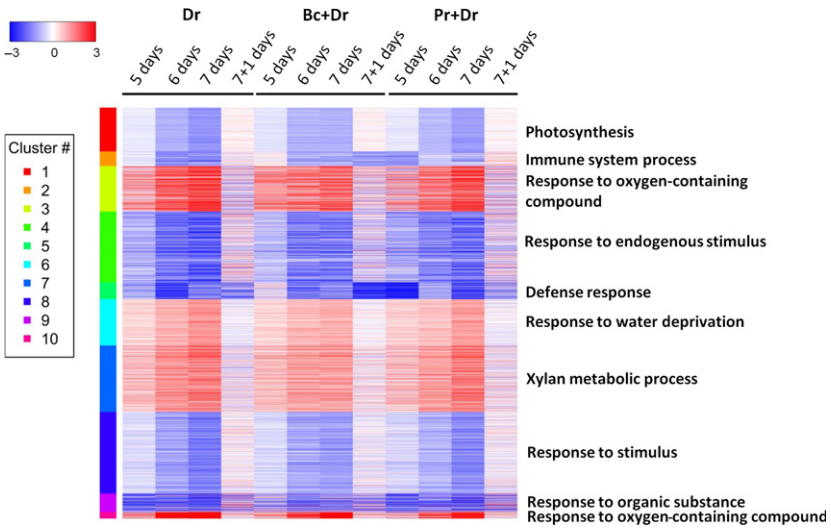

Figure 12. Dynamics of the expression of the drought set of DEGs during single and sequential double stresses.
Heatmap showing the expression patterns over time of the 4032 drought-induced DEGs during a 7-day period of water withhold and 1 day after re-watering on control (Dr), *B. cinerea* pre-infected (Bc + Dr), or *P. rapae* pre-infested (Pr + Dr) Arabidopsis plants. The drought-responsive DEGs were clustered using mclust yielding 10 clusters (colored bars on the left). On the right side, the most significant GO term for each cluster (full data set in Table S7). Blue–red color key for change in gene expression level: $-3 > \log_2$ fold change >3.

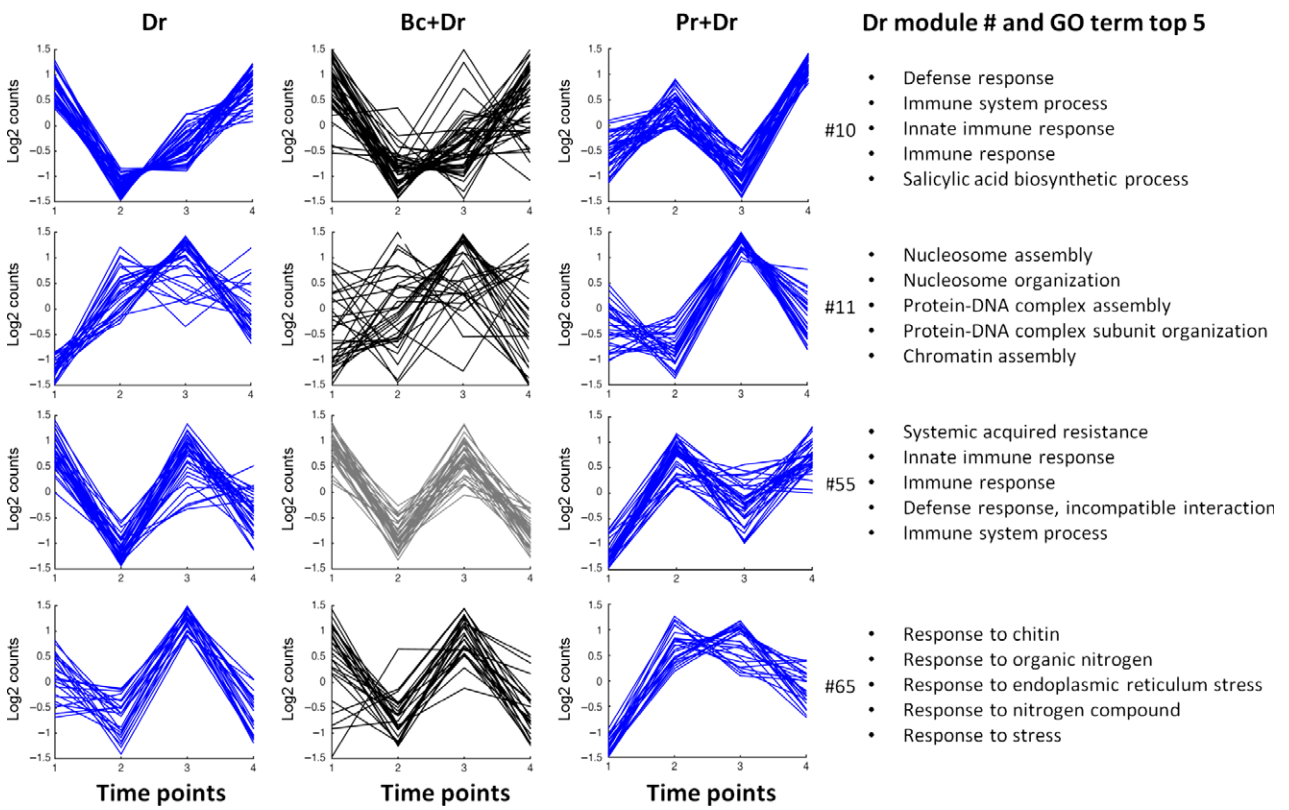

Figure 13. Expression patterns of selected Wigwams modules from the drought set of DEGs during single and sequential stress conditions.
A selection of Wigwams modules of co-expressed gene clusters is depicted that show a different pattern in one or both of the sequential stresses *B. cinerea*-drought (Bc + Dr) and *P. rapae*-drought (Pr + Dr) in comparison to the single stress drought (Dr). The modules represent standardized patterns of differential gene expression over time ($\log_2$ counts). Blue-colored graphs indicate modules of which the genes are significantly co-expressed over time in the given stress condition. In the black-colored graphs, the genes in the module are not significantly co-expressed. Time points 1, 2, 3 and 4 represent 5, 6, 7 and 7 + 1 day after onset of water withhold, in which the 7 + 1 day time point represents plants that were re-watered for 1 day after the 7-day drought period. The top 5 GO terms with highest significance in the respective modules are given (full data set for all Wigwams modules is presented in Table S5).

differently in the sequential double stress profile in comparison with the single stress treatment; (iv) plant hormone-related biological processes play a dominant role in the interaction between the studied stress-induced transcriptomes supporting previous findings (Rejeb *et al.*, 2014); and (v) a previous stress can affect the outcome of a response to a sequential second stress, resulting in altered plant resistance.

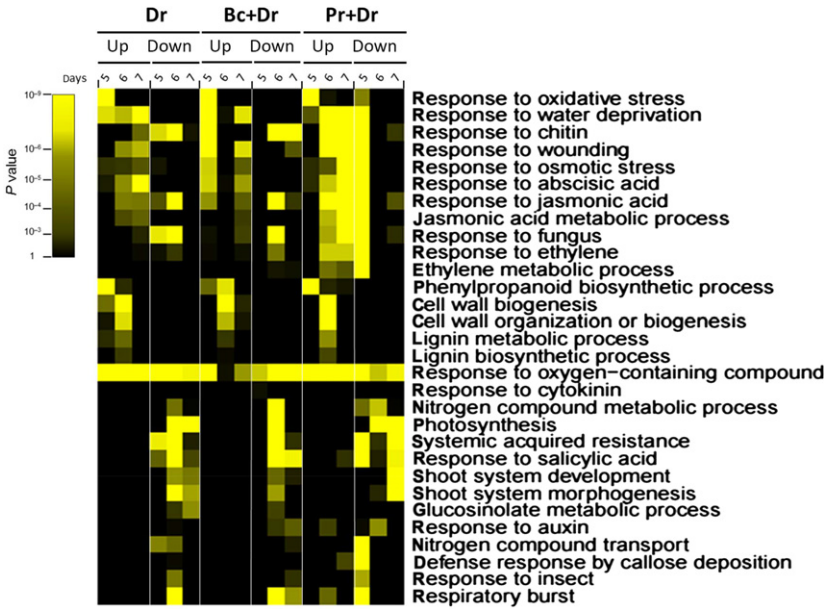

Figure 14. Timing of GO term overrepresentation patterns in drought single and sequential stress data sets.
Heatmap represents the *P* values of GO term overrepresentation of drought-responsive DEG sets (corresponding to the gene lists in Table S1) that become significantly activated (up) or repressed (down) for the first time at the given stress conditions and time points. Color index of *P* values represents level of significance. On the right, overrepresented GO terms. Bc, *B. cinerea*; Pi, *P. rapae*; Dr, drought; days, day after water withhold.

Transcriptome profiles of different single stress responses show significant overlap

The expression profiles induced by the single stresses *B. cinerea* infection, *P. rapae* infestation, and drought are clearly different in timing and numbers of activated and repressed genes (Figure 2). Clustering of the profiles of all DEGs (7355) from the three single stress responses shows that, in general, genes related to stress-related processes are overrepresented in the activated gene sets, while genes related to growth become repressed (Figure 3). This confirms the notion that plants under stress prioritize appropriate adaptive responses over growth (Vos *et al.*, 2013a, 2015). One-third of all DEGs under single stress conditions was also differentially expressed under one or both of the other single stress conditions (Figure 4a). A set of 394 DEGs was differentially expressed in response to all three single stresses and clustering of their expression profiles clearly shows that the expression of the genes in the 12 distinguished clusters is often regulated in opposite directions (activated or repressed), depending on the nature of the stress (Figure 4b). Only one cluster, containing an overrepresentation of genes related to the GO term 'response to oxygen-containing compound' shows a general activation of genes under all three single stress conditions, highlighting that production and responsiveness to oxygen-containing compounds are central to stress responses in general. Overall, these results indicate that a significant proportion of the stress-related transcriptome is engaged by all three stresses tested. The differential stress-type specific activation or repression of genes suggests their positive versus negative function in different adaptive stress responses. When a plant experiences a combination of stresses, this may cause synergistic or antagonistic effects on the level of tolerance to the stresses at hand.

The transcriptome is rewired to the last stress response, but the prior stress leaves first-stress signatures in the second stress profile

Analysis of the dynamics of both the single and sequential double stress transcriptome profiles showed that the transcriptome profiles of all possible double stress combinations were remarkably similar to those of the last encountered stress if applied individually (Figures 4c, 5, 9, and 12). In analogy, 1 day after re-watering, the drought-induced transcriptome was largely reset to the non-stressed condition (Figure 12). Apparently, plants are highly plastic in their capacity to adapt to changes in their biotic and abiotic environment, and swiftly rewire their transcriptome to the latest stress encountered. Nevertheless, it has been demonstrated that prior exposure to biotic or abiotic stresses can have dramatic effects on the outcome of the response to a second stress (Rejeb *et al.*, 2014).

To identify genes and biological processes that could contribute to the interaction between different stress responses, different types of analyses have been carried out. When globally inspecting the transcriptional profiles of the single and sequential double stress profiles, it is difficult to pinpoint obvious effects of a first stress on the dynamics of the transcriptional response to a second stress. Using mclust clustering of the transcriptional profiles of the *B. cinerea*, *P. rapae* and drought sets of DEGs (Figures 5, 9 and 12) different gene clusters were identified that showed distinct behavior over time during the single and sequential double stress responses. However, in this way relatively

small differences between the single and sequential stresses were detected. In order to better pinpoint co-expressed gene clusters that represent first-stress signatures in the second stress transcriptome profiles, we used the bioinformatics tool Wigwams (Polanski *et al.*, 2014). We were able to dissect the transcriptional profiles into modules of co-regulated genes in time across different conditions. This approach highlighted modules whose gene expression patterns differed from the single stress profile in one or both of the sequential double stress profiles (Figures 6, 10, 13, and S1–S3). Future studies should reveal the role of candidate genes in these clusters in shaping the outcome of the adaptive stress responses in the sequential dual stress conditions. Moreover, the Wigwams modules of co-regulated genes could aid in dissecting the regulatory circuitry underlying plant responses to combinatorial stresses, e.g. by analyzing the representation of transcription factor binding motifs in the promoters of the gene modules.

Hormone-related responses prevail in biological processes that are differentially enriched in the double stress transcriptional profiles

Zooming in on the biological processes that are differentially enriched among the transcriptional profiles of the single versus the sequential double stresses, we monitored GO term enrichment at the time points of first differential expression of all genes. Plots of all biological GO terms that become significantly represented in the set of DEGs at the different time points provide a landscape of the timing at which these biological processes significantly change (Figures 7, 11 and 14). Interestingly, among all biological processes that become clearly more enriched in the sequential double stresses over their respective single stresses (either in activated or repressed DEGs) are GO terms related to the response to the stress-related hormones JA, ABA, SA, and ET and occasionally to auxin. This observation suggests that responses to these hormones are likely to play a central role in the interaction between the signaling pathways that regulate the adaptive responses to the sequential double stresses. In the past, JA, ABA, ET, and SA have been demonstrated to be crucial positive or negative regulators of plant resistance against *B. cinerea* (JA, ET, and SA; Thomma *et al.*, 1998, 1999; El Oirdi *et al.*, 2011; Vos *et al.*, 2015), *P. rapae* (JA and ABA; De Vos *et al.*, 2006; Bodenhausen and Reymond, 2007; Vos *et al.*, 2013b), and drought stress (ABA; Yamaguchi-Shinozaki and Shinozaki, 2006). Hence, interactions between the different hormone-controlled signaling pathways may be decisive in the outcome of the adaptive response when two stresses are encountered sequentially.

Effect of prior stress on level of resistance to subsequent stress

Classic examples of interactions between defense pathways are the different forms of induced resistance that are triggered by pathogens, insect herbivores and beneficial microbes as they all change the outcome of the defense response against a subsequent invasion by another pathogen or insect in a positive or negative manner (De Vos *et al.*, 2006; Howe and Jander, 2008; Poelman *et al.*, 2008; Van Oosten *et al.*, 2008; Pieterse *et al.*, 2014; Vos *et al.*, 2015). Also for abiotic stresses effects on the level of resistance against other abiotic and biotic stresses are documented (Fujita *et al.*, 2006; Rejeb *et al.*, 2014). For the combinations of sequential stresses that were investigated here, we found that prior infestation by *P. rapae* caterpillars changed the level of resistance against *B. cinerea* (Figure 8), even though the global transcriptional profiles induced by *B. cinerea* as single or second stress did not differ dramatically (Figure 5). It can thus be concluded that subtle first-stress signatures in the double stress transcriptional profile may have significant effects on the outcome of the adaptive response to the second stress, although it cannot be excluded that non-transcriptional changes may also contribute to changes in the level of resistance against the second stress. Previously, it was shown that herbivory on Arabidopsis by *P. rapae* results in a systemic increase in the levels of JA, and that this can prime systemic tissues for enhanced JA-dependent anti-herbivory defenses (Vos *et al.*, 2013b). Since JA-regulated defenses play a major role in resistance against the necrotrophic pathogen *B. cinerea* as well, herbivory-induced priming of JA responsiveness may contribute to the enhanced resistance level against *B. cinerea* (Figures 7 and 8).

This study was aimed at analyzing the dynamics of gene expression patterns in response to a set of single and sequential double stresses. Future research will be focused on biological validation of candidate genes in the Wigwams modules with putative major roles in shaping the outcome of sequential double stresses. Knowledge on how plants cope with different stresses simultaneously or in sequence will aid in breeding for multi-stress tolerant crops.

EXPERIMENTAL PROCEDURES

Plant cultivation

Seeds of *Arabidopsis thaliana* accession Col-0 were sown in cultivation containers filled with autoclaved river sand. Sand was supplied with half-strength Hoagland solution containing Sequestrene as described (Van Wees *et al.*, 2013). In order to attain 100% relative humidity (RH) for germination, cultivation containers were enclosed in a tray with water and covered with a transparent lid. Seeds were stratified for 2 days at 4°C in the dark to ensure a homogeneous germination after which the tray was moved to a growth chamber ($t = 0$) with an 8-h day/16-h night rhythm, a temperature of 21°C, and a light intensity of 100 μmol m^{-2} sec^{-1}. After 10 days, the lids of the trays were slightly opened and gradually removed over a 4-day period to adjust to the 70% RH present in the growth chamber. Fourteen-day-old seedlings were transplanted to individual pots containing

a mixture of river sand and potting soil (1:1 (v:v)). Pots were supplied with water from the bottom up three times per week. At an age of 3 weeks the plants were supplied once with half-strength Hoagland solution.

Rearing of *P. rapae* and treatment with *P. rapae* caterpillars

Pieris rapae caterpillars were reared on cabbage plants (*Brassica oleracea* convar. *capitata* var. *alba*) under greenhouse conditions (24°C, with natural daylight). Butterflies were supplied with flowering plants such as *Lantana camara* for their (nectar) food. When flowers were scarce, additional food (solution of 20% honey and 10% sucrose) was offered to the butterflies. Inbreeding of the population was minimalized by adding wild butterflies and caterpillars from the Dutch Flevopolder to the existing population. After starving for 1 h, first-instar (L1) larvae were placed on Arabidopsis leaves using a fine paint brush as described (Van Wees *et al.*, 2013).

Cultivation of *B. cinerea* and treatment with *B. cinerea* spores

Botrytis cinerea strain B05.10 (Staats and Van Kan, 2012) was grown on half-strength Potato Dextrose Agar (PDA; Difco™ BD Diagnostics, Franklin Lakes, NJ, USA) plates containing penicillin (100 μg ml^{-1}) and streptomycin (200 μg ml^{-1}) for 2 weeks at room temperature. Spores were collected, filtered through glass wool, and re-suspended in half-strength Potato Dextrose Broth (PDB; Difco™ BD Diagnostics) to a final density of 1×10^5 spores ml^{-1}. After a 3-h incubation period, the spores were used for inoculation by applying 5-μl droplets on Arabidopsis leaves as described (Van Wees *et al.*, 2013). For the RNA-Seq analysis, four droplets were applied on a single leaf, while for disease resistance assays, a single droplet was administered to the leaf.

Single and sequential double stress treatments

Single and sequential double stress treatments were applied according to the schedule shown in Figure 1. Developmental leaf number 8 was treated with the second stress and harvested for RNA-Seq analysis. Individual leaves were numbered from oldest to youngest. For single and sequential double stress treatments in which *B. cinerea* was the second stress, developmental leaf number 8 of 5-week-old plants was inoculated with *B. cinerea* by pipetting four 5-μl droplets of spore suspension (1×10^5 spores ml^{-1}) onto the leaf. Plants were kept at 100% RH for the remaining time period. Mock-treated plants received droplets of half-strength PDB and were kept at 100% RH. Pre-treatment with drought was started when plants were 4 weeks old by withholding water for 7 days, after which plants were re-watered and allowed to recover for 1 day before plants were inoculated with *B. cinerea. P. rapae* pre-treatment was started 1 day prior to *B. cinerea* inoculation by placing a single *P. rapae* L1 caterpillar on leaf 7 and allowing it to feed for 1 day. Only plants of which leaf number 8 was undamaged were used for inoculation with *B. cinerea* as second stress. Leaf number 8 was harvested at 6, 12, 18, and 24 h after inoculation with *B. cinerea.*

For single and sequential double stress treatments in which *P. rapae* herbivory was the second stress, two *P. rapae* L1 larvae were transferred to developmental leaf number 8 of 5-week-old plants. Pre-treatment with drought was achieved as described above for the *B. cinerea* experimental set-up. *B. cinerea* pre-treatment was performed 1 day prior to introduction of *P. rapae* by inoculating leaves 6 and 7 with one 5-μl droplet of *B. cinerea* spore suspension (1×10^5 spores ml^{-1}) per leaf and placing the plants at 100% RH for 1 day. A mock treatment for the *B. cinerea* pre-treatment was included by placing droplets with half-strength PDB on the leaves and keeping the plants at 100% RH for 1 day. Leaf number 8 was harvested at 3, 6, 12 and 24 h after the start of *P. rapae* feeding. When leaf number 8 was not damaged by *P. rapae* (because it had moved to another leaf), the next-closest *P. rapae*-damaged leaf was harvested.

For single and sequential double stress treatments in which drought was the second stress, 4-week-old plants were refrained from watering for 7 days. After 7 days of water withhold, plants were re-watered and allowed to recover for 1 day. *B. cinerea* pre-treatment was performed at the beginning of day 1 of the drought period by inoculating leaves 6 and 7 with one 5-μl droplet of *B. cinerea* spore suspension (1×10^5 spores ml^{-1}) per leaf and placing the plants at 100% RH for 1 day. A mock treatment for the *B. cinerea* pre-treatment was included by placing droplets with half-strength PDB on the leaves and keeping the plants at 100% RH for 1 day. *P. rapae* pre-treatment was performed at the same time as the *B. cinerea* pre-treatment by placing one *P. rapae* caterpillar on leaf 7 and allowing it to feed on the plant for 1 day. Only plants of which leaf number 8 was undamaged were used to harvest leaf 8. Leaf number 8 was harvested at 5, 6, 7, and 7 + 1 days after the onset of water withhold (with 7 + 1 representing the time point of 1 day after re-watering).

For each treatment and time point, three biological replicates were used for RNA-Seq analysis. Each of the three biological replicates consisted of four pooled 'number 8' leaves harvested from four similarly-treated plants. For all treatments in which *B. cinerea* inoculation was used as first or second stress, a mock treatment was performed in which plants were inoculated with droplets of half-strength PDB and placed at 100% RH for 1 day. For all treatments without *B. cinerea*, controls consisted of untreated plants. After harvest, leaf samples were immediately frozen in liquid nitrogen and stored at -80°C.

Experimental design

The experiment was carried out in a fully randomized factorial design with two factors; time and treatment, with time having four levels (four time points analyzed per stress combination) and treatment having five levels (control, mock, two different first stresses per sequential stress, and one single stress). The climate chamber space was divided in three blocks, in which time was randomized. Within every time point, treatments were assigned randomly to the plants. RNA extraction was carried out in batches of approximately 20 randomly chosen samples.

RNA extraction, library preparation, and RNA-Seq alignment

RNA was extracted using the Plant RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. All samples were treated with DNase I on column using the Qiagen RNase-Free DNase Set. Quality of RNA was checked by determining the RNA Integrity Number (RIN) with an Agilent 2100 bioanalyzer and RNA LabChip (Agilent Technologies, Santa Clara, CA, USA). For the library preparation samples with a RIN value ≥6 were used. The samples were processed according to the TruSeq Stranded mRNA HT Sample Prep Kit from Illumina (Illumina Inc., San Diego, CA, USA). 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, poly-A RNA was fragmented using divalent cations under elevated temperature. First-strand cDNA was synthesized using

random primers. Strand specificity was 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. Samples were sequenced with an Illumina Hi-seq 2000 sequencer using three sequencing runs. Samples were randomly assigned to seven lanes of the Illumina flow cells within each run.

Processing of raw sequencing data, alignment of the RNA-Seq data to the Arabidopsis genome, and downstream processing was performed as described (Van Verk *et al.*, 2013). RNA-Seq reads were aligned to the Arabidopsis genome (TAIR version 10) using TopHat v2.0.4 (Trapnell *et al.*, 2009) with parameters: 'transcriptome-mismatches 3', 'N 3', 'bowtie1', 'no-novel-juncs', 'genome-read-mismatches 3', 'read-mismatches 3', 'G', 'min-intron-length 40', 'max-intron-length 2000.' Gene expression levels were calculated by counting the number of mapped reads per annotated gene model using HTSeq-count v0.5.3p9 (Anders *et al.*, 2014). For downstream analyses, raw read counts were normalized for between sample differences in sequencing depth (Love *et al.*, 2014). Differential gene expression was calculated using DESeq2 (Love *et al.*, 2014) for all stress treatments and time points relative to the appropriate non-stress-treated control/mock treatment that was cultivated and harvested in exactly the same way as the stress-treated samples. The raw *P. rapae* RNA-Seq data have been used in a previous study (Davila Olivas *et al.*, 2016). In the study of Davila Olivas *et al.*, the raw *P. rapae* RNA-Seq reads were analyzed independently of this study with the specific goal to identify *P. rapae*-responsive genes that are affected by prior drought stress or *B. cinerea* infection and possibly link them to effects on changes in *P. rapae* resistance. In the present study, the raw *P. rapae* RNA-Seq reads were used in the larger framework of analyzing global dynamics of gene expression profiles during multiple combinatorial stress conditions in which also *B. cinerea* and drought stress were analyzed as second stresses. All raw RNA-Seq read data are deposited in the NCBI Short Read Archive (http:// www.ncbi.nlm.-nih.gov/sra/) under the BioProject accession code PRJNA315516.

Gene ontology analysis

To identify enrichment of GO terms in the different sets of DEGs, 'Go term finder' (Boyle *et al.*, 2004) analysis was performed using an *A. thaliana* gene association file downloaded from ftp.geneontology.org on 2 May 2013. The default background set was used (all 30504 transcripts in the database that have GO annotations). GO term finder tests for overrepresentation of GO categories using the hypergeometric distribution and FDR for multiple testing (P-value ≤ 0.05). Figures showing heatmaps of P values were generated using the R package (version 3.2.1; http://www.r-project.org/).

Clustering

Hierarchical clustering of the core set of single stress DEGs was performed on $\log_2$ fold-change expression values using the R function hclust with a cosine similarity metric and average linkage. The cutree function was used with a visually determined cut height to partition the resulting dendrogram into clusters. Clustering of the core set of single stress DEGs and shared main treatment datasets was performed using model-based clustering package mclust version 4 in R (Fraley *et al.*, 2012) with the number of clusters optimized in the range 1 to 10 using the Bayesian information criterion.

Wigwams analysis

To identify modules of co-expressed genes across single and sequential stresses the Wigwams algorithm was applied (Polanski *et al.*, 2014), using $\log_2$ transformed expression values for the DEGs of each single stress across their respective sequential double stresses. For each main treatment, Wigwams was run with default arguments to partition genes into modules that indicate co-expression in subsets of the relevant main treatments.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Expression patterns of all 35 Wigwams modules from the *B. cinerea* set of DEGs during single and sequential stress conditions.

Figure S2. Expression patterns of all 93 Wigwams modules from the *P. rapae* set of DEGs during single and sequential stress conditions.

Figure S3. Expression patterns of all 72 Wigwams modules from the drought set of DEGs during single and sequential stress conditions.

Table S1. Differentially expressed genes (DEGs) of *Arabidopsis thaliana* (AGI numbers of DEGs; FDR <0.05; >2-fold) in response to *B. cinerea* infection, *P. rapae* infestation, drought stress, and their six sequential combinations at four consecutive time points.

Table S2. Enriched GO terms in gene clusters shown in Figure 3 (Clustering of all DEGs of the single stresses).

Table S3. Enriched GO terms in gene clusters shown in Figure 4(b) (Shared core DEGs between the three single stress responses).

Table S4. Enriched GO terms in gene clusters shown in Figure 5 (Dynamics of the expression of the *B. cinerea* set of DEGs during single and sequential double stresses).

Table S5. Enriched GO terms in Wigwams modules of *B. cinerea*, *rapae*, and Drought set of DEGs.

Table S6. Enriched GO terms in gene clusters shown in Figure 9 (Dynamics of the expression of the *P. rapae* set of DEGs during single and sequential double stresses).

Table S7. Enriched GO terms in gene clusters shown in Figure 12 (Dynamics of the expression of the drought set of DEGs during single and sequential double stresses).

REFERENCES

Agrawal, A.A. (2011) Current trends in the evolutionary ecology of plant defence. *Funct. Ecol.* **25**, 420–432.

Anders, S., Pyl, P. and Huber, W. (2014) HTSeq-a Python framework to work with high-throughput sequencing data. *Bioinformatics*, **31**, 166–169.

Appel, H.M., Maqbool, S.B., Raina, S. *et al.* (2014) Transcriptional and metabolic signatures of Arabidopsis responses to chewing damage by an insect herbivore and bacterial infection and the consequences of their interaction. *Front. Plant Sci.* **5**, 441.

Atkinson, N.J. and Urwin, P.E. (2012) The interaction of plant biotic and abiotic stresses: from genes to the field. *J. Exp. Bot.* **63**, 3523–3543.

Atkinson, N.J., Lilley, C.J. and Urwin, P.E. (2013) Identification of genes involved in the response of Arabidopsis to simultaneous biotic and abiotic stresses. *Plant Physiol.* **162**, 2028–2041.

Birkenbihl, R.P., Diezel, C. and Somssich, I.E. (2012) Arabidopsis WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward *Botrytis cinerea* infection. *Plant Physiol.* **159**, 266–285.

Bodenhausen, N. and Reymond, P. (2007) Signaling pathways controlling induced resistance to insect herbivores in Arabidopsis. *Mol. Plant Microbe Interact.* **20**, 1406–1420.

Boyle, E.I., Weng, S.A., Gollub, J., Jin, H., Botstein, D., Cherry, J.M. and Sherlock, G. (2004) GO:TermFinder – open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. *Bioinformatics*, **20**, 3710–3715.

Bray, E.A. (1997) Plant responses to water deficit. *Trends Plant Sci.* **2**, 48–54.

Broekgaarden, C., Caarls, L., Vos, I.A., Pieterse, C.M.J. and Van Wees, S.C.M. (2015) Ethylene:traffic controller on hormonal crossroads to defense. *Plant Physiol.* **169**, 2371–2379.

Caarls, L., Pieterse, C.M.J. and Van Wees, S.C.M. (2015) How salicylic acid takes transcriptional control over jasmonic acid signaling. *Front. Plant Sci.* **6**, 11.

Clauw, P., Coppens, F., De Beuf, K., Dhondt, S., Van Daele, T., Maleux, K., Storme, V., Clement, L., Gonzalez, N. and Inzé, D. (2015) Leaf responses to mild drought stress in natural variants of Arabidopsis. *Plant Physiol.* **167**, 800–816.

Cyr, H. and Pace, M.L. (1993) Magnitude and patterns of herbivory in aquatic and terrestrial ecosystems. *Nature*, **361**, 148–150.

Davila Olivas, N.H., Coolen, S., Huang, P. *et al.* (2016) Effect of prior drought and pathogen stress on Arabidopsis transcriptome changes to caterpillar herbivory. *New Phytol.* **210**, 1344–1356. doi:10.1111/nph.13847. [Epub ahead of print].

De Vos, M., Van Oosten, V.R., Van Poecke, R.M.P. *et al.* (2005) Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Mol. Plant Microbe Interact.* **18**, 923–937.

De Vos, M., Van Zaanen, W., Koornneef, A., Korzelius, J.P., Dicke, M., Van Loon, L.C. and Pieterse, C.M.J. (2006) Herbivore-induced resistance against microbial pathogens in Arabidopsis. *Plant Physiol.* **142**, 352–363.

Dean, R., Van Kan, J.A.L., Pretorius, Z.A. *et al.* (2012) The Top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.* **13**, 414–430.

Diaz, J., Ten Have, A. and Van Kan, J.A.L. (2002) The role of ethylene and wound signaling in resistance of tomato to *Botrytis cinerea*. *Plant Physiol.* **129**, 1341–1351.

El Oirdi, M., Abd El Rahman, T., Rigano, L., El Hadrami, A., Rodriguez, M.C., Daayf, F., Vojnov, A. and Bouarab, K. (2011) *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato. *Plant Cell*, **23**, 2405–2421.

Ferrari, S., Galletti, R., Denoux, C., De Lorenzo, G., Ausubel, F.M. and Dewdney, J. (2007) Resistance to *Botrytis cinerea* induced in Arabidopsis by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires PHYTOALEXIN DEFICIENT3. *Plant Physiol.* **144**, 367–379.

Fraley, C., Raftery, A.E. and Scrucca, L. (2012) *Mclust Version 4 for R: Normal Mixture Modeling and Model-Based Clustering, Classification, and Density Estimation: Department of Statistics*. Washington: University of Washington.

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Curr. Opin. Plant Biol.* **9**, 436–442.

Geraats, B.P.J., Bakker, P.A.H.M. and Van Loon, L.C. (2002) Ethylene insensitivity impairs resistance to soilborne pathogens in tobacco and *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* **15**, 1078–1085.

Giron, D., Frago, E., Glevarec, G., Pieterse, C.M.J. and Dicke, M. (2013) Cytokinins as key regulators in plant–microbe–insect interactions: connecting plant growth and defence. *Funct. Ecol.* **27**, 599–609.

Glazebrook, J. (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* **43**, 205–227.

Hopkins, R.J., Van Dam, N.M. and Van Loon, J.J.A. (2009) Role of glucosinolates in insect-plant relationships and multitrophic interactions. *Annu. Rev. Entomol.* **54**, 57–83.

Howe, G.A. and Jander, G. (2008) Plant immunity to insect herbivores. *Annu. Rev. Plant Biol.* **59**, 41–66.

Huang, D.Q., Wu, W.R., Abrams, S.R. and Cutler, A.J. (2008) The relationship of drought-related gene expression in *Arabidopsis thaliana* to hormonal and environmental factors. *J. Exp. Bot.* **59**, 2991–3007.

Ingram, J. and Bartels, D. (1996) The molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 377–403.

Johnson, M.T.J. (2011) Evolutionary ecology of plant defences against herbivores. *Funct. Ecol.* **25**, 305–311.

Kazan, K. and Lyons, R. (2014) Intervention of phytohormone pathways by pathogen effectors. *Plant Cell*, **26**, 2285–2309.

Kissoudis, C., Van de Wiel, C., Visser, R.G.F. and Van der Linden, G. (2014) Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk. *Front. Plant Sci.* **5**, 207.

Koornneef, A., Leon-Reyes, A., Ritsema, T., Verhage, A., Den Otter, F.C., Van Loon, L.C. and Pieterse, C.M.J. (2008) Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. *Plant Physiol.* **147**, 1358–1368.

Laluk, K. and Mengiste, T. (2010) Necrotroph attacks on plants: wanton destruction or covert extortion? *Arabidopsis Book*, **8**, e0136.

Liu, S., Kracher, B., Ziegler, J., Birkenbihl, R.P. and Somssich, I.E. (2015) Negative regulation of ABA signaling by WRKY33 is critical for Arabidopsis immunity towards *Botrytis cinerea* 2100. *eLife*, **4**, e07295.

Love, M.I., Huber, W. and Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550.

Mohr, P.G. and Cahill, D.M. (2003) Abscisic acid influences the susceptibility of *Arabidopsis thaliana* to *Pseudomonas syringae* pv. *tomato* and *Peronospora parasitica*. *Funct. Plant Biol.* **30**, 461–469.

O'Brien, J.A. and Benková, E. (2013) Cytokinin cross-talking during biotic and abiotic stress responses. *Front. Plant Sci.* **4**, 451.

Pel, M.J.C. and Pieterse, C.M.J. (2013) Microbial recognition and evasion of host immunity. *J. Exp. Bot.* **64**, 1237–1248.

Pieterse, C.M.J., Leon-Reyes, A., Van der Ent, S. and Van Wees, S.C.M. (2009) Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* **5**, 308–316.

Pieterse, C.M.J., Van der Does, D., Zamioudis, C., Leon-Reyes, A. and Van Wees, S.C.M. (2012) Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* **28**, 489–521.

Pieterse, C.M.J., Zamioudis, C., Berendsen, R.L., Weller, D.M., Van Wees, S.C.M. and Bakker, P.A.H.M. (2014) Induced systemic resistance by beneficial microbes. *Annu. Rev. Phytopathol.* **52**, 347–375.

Poelman, E.H., Broekgaarden, C., Van Loon, J.J.A. and Dicke, M. (2008) Early season herbivore differentially affects plant defence responses to subsequently colonizing herbivores and their abundance in the field. *Mol. Ecol.* **17**, 3352–3365.

Polanski, K., Rhodes, J., Hill, C. *et al.* (2014) Wigwams: identifying gene modules co-regulated across multiple biological conditions. *Bioinformatics*, **30**, 962–970.

Prasch, C.M. and Sonnewald, U. (2013) Simultaneous application of heat, drought, and virus to Arabidopsis plants reveals significant shifts in signaling networks. *Plant Physiol.* **162**, 1849–1866.

Ramegowda, V. and Senthil-Kumar, M. (2015) The interactive effects of simultaneous biotic and abiotic stresses on plants: Mechanistic understanding from drought and pathogen combination. *J. Plant Physiol.* **176**, 47–54.

Rasmussen, S., Barah, P., Suarez-Rodriguez, M.C., Bressendorff, S., Friis, P., Costantino, P., Bones, A.M., Nielsen, H.B. and Mundy, J. (2013) Transcriptome responses to combinations of stresses in Arabidopsis. *Plant Physiol.* **161**, 1783–1794.

Rehrig, E.M., Appel, H.M., Jones, A.D. and Schultz, J.C. (2014) Roles for jasmonate- and ethylene-induced transcription factors in the ability of Arabidopsis to respond differentially to damage caused by two insect herbivores. *Front. Plant Sci.* **5**, 407.

Rejeb, I.B., Pastor, V. and Mauch-Mani, B. (2014) Plant responses to simultaneous biotic and abiotic stress: molecular mechanisms. *Plants*, **3**, 458–475.

Reymond, P. and Farmer, E.E. (1998) Jasmonate and salicylate as global signals for defense gene expression. *Curr. Opin. Plant Biol.* **1**, 404–411.

Reymond, P., Weber, H., Damond, M. and Farmer, E.E. (2000) Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. *Plant Cell*, **12**, 707–719.

Reymond, P., Bodenhausen, N., Van Poecke, R.M.P., Krishnamurthy, V., Dicke, M. and Farmer, E.E. (2004) A conserved transcriptional pattern in response to a specialist and a generalist herbivore. *Plant Cell*, **16**, 3132–3147.

Riera, M., Valon, C., Fenzi, F., Giraudat, J. and Leung, J. (2005) The genetics of adaptive responses to drought stress: abscisic acid-dependent and abscisic acid-independent signalling components. *Physiol. Plant.* **123**, 111–119.

Rivero, R.M., Mestre, T.C., Mittler, R., Rubio, F., Garcia-Sanchez, F. and Martinez, V. (2014) The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. *Plant Cell Environ.* **37**, 1059–1073.

Robert-Seilaniantz, A., Grant, M. and Jones, J.D.G. (2011) Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annu. Rev. Phytopathol.* **49**, 317–343.

Rowe, H.C., Walley, J.W., Corwin, J., Chan, E.K.F., Dehesh, K. and Kliebenstein, D.J. (2010) Deficiencies in jasmonate-mediated plant defense reveal quantitative variation in *Botrytis cinerea* pathogenesis. *PLoS Pathog.* **6**, e1000861.

Santino, A., Taurino, M., De Domenico, S., Bonsegna, S., Poltronieri, P., Pastor, V. and Flors, V. (2013) Jasmonate signaling in plant development and defense response to multiple (a)biotic stresses. *Plant Cell Rep.* **32**, 1085–1098.

Schachtman, D.P. and Goodger, J.Q.D. (2008) Chemical root to shoot signaling under drought. *Trends Plant Sci.* **13**, 281–287.

Sewelam, N., Oshima, Y., Mitsuda, N. and Ohme-Takagi, M. (2014) A step towards understanding plant responses to multiple environmental stresses: a genome-wide study. *Plant Cell Environ.* **37**, 2024–2035.

Sham, A., Moustafa, K., Al-Ameri, S., Al-Azzawi, A., Iratni, R. and AbuQamar, S. (2015) Identification of Arabidopsis candidate genes in response to biotic and abiotic stresses using comparative microarrays. *PLoS ONE*, **10**, e0125666.

Shinozaki, K., Yamaguchi-Shinozaki, K. and Seki, M. (2003) Regulatory network of gene expression in the drought and cold stress responses. *Curr. Opin. Plant Biol.* **6**, 410–417.

Soler, R., Erb, M. and Kaplan, I. (2013) Long distance root-shoot signalling in plant-insect community interactions. *Trends Plant Sci.* **18**, 149–156.

Staats, M. and Van Kan, J.A.L. (2012) Genome update of *Botrytis cinerea* strains B05.10 and T4. *Eukaryot. Cell*, **11**, 1413–1414.

Stam, J.M., Kroes, A., Li, Y.H., Gols, R., Van Loon, J.J.A., Poelman, E.H. and Dicke, M. (2014) Plant interactions with multiple insect herbivores: from community to genes. *Annu. Rev. Plant Biol.* **65**, 689–713.

Suzuki, N., Rivero, R.M., Shulaev, V., Blumwald, E. and Mittler, R. (2014) Abiotic and biotic stress combinations. *New Phytol.* **203**, 32–43.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A. and Broekaert, W.F. (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. *Proc. Natl Acad. Sci. USA*, **95**, 15107–15111.

Thomma, B.P.H.J., Eggermont, K., Tierens, K.F.M. and Broekaert, W.F. (1999) Requirement of functional *ethylene-insensitive 2* gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea*. *Plant Physiol.* **121**, 1093–1102.

Trapnell, C., Pachter, L. and Salzberg, S.L. (2009) TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics*, **25**, 1105–1111.

Van Kan, J.A.L. (2006) Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends Plant Sci.* **11**, 247–253.

Van Oosten, V.R., Bodenhausen, N., Reymond, P., Van Pelt, J.A., Van Loon, L.C., Dicke, M. and Pieterse, C.M.J. (2008) Differential effectiveness of microbially induced resistance against herbivorous insects in Arabidopsis. *Mol. Plant Microbe Interact.* **21**, 919–930.

Van Verk, M.C., Hickman, R., Pieterse, C.M.J. and Van Wees, S.C.M. (2013) RNA-Seq: revelation of the messengers. *Trends Plant Sci.* **18**, 175–179.

Van Wees, S.C.M., Van Pelt, J.A., Bakker, P.A.H.M. and Pieterse, C.M.J. (2013) Bioassays for assessing jasmonate-dependent defenses triggered by pathogens, herbivorous insects, or beneficial rhizobacteria. *Methods Mol. Biol.* **1011**, 35–49.

Verhage, A., Vlaardingerbroek, I., Raaymakers, C., Van Dam, N., Dicke, M., Van Wees, S.C.M. and Pieterse, C.M.J. (2011) Rewiring of the jasmonate signaling pathway in Arabidopsis during insect herbivory. *Front. Plant Sci.* **2**, 47.

Vos, I.A., Pieterse, C.M.J. and Van Wees, S.C.M. (2013a) Costs and benefits of hormone-regulated plant defences. *Plant. Pathol.* **62**, 43–55.

Vos, I.A., Verhage, A., Schuurink, R.C., Watt, L.G., Pieterse, C.M.J. and Van Wees, S.C.M. (2013b) Onset of herbivore-induced resistance in systemic tissue primed for jasmonate-dependent defenses is activated by abscisic acid. *Front. Plant Sci.* **4**, 539.

Vos, I.A., Moritz, L., Pieterse, C.M.J. and Van Wees, S.C.M. (2015) Impact of hormonal crosstalk on plant resistance and fitness under multi-attacker conditions. *Front. Plant Sci.* **6**, 639.

Wang, W. and Wang, Z.-Y. (2014) At the intersection of plant growth and immunity. *Cell Host Microbe*, **15**, 400–402.

Wasternack, C. (2015) How jasmonates earned their laurels: past and present. *J. Plant Growth Regul.* **34**, 761–794.

Windram, O., Madhou, P., McHattie, S. *et al.* (2012) Arabidopsis defense against *Botrytis cinerea:* Chronology and regulation deciphered by high-resolution temporal transcriptomic analysis. *Plant Cell*, **24**, 3530–3557.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant Biol.* **57**, 781–803.

Yasuda, M., Ishikawa, A., Jikumaru, Y. *et al.* (2008) Antagonistic interaction between systemic acquired resistance and the abscisic acid-mediated abiotic stress response in Arabidopsis. *Plant Cell*, **20**, 1678–1692.