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RNAseq-based transcriptome analysis of *Lactuca sativa* infected by the fungal necrotroph *Botrytis cinerea*

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ABSTRACT

The fungal pathogen *Botrytis cinerea* establishes a necrotrophic interaction with its host plants, including lettuce (*Lactuca sativa*), causing it to wilt, collapse and eventually dry up and die, which results in serious economic losses. Global expression profiling using RNAseq and the newly sequenced lettuce genome identified a complex network of genes involved in the lettuce–*B. cinerea* interaction. The observed high number of differentially expressed genes allowed us to classify them according to the biological pathways in which they are implicated, generating a holistic picture. Most pronounced were the induction of the phenylpropanoid pathway and terpenoid biosynthesis, whereas photosynthesis was globally down-regulated at 48 h post-inoculation. Large-scale comparison with data available on the interaction of *B. cinerea* with the model plant *Arabidopsis thaliana* revealed both general and species-specific responses to infection with this pathogen. Surprisingly, expression analysis of selected genes could not detect significant systemic transcriptional alterations in lettuce leaves distant from the inoculation site. Additionally, we assessed the response of these lettuce genes to a biotrophic pathogen, *Bremia lactucae*, revealing that similar pathways are induced during compatible interactions of lettuce with necrotrophic and biotrophic pathogens.

Key-words: *Bremia lactucae*; grey mould; lettuce; transcriptomics.

INTRODUCTION

The fungal pathogen *Botrytis cinerea* (teleomorph *Botryotinia fuckeliana*) causes disease in more than 200 plant species, including numerous economically important crops (Elad *et al*. 2004). It attacks many organs, including leaves, stems, fruits and flowers, during both pre- and post-harvest (Leroux 2004). *B. cinerea* is difficult to control due to its wide host range, its survival for long periods of time as sclerotia in crop debris and the development of multidrug-resistant strains (Williamson *et al*. 2007). The fungus has a predominantly necrotrophic lifestyle, which involves killing plant host cells by diverse phytotoxic compounds and cell wall-degrading enzymes, after which it extracts nutrients from the dead cells (Williamson *et al*. 2007; Lazniewska *et al*. 2010). *B. cinerea* has become a model pathogen for the molecular study of this type of plant–pathogen interaction (Williamson *et al*. 2007; Mengiste 2012).

Plant defence mechanisms against necrotrophic pathogens such as *B. cinerea* are considered to be complex and differ from those that are effective against biotrophs. Until recently, defence responses against necrotrophs were thought to be mediated almost exclusively by pathways that depend on the phytohormones ethylene (ET) and jasmonic acid (JA), whereas salicylic acid (SA)-dependent responses and systemic acquired resistance (SAR) were not assumed to play a role (Glazebrook 2005). However, the induction of SA-related genes [e.g. *pathogenesis-related* (*PR*) *1* and *5*] and increased susceptibility to *B. cinerea* of transgenic *Arabidopsis thaliana* NahG plants, which are compromised in SA accumulation, have been reported (Govrin & Levine 2002; Ferrari *et al*. 2003). Other phytohormones including auxin, abscisic acid (ABA) and gibberellic acid (GA) are also known to be involved in defence responses against *B. cinerea* (Elad 1997; Audenaert, De Meyer & Hofte, 2002; Llorente *et al*. 2008; Grant & Jones 2009). Moreover, *B. cinerea* synthesizes plant hormone analogues and elicitors that induce these pathways, thereby possibly altering the hormone balance in infected plant tissues and affecting the plant's susceptibility to pathogen development (Sharon *et al*. 2004; El Oirdi *et al*. 2011). Induction of signalling pathways leads to cell wall modifications, production of PR-proteins and induction of the phenylpropanoid pathway, the latter resulting in the synthesis of secondary metabolites, which act as antimicrobial compounds as well as signal molecules in plant–microbe interactions (Lazniewska *et al*. 2010; Naoumkina *et al*. 2010).

One of the earliest defence responses of plants to *B. cinerea* infection is the generation of an oxidative burst, which is characterized by the rapid generation of different reactive oxygen species (ROS) (Lamb & Dixon 1997). These ROS can act directly against the pathogen through their toxic activity (Chen & Schopfer 1999) or indirectly through defence signalling (Torres 2010) and by strengthening the physical barriers in the plant (Huckelhoven 2007). A typical result of this oxidative burst is the generation of a hypersensitivity response (HR), including localized cell death (Levine *et al*. 1994). HR is an important component of defence against biotrophic pathogens; however, it can be beneficial for necrotrophs, such as *B. cinerea*, that feed on dead tissue (Govrin & Levine 2000). Moreover, *B. cinerea* can even induce the

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generation of ROS during interaction with its host plant (Schouten *et al*. 2002). Consequently, plant resistance to *B. cinerea* has been reported to depend on the balance between cell death and survival by counteracting the toxic effects of ROS (van Baarlen *et al*. 2007).

So far, large-scale transcriptional profiling of the plant response following inoculation with *B. cinerea* has only been reported for the model plant *A. thaliana* and tomato (AbuQamar *et al*. 2006; Asselbergh *et al*. 2007; Ferrari *et al*. 2007; Cantu *et al*. 2009; Birkenbihl, Diezel & Somssich 2012; Mathys *et al*. 2012; Mulema & Denby 2012; Windram *et al*. 2012). Up-regulation of genes involved in general biotic stress (e.g. *PR*-genes), oxidative stress, the phenylpropanoid pathway, and hormone biosynthesis and signalling were observed in all of these studies. Small-scale transcriptional studies have been performed in other plant species as well. For example, Repka (2006) confirmed the induction of the phenylpropanoid pathway and *PR*-genes (of family 1, 2, 3, 8 and 9) in grapevine after treatment with *B. cinerea*-derived elicitors. Similarly, a chitinase-encoding gene and a JA-biosynthesis gene were found to be induced during the *B. cinerea* infection of *Brassica napus* (Sarosh, Danielsson & Meijer 2009). In strawberry, induction of *PR*-genes (of family 1 and 5), a JA-biosynthesis gene and a phenylpropanoid pathway gene was observed (Harel *et al*. 2012). It is thus likely that homologous genes and similar defence pathways are induced in other host plants, but this has not been documented on a genome-wide scale.

The present study analyses changes in the lettuce transcriptome following inoculation with *B. cinerea*. Together with *Rhizoctonia solani*, *Sclerotinia* spp. and *Pythium* spp., *B. cinerea* is one of the main causes responsible for basal rot in lettuce greenhouses, especially in winter (Wareing *et al*. 1986; Nordskog *et al*. 2008; Van Beneden *et al*. 2009). Sowley, Dewey & Shaw (2010) demonstrated that *B. cinerea*, initially present in lettuce seeds, can spread to newly produced tissue. Furthermore, they showed that asymptomatic lettuce plants can harbour *B. cinerea* mycelium in roots, stems and leaves. This type of growth is atypical for a pathogen regarded as exhibiting a purely necrotrophic lifestyle (Sowley *et al*. 2010). Therefore, a more detailed understanding of this host–pathogen interaction is warranted.

In this study, the response of lettuce (*Lactuca sativa* cv. Salinas) induced by *B. cinerea* was determined via a detailed transcriptomic analysis using RNA sequencing (RNAseq). For this purpose, we used the recently sequenced lettuce genome as the reference sequence (http://lgr.genomecenter.ucdavis.edu). By including three different time points, we provide comprehensive insight in molecular mechanisms involved at different steps in the response of lettuce to *B. cinerea* infection. Both gene ontology (GO) and overviews of metabolism using MapMan were conducted to interpret the results. In addition, the expression of a selected set of genes was measured using qRT-PCR on uninoculated leaves of the same set of plants, allowing comparison of local versus systemic responses. Finally, qRT-PCR was likewise used to evaluate whether the observed responses to the necrotrophic *B. cinerea* also occur after inoculation with the biotrophic oomycete, *Bremia lactucae*, the causal agent of lettuce downy mildew and one of the most important lettuce pathogens in terms of economic loss (Crute 1992; Lebeda *et al*. 2008). To our knowledge, this is the first report of a complete quantitative transcriptome analysis of lettuce.

MATERIALS AND METHODS

Biological materials

Cultivation and spore harvesting of *B. cinerea* strain B05.10 (provided by Rudi Aerts, Katholieke Hogeschool Kempen, Belgium) was performed as described previously (Broekaert *et al*. 1990). *B. lactucae* isolate Bl:26 was provided by Monica Höfte (Universiteit Gent, Belgium) and was maintained on lettuce plants during the described disease assays. Seeds of *L. sativa* cv. Salinas were obtained from Rijk Zwaan (http://www.rijkzwaan.nl).

B. cinerea disease assays

L. sativa cv. Salinas plants were grown for 5 weeks (8–10 leaves per plant) in soil ('DCM potgrond voor Zaaien en Stekken', DCM, Sint-Katelijne-Waver, Belgium) in a growth chamber with 21 °C, 75% humidity and a 12 h daylight cycle with a light intensity of approximately 120 μmol m^{-2} s^{-2}. Four 5 μL drops of a *B. cinerea* spore suspension (5×10^5 mL^{-1} in $^1/_2$ PDB) were inoculated onto two leaves per plant. Mock-inoculated plants received only $^1/_2$ PDB. Plants were kept in transparent sealed boxes to retain almost 100% humidity after inoculation. For RNAseq analysis of the local response to *B. cinerea*, two inoculated leaves of four individual plants were collected for each treatment at 12, 24 and 48 h post-inoculation (hpi) in three independent experiments. For analysis of the systemic response, two uninoculated leaves of inoculated plants were collected for each treatment at 48, 72 and 96 hpi, also in three independent experiments. The leaves collected at 48 hpi are coming from the same plants as the ones used for the local response analysis, and the ones collected at 72 and 96 hpi are from different plants but collected during the same experiments. Plant samples were immediately snap-frozen and crushed in liquid nitrogen and stored at –80 °C until RNA extraction was performed.

B. lactucae disease assays

L. sativa cv. Salinas plants were grown in soil ('DCM potgrond voor Zaaien en Stekken', DCM, Sint-Katelijne-Waver, Belgium) in a growth chamber with 16 °C, 75% humidity and a 12 h daylight cycle with a light intensity of approximately 120 μmol m^{-2} s^{-2}. Five-week-old plants (8–10 leaves per plant) were spray-inoculated with a spore suspension of *B. lactucae* isolate Bl:26 (5×10^5 spores mL^{-1} in H_2O). Mock-inoculated plants were spray-inoculated with water only. Plants were kept in transparent sealed boxes to retain almost 100% humidity after inoculation. Two inoculated leaves from each of four plants were collected for each treatment at

8 dpi in three independent experiments. Plant samples were immediately snap-frozen and crushed in liquid nitrogen and stored at –80 °C until RNA extraction was performed.

Illumina sequencing and data processing

Total RNA was isolated using a combination of Plant RNA Reagent® (Invitrogen Paisley, UK; http://www.invitrogen.com) and a Qiagen RNeasy kit (Qiagen, Valencia, CA, USA; http://www.qiagen.com). RNA quantity and quality control was performed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA; http://www.nanodrop.com) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA; http://www.agilent.com). Ten micrograms of total RNA from all samples were used to make individual bar-coded libraries as described by Zhong *et al*. (2011) using NEXTflex™ PCR-Free Barcodes (Bioo Scientific, Austin, TX, USA; http://www.biooscientific.com). The individual libraries were pooled using the same amount of each library and the quality of the final library pool was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies) and sequenced at the Expression Analysis Core Facility (University of California Davis, Davis, CA, USA) in one lane of an Illumina HiSeq 2000 sequencer (single end, 100 bp; (Illumina, San Diego, CA, USA). Raw Illumina sequence reads (available from the Sequence Read Archive – accession number SRA059059) were processed using the allPrep script (Meric Lieberman, University of California Davis; http://comailab.genomecenter.ucdavis.edu/index.php/Barcoded_data_preparation_tools) including splitting by bar code, removal of adapter sequences, and filtering and trimming for quality. Hereby reads containing an 'N' were removed and reads were trimmed from both ends until the average of all 5 bp sliding windows reached a Phred score of 20 or higher. All sequences shorter than 26 bases were discarded.

Data analysis

All high-quality reads of each sample were mapped using CLC Genomics Workbench software (CLCbio, Aarhus, Denmark; http://www.clcbio.com) to the predicted gene models of *L. sativa* cv. Salinas (http://lgr.genomecenter.ucdavis.edu) and genes of *B. cinerea* B05.10 downloaded from http://www.broadinstitute.org (*Botrytis cinerea* Sequencing Project, Broad Institute of Harvard and MIT). At least 95% of the bases were required to align to the reference and a maximum of two mismatches were allowed. Differential gene expression was inferred based on the total mapping counts using the edgeR package (version 2.6.8; Robinson, McCarthy & Smyth 2010), implemented in R (R Development Core Team 2011), taking into account a batch effect between the different biological experiments. Only genes that were detected in at least three biological replicates of one condition, above the detection threshold of 1 count per million, were used in this analysis. A gene-wise dispersion was estimated using default settings for a generalized linear model. Genes were considered differentially expressed (DE) if they possessed an absolute value of $\log_2$-fold change ≥ 1 and an adjusted P-value ≤ 0.05 (with application of the Benjamini and Hochberg correction for multiple testing; Benjamini & Hochberg 2000) (Supporting Information Appendix S1).

Corresponding *A. thaliana* orthologues for all detected lettuce genes were determined based on the Reciprocal Smallest Distance algorithm (Wall *et al*. 2003) (Supporting Information Appendix S1). The applied parameters are (1) a minimal expected (E)-value of 10^{-10} for BLASTp between translated lettuce gene models and the *A. thaliana* proteins downloaded from http://www.arabidopsis.org and (2) the divergent parameter (div = 0.5), which defines the shape parameter of the gamma distribution to estimate the phylogenetic distances among all sequences in the dataset. Each lettuce gene model was also submitted to the BLAST2GO program (http://www.blast2go.org/, Bioinformatics and Genomics Department, Centro de Investigación Príncipe Valencia, Spain; Conesa *et al*. 2005; Conesa & Götz, 2008) for functional annotation. This program retrieves the GO terms for the gene models using the structured vocabulary provided by the Gene Ontology project (http://www.geneontology.org/). Default settings were applied, but the minimal E-value was lowered to a more stringent value of 10^{-10} (van der Merwe, Franchini & Roodt-Wilding 2011; Garcia *et al*. 2012; Zhang *et al*. 2012). GO enrichment analysis was performed using the Wallenius non-central hypergeometric distribution in the Goseq package (version 1.10.0; Young *et al*. 2010). For the *A. thaliana* dataset of Ferrari *et al*. (2007), we performed GO analysis using the bioinformatics resource DAVID, which is suitable for microarray data (Huang, Sherman & Lempicki 2009a,b). For pathway analysis of both lettuce and *A. thaliana* datasets, we used the MapMan tool (Thimm MapMan *et al*. 2004). A MapMan mapping file was specifically generated for the lettuce genes by the Mercator tool (http://mapman.gabipd.org/web/guest/app/mercator), which bins all genes according to hierarchical ontologies after searching a variety of databases. Default parameters were retained, additionally ORYZA TIGR5 rice protein and IPR Interpo scans were selected and the 'unassigned' bin was considered with equal weight when assigning bin codes (May *et al*. 2008; Howell *et al*. 2009).

qRT-PCR

Primers were developed using Primer3 software (Supporting Information Appendix S2; Rozen & Skaletsky 2000). RNA extraction, DNase treatment and reverse transcription were carried out as described previously (Mirouze *et al*. 2006). The qRT-PCR analysis was carried out using the StepOnePlus System and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA; http://www.appliedbiosystems.com) as described by Mathys *et al*. (2012). Two normalization genes [*β-tubulin* (Lsa017612.1) and *adenine phosphoribosyltransferase 1* (Lsa039252.1)] were used (Argyris *et al*. 2008). Relative $\log_2$ induction ratios of treated samples compared with mock-treatment were calculated based on the ΔΔCt method (Livak & Schmittgen 2001).

RESULTS

Analysis of RNAseq data of lettuce after inoculation with *B. cinerea*

To identify lettuce genes that are differentially expressed during the response to *B. cinerea*, leaves were inoculated with *B. cinerea* or mock-inoculated. Successful infection was characterized by the appearance of soft rot symptoms at 48 hpi. At 12, 24 and 48 hpi, inoculated leaf samples were collected and RNAseq was performed. A total of 166 300 114 high-quality reads (average length = 99 bp) was generated using an Illumina HiSeq 2000 sequencer. Each sample and each biological condition was represented by an average of 9.2 million and 27.6 million reads, respectively (Table 1). For each sample, ~77% of the reads could be mapped to the lettuce genome reference; and of these mapped reads, ~92% matched uniquely. The amount of reads mapped to the *B. cinerea* reference increased over time in the samples inoculated with *B. cinerea*, with on average 5.5% mapped reads at 48 hpi (Table 1). The genes identified from *B. cinerea* are listed in Supporting Information Appendix S3.

The relation between gene expression levels in the three biological replicates and at the different time points is shown in Fig. 1. Overall, these data agree on the biological distance between all samples. There is a high correlation between the three samples at 48 hpi with *B. cinerea*, which are different from all other samples. Furthermore, samples taken at 12 hpi (collected in the dark) are clearly dissimilar from samples taken at 24 and 48 hpi control samples (both collected in the light). This reflects global diurnal fluctuations in transcript profiles of plants harvested in dark or light conditions. Moreover, there is a high correlation between both mock-inoculated samples and samples inoculated with *B. cinerea* within a same biological experiment at 12 hpi. At this time point, this correlation is higher than between biological replicates of one condition, predicting only small transcriptional changes at 12 hpi. Finally, these representations support the need to take into account a batch effect between biological replicates, as there is a high correlation between samples belonging to the same experiment at all time points, with especially experiment 1 being slightly different from the other two (Fig. 1a,b).

A total of 19 612 genes (representing 44% of the total number of annotated genes) were detected above the detection threshold of 1 count per million in at least the three biological replicates of one condition. At the three different time points after inoculation with *B. cinerea* (12, 24 and 48 hpi), we detected 1, 139 and 4598 differentially expressed (DE) genes, respectively (Fig. 2 and Supporting Information Appendix S1). More up-regulated genes than down-regulated genes were observed at all time points. Of the 127 genes up-regulated at 24 hpi, 115 genes were also up-regulated at 48 hpi (Fig. 2a). In contrast, only 1 of 12 down-regulated genes at 24 hpi was also down-regulated at 48 hpi (Fig. 2b).

To validate the RNAseq results, expression levels of a selected set of up- and down-regulated and unaffected genes was analysed by qRT-PCR (Fig. 3). A close correlation ($R = 0.89$) was observed between $\log_2$-fold changes measured by RNAseq and qRT-PCR.

Biological responses of lettuce after inoculation with *B. cinerea*

To facilitate the global analysis of gene expression profiles and evaluate the potential functions of the large number of genes that showed significant differential expression at 48 hpi, DE genes were classified using GO (Gene Ontology Consortium 2000). GO terms were assigned to the lettuce gene models using BLAST2GO. For 96% of the genes above the detection threshold, a blast hit was obtained; and to 72% of these genes, one or more GO terms could be assigned. Over-representations of GO terms in the set of differentially up- and down-regulated genes were evaluated to indicate which biological processes, molecular functions and cellular components were most affected after inoculation with *B. cinerea*.

The biological processes that are significantly ($P \leq 0.005$) enriched in the set of up-regulated genes (Supporting Information Appendix S4) include many defence responses, for example, responses to chitin, wounding, oxidative stress, fungus and respiratory burst. In accordance to this, signalling mediated by or responses to the major defence-related phytohormones (JA, SA and ET) were also significantly induced; but interestingly, ABA-mediated signalling was the most pronounced affected hormonal pathway. Furthermore, biosynthesis of secondary metabolites, via the phenylpropanoid pathway (mainly lignin biosynthesis) and terpenoid biosynthesis, are induced. GO terms for molecular function that were up-regulated include genes that code for proteins with haem-binding activity, electron carrier activity and oxidoreductase activity. Several significantly induced GO terms representing cellular components associate with the cell wall, plasma- and endomembranes, proteasome complex and mitochondrion.

The biological processes that are significantly enriched in the set of down-regulated genes (Supporting Information Appendix S4) include many processes related to photosynthesis, carbohydrate metabolic processes and the response to auxin. More vital plant processes are down-regulated, demonstrated by the enrichment of water and lipid transport, syncytium formation, cuticle development, cutin biosynthetic process and circadian rhythm. Analysis of the GO terms representing molecular function and cellular components provides the same insight with most notably the down-regulation of numerous GO terms related to photosynthesis.

Subsequently, transcriptional changes at 48 hpi were visualized via the MapMan tool (Thimm *et al*. 2004). Figure 4 shows a general overview of metabolic changes and clearly confirms the up-regulation of secondary metabolism and down-regulation of photosynthesis-related processes. With respect to secondary metabolism, MapMan reveals the up-regulation of genes involved in the production of almost all secondary metabolites, with the exception of chalcone-related genes, which are all down-regulated or not affected (Supporting Information Fig. S1). In addition, MapMan

Table 1. Summary of read mapping

Time point and treatment	Experiment	Total high-quality reads generated	Total reads per biological condition	Total reads mapped to lettuce reference	%	Reads mapped uniquely to lettuce reference	%	Reads mapped to *Botrytis cinerea* reference	%
12 hpi control	1	12 100 638	27 735 685	9 498 975	78.5	9 047 889	74.8	8 436	0.07
	2	7 547 458		5 757 034	76.3	5 358 025	71.0	13 056	0.17
	3	8 087 589		6 367 418	78.7	5 901 039	73.0	6 053	0.07
12 hpi inoculated	1	7 831 383	24 340 257	6 250 384	79.8	5 956 788	76.1	10 979	0.14
	2	7 291 483		5 606 716	76.9	5 208 112	71.4	12 402	0.17
	3	9 217 391		7 294 081	79.1	6 767 799	73.4	10 282	0.11
24 hpi control	1	7 444 387	23 911 431	5 873 429	78.9	5 347 308	71.8	6 349	0.09
	2	8 064 507		6 357 800	78.8	5 718 809	70.9	13 547	0.17
	3	8 402 537		6 778 635	80.7	6 157 456	73.3	5 087	0.06
24 hpi inoculated	1	8 373 577	22 711 734	6 596 149	78.8	6 053 263	72.3	76 815	0.92
	2	6 891 847		5 451 307	79.1	4 908 831	71.2	22 543	0.33
	3	7 446 310		5 726 377	76.9	5 158 363	69.3	51 616	0.69
48 hpi control	1	10 494 058	31 107 629	8 232 847	78.5	7 435 717	70.9	16 971	0.16
	2	10 492 838		8 079 906	77.0	7 253 414	69.1	34 298	0.33
	3	10 120 733		7 632 570	75.4	6 777 861	67.0	30 710	0.30
48 hpi inoculated	1	20 726 205	36 493 378	15 413 395	74.3	14 494 556	69.9	981 495	4.74
	2	8 742 262		6 289 516	71.9	5 774 747	66.1	324 147	3.71
	3	7 024 911		5 003 424	71.2	4 631 264	65.9	566 381	8.06
Average		9 238 895		7 122 776		6 552 847			
Total		166 300 114		128 209 964	77.1	117 951 241	70		

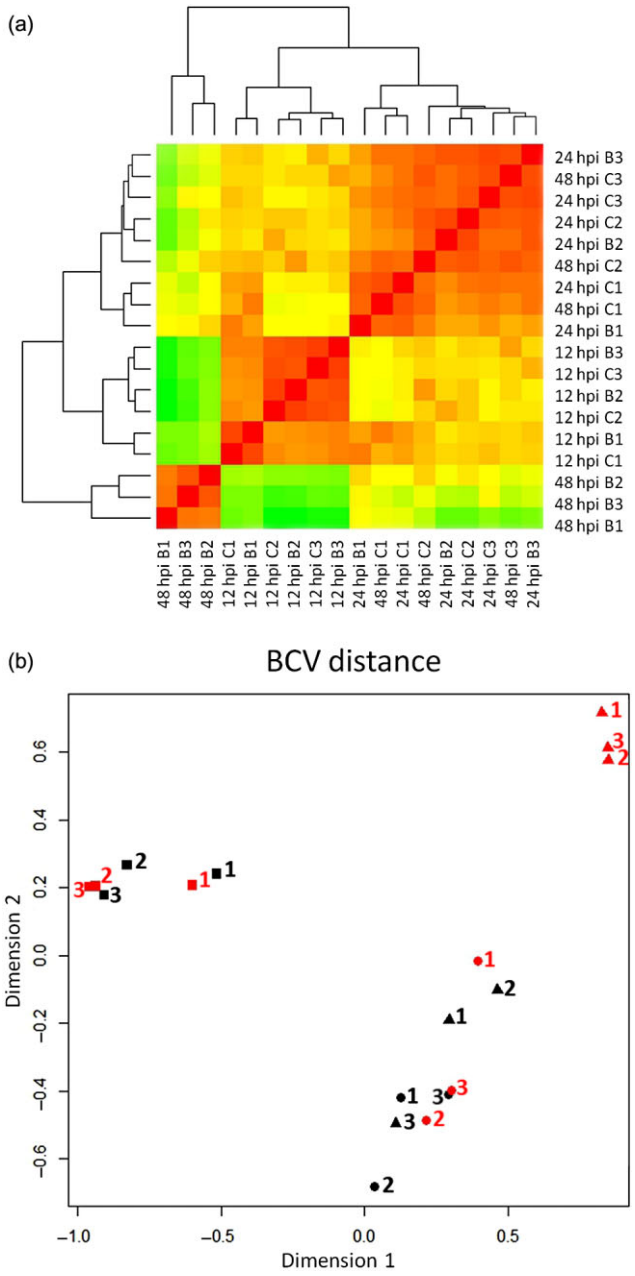

Figure 1. Correlation between biological replicates. (a) Heat-map of Spearman's correlation of the transcript expression levels from all samples compared against each other, represented by a coloured field ranging from green (0.7) to red (1). hpi, hours post-inoculation; C, control; B, inoculated with *B. cinerea*; and 1–3 indicates the experiment. Raw count data were normalized using the normalization factor generated by edgeR (Robinson *et al*. 2010), while additionally adjusting for library size. (b) Multidimensional scaling plot generated in edgeR. Distances on the plot correspond to the biological coefficient of variation (BCV) between each pair of samples. Samples collected at 12, 24 and 48 hpi are represented by a square, circle and triangle, respectively. Black, control; red, inoculated with *B. cinerea*; and 1–3 indicates the experiment.

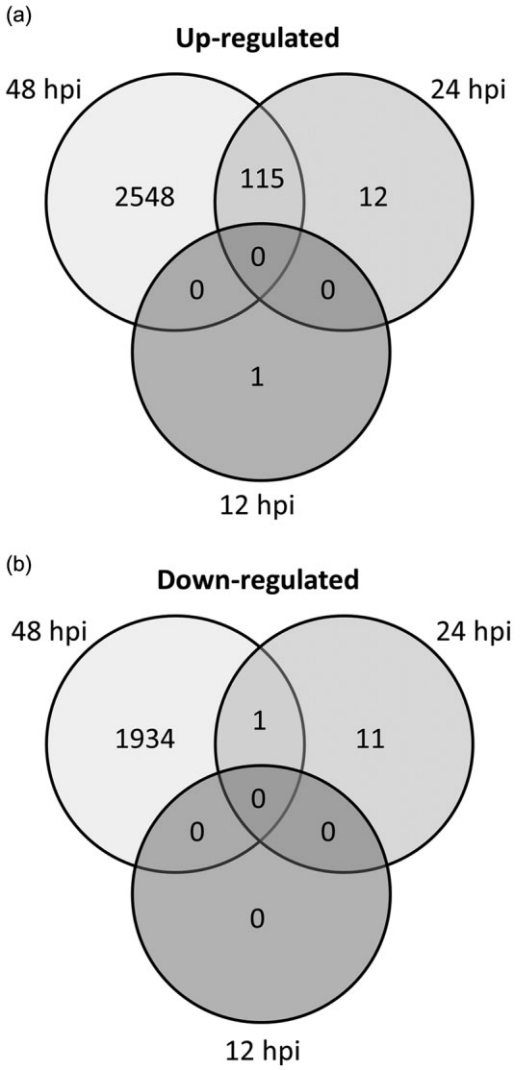

Figure 2. Venn diagrams showing common genes significantly (a) up- and (b) down-regulated at 12, 24 and 48 hpi. DE genes were filtered with a cut-off of $\log_2$-fold change ≥ 1 and FDR-corrected *P*-value ≤ 0.05.

indicates the involvement of metabolism of all known phytohormones in the response of lettuce to *B. cinerea* (Supporting Information Fig. S1).

Comparison of the locally induced response to *B. cinerea* in lettuce and *A. thaliana*

In the past, whole-genome expression studies focusing on plant leaf-*B. cinerea* interaction that generated lists of DE genes at comparable time points as our study have been performed for *A. thaliana* [18 and 48 hpi (Ferrari *et al*. 2007); 12 and 24 hpi (Mulema & Denby 2012); 14 hpi (Birkenbihl *et al*. 2012)] and tomato [8 hpi (Asselbergh *et al*. 2007)].

At 12 hpi, we detected 1 DE gene in lettuce (Fig. 2 and Supporting Information Appendix S1), which according to the BLAST2GO description encodes a predicted protein without further information. However, when looking at the

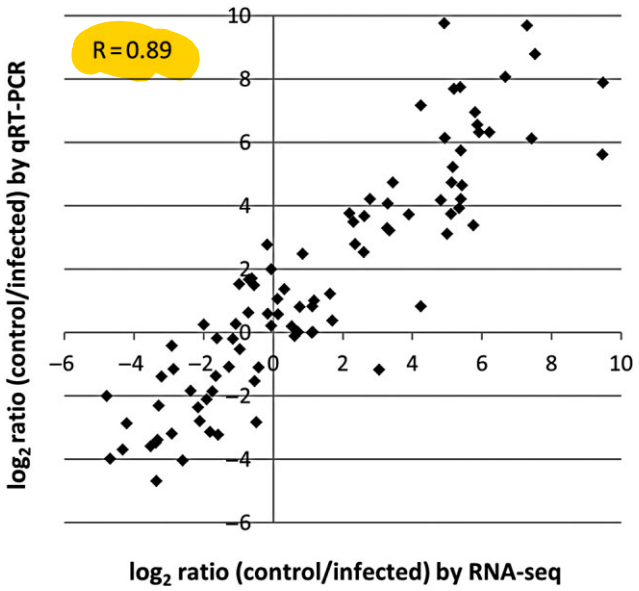

Figure 3. Correlation between RNAseq and qRT-PCR. The relative expression levels were obtained by RNAseq using edgeR and by qRT-PCR using the ΔΔCt method. The Pearson's correlation coefficient between relative expression levels is shown.

blast hits in detail (data not shown), it appears that this gene contains a DUF538 (domain of unknown function 538) domain. Birkenbihl *et al*. (2012) also detected the induction of one DUF538 family gene in *A. thaliana* at 14 hpi, but no DUF538 family genes were induced in other comparable studies in *A. thaliana* at 12 and 18 hpi, nor in tomato at 8 hpi (Asselbergh *et al*. 2007; Ferrari *et al*. 2007; Mulema & Denby 2012).

At 24 hpi, for 7 of the 35 up-regulated genes in lettuce that have an *A. thaliana* orthologue (20%), the orthologue is also up-regulated in the *A. thaliana* data reported by Mulema & Denby (2012) but none of the down-regulated genes are similar (Supporting Information Appendix S5). Remarkable is the significant amount of down-regulated photosynthesis-related genes in the *A. thaliana* study at this time point, which is only observed at 48 hpi in our lettuce study. This observation, together with the almost 20-fold higher amount of detected DE genes in *A. thaliana*, suggests that the *B. cinerea* infection was already more advanced in the *A. thaliana* experiment. Therefore, a comparison with the *A. thaliana*–*B. cinerea* results of Ferrari *et al*. (2007), who have a more similar experimental regime as our study, might be more appropriate. No 24 hpi time point is available in their study, but the amount of DE genes at 18 hpi in this analysis is comparable to our 24 hpi time point (139 and 154 DE genes, respectively), indicating that these are appropriate datasets for such comparative evaluation. However, for only 6 of the 35 *B. cinerea*-induced lettuce genes that have an *A. thaliana* orthologue (17%), the orthologue was also induced in *A. thaliana* (Supporting Information Appendix S4). When the three plant–*B. cinerea* transcriptomic studies performed at 18 and 24 hpi with *B. cinerea* (Ferrari *et al*. 2007; Mulema & Denby 2012; our RNAseq data) are compared, we identify two up-regulated lettuce genes of which the *A. thaliana* orthologue {AT1G74360/Lsa004290.1 [a leucine-rich repeat (LRR) protein kinase encoding gene] and AT4G17500/Lsa016859.1 [*ethylene response factor 1* (*ERF1*)]} is similarly induced.

Comparing the lettuce data at 48 hpi with *A. thaliana*–*B. cinerea* data at 48 hpi (Ferrari *et al*. 2007) revealed that for 327 of the 1048 up-regulated genes in lettuce that have an *A. thaliana* orthologue (Supporting Information Appendix S5), the orthologue was also induced in *A. thaliana*, whereas for 469 of the 933 down-regulated genes in lettuce that have an *A. thaliana* orthologue, the orthologue was also repressed in *A. thaliana* (31 and 50%, respectively). For the *A. thaliana* dataset of Ferrari *et al*. (2007) at 48 hpi, we performed GO analysis (Supporting Information Appendix S6) and created a MapMan overview for general metabolism, secondary metabolism and hormone pathways (Supporting Information Figs S2 & S3). A large similarity in transcriptional changes was observed between lettuce and *A. thaliana*, affecting stress responses and phytohormone signalling, in both plant species (Supporting Information Appendix S6, Figs S2 & S3). However, some differences are observed, as the very pronounced up-regulation of terpenoid biosynthesis via the mevalonic acid (MVA) pathway in lettuce was not detected in *A. thaliana* (Ferrari *et al*. 2007). Interestingly, AT1G74360/Lsa004290.1 (*LRR protein kinase*) and AT4G17500/Lsa016859.1 (*ERF1*) are also induced in both plant species at 48 hpi.

Comparison of the local and systemic response of lettuce to inoculation with *B. cinerea*

Using qRT-PCR, we examined gene expression changes induced after inoculation with *B. cinerea* in non-inoculated leaves of inoculated plants (systemic leaves). Because the local response was most pronounced at 48 hpi, systemic leaves were analysed at 48 hpi but also at 72 and 96 hpi, allowing additional time for the elicitation of a response in the plant parts distant from the infection site. These experiments were performed on a diverse set of genes representing several plant hormone-signalling and defence-related pathways (Table 2). Most of these genes were also differentially expressed in local leaves. Surprisingly, none of the analysed genes showed significant differential expression in the systemic leaves (Fig. 5).

Comparison between the local response of lettuce to *B. cinerea* and *B. lactucae*

Transcriptional changes induced by necrotrophic pathogens, like *B. cinerea*, are expected to differ from those induced by biotrophic pathogens. To make such a comparison in lettuce, we inoculated lettuce leaves with the compatible isolate of *B. lactucae* Bl:26. At 8 d post-inoculation (dpi), we could detect significant changes in gene expression of a selected set of genes belonging to different stress-related pathways using qRT-PCR (Fig. 6). At this time point, we could visually detect sporulation of *B. lactucae* and the first disease symptoms,

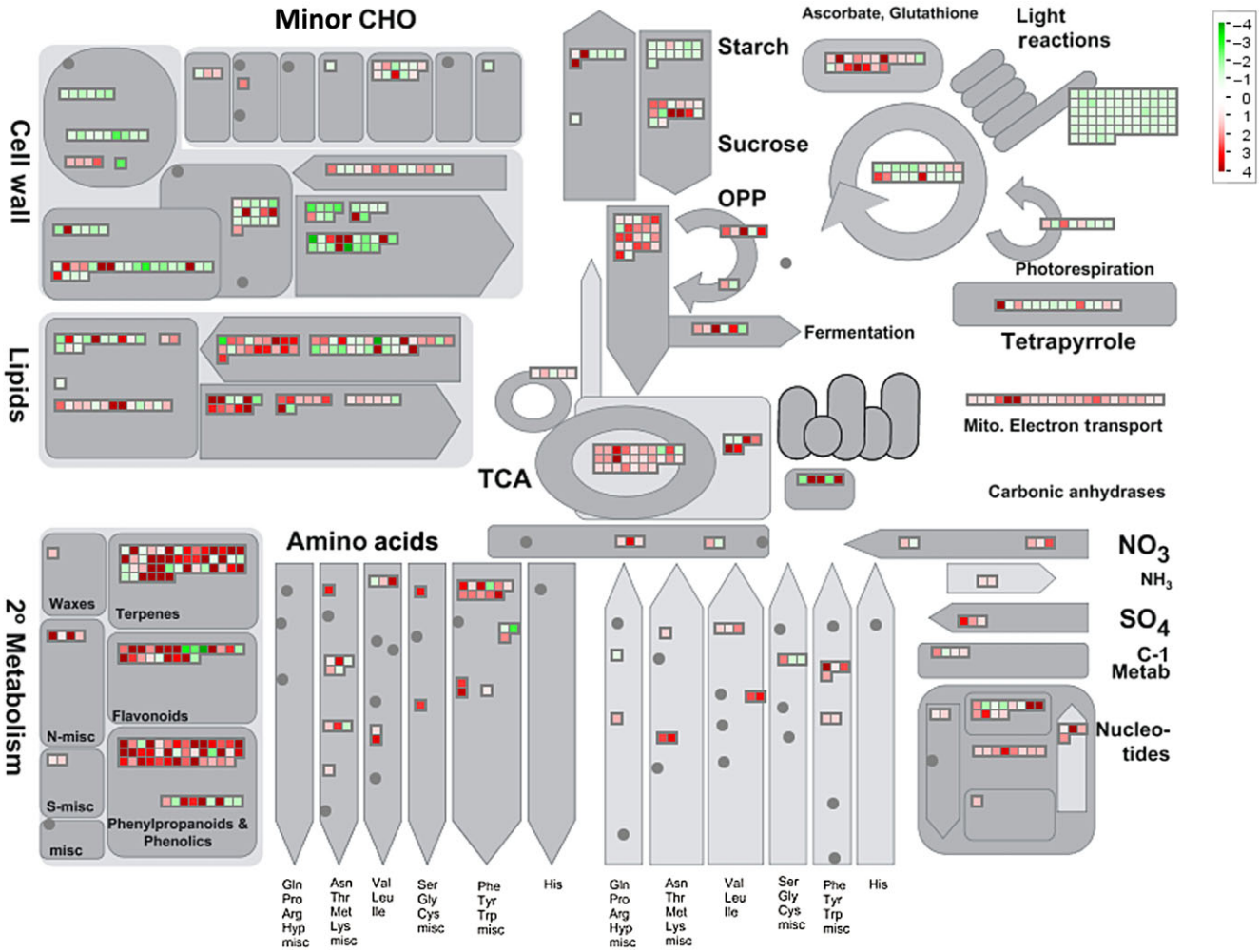

Figure 4. Overview of metabolic changes in infected leaves at 48 hpi, visualized by MapMan. Genes significantly up- and down-regulated in infected leaves relative to mock-inoculated leaves are indicated in red and green, respectively. Scale bars display $\log_2$-fold changes. Only significant changes are displayed. CHO, carbohydrates; OPP, oxidative pentose phosphate pathway; TCA, tricarboxylic acid cycle.

making this a good time point for comparison with the RNAseq data of the local lettuce–*B. cinerea* interaction at 48 hpi. The same genes as in the analysis of the systemic lettuce response to *B. cinerea* were used (Table 2).

In general, there is a high correlation between gene expression changes induced by *B. cinerea* and the compatible isolate of *B. lactucae*, for nearly all genes. However, as expected, some important differences were also detected. Firstly, the *PR1-like* gene *PRB1* (Lsa018589.1/AT2G14580) is significantly more induced after inoculation with *B. lactucae* compared to inoculation with *B. cinerea* (fold changes of 32 and 35, respectively). Interestingly, the expression of *EDS1* (Lsa005556.1/AT3G48090), a gene involved in the SA-pathway (Van Leeuwen *et al*. 2007), is up-regulated threefold after *B. lactucae* inoculation, but remains unaffected after inoculation with *B. cinerea*. It should be noted, however, that the difference of induction caused by the two pathogens is not significant for this gene. Secondly, the induction of a lipoxygenase-encoding gene (Lsa036946.1) is 13 times lower after inoculation with *B. lactucae* compared to inoculation with *B. cinerea* (significant difference in fold changes of 16 and 207, respectively). However, no significant difference in gene expression changes caused by *B. lactucae* and *B. cinerea* could be observed for all other analysed genes.

DISCUSSION

Local responses of lettuce after inoculation with *B. cinerea*

In this study, we analysed changes in the lettuce transcriptome in response to inoculation with the necrotrophic pathogen *B. cinerea* at three different time points. The recent completion of the lettuce genome sequence (http://lgr.genomecemnter.ucdavis.edu) allowed the use of RNAseq rather than microarrays, which favoured the detection of previously undiscovered genes. In total, 77% of the reads could be assigned to lettuce genes and were used for gene expression profiling (Table 1). Moreover, a very close correlation

Table 2. Genes used for qRT-PCR analyses of systemic lettuce leaves after inoculation with *Botrytis cinerea* and local leaves after inoculation with *Bremia lactucae*

Lettuce ID	BLAST2GO description	*A. thaliana* orthologue	Full *Arabidopsis thaliana* gene name	Gene abbreviation[a]	Local effect at 48 hpi (RNAseq)
Lsa018589.1	Pathogenesis-related protein 1	AT2G14580	*Basic pathogenesis-related 1*	*PRB1*	Up
Lsa005556.1	Enhanced disease susceptibility 1	AT3G48090	*Enhanced disease susceptible 1*	*EDS1*	Unaffected
Lsa003507.1	Isochorismate synthase	AT1G18870	*Isochorismate synthase 2*	*ICS2*	Down
Lsa044239.1	Phenylalanine ammonia lyase	AT2G37040	*Phenylalanine ammonium lyase 1*	*PAL1*	Up
Lsa002977.1	ssDNA-binding transcriptional regulator	AT2G02740	*Whirly 3*	*WHY3*	Unaffected
Lsa036946.1	Lipoxygenase	No		*LOX*	Up
Lsa025821.1	Allene oxide synthase, chloroplastic	No		*AOS*	Up
Lsa040211.1	12-oxophytodienoate reductase 3	AT2G06050	*Oxophytodienoate reductase 3*	*OPR3*	Unaffected
Lsa016859.1	Ethylene-responsive element binding protein1 homolog	AT4G17500	*Ethylene response factor 1*	*ERF1*	Up
Lsa000125.1	Gibberellin-regulated protein	No		*GRP*	Down
Lsa010697.1	Abscisic insensitive 1b	AT1G72770	*Hypersensitive to ABA 1-like*	*HAB1*	Unaffected
Lsa036022.1	Mitogen-activated protein kinase	No		*MPK*	Up
Lsa014258.1	Chalcone synthase	AT5G13930	*Chalcone synthase*	*CHS*	Down
Lsa018983.1	Hydroxycinnamoyl transferase	No		*HCT*	Up
Lsa037824.1	Dihydroflavonol 4-reductase	AT5G42800	*Dihydroflavonol 4-reductase*	*DFR*	Up
Lsa004786.1	Fatty acid hydroperoxide lyase	AT4G15440	*Hydroperoxide lyase 1*	*HPL1*	Down
Lsa032832.1	Pleiotropic drug resistance protein 1-like	No		*PDR*	Up
Lsa035434.1	Heat shock protein	No		*HSP*	Up
Lsa042448.1	MYB-related transcription factor	AT1G06180	*MYB-domain protein 13*	*MYB13*	Up
Lsa037249.1	β-Glucanase	No		*BG*	Up
Lsa035144.1	Glutathione S-transferase	No		*GST*	Unaffected

[a]Gene abbreviations are assigned based on the corresponding *A. thaliana* orthologue abbreviation on the TAIR (the *Arabidopsis* information resource) website (http://www.arabidopsis.org) or is self-assigned when no *A. thaliana* orthologue exists.

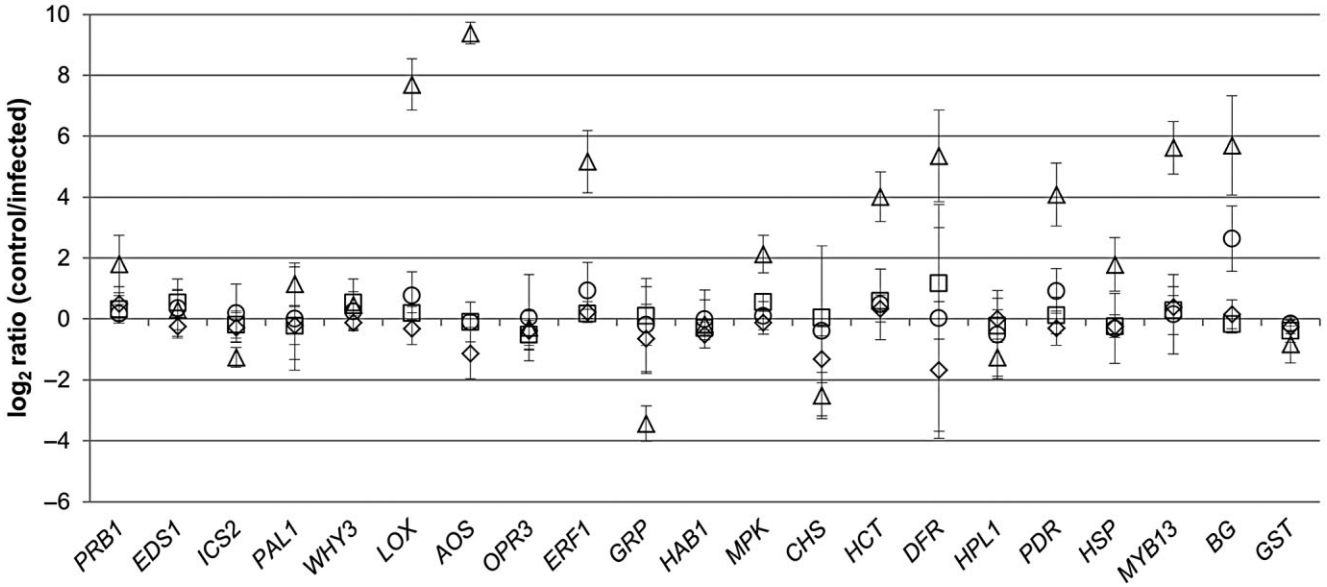

Figure 5. Comparison between local and systemic response of lettuce to *Botrytis cinerea*. Genes are represented by the corresponding abbreviation as denoted in Table 2. The relative expression levels are obtained by qRT-PCR using the ΔΔCt method for systemic samples and by RNAseq using edgeR for the local samples. The values are means ± SE of three replicates. Squares, diamonds and circles refer to systemic leaves at 48, 72 and 96 hpi, respectively. Triangles refer to local leaves 48 hpi after inoculation. Significance of induction was tested in a Student's *t*-test (absolute value of fold change ≥ 2 and $P \leq 0.05$).

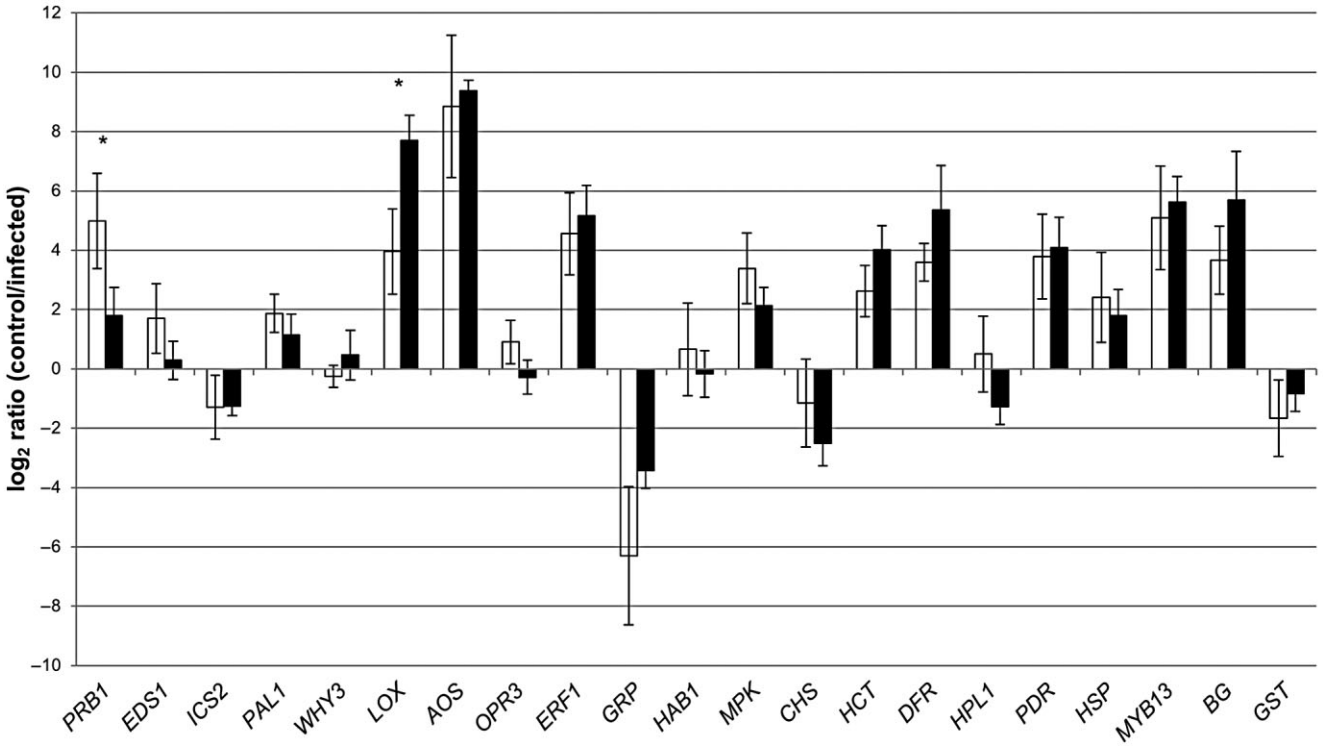

Figure 6. Comparison between the local response of lettuce to *Botrytis cinerea* and *Bremia lactucae*. Genes are represented by the corresponding abbreviation as denoted in Table 2. The relative expression levels are obtained by qRT-PCR using the ΔΔCt method for leaf samples inoculated with *B. lactucae* (white) and by RNAseq using edgeR for leaf samples inoculated with *B. cinerea* (black). The values are means ± SE of three replicates. Significance of the difference between both inductions was tested in a Student's *t*-test (*$P \leq 0.05$).

was observed between relative expression levels measured with RNAseq and qRT-PCR (Fig. 3), validating the RNAseq methodology described here for quantitative analysis of the lettuce transcriptome.

In lettuce, we detected a total of 4616 DE genes after inoculation with *B. cinerea* (Fig. 2 and Supporting Information Appendix S1), of which only one was differentially expressed at the earliest time point (12 hpi). When we compare our data at 24 and 48 hpi with available *A. thaliana–B. cinerea* transcriptomic studies performed at comparable time points (Ferrari *et al*. 2007; Mulema & Denby 2012), we detected only a partial overlap between DE genes (17–50% when only lettuce genes that have an *A. thaliana* orthologue were considered) (Supporting Information Appendix S5). Only two genes [AT1G74360/Lsa004290.1 (coding for an LRR protein kinase) and AT4G17500/Lsa016859.1 (*ERF1*)] were induced in all three studies at both 18/24 and 48 hpi, which was also confirmed by a very recent study performed by Windram *et al*. (2012) who showed a significant induction of these genes in *A. thaliana* as early as 16 and 14 hpi with *B. cinerea*, respectively. Their study consisted of a detailed transcriptome analysis of the local *A. thaliana–B. cinerea* interaction covering 24 time points within the first 48 hpi, using the time series structure of the data in determining differential expression rather than a time point-based statistic. They revealed a detailed chronology of defence responses, including the sequential involvement of different phytohormones. The ET-pathway was identified as the first to be induced, which is consistent with our lettuce data, because several of the genes differentially expressed at 24 hpi are involved in the ET-pathway.

Our lettuce RNAseq data provide further data demonstrating that the plant response to *B. cinerea* at 48 hpi involves many genes and is very complex. Global plant defence responses observed in the data of Windram *et al*. (2012) using GO annotations correlate well with our data in lettuce. Important defence-related phytohormone-signalling pathways are affected, including the ET-, JA- and SA-pathway. Interestingly, the most significantly induced hormonal pathway in lettuce is the one mediated by ABA (Supporting Information Appendix S4). GO analysis of the data of Ferrari *et al*. (2007) confirms this induction of ABA-mediated signalling at 48 hpi with *B. cinerea* in *A. thaliana* (Supporting Information Appendix S6). An induction of ABA-responsive genes is also observed by Windram *et al*. (2012) in *A. thaliana*, although already at 24 hpi. In contrast, ABA catabolism is over-represented among genes induced at 20 hpi in their data, which is followed by a negative regulation of ABA signalling at 22 hpi (Windram *et al*. 2012). These results illustrate that the involvement of ABA in the plant defence response to *B. cinerea* is not straightforward and is consistent with other contradictory results on the involvement of ABA in resistance to *B. cinerea* (Audenaert *et al*. 2002; AbuQamar *et al*. 2009). In the next part, we highlight some other aspects of the RNAseq

data at 48 hpi, describing important steps in the lettuce response to *B. cinerea*.

Pathogen recognition

The perception of conserved microbial-associated molecular patterns (MAMPs), including plant-derived degradation products of microbial activity such as oligogalacturonides (OGs) by plant receptors designated as pattern recognition receptors (PRRs) induces a first layer of basal defence or MAMP-triggered immunity to arrest pathogen infection (Boller & Felix 2009). PRRs include the membrane localized receptor-like kinase-family (RLKs), which is the largest family of receptors in the plant genome (Tor, Lotze & Holton 2009). Several RLKs have previously been described to be involved in plant–microbe interactions (Song *et al*. 1995; Gomez-Gomez & Boller 2000; Sun *et al*. 2004; Zipfel *et al*. 2004, 2006). Based on statistical analysis of *A. thaliana* gene expression data, RLKs are more likely to have altered expression in response to biotic stress than other genes (Chae *et al*. 2009; Lehti-Shiu *et al*. 2009), which was confirmed for lettuce by our RNAseq data, as described hereafter.

The OG receptor WAK (wall-associated kinase) is an RLK that has been characterized in relation to the immune responses against *B. cinerea*. Moreover, over-expressing WAK1 kinase enhanced resistance to *B. cinerea* in *A. thaliana* (Brutus *et al*. 2010). He, He & Kohorn (1998) confirmed that induced expression of *WAK1* is necessary for *A. thaliana* survival during general pathogen-induced plant responses induced by phytohormones. Our RNAseq data indicate that 10 out of 21 detected lettuce *WAKs* are up-regulated and only two are down-regulated (Supporting Information Appendix S1).

Perception of microbial signals accomplished by PRRs, additionally involves leucine-rich repeat RLKs (*LRR-RLKs*; Tor *et al*. 2009). Our RNAseq data detected several *LRR-RLKs* with different expression patterns after *B. cinerea* inoculation. One up-regulated *LRR-RLK* is a *BRI-like* (*brassinosteroid insensitive*; Lsa034184.1) gene, coding for an *LRR-RLK* that forms heterodimeric complexes with another *LRR-RLK*, namely *BAK1* (BRI1-associated receptor kinase 1) in *A. thaliana. BAK1* constitutes a negative control element of microbial infection-induced cell death in plants (Kemmerling *et al*. 2007), because in *BAK1* mutants, programmed cell death was triggered after inoculation with *B. cinerea*, which resulted in higher susceptibility to the pathogen. However, in contrast with the *BRI-like* gene, no *BAK1* gene was detected in lettuce. Interestingly, one of the genes that was up-regulated in all compared lettuce/*A. thaliana–B. cinerea* interaction studies is also an *LRR-RLK* (AT1G74360/Lsa004290.1).

One of the largest RLK groups is formed by the cysteine-rich receptor-like kinases (CRKs), which are less extensively studied but have been suggested to play important roles in the regulation of pathogen defence and programmed cell death in *A. thaliana* (Czernic *et al*. 1999; Chen *et al*. 2004). *CRKs* are induced by oxidative stress, pathogen attack (*Pseudomonas syringae* and *Ralstonia solanacearum*) and application of SA (Czernic *et al*. 1999; Chen, Du & Chen 2003; Chen *et al*. 2004). We detected 28 *CRKs* during RNAseq analysis of *B. cinerea* inoculated lettuce. For 15 of these *CRK*s, the expression is induced and none are repressed.

These data suggest putative roles for *PRR-RLKs* in mediating events in the response of plants to *B. cinerea* and their importance for fast pathogen recognition before entrance into plant cells and structures. It suggests that the induction of genes coding for these proteins is an attempt of the plant to recognize the intruder to establish a proper defence response.

ROS

Oxidative burst, characterized by the rapid generation of ROS (Lamb & Dixon 1997), belongs to the early events of resistant and susceptible plant responses to *B. cinerea* (Govrin & Levine 2000). It plays an important role in host cell death, which indicates a successful infection, as necrotrophic pathogens such as *B. cinerea* benefit from dead tissue (Govrin *et al*. 2006). Additionally, it has been established that during plant–pathogen interactions, ROS can act as signalling molecules, with specific targets being key to initiate cellular responses that can lead to tolerance (Hancock *et al*. 2002). Because of these dual functions of ROS, controlled generation is of tremendous importance and plants possess several scavenging and antioxidant mechanisms for oxidative stress protection. ROS are eliminated directly by several antioxidant enzymes and also the ascorbate-glutathione cycle is an important antioxidant mechanism in plants that consists of a series of reactions resulting in the removal of H_2O_2 (Noctor & Foyer 1998; Kuzniak 2010), which is clearly induced in our data at 48 hpi (Fig. 4). Among the highest up-regulated genes at 48 hpi in lettuce, we detect many genes involved in oxidative stress and cell death. For example, the third highest induced gene, Lsa018575.1, encodes a pathogen-inducible α-dioxygenase. Enzymes with this activity have been shown to be involved in protection against oxidative stress and cell death (Sanz, Moreno & Castresana 1998; Ponce de León *et al*. 2002; Hamberg *et al*. 2003). *BOS1* (*Botrytis susceptible 1*), encoding an R2R3 MYB transcription factor, appears to restrict necrosis triggered by *B. cinerea* (Mengiste *et al*. 2003; Veronese *et al*. 2004). Interestingly, according to our RNAseq data, this gene (Lsa012088.1/AT3G06490) too is among the highest induced at 48 hpi. The major source for this ROS production in the plant are NADPH oxidases, also called respiratory burst oxidase homologues (rboh; Torres 2010). It was demonstrated in *A. thaliana* that both *rbohD* and *rbohF* are required for the accumulation of ROS and they were proven to be important for plant defence (Torres, Dangl & Jones 2002). In lettuce, *rbohD* (Lsa002796.1/AT5G47910) was induced at 48 hpi with *B. cinerea*, but *rbohF* (Lsa018309.1/AT1G64060) was unaffected.

Terpenoid biosynthesis

One of the most remarkable differences observed between the *A. thaliana* and lettuce response to inoculation with

B. cinerea is the induction of terpenoid biosynthesis genes, which was only significant in lettuce. Terpenoids consist of a large class of chemical compounds produced by most, if not all, living organisms. Over 40 000 different terpenoid compounds have been characterized, most of them of plant origin (Yu & Utsumi 2009). Some of these compounds are present in almost all plant species, and they are produced in primary metabolism, including some phytohormones such as ABA and structural membrane components such as sterols (Yu & Utsumi 2009). However, the majority of plant terpenoids are secondary metabolites, active as direct defensive compounds and found only in certain species or taxonomically related groups (Croteau, Kutchan & Lewis 2000; Yu & Utsumi 2009). Members of the Compositae (including lettuce, artichoke and sunflower) are known to accumulate high levels of terpenoids, especially sesquiterpenes, as defence compounds (Seaman 1982; Bennett *et al*. 2002; Goepfert *et al*. 2009; Menin *et al*. 2012). Sesquiterpenes are derived from the five-carbon building blocks isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (Yu & Utsumi 2009), which can be synthesized through two independent pathways. In the cytosol, IPP is derived from the classic MVA pathway starting with the condensation of acetyl-CoA (Newman & Chappell 1999), whereas in plastids, IPP is generated from pyruvate and glyceraldehyde 3-phosphate through the non-MVA or methylerythritol phosphate (MEP) pathway (Eisenreich *et al*. 1998; Lichtenthaler, 1999). Usually, the cytosolic pool of IPP provides the precursor for the production of sesquiterpenes and triterpenes, while the plastidial pool of IPP serves as the precursor for the production of monoterpenes, diterpenes and tetraterpenes (Yu & Utsumi 2009). Our data show a clear induction of the MVA pathway, including up-regulation of 14 genes coding for enzymes involved in every step from acetyl-CoA to IPP (Supporting Information Fig. S1 and Appendix S1). Moreover, one sesquiterpene cyclase (Lsa013589.1) and eight out of nine detected sesquiterpene synthases (seven germacrene A synthases: Lsa002309.1, Lsa002515.1, Lsa011995.1, Lsa011993.1, Lsa010684.1/AT5G23960, Lsa002307.1 and Lsa002308.1 and one amorphadiene synthase: Lsa013591.1) are all significantly induced (Supporting Information Fig. S1 and Appendix S1). The induction of such germacrene A synthases was shown to be directly correlated with the accumulation of the phytoalexin sesquiterpene lettucenin A (derived from germacrene A) in lettuce cotyledons after infection with *B. lactucae* (Bennett *et al*. 2002). In 1994, Bennett and co-workers already demonstrated the production of lettucenin A in lettuce after *B. cinerea* infection (Bennett *et al*. 1994). They additionally showed that this sesquiterpene possesses considerable antifungal activity against this pathogen *in vitro*, as was later shown for sesquiterpenes from other plants too (Wedge, Galindo & Macias 2000; Zhang *et al*. 2008). Although sesquiterpene production is highly induced in the cultivar used in this study, it did not prevent successful infection by *B. cinerea*. Interestingly, one of the most important phytotoxins (botrydial) produced by this pathogen is also a sesquiterpene compound (Rossi *et al*. 2011).

Photosynthesis

At 48 hpi, a significant down-regulation of photosynthesis-related genes was observed in leaves inoculated with *B. cinerea* according to both GO analysis and MapMan (Fig. 4 and Supporting Information Appendix S4). This phenomenon has been noticed previously for several plant–pathogen interactions and often correlates with an induction of *PR*-genes (Chou *et al*. 2000; Scharte, Schon & Weis 2005; Swarbrick, Schulze-Lefert & Scholes 2006; Bonfig *et al*. 2010; Gyetvai *et al*. 2012; Milli *et al*. 2012). Also in lettuce, Klosterman *et al*. (2011) detected a down-regulation of photosynthesis-related genes and up-regulation of *PR*-genes (*PR1*, *PR3* and *PR5*) after infection with *Verticillium dahliae*. The allocation of resources for the onset of immune reactions and biosynthesis of protective compounds causes a demand for carbohydrates and energy in the infected tissue, which becomes satisfied through increased activities of cell wall invertases, hexose transporters, the oxidative pentose phosphate (OPP) pathway and respiratory metabolism (Bolton, 2009; Bilgin *et al*. 2010; Kangasjarvi *et al*. 2012). Our RNAseq data of lettuce at 48 hpi support these findings because (1) the only gene detected with RNAseq encoding a cell wall invertase (Lsa015654.1/AT3G52600) is up-regulated and (2) *G6PD6* (*glucose-6-phosphate dehydrogenase 6*; Lsa022357.1/AT5G40760), which codes for an enzyme that controls NADPH provision via the oxidative pentose phosphate pathway, is up-regulated. MapMan (Fig. 4) reveals a clear induction of the OPP pathway and all parts of respiratory metabolism (including glycolysis, the tricarboxylic acid (TCA) cycle and mitochondrial electron transport). The latter is also confirmed by the GO analysis (Supporting Information Appendix S4). Such reprogramming of primary carbon metabolism may further repress photosynthesis, enhance expression of defence-related genes and favour the production of compounds with antimicrobial activity (Bolton 2009; Kangasjarvi *et al*. 2012).

Another process down-regulated in lettuce according to the GO analysis at 48 hpi is the circadian rhythm. This was also observed in *A. thaliana* by Windram *et al*. (2012), who clearly observed a moderation of the oscillating expression of core clock components starting at 24 hpi. This may reflect an attempt of the pathogen to hamper the plant's defence response, as it is known that the expression of several defence-related genes is modulated by circadian rhythm (Wang *et al*. 2011; Windram *et al*. 2012).

Systemic response of lettuce to inoculation with *B. cinerea*

In order to compare the defence response in systemic versus local leaves of lettuce inoculated with *B. cinerea*, we analysed the expression of a set of 24 genes (Fig. 5), selected based on their involvement in diverse defence-related pathways (Table 2). Almost all of these genes were found to be highly affected in local leaves inoculated with *B. cinerea*. Surprisingly, we did not observe significant changes in gene expression in systemic leaves of lettuce inoculated with *B. cinerea*. Few studies have focused on a systemic response of plants to

B. cinerea inoculation. Only one large-scale transcriptional analysis (Mathys *et al*. 2012) investigated this response in *A. thaliana*, and in contrast to our data, they observed significant changes for 7 of the 12 lettuce genes with a corresponding *A. thaliana* orthologue mentioned in this study (*EDS1*, *PAL1*, *OPR3*, *ERF1*, *DFR* and *MYB13* are induced, and *CHS* is repressed in their data) at 48 hpi. In contrast, Govrin & Levine (2002) reported that *B. cinerea* did not induce SAR or production of phytoalexins in systemic leaves of *A. thaliana* after inoculation with *B. cinerea*. The absence of significant systemic differential gene expression in this small-scale study is striking and should be further investigated on a larger scale.

Comparison of genes locally induced by the necrotroph *B. cinerea* versus the biotroph *B. lactucae*

In contrast to *B. cinerea*, the oomycete *B. lactucae* has a biotrophic lifestyle. Biotrophic pathogens have evolved infection mechanisms to invade plants with minimal damage to host cells and exploit them as living substrates (Mendgen & Hahn 2002). Surprisingly, we observed a high general correlation between gene expression changes induced by *B. cinerea* and *B. lactucae* for the selected set of genes (Fig. 6). However, as expected, a few significant differences were also detected including higher expression in *B. lactucae* infected plants of the *PR1-like* gene *PRB1* (Lsa018589.1/AT2G14580) and *EDS1* (Lsa005556.1/AT3G48090), the latter is involved in the SA-pathway, which is known to be important in resistance to biotrophs. We additionally detected higher expression of a lipoxygenase-encoding gene in plants inoculated with *B. cinerea*. Lipoxygenases are known to be involved in synthesis of signalling compounds such as green leaf volatiles and JA (Joo & Oh 2012; the latter is associated with resistance to necrotrophs (Glazebrook 2005). However, no difference in gene expression changes caused by *B. lactucae* and *B. cinerea* could be observed for other genes of the SA- and JA-pathways. The induction of genes involved in multiple disease signalling pathways corresponds to data obtained from a transcriptional study analysing the compatible interaction between maize and the biotrophic pathogen *Ustilago maydis*, revealing rather unspecific defence reactions as well, including changes in multiple hormone signalling pathways and secondary metabolism (Doehlemann *et al*. 2008). Our results confirm that compatible lettuce interactions with biotrophic and necrotrophic pathogens, leading to successful infection, induce the same pathways. A whole-transcriptome analysis of compatible and incompatible lettuce–*B. lactucae* and lettuce–*B. cinerea* interactions would reveal more insights into the differences between responses in lettuce induced by necrotrophic and biotrophic pathogens.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. MapMan: secondary metabolism and phytohormones in lettuce at 48 hpi. Genes that are up-regulated in infected tissues are indicated in red and genes down-regulated are indicated in green. The scale bar displays changes in gene expression as $\log_2$-fold change. Only significant changes are displayed. MVA = mevalonic acid, IAA = indole-3-acetic acid, ABA = abscisic acid, BA = butyric acid, SA = salicylic acid and GA = gibberellic acid.

Figure S2. MapMan: general overview of metabolism in *A. thaliana* at 48 hpi [data of Ferrari *et al*. (2007)]. Genes that are up-regulated in infected tissues are indicated in red and genes down-regulated are indicated in green. The scale bar displays changes in gene expression as $\log_2$-fold change. CHO = carbohydrates, OPP = oxidative pentose phosphate pathway and TCA = tricarboxylic acid cycle.

Figure S3. MapMan: secondary metabolism and phytohormones in *A. thaliana* at 48 hpi [data of Ferrari *et al*. (2007)]. Genes that are up-regulated in infected tissues are indicated in red and genes down-regulated are indicated in green. The scale bar displays changes in gene expression as $\log_2$-fold change. Only significant changes are displayed. MVA = mevalonic acid, IAA = indole-3-acetic acid, ABA = abscisic acid, BA = butyric acid, SA = salicylic acid and GA = gibberellic acid.

Appendix S1. Log_2-fold change of lettuce genes in response to *B. cinerea* infection. Only genes detected above the detection threshold of 1 count per million are given and genes with significant changes in expression are indicated in red (FDR-adjusted P-value ≤ 0.05). The table comprises lettuce gene IDs and their corresponding BLAST2GO description and *A. thaliana* orthologue. The different worksheets contain the gene lists for the different time points analysed (12, 24 and 48 hpi).

Appendix S2. Primers used for qRT-PCR. The table contains lettuce ID and primer sequences of the primers used for qRT-PCR shown in Fig. 3, 5 and 6.

Appendix S3. *Botrytis cinerea* genes identified with RNAseq. The number of total reads mapped to each *B. cinerea* gene for each *B. cinerea*-infected sample is given.

Appendix S4. GO terms differentially regulated in lettuce at 48 hpi. The P-value is calculated with the Wallenius non-central hypergeometric distribution, using Goseq. Different worksheets contain the GO terms that associate with biological processes, molecular function and cellular components, up- and down-regulated, respectively.

Appendix S5. Common DE genes in lettuce and *A. thaliana* after inoculation with *B. cinerea*. The different worksheets contain (i) common DE genes in lettuce and *A. thaliana* at 24 hpi with *B. cinerea* (Mulema & Denby 2012), (ii) common DE genes in lettuce at 24 hpi and *A. thaliana* at 18 hpi with *B. cinerea* (Ferrari *et al*. 2007) and (iii) common DE genes in lettuce and *A. thaliana* at 48 hpi with *B. cinerea* (Ferrari *et al*. 2007).

Appendix S6. GO terms differentially regulated in *A. thaliana* at 48 hpi (data of Ferrari *et al*. (2007)). The P-value is calculated with a Fisher's exact test using DAVID. Different worksheets contain the GO terms that associate with biological processes, molecular function and cellular components, up- and down-regulated, respectively.