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Transcriptome Arofile of *Brassica rapa* L. Reveals the Involvement of Jasmonic Acid, Ethylene, and Brassinosteroid Signaling Pathways in Clubroot Resistance

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Abstract: *Plasmodiophora brassicae* is a protozoan pathogen that causes clubroot disease in cruciferous plants, particularly Chinese cabbage (*Brassica rapa*). A previous study identified a clubroot resistance gene (*CRd*) conferring race-specific resistance to *P. brassicae*. However, the defense mechanisms of *B. rapa* against virulent vs. avirulent *P. brassicae* are poorly understood. In this study, we carried out a global transcriptional analysis in the clubroot-resistant Chinese cabbage inbred line "85–74" carrying the *CRd* gene and inoculated with avirulent (LAB-4) or virulent (SCCD-52) *P. brassicae*. RNA sequencing showed that "85–74" responded most rapidly to SCCD-52 infection, and the number of differentially expressed genes was much higher in SCCD-52-treated as compared to LAB-4-treated plants (5552 vs. 304). Transcriptome profiling revealed that plant hormone signal transduction and plant–pathogen interaction pathways played key roles in the late stages of *P. brassicae* infection. Genes relating to the salicyclic acid (SA), jasmonic acid (JA)/ethylene (ET), and brassinosteroid (BR) signaling pathways were up-regulated relative to untreated plants in response to LAB-4 infection at 8, 16, and 32 days post-inoculation (dpi) whereas JA, ET, and BR signaling-related genes were not activated in response to SCCD-52, and SA signaling-related genes were up-regulated in both LAB-4 and SCCD-52, suggesting that SA signaling is not the key factor in host resistance to avirulent *P. brassicae*. In addition, genes associated with phosphorylation and Ca^{2+} signaling pathways were down-regulated to a greater degree following LAB-4 as compared to SCCD-52 infection at 8 dpi. These results indicate that effector-triggered immunity in "85–74" is more potently activated in response to infection with avirulent *P. brassicae* and that JA, ET, and BR signaling are important for the host response at the late stage of infection. These findings provide insight into *P. brassicae* pathotype-specific defense mechanisms in cruciferous crops.

Keywords: *Plasmodiophora brassicae*; Clubroot disease; *Brassica rapa*; RNA-seq; Avirulent pathogen; Virulent pathogen

1. Introduction

Plants have evolved immune responses consisting of initial pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and subsequent effector-triggered immunity (ETI) [1] for protection against pathogens such as bacteria, viruses, and fungi [1–4]. Most pathogens are inhibited by the recognition of PAMPs by pattern recognition receptors (PRRs) localized on the plasma membrane. However, pathogens can suppress PTI by secreting effectors into host cells. ETI is mediated by

intracellular receptors encoded by plant disease resistance genes (R genes) that recognize the effector protein and subsequently activate downstream immune responses to prevent pathogen infection [5].

The plant hormones ethylene (ET), jasmonic acid (JA), and salicyclic acid (SA) play a central role in the regulation of plant immune responses [6]. JA/ET pathways are critical for systemic resistance against Chilli veinal mottle virus in tobacco, whereas the SA pathway plays a minor role at an early stage of infection [7]. Infection by *Fusarium oxysporum* (*Fol*) activates both the ET and SA pathways in tomato but have antagonistic functions, whereas JA signaling has no discernible effect on susceptibility to *Fol*. [8]. The ET response enhances susceptibility to *Fol* infection and disease development, whereas the SA response blocks colonization through constitutive activation. In general, SA signaling positively regulates plant defense against biotrophic pathogens [9,10], but ET/JA pathways target necrotrophic pathogens and herbivorous pests [11,12]. However, several exceptions have been reported. For example, the SA pathway is required for plant resistance to specific necrotrophic pathogens (*Plectosphaerella cucumerina*), while ET and JA signaling mediate resistance to some biotrophic pathogens (*Pseudomonas syringae* and *Peronospora parasitica*) [13,14].

Clubroot is a soil-borne disease caused by *Plasmodiophora brassicae*, an obligate biotrophic protist that specifically infects *Brassica* crops [15]. Clubroot disease jeopardizes crop cultivation and yield by causing the development of large galls on the roots that reduce the plant's ability to absorb water and nutrients from the soil. Although clubroot resistance (CR) genes have been identified, mainly in *Brassica rapa* [16–23], the detailed mechanism of CR remains unclear. However, the suppression of clubroot by exogenous SA was also reported in broccoli (*Brassica oleracea*) [24]. The SA and JA pathways function antagonistically in the defense response to plant pathogens, whereby the accumulation of JA inhibits that of SA and vice versa [25]. It was also shown that *Botrytis cinerea* regulates both pathways to enable infection of tomato [26]. A novel CR gene (*CRd*) conferring complete resistance to a specific pathotype of *P. brassicae* was recently identified in *B. rapa* [23]. However, the mechanism of action of the *CRd* gene product and its association with phytohormone signaling at different developmental stages of *P. brassicae* has yet to be elucidated, and we suspect that the hormonal signal transduction pathway involved in clubroot disease resistance may be distinct due to the different CR genes.

RNA sequencing (RNA-seq) is a powerful tool for detecting genes related to a specific trait at the whole-genome level [27]; it has been widely employed to study plant–pathogen interactions, including that between *Brassica* and *P. brassicae*. Genes involved in the SA signaling pathway were induced in *Arabidopsis thaliana* [28], *B. rapa* (contained *CRb* gene) [29,30], and *B. oleracea* [31] following early infection by *P. brassicae*, whereas the JA pathway was found to be involved in the defense response of *B. napus*, which harbors the *Rcr1* gene [32]. In contrast, JA signaling was suppressed in *B. rapa* [29]. A recent study showed that both the JA and SA pathways contribute to CR in *Arabidopsis* [33]. The observed differences could be due to variations among *Brassica* species or in the CR genes used in previous studies. Other plant hormones such as ET and brassinosteroid (BR) have also been implicated in the defense response to clubroot [28,30,32]. Additionally, changes in the expression of genes related to metabolism, cell wall modification, nucleotide binding site leucine-rich repeat (NBS–LRR) proteins, Ca^{2+} signaling, defense-related callose deposition, chitin metabolism, and the pathogenesis-related (PR) pathway have been reported upon infection of *B. rapa* with *P. brassicae* [29,32,34–39].

It has been reported that two potentially novel systemic signaling pathways: a systemic response elicited by hypersensitive response (HR)that leads to enhanced *Trichoplusia ni* resistance and overrides the SA-mediated increase in *T. ni* susceptibility, and a SA-independent systemic response induced by virulent pathogens that leads to enhanced susceptibility to *T. ni* [40]. Previous studies have examined the mechanism of CR by treating CR germplasm with an avirulent pathotype of *P. brassicae*. However, it has not been reported that virulent pathotype of *P. brassicae* was used in infect CR materials. *P. brassicae* is an obligate biotrophic pathogen, and it is unclear that the mechanism of SA, JA, and other hormone signal pathways participating in clubroot resistance was different when avirulent and virulent pathotype of *P. brassicae* infected CR plants at different development stages. In this study, a Chinese cabbage inbred line harboring the *CRd* gene [23] was inoculated with either an avirulent or virulent

pathotype of *P. brassicae* to investigate the involvement of plant hormone signaling in CR and identify relevant genes by whole-genome transcriptional analysis.

2. Materials and Methods

2.1. Plant Material

The clubroot-resistant inbred line "85–74" harboring the *CRd* gene was used in this study. All plants were grown in 72-well multipots and maintained in a climate-controlled room at 20 °C–25 °C under a 16-h photoperiod.

2.2. Pathogen Inoculation and Tissue Sampling

To investigate the defense mechanism of CR Chinese cabbage inbred line "85–74" infected with avirulent or virulent strains of *P. brassicae*, 11 pathotypes collected from different epidemic areas in China were screened. "85–74" harboring the *CRd* locus was found to be resistant to the LAB-4 pathotype but susceptible to the SCCD-52 pathotype of *P. brassicae*, which was confirmed in more than three trials. Therefore, LAB-4 and SCCD-52 were used to infect "85–74". The clubroot pathotypes LAB-4 and SCCD-52, defined as pathotype 4 of *P. brassicae* [41], were isolated from a highly susceptible Chinese cabbage plant and stored at −20 °C. For inoculation, 1 ml of LAB-4 or SCCD-52 spore suspension (1.0×10^7 spores/mL) was applied to the stem base of 30-day-old Chinese cabbage plants. The inoculated and uninfected control plants were maintained in a climate-controlled room at 20 °C–25 °C under a 16-h photoperiod. The soil was kept moist during the treatment period. The roots of "85–74" were sampled at 0 h and 4 and 8 days post-inoculation (dpi) for analysis of genes that are differently expressed between plants infected with LAB-4 compared with SCCD-52. The roots of 30 plants were sampled at each time point, with three biological replicates. Ten plants for each replicate were pooled for RNA extraction. The roots were washed with distilled water and immediately frozen in liquid nitrogen and stored at −80 °C. To confirm successful infection, 36 plants were maintained in the climate chamber for 32 days after inoculation.

2.3. RNA Isolation, cDNA Library Construction, and Sequencing

Total RNA in each sample was isolated from approximately 0.1 g of fresh roots using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Takara Bio, Dalian, China) to remove any contaminating genomic DNA. The RNA concentration of each sample was determined using a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Samples with a 260/280 ratio of 1.9–2.1 and a 260/230 ratio ≥ 2.0 were considered to be of high quality and were used for experiments. The integrity of the purified RNA was verified by 1.0% (w/v) agarose gel electrophoresis. The cDNA library was prepared and sequenced by Gene Denovo Technology Co. (Guangzhou, China). The mRNA was enriched from total RNA using magnetic beads with oligo(dT) and then fragmented. Using mRNA as a template, a single strand of cDNA was synthesized with a six-base random primer followed by synthesis of double-stranded cDNA, which was purified, subjected to end repair, and mixed with Solexa adapters. Suitable fragments were recovered from an agarose gel. Using the adapter sequences, the double-stranded cDNA fragments were amplified by polymerase chain reaction (PCR). The constructed libraries were sequenced on a Hiseq2000 platform (Illumina, San Diego, CA, USA).

2.4. RNA-seq Data Analysis and Differentially Expressed Genes (DEGs) Identification

To obtain high-quality clean reads for analysis, the reads were filtered in multiple steps as follows. Those containing the join were removed and the join and downstream portion were excised. If the length of the read after truncation was less than 50 bp, the read was discarded; otherwise, it was retained. Reads with only A bases, an N ratio greater than 10%, or of low quality (i.e., those in which the number of bases with a mass value of Q ≤ 20 was over 50%) were removed. Given that

contaminating rRNA can affect subsequent analysis, the read alignment tool bowtie2 v.2.2.8 was used to compare high-quality clean reads to the rRNA sequence of *B. rapa* (mismatch number: 0). Reads of aligned rRNAs were removed and the data were retained for subsequent analysis. Reads filtered for rRNA were aligned to the reference genome of Chinese cabbage using TopHat2 v.2.1.1 software [42]. The gene expression levels of all libraries were estimated by calculating read density as reads per kilobase per million mapped reads [43]. DEG identification was performed using edgeR software (https://bioconductor.org/packages/release/bioc/html/edgeR.html); the screening conditions were false discovery rate (FDR) < 0.05 and |log2 fold change (FC)| > 1. Patterns of genes that were differentially expressed at the various time points are represented in Venn diagrams.

2.5. Functional Annotation, Gene Ontology (GO) Enrichment, and Kyoto Encyclopedia of Genes and Genomes (KEGG) Analysis of DEGs

GO analysis and KEGG pathway analysis were carried out for the identified DEGs of LAB-4 vs. control and LAB-4 vs. SCCD-52 at 4 and 8 dpi. GO annotation was performed using Blast2GO software [44]. The GO database includes three ontologies: molecular function, cellular component, and biological process. KOBAS v.2.0 software was used to identify the significantly enriched pathways corresponding to the DEGs [45]. Pathways with a corrected p value < 0.05 were considered statistically significant [46].

2.6. Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR verification of DEGs was performed using the specific primers provided for Master Cyclereprealplex (Eppendorf, Hamburg, Germany) and SYBR Premix Ex Taq II (Perfect Real Time) (Takara Bio). The primers were designed using Premier 5.0 software and are listed in Supplementary Table S1. Each reaction mixture (20 µL) contained 10 µL of SYBR Premix ExTaq (2×), 0.5 µL of PCR forward/reverse gene-specific primers (10 µM), and diluted cDNA (2 µL). Three experimental replicates were prepared for each gene using different cDNAs synthesized from three biological replicates. The PCR program was as follows: 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 25 s, and 72 °C for 30 s. The specificity of amplification was confirmed by melting curve analysis after 40 cycles. Analysis of gene expression was performed for all samples at 4, 8, 16, and 32 dpi of "85–74" with LAB-4. The expression levels of genes investigated in this study were calculated with the −ΔΔCt method [47], where $\Delta\Delta Ct = (CT_{gene} - CT_{actin})_{treat} - (CT_{gene} - CT_{actin})_{control}$.

3. Results

3.1. P. brassicae Physiological Race Selection and Phenotype Identification

The Chinese cabbage inbred line "85–74" harboring the *CRd* locus is resistant to the LAB-4 pathotype of *P. brassicae* (Figure 1a). To identify an antagonist of *CRd*, more than 10 pathotypes collected from different epidemic areas in China were screened. The SCCD-52 pathotype successfully infected "85–74", which was confirmed in more than three trials (Figure 1b and Table 1). Therefore, LAB-4 (avirulent) and SCCD-52 (virulent) were used to inoculate "85–74", and three replicates of each pathotype at each time point were collected for RNA-seq.

Figure 1. Phenotype of roots in "85–74" plants infected with *P. brassicae*. Photograph of "85–74" at 30 dpi with LAB-4 (**a**) and SCCD-52 (**b**).

Table 1. Resistance of Chinese cabbage to different pathotypes of *P. brassicae.*

Material	Local Pathotype										
	LAB-4	**LAB-7**	**LAB-16**	**LAB-19**	**AHXC-68**	**LNXM-1**	**HBLC-31**	**AHHS-65**	**HBSY-32**	**SCCD-52**	**SCCQ-61**
85–74	−	−	−	−	−	−	−	−	−	+	−
BJN3-1	+	+	+	+	+	+	+	+	+	+	+

+, Susceptible reactions; −, resistant reactions.

3.2. RNA-seq Alignment and Mapping to the Reference Genome

RNA-seq generated 4.0–5.4 × 10^6 clean reads after preprocessing and quality control. The content of base *GUANINE* and *CYTOSINE* (GC) was approximately 47%, and Q30 values of all the samples were >95%, indicating that the sequencing quality met the requirements for subsequent analysis (Supplementary Table S2). Most reads were successfully aligned to the *B. rapa* genome (http://brassicadb.org/brad/). A high percentage (~75%–77%) of mapped reads from each inoculated sample aligned to a single position. The mapping ratio was approximately 77%–78% of the total number of sequenced reads for the virulent pathotype SCCD-52 and avirulent pathotype LAB-4. The expression levels of unigenes showed similar distribution patterns among the 17 samples, suggesting a lack of bias in the cDNA library construction (Supplementary Figures S1 and S2).

3.3. Analysis of DEGs in Response to P. brassicae

To investigate changes in the transcriptome during infection with the two types of pathogen, genes that were differentially expressed between the two *P. brassicae* pathotypes and control samples were analyzed at 4 and 8 dpi. The number of DEGs (FDR < 0.05 and |log2FC| > 1) was determined based on comparison with control samples (i.e., plants that were not inoculated with either *P. brassicae* pathotype) at the indicated time points. At 4 dpi, there were 304 DEGs in plants inoculated with LAB-4, which was significantly lower than the 5552 DEGs in those inoculated with SCCD-52 (Figure 2a,b, and Supplementary Table S3). The large difference in the number of DEGs indicated that *B. rapa* responds rapidly to infection with a virulent *P. brassicae* pathotype, even with the *CRd* locus. With LAB-4, the number of DEGs (3066) increased with longer post-inoculation times. However, with SCCD-52 there were fewer DEGs (1152) at 8 dpi compared with the earlier time point (Figure 2a,b, and Supplementary Table S4). In addition, the change in the proportion of up-regulated DEGs over time varied for the two pathotypes of *P. brassicae*, with a decrease from 77.2% to 60.7% for SCCD-52 and an increase from 74.6% to 76.6% for LAB-4. These results highlight the distinct responses of "85–74" containing *CRd* to pathogens exhibiting varying degrees of virulence. We examined DEGs specific to each of the two pathotypes at the two time points (4 and 8 dpi) and identified 27 and 5552 that were exclusively expressed at 4 dpi in plants inoculated with LAB-4 and SCCD-52, respectively (Figure 3a). The number of DEGs specifically expressed in samples inoculated with LAB-4 (2573) was significantly increased at 8 dpi (Figure 3b).

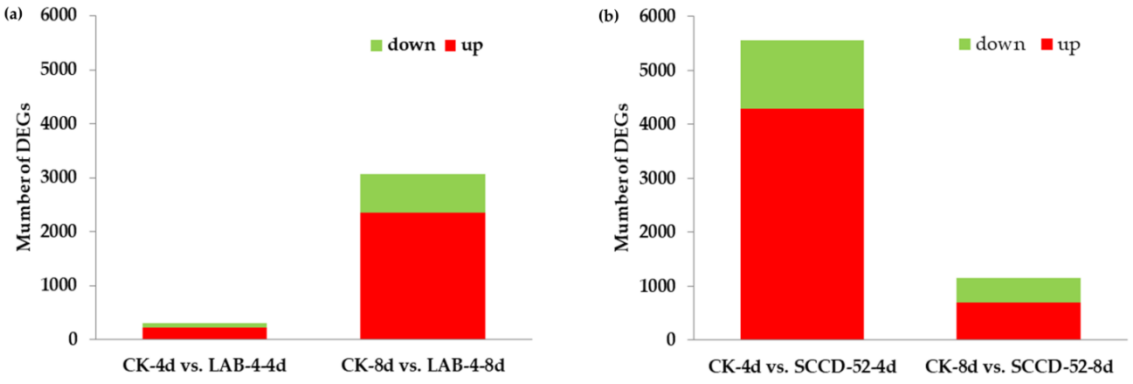

Figure 2. Comparison of the number of DEGs between control plants and plants infected with the two pathotypes of *P. brassicae* at 4 and 8 dpi. Number of DEGs based on the comparison between control plants and plants infected with LAB-4 (**a**) and SCCD-52 (**b**) at 4 and 8 dpi. Green and red represent up- and down-regulated DEGs, respectively.

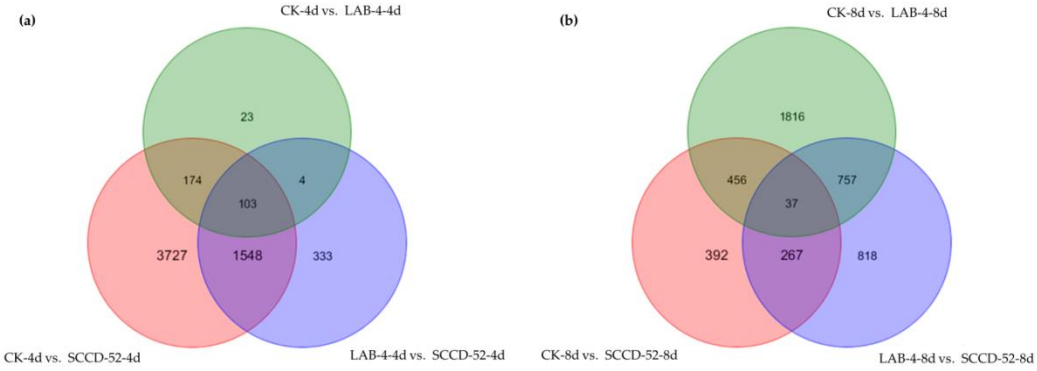

Figure 3. Number of DEGs in different samples at 4 and 8 dpi. Number of DEGs at 4 dpi (**a**) and 8 dpi (**b**). Green, red, and purple represent control vs. LAB-4, control vs. SCCD-52, and LAB-4 vs. SCCD-52, respectively.

3.4. Functional Enrichment Analysis

Given the difference in the number of DEGs in response to the two *P. brassicae* pathotypes at 4 dpi, we examined KEGG pathway enrichment at this time point. The top 20 enriched pathways are shown in Supplementary Table S4. A comparison between LAB-4 and control samples revealed that the DEGs were enriched in "plant hormone signal transduction" (ko04075), "phenylpropanoid biosynthesis" (ko00940), "nitrogen metabolism" (ko00910), and "plant–pathogen interaction" (ko04626) pathways ($p < 0.05$) (Supplementary Tables S5, S6, and Figure 4a). Notably, the "plant–pathogen interaction" pathway was not identified as an enriched pathway in the comparison between SCCD-52 and LAB-4 samples at the same time point (Figure 4c), suggesting that the *CR* gene is an early marker for *P. brassicae* infection. Given that changes in DEG profiles as a function of post-inoculation time differed for the two *P. brassicae* pathotypes, we also performed KEGG pathway enrichment analysis at 8 dpi. The top 20 enriched pathways are shown in Supplementary Tables S7 and S8. "Plant hormone signal transduction" was significantly enriched (with the highest RichFactor) in the comparison between LAB-4 and control samples, and the number of DEGs was higher at this time point than at 4 dpi (Figure 4b). Thus, plant hormone signaling likely plays an important and sustained role in the resistance to avirulent *P. brassicae*. The number of DEGs in the "plant–pathogen interaction" pathway was also increased; however, this pathway was not enriched in SCCD-52-infected plants at 8 dpi. Additionally, "plant hormone signal transduction" was enriched in the comparison between avirulent LAB-4 and virulent SCCD-52 (Figure 4d). Thus, plant hormone signaling mediates the resistance of Chinese cabbage to clubroot.

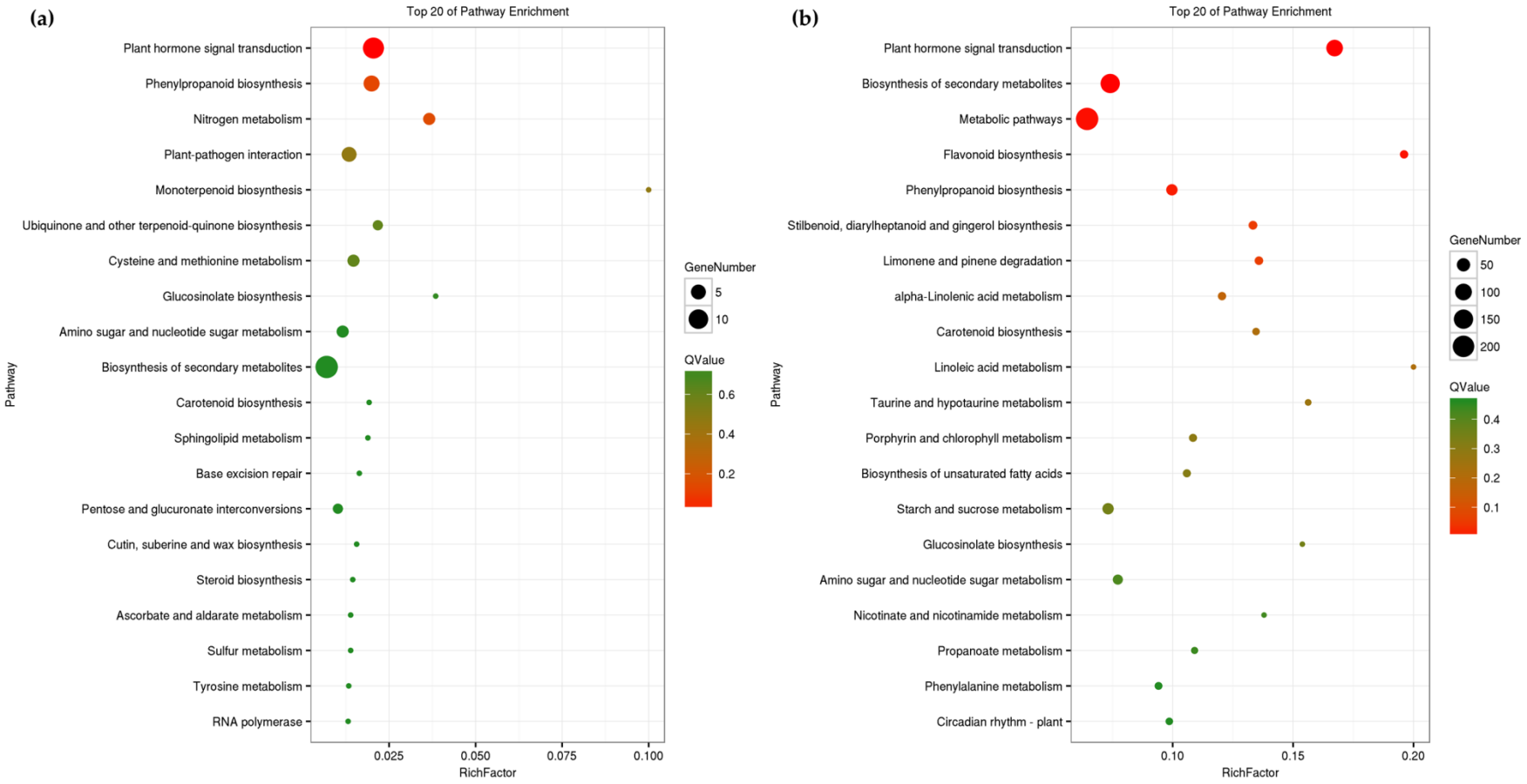

Figure 4. *Cont.*

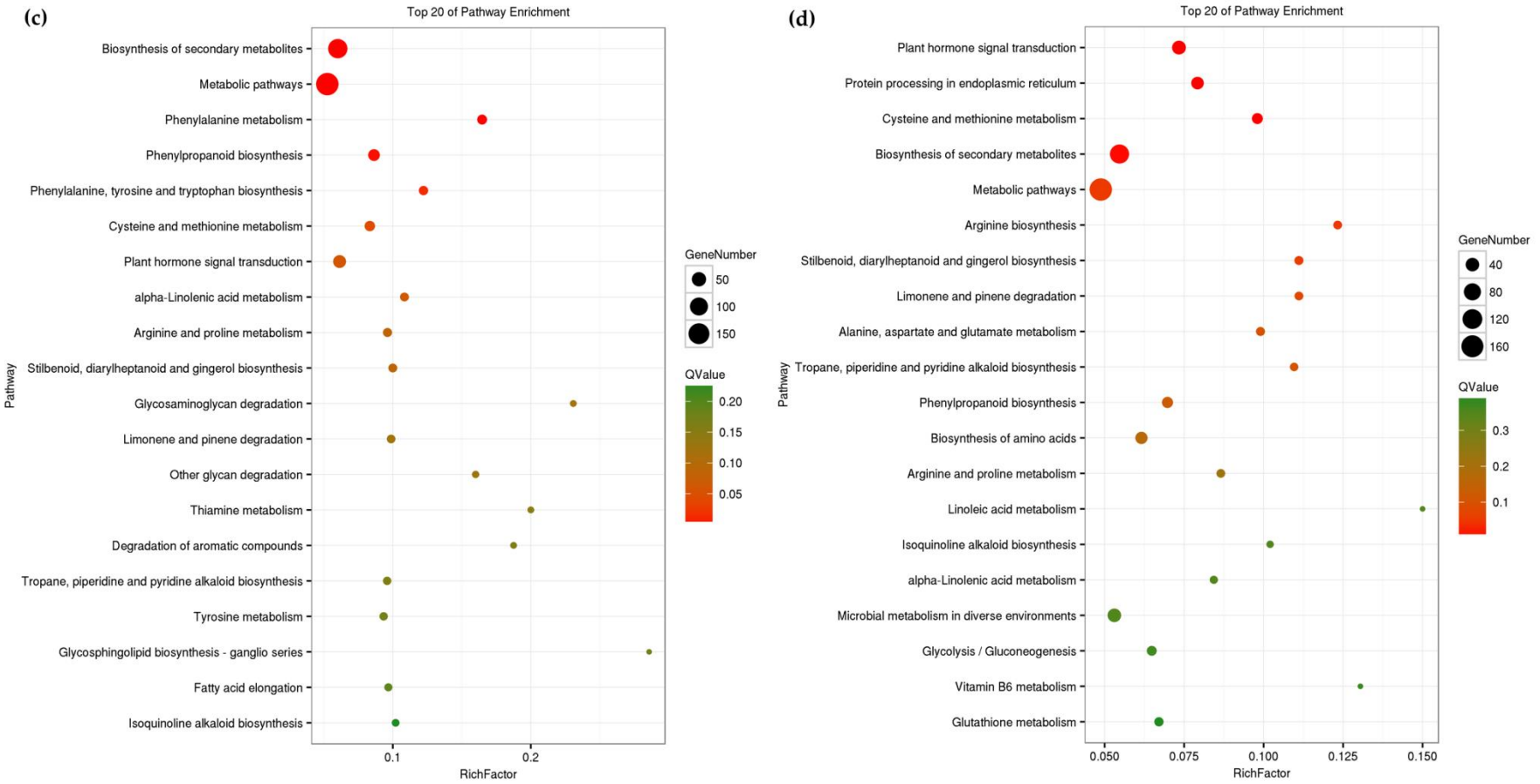

Figure 4. Top 20 KEGG pathways of DEGs in response to *P. brassicae* infection at 4 and 8 dpi. KEGG pathways are listed on the left, whereas Q values and gene numbers are shown on the right. Top 20 enriched KEGG pathways in the LAB-4 vs. control comparison at 4 dpi (**a**) and 8 dpi (**b**); and in the LAB-4 vs. SCCD-52 comparison at 4 dpi (**c**) and 8 dpi (**d**).

3.5. GO Analysis of DEGs during P. brassicae Infection

To further investigate the biological functions of the DEGs and verify the results of the KEGG analysis, the GO classifications of DEGs in the LAB-4 vs. control and LAB-4 vs. SCCD-52 comparisons at the two time points were examined in the context of all genes in the *B. rapa* genome (Figure 5a,b). The top five subcategories in the biological process class were "cellular process", "metabolic process", "single-organism process", "response to stimulus", and "biological regulation". The most frequently represented molecular function categories were "binding", "catalytic activity", and "nucleic acid binding transcription factor activity"; and the most highly represented cellular component categories were "cell", "cell part", and "organelle". The top 10 GO terms in the biological process category for the LAB-4 vs. control comparison are shown in Tables 2 and 3. Similar to the results of the KEGG pathway enrichment analysis, "hormone-mediated signaling pathway", "cellular response to hormone stimulus", "regulation of meristem development", and "cellular response to endogenous stimulus" were represented at both time points, and the number of DEGs was significantly higher at 8 dpi. On the other hand, "JA metabolic process", "regulation of phosphorelay signal transduction system", and "regulation of intracellular signal transduction" were enriched at 4 and 8 dpi in the LAB-4 vs. SCCD-52 comparison (Tables 4 and 5). This suggests that plant hormone signal transduction plays a key role in the defense response to *P. brassicae*. The relative expression levels of pathway genes at the two time points are shown in Figure 6.

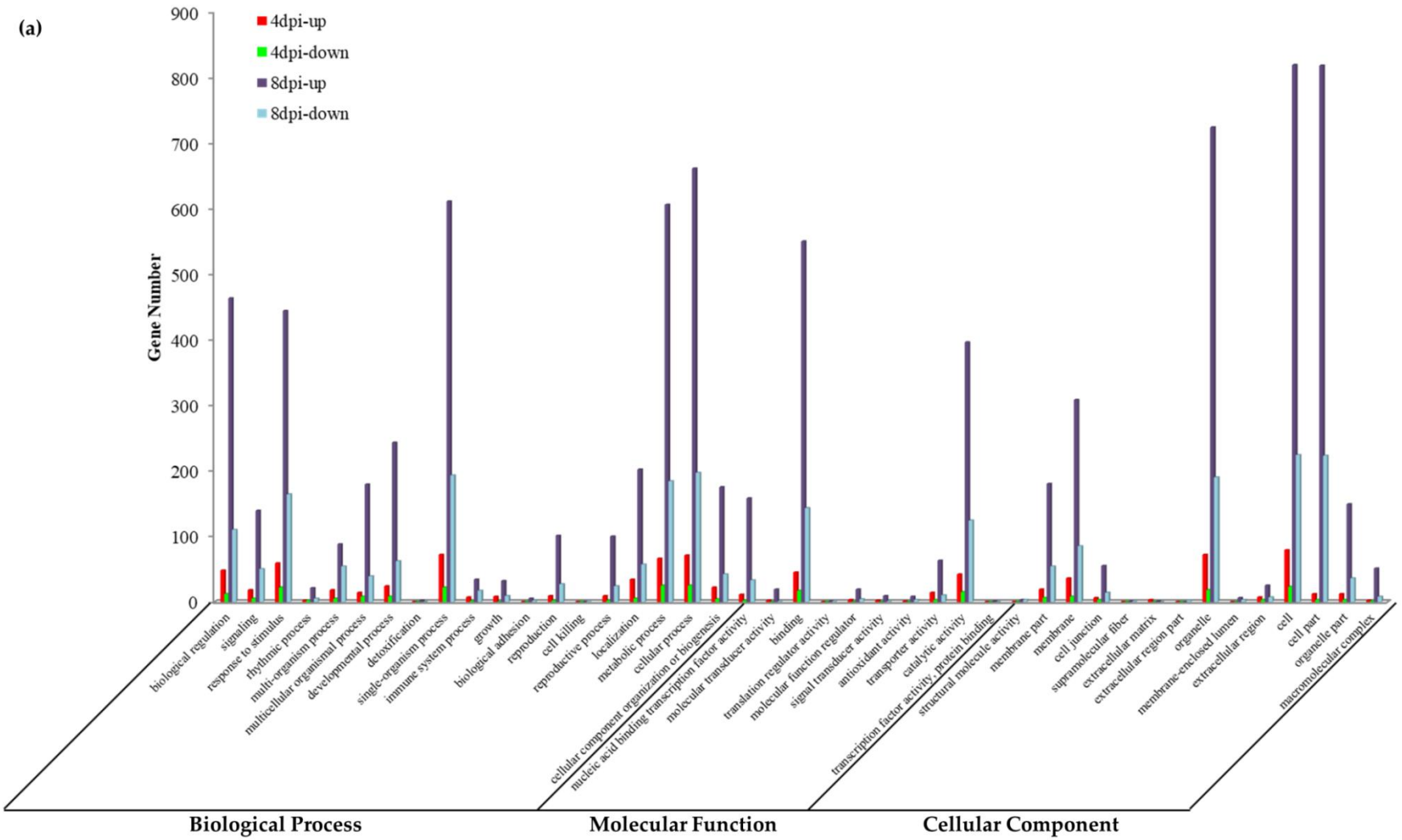

Figure 5. *Cont.*

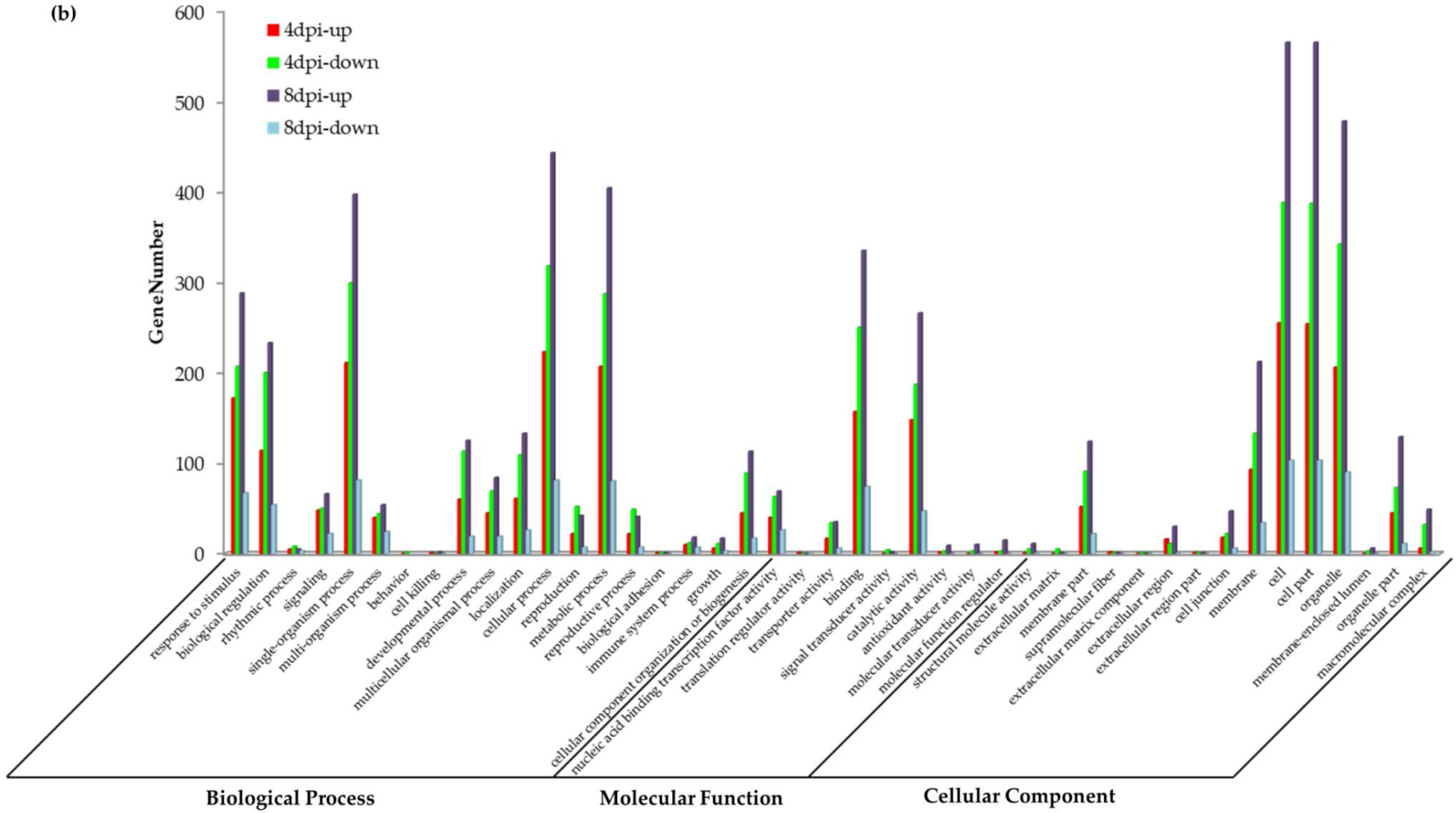

Figure 5. GO categories of DEGs in LAB-4 and SCCD-52. Unigenes were mapped to three main categories: biological process, molecular function, and cellular component. (**a**) LAB-4 vs. control; (**b**) LAB-4 vs. SCCD-52. The y-axis shows the number of annotated DEGs.

Table 2. Top 10 enriched GO terms in the LAB-4 vs. control comparison at 4 dpi.

Term	Gene Number	GO ID
Response to stimulus	81	GO:0050896
Ion transport	26	GO:0006811
Response to chemical	54	GO:0042221
Regulation of meristem development	10	GO:0048509
Hormone-mediated signaling pathway	18	GO:0009755
Cellular response to hormone stimulus	18	GO:0032870
Cellular response to endogenous stimulus	18	GO:0071495
Fluid transport	8	GO:0042044
Regulation of pH	2	GO:0006885
Cellular monovalent inorganic cation homeostasis	2	GO:0030004

Table 3. Top 10 enriched GO terms in the LAB-4 vs. control comparison at 8 dpi.

Term	Gene Number	GO ID
Biological regulation	573	GO:0065007
Regulation of biological process	526	GO:0050789
Regulation of meristem development	55	GO:0048509
Response to endogenous stimulus	209	GO:0009719
Response to hormone	174	GO:0009725
Regulation of cellular process	345	GO:0050794
Hormone-mediated signaling pathway	111	GO:0009755
Cellular response to hormone stimulus	111	GO:0032870
Cellular response to endogenous stimulus	111	GO:0071495
Enzyme linked receptor protein signaling pathway	27	GO:0007167

Table 4. Top 10 enriched GO terms in the LAB-4 vs. SCCD-52 comparison at 4 dpi.

Term	Gene Number	GO ID
Response to chemical acid	116	GO:0001101
Response to stimulus	379	GO:0050896
Jasmonic acid metabolic process	24	GO:0009694
Transition metal ion homeostasis	12	GO:0055076
Inorganic anion transport	27	GO:0015698
Transition metal ion transport	19	GO:0000041
Response to external stimulus	127	GO:0009605
Cation homeostasis	16	GO:0055080
Anion transport	48	GO:0006820
Secondary metabolic process	53	GO:0019748

Table 5. Top 10 enriched GO terms in the LAB-4 vs. SCCD-52 comparison at 8 dpi.

Term	Gene Number	GO ID
Regulation of phosphorelay signal transduction system	7	GO:0070297
Response to heat	19	GO:0009408
Response to light intensity	31	GO:0009642
Response to chemicals	233	GO:0042221
Regulation of intracellular signal transduction	8	GO:1902531
Response to endogenous stimulus	114	GO:0009719
Response to reactive oxygen species	30	GO:0000302
Regulation of organic acid transport	4	GO:0032890
Regulation of amine transport	4	GO:0051952
Regulation of amino acid transport	4	GO:0051955

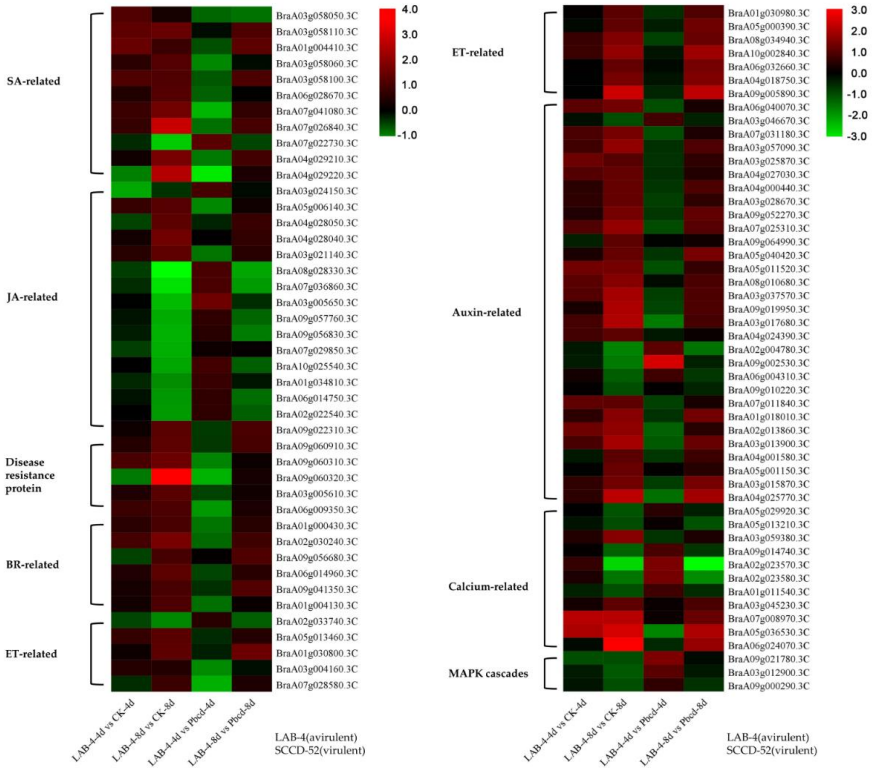

Figure 6. Heat maps of DEGs involved in the host defense response to *P. brassicae* infection. In the LAB-4 (avirulent) vs. control and SCCD-52 (virulent) vs. control comparisons, green and red indicate down- and up-regulated DEGs, respectively.

3.6. DEGs Involved in Resistance to P. brassicae

The results of the KEGG and GO analyses showed that "plant hormone signal transduction" and "plant–pathogen interaction" pathways are involved in the immune response to *P. brassicae*. We further examined 94 genes related to the two pathways including 30 related to auxin, 15 to JA, 12 to ET, 11 to SA, 11 to Ca^{2+}, six to BR, and three to the phosphorylation signaling along with five related to disease resistance. A heat map generated based on the fold expression level of control plants and those infected with avirulent (LAB-4) and virulent (SCCD-52) *P. brassicae* showed that genes related to SA, ET, and BR and signal transduction as well as auxin-responsive genes were up-regulated at 8 dpi (Figure 6 and Supplementary Table S9). The expression of genes involved in JA regulation increased whereas that of genes encoding JA inhibitory factors decreased at the late stage of infection; meanwhile, phosphorylation signaling pathway-related genes was activated at 4 dpi in LAB-4. However, as in plants lacking the *CR* locus, CR *B. rapa* was susceptible to infection with the virulent SCCD-52 pathotype [30]. These results imply that PTI plays an important role in the resistance of *B. rapa* to *P. brassicae* at the late stage of infection, which may involve induction of plant hormone signal transduction and plant–pathogen interactions.

3.7. JA, ET, and BR Signaling Are Important for Host Response at the Late Stage of Infection with Avirulent LAB-4

We analyzed plant hormone signal transduction pathways that were differentially activated between LAB-4- or SCCD-52-infected plants and controls at 8 dpi. There was a clear difference in plant hormone signaling pathway activation between plants infected with avirulent LAB-4 vs. virulent SCCD-52 (Figure 7a,b). In the presence of the *CRd* gene, SA-, ET-, JA-, and BR-related genes were up-regulated upon LAB-4 infection with the exception of jasmonate ZIM-domain (*JAZ*), which is a JA inhibitory factor. In contrast, only SA-related genes were up-regulated in response to SCCD-52

infection. Although the SA signal transduction pathway is up-regulated during the infection of both *P. brassicae*, while the host does not show resistance when only SA is up-regulated in SCCD-52 (virulent). These results suggest that resistance to avirulent LAB-4 is not due to accumulation of SA and that JA, ET, and BR signal transduction pathways are more important for the host response to avirulent pathogen at the late stage of infection.

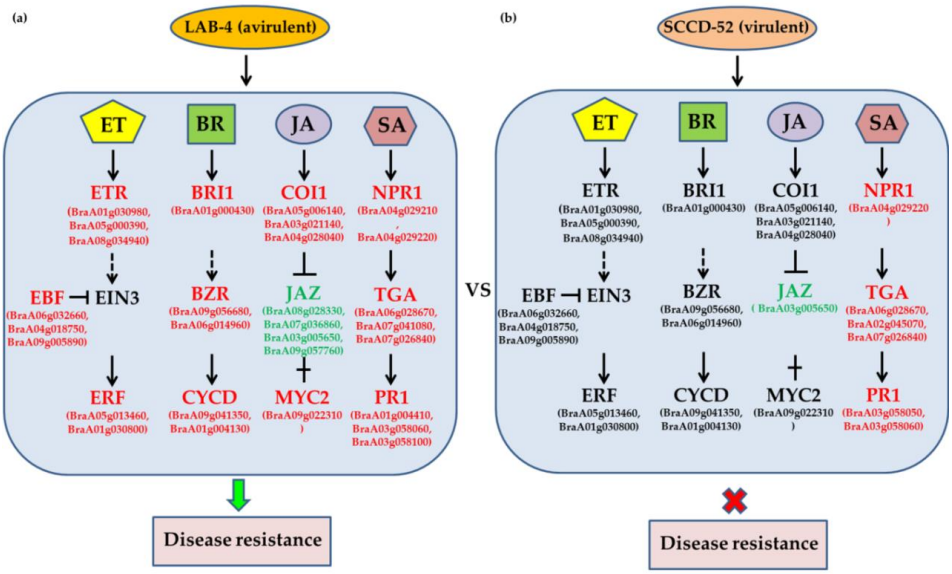

Figure 7. Changes in plant hormone signal transduction upon infection with different pathogens in the presence of the *CRd* gene. (**a**) LAB-4 compared with control at 8 dpi. (**b**) SCCD-52 compared with control at 8 dpi. Green and red indicate down- and up-regulated genes, respectively.

3.8. Validation of DEGs by RT-qPCR

To validate the RNA-seq results, the expression levels of DEGs related to the SA, JA, ET, and BR signaling pathways and disease resistance in LAB-4-infected and control plants were analyzed by RT-qPCR (Figure 8 and Supplementary Table S8) at 4 and 8 dpi and two additional time points (16 and 32 dpi). The *BraA06g028670.3C*, *BraA07g041080.3C*, and *BraA07g026840.3C* genes encoding the SA-responsive transcription factors (TGA) and *BraA04g029210.3C* and *BraA04g029220.3C* genes encoding SA regulatory proteins (*NONEXPRESSOR OF PR GENES 1*, *NPR1*) were induced while *BraA01g004410.3C*, *BraA03g058060.3C*, and *BraA03g058100.3C* (*PR1*) genes were overexpressed upon LAB4 infection [48,49]. The JA signaling-associated genes *BraA05g006140.3C*, *BraA03g021140.3C*, and *BraA04g028040.3C* (*CORONATINE-INSENSITIVE 1*, *COI1*)—which mediates JA signaling by promoting hormone-dependent ubiquitination and degradation of JAZ transcriptional repressor proteins [50,51]—and the *BraA09g022310.3C* gene (*MYC2*) encoding a related transcription factor [52] were up-regulated at each of the examined time points [53]. The ET receptor genes *BraA01g030980.3C*, *BraA05g000390.3C*, *BraA08g034940.3C*, and *BraA10g002840.3C* (*ETR*); EIN3-binding F-box protein-encoding genes *BraA06g032660.3C* (*EBF2*), *BraA09g005890.3C* (*EBF2*), and *BraA04g018750.3C* (*EBF1*); and ET response factor (ERF)-encoding genes *BraA05g013460.3C* (*ERF15*) and *BraA01g030800.3C* (*ERF1B*) were also up-regulated. The BR receptor gene *BraA01g000430.3C* (*BR-INSENSITIVE 1*, *BRI1*), BR-activated transcription factor-encoding genes *BraA09g056680.3C* and *BraA06g014960.3C* (*BRASSINAZOLE-RESISTANT2*, *BZR2*), and cell division-related genes *BraA09g041350.3C* and *BraA01g004130.3C* (*D-TYPE CYCLIN 3*, *CYCD3*)—which have similar expression patterns—were aberrantly expressed, as in the case of auxin-related genes. The levels of disease resistance genes *BraA09g060310.3C* (*RESISTANT TO P. SYRINGAE* 2, *RPS2*), *BraA09g060320.3C* (*RPS2*), *BraA06g009350.3C* (*RESISTANT TO P. SYRINGAE* 5, *RPS5*), and *BraA03g005610.3C* (*PBS1*) increased at 8, 16, and 32 dpi (Figure 8). The RT-qPCR results were mostly consistent with those obtained by RNA-seq and confirm the involvement of specific genes and pathways in LAB-4 infection.

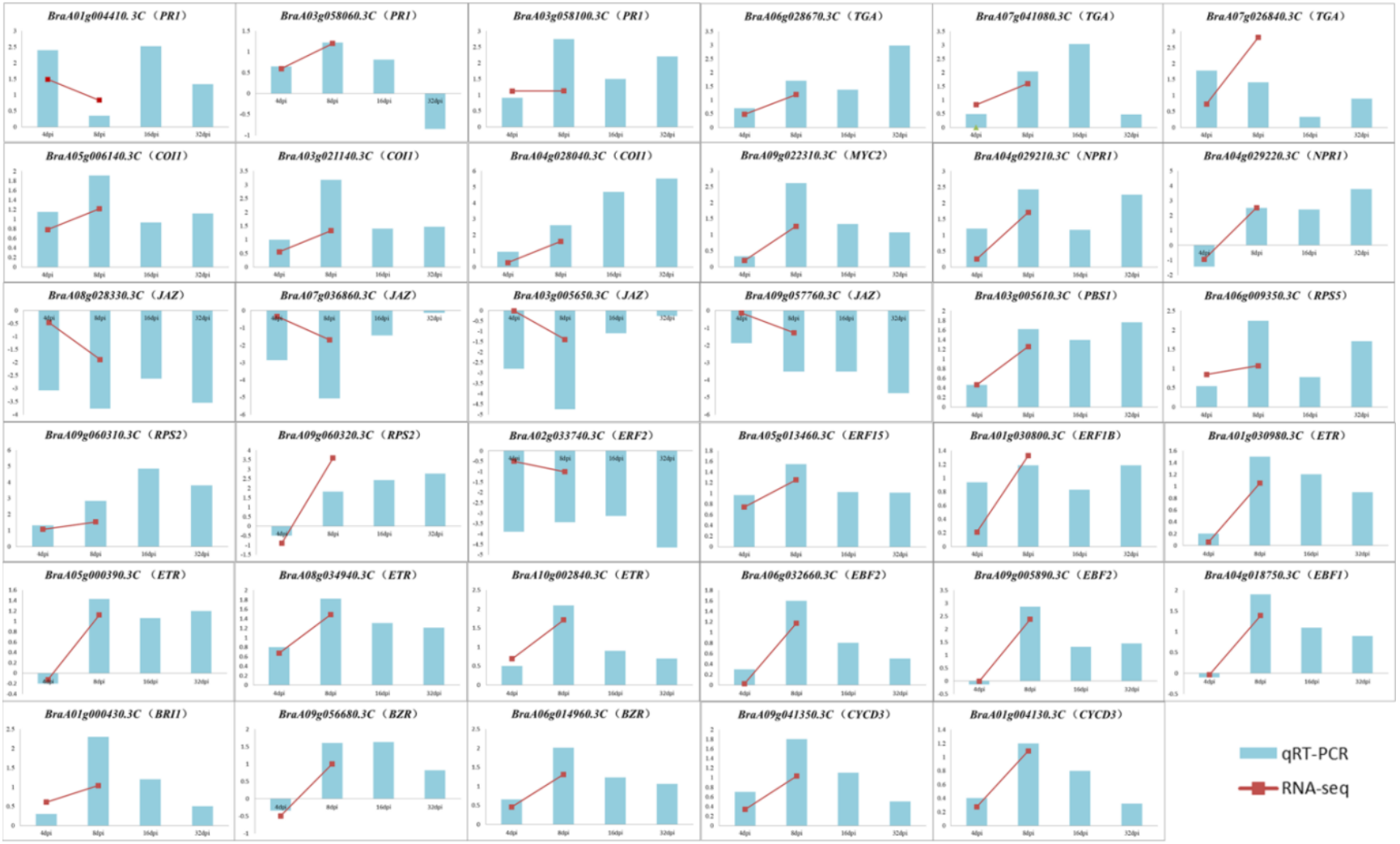

Figure 8. RT-qPCR validation of RNA-seq data. DEGs analyzed by RT-qPCR showed the same trend as those identified by RNA-seq. The y-axis shows the fold difference between the avirulent pathogen LAB-4 and control, with positive and negative values indicating up- and down-regulation, respectively.

4. Discussion

The host defense response to *P. brassicae* infection and clubroot progression has been investigated in genomic studies. More than 1000 genes were reported to be differentially expressed in clubroot-infected *Arabidopsis* roots compared with those of uninfected plants [38]. In the current study, the *B. rapa* disease-resistant variety "85–74" harboring the *CRd* gene [23] was inoculated with avirulent LAB-4 or virulent SCCD-52 *P. brassicae*; transcriptome sequencing revealed a large number of DEGs at 0 h and 4 and 8 dpi. The two major KEGG pathways represented by the DEGs were "plant hormone signal transduction" and "plant–pathogen interaction"; 94 DEGs in these pathways were identified by searching data from the literature and through a keyword search of the *B. rapa* genome database, and are discussed in detail below.

4.1. Expression of Phosphorylation-Related and Ca^{2+} Signaling Pathway Genes Is Altered by P. brassicae Infection

PRRs on the surface of plant cells recognize pathogens by sensing PAMPs, thereby inducing PTI. Key PRR genes activated by PAMPs include *flagellin sensing 2* (*FLS2*), which transduces signals that trigger PTI [54]. Upon pathogen infection, WRKY-type transcription factors are up-regulated whereas DEGs encoding members of other transcription factor families are down-regulated [55]. WRKY-type transcription factors function in a complex defense response network as both positive and negative regulators of plant immunity [56]. Mitogen-activated protein kinase (MAPK) signaling components include three proteins activated by sequential phosphorylation—namely MAPK, MAP2K, and MAP3K [57]; MAPK activation is one of the earliest signaling events following pathogen detection [58]. In this study, the levels of *BraA09g021780.3C* (FLS2), *BraA03g012900.3C* (MKK6), and *BraA09g000290.3C* (WRKY22) were lower in LAB-4-infected plants than in controls (Figure 6 and Supplementary Table S7), but were higher in plants infected with avirulent (LAB-4) as compared to virulent (SCCD-52) *P. brassicae* at 4 dpi. This indicates that the phosphorylation signaling pathway was responding to the stress caused by the pathogen at a relatively early time point after infection. Genes associated with Ca^{2+} influx and Ca^{2+} signaling pathways are up-regulated in root tissue of resistant as compared to susceptible *B. rapa* [29,31]. Ca^{2+} is an important secondary messenger in signal transduction pathways that regulates plant responses to abiotic and biotic stresses [59]. The *B. rapa CALMODULIN-LIKE 39* (*CML 39*) (*BraA02g023570.3C* and *BraA02g023580.3C*), *CML 42-LIKE* (*BraA01g011540.3C*), and *CYCLIC NUCLEOTIDE-GATED ION CHANNEL* (*CNGC*) (*BraA05g029920.3C*) genes were up-regulated upon infection with the avirulent pathogen (LAB-4) as compared to the virulent pathogen (SCCD-52) at 4 dpi, whereas the *CML 41-* (*BraA03g045230.3C*), *CML 22-* (BraA07g008970.3C), *CML 36-* (*BraA05g036530.3C*), and *CML 47-* (BraA06g024070.3C)-like genes were up-regulated at 8 dpi (Figure 6 and Supplementary Table S7), indicating that Ca^{2+} signaling contributes to the plant defense response.

4.2. Auxin and BR-Related Genes Involved in P. brassicae Infection

A microarray analysis of genes expressed during the primary phase of the *P. brassicae* life cycle in *Arabidopsis* root showed that the levels of several genes related to signal transduction and primary and secondary metabolism were altered [34]. A similar analysis of *Arabidopsis* root during different stages of *P. brassicae* development showed that genes related to auxin, cytokinin, and BR metabolism and signaling were up-regulated [37]. In this study, only five of 30 auxin-related genes were down-regulated whereas 25 were up-regulated in LAB-4-infected *B. rapa* relative to SCCD-52-infected and control plants at 8 dpi; however, all of the auxin-related genes were down-regulated at 8 dpi (Figure 6 and Supplementary Table S7). BR hormones function in disease resistance in both tobacco and rice [60], and studies in *Arabidopsis* have demonstrated that BR signaling antagonizes PTI [61–63]. Disruption of *BRI1* increases disease resistance against necrotrophic and hemibiotrophic pathogens that have a short asymptomatic phase, but has no effect on biotrophic pathogens or those with a prolonged asymptomatic phase [64]. *BRI1 KINASE INHIBITOR 1* (*BKI1*) inhibits the transphosphorylation-mediated activation

of the BRI1-BAK1 receptor complex [65–67]. In addition, the BR-activated transcription factor BZR represses the expression of genes such as FLS2 and *SUPPRESSOR OF NPR1-1, CONSTITUTIVE 1* (*SNC1*) that are directly involved in the defense against pathogens [61,68]. In this study, six BR-related genes were up-regulated at 8 dpi, including one *BRI1* (*BraA01g000430.3C*), one *BKI1* (*BraA02g030240.3C*), and two *BZR1* (*BraA09g056680.3C* and *BraA06g014960.3C*) genes (Figure 6 and Supplementary Table S7), indicating that PTI is suppressed at the late stage of infection with *P. brassicae*.

4.3. Up-regulation of Disease Resistance Genes in the Late Stage of Infection

Plant R gene products directly or indirectly mediate the recognition of pathogen effector proteins and trigger downstream signaling cascades in innate immunity [1,69]. However, screening for R gene variants conferring resistance to a specific pathogen or new pathogen races is complicated and labor-intensive [70]. In the present study, three *RPS2* (*BraA09g060910.3C*, *BraA09g060310.3C*, and *BraA09g060320.3C*), one *PBS1* (*BraA03g005610.3C*), and one *RPS5* (*BraA06g009350.3C*) gene were up-regulated at 8 dpi (Figure 6 and Supplementary Table S7). *RPS2* and *RPS5* belong to the leucine-rich repeat class and nucleotide binding site leucine-rich repeat class, respectively, of plant R genes [71]; and *PBS1* functions in the recognition of pathogen avirulence proteins [72]. Thus, *PBS1*, *RPS2*, and *RPS5* in *B. rapa* may be involved in the resistance to *P. brassicae* infection. The *CRd* locus was mapped to an interval of approximately 60.4 kb on chromosome A03; four TIR–NBS–LRR candidate genes have been identified in this region [23]. However, the expression levels of these genes did not differ between plants infected with avirulent (LAB-4) as compared to virulent (SCCD-52) *P. brassicae*. This clubroot resistance was most likely due to sequence differences between the *CR* regions of resistant and susceptible varieties of *B. rapa*.

4.4. JA, ET, and BR Signaling Pathways Play a Critical Role in the Response to Avirulent P. brassicae at the Late Stage of Infection

SA, JA, and ET mediate the three major disease resistance signaling pathways. In plants, SA is a hormone that is involved in the defense response to nutrient pathogens, whereas JA and ET are associated with the response to saprophytic pathogens [73]. In the current study, four *COI1* genes and one *MYC2* gene encoding JA-related transcription factors were up-regulated whereas 11 *JAZ* genes encoding JA-related transcriptional repressors were down-regulated at 8 dpi in plants infected with LAB-4 as compared to SCCD-52. The JA signaling pathway was inhibited at the early stage but activated at the late stage of infection. Two downstream regulatory genes of SA signaling encoding the transcription factors TGA1 and NPR1 were up-regulated at 8 dpi, whereas the *PR1* gene was up-regulated at each time point examined. Meanwhile, the levels of the ET signaling-related genes *ETR*, *EBF*, and *ERF* increased at 8 dpi (Figure 6 and Supplementary Table S7). In contrast to other studies, we found that at the late stage of infection with LAB-4 the SA, JA, ET, and BR signaling pathways were induced; at 8 dpi, the SA signaling pathway was activated upon infection with both LAB-4 and SCCD-52, whereas the JA, ET, and BR signaling pathways were only induced in the former instance (Figure 8A,B). A previous study reported that the non-pathogenic rhizosphere-colonizing bacteria *Pseudomonas* spp. trigger a similar response known as rhizobacteria-induced systemic resistance that involves JA but not SA signaling [60]. The discrepancies between our results and those of other studies may be due to the different *Brassica* species or CR genes that were used. Based on these findings, we propose that JA, ET, and BR signaling play key roles in CR in *B. rapa* "85–74" infected with either avirulent (LAB-4) or virulent (SCCD-52) *P. brassicae*.

5. Conclusions

In this study, we analyzed the transcriptome profiles of disease-resistant *B. rapa* seedling roots following infection with avirulent (LAB-4) or virulent (SCCD-52) *P. brassicae*. We showed that the primary response of *B. rapa* to infection involves plant hormone signal transduction and plant–pathogen interactions. ETI was enhanced in resistant "85–74", while ET/JA and BR signaling pathways were

found to play important roles in the late stage of infection with avirulent LAB-4. These results provide insight into the mechanisms of *B. rapa* resistance to clubroot disease, which can improve disease management in *B. rapa* and other economically important crop species.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/10/589/s1, Figure S1: Abundance distribution map of all sample genes. The *x*-axis is log10 FPKM, and the higher the value, the higher the gene expression level; the *y*-axis is the abundance of the gene, i.e., the number of genes corresponding to the expression of the horizontal axis/detecting the total number of expressed genes; Each color in the figure represents a sample; the peak of the distribution curve represents the region where the gene expression of the entire sample is most concentrated, Figure S2: Comparison of gene expression levels between groups. The *y*-axis is the expression level of the gene, and each box represents a sample group. The line in the middle of the box indicates the median, and the top and bottom sides of the box are the upper and lower quartiles. From the box plot, you can visually compare the expression levels of genes between different groups, Table S1: qRT-PCR primer information, Table S2: RNA-seq data evaluation statistical, Table S3: Ck-4d-vs-LAB-4-4d differentially expressed genes and Ck-4d-vs-SCCD-52-4d differentially expressed genes, Table S4: Ck-8d-vs-LAB-4-8d differentially expressed genes and Ck-8d-vs-SCCD-52-8d differentially expressed genes, Table S5: Top 20 enriched KEGG pathways about CK-vs-LAB-4 at 4 dpi, Table S6: Top 20 enriched KEGG pathways about LAB-4-vs-SCCD-52 at 4 dpi, Table S7: Top 20 enriched KEGG pathways about CK-vs-LAB-4 at 8 dpi, Table S8: Top 20 enriched KEGG pathways about LAB-4-vs-SCCD-52 at 8 dpi, Table S9: Heat map genes information.

Author Contributions: Conceptualization, Z.P.; methodology, P.F., Z.Z. and W.P.; software, P.F. and Y.Z.; validation, P.F.; formal analysis, P.F. Z.Z. and X.L.; investigation, Y.P and Y.Z..; resources, Y.P.; data curation, Z.P., P.F. and Y.P.; writing—original draft preparation, P.F.; writing—review and editing, Z.P., P.F., Z.Z., W.P. and X.L.; supervision, Z.P.; project administration, Z.P.; funding acquisition, Z.P.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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