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Full Length Article

Transcriptome Analysis of a Highly Resistant Chinese Cabbage Variety in Response to *Plasmodiophora brassicae* during Early and Late Stages of Root Hair Infection

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Abstract

Plasmodiophora brassicae (P. brassicae) is widely distributed in the field. To understand the early and late defense responses induced by *P. brassicae* infection in *Brassica rapa*, RNA-Seq was used to perform high-throughput transcriptome analysis of roots of a highly resistant Chinese cabbage variety that does not form root galls. There are totally 153 and 99 differentially expressed genes (DEGs) between inoculated and healthy roots at 4 and 10 days post inoculation (dpi), respectively. The functions of the DEGs were annotated. Data showed that most DEGs were involved in metabolic processes, cell wall modification, peroxidase activity, signal transduction and defense. There were 71 DEGs associated with plant–pathogen interactions, including genes associated with hormone signalling, pathogenesis, peroxidase, oxidoreductase, chitinase, cell wall modification, and trehalose biosynthetic and metabolic processes. The expression levels of resistance proteins and pathogenesis-related (PR) genes mediated by salicylic acid (SA) were high in the inoculated roots, especially at 4 dpi, suggesting that SA signalling during early infection was important for resistance to *P. brassicae* in this highly resistant Chinese cabbage variety. These results provide theoretical basis for studying the molecular mechanism of clubroot resistance.

Keywords: *Brassica rapa*; *P. brassicae*; RNA-Seq; Clubroot; Disease resistance

Introduction

Clubroot, caused by *Plasmodiophora brassicae*, is one of the main diseases of *Brassica* crops in the field (Peng *et al.* 2016). Genes involved in clubroot resistance in *Brassica rapa* have been reported (Ueno *et al.* 2012; Chen *et al.* 2013; Hatakeyama *et al.* 2013; Chen *et al.* 2016; Lovelock *et al.* 2016; Zhang *et al.* 2016; Zhao *et al.* 2017), but much less is still known about the resistance mechanisms of *Brassica rapa* to *P. brassicae* infection.

Two innate immune systems in plants exist to prevent the invasion of pathogens (Jones and Dang 2006). The first, pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), can be suppressed by pathogen effectors. Resistance (*R*) genes in plants recognized pathogen effectors. Then the second defense response (effector-triggered immunity, ETI) is triggered. Many defense responses, such as hormone signalling (Moore *et al.* 2011), are activated via transcriptional regulation during the PTI and ETI processes. Defense responses are triggered by chitin (a PAMP of *P. brassicae*) in plants (Schwelm *et al.* 2015). However, these immune systems have not been fully studied in the *Brassica rapa* response to *P. brassicae*. Two stages occur during the infection process of *Brassica* crops by *P. brassicae*: first is root hair infection and the second is cortex infection (Wang *et al.* 2011a). Primary infection has been observed in resistant *Brassica* strains without secondary infection, indicating that *P. brassicae* was blocked at the secondary infection stage (Deora *et al.* 2013).

Auxin, glucosinolates, brassinosteroids and ethylene have proved to play an important role in symptomatic development of swelling in *Arabidopsis* roots (Ludwig-Müller 2009; Knaust and Ludwig-Müller 2013). Hormones are used to promote the elongation of root and the gall formation in the clubroot (Ludwig-Müller and Schuller 2008; Ludwig-Müller *et al.* 2009; Ludwig-Müller 2014). The cytokinin are significantly up-regulated during the stage of cortex infection (Ludwig-Müller *et al.* 2009), but are down-regulated at the stage of root tumor formation (Malinowski *et al.* 2016). The stretch and division of host cell was inhibited or promoted in the process of incompatibility interaction (Jubault *et al.* 2013). Auxin is

up-regulated in two ways after infection by *P. brassicae*. Indole-thioglycoside is an intermediate product in the synthesis pathway of auxin. Indole-thioglucoside is up-regulated in host plant after infection by *P. brassicae* (Ludwig-Müller 2009). Cyanide hydrolysis enzyme is also a key enzyme in auxin synthesis pathway. Cyanide hydrolysis enzyme is up-regulated in host plant infected by *P. brassicae* (Siemens *et al.* 2006). The variable transcriptional activation of cyanide hydrolysis enzyme gene might be involved in the up-regulation of auxin in the pathogenetic period of pathogen (Ando *et al.* 2008). The cytokinin produced by *P. brassicae* might promote the formation of meristem in the areas of epithelial cell in host plant (Devos *et al.* 2006; Ludwig-Müller *et al.* 2009). Other substances reported to be involved in pathogenic process include flavones (Päsold *et al.* 2010), and brassinolide (Schuller *et al.* 2014).

It is likely that *P. brassicae* could also complete root hair infection in incompatible interaction. So the difference between incompatible and compatible interaction in the infection process is mainly reflected in cortex infection. A large number of root hair infections could be formed after artificial inoculation of resistant rapeseed (Deora *et al.* 2013). However, only a small amount of secondary plasma mass of *P. brassicae* was observed in the cortex at 12 day post inoculation (dpi). At 18 and 24 dpi, cortical infection was extremely difficult to be observed. Two types of callose deposits were observed in resistant hosts (Donald *et al.* 2016). One is localized deposition at the base of the infected root hair cells, and the other is in the entire root hair cells and epithelial cells. In transcriptome analyses of host on the resistance genes Rcr1, callose deposition related genes are also increased (Chu *et al.* 2014). Kobelt *et al.* (2000) found that the host containing the RPB1 gene could limit the infection of *P. brassicae* by hypersensitivity reaction. But no hypersensitivity reaction was found in Canadian canola (Deora *et al.* 2013).

Differentially expressed genes (DEGs) have been analysed to gain a better understanding of host–pathogen interactions, including in transcriptomic approaches focusing on the interactions between *Arabidopsis* and *P. brassicae* (Devos *et al.* 2006). Many studies have reported the early infection (Agarwal *et al.* 2011), but the activity of abnormal cells was inhibited at the late stages of infection in *Arabidopsis thaliana* (Jubault *et al.* 2013).

In *Arabidopsis*, both the salicylic acid (SA) and jasmonic acid (JA) pathways are thought to contribute resistance to the *P. brassicae* (Lemarié *et al.* 2015). Compared with susceptible plants, resistant plants exhibited up-regulated expression of genes involved in the JA and ethylene (ET) pathways at 15 days post inoculation (dpi) (Chu *et al.* 2014).

SA signaling is involved in plant resistance to *P. brassicae*. The expression of PR5 and PR2, genes in response to salicylic acid, were up-regulated significantly in *Arabidopsis* resistance varieties (Lemarié *et al.* 2015). Treatment with exogenous SA could reduce the root swelling (Lovelock *et al.* 2013). Genes encoding methyl salicylate were involved in the host defenses (Manoharan *et al.* 2016). Chen *et al.* (2016) also found that SA-mediated signal transduction pathway was involved in host defences in transcriptome analysis of the nearly isogenic line in Chinese cabbage resistant to clubroot. But in transcriptome analysis of the resistant varieties, the genes expression related to SA pathway were not increased (Chu *et al.* 2014). In addition, JA pathway was also involved in the host resistance to *P. brassicae*. In *Arabidopsis* resistance varieties, expression of ARGAH2 and THI2.1 response to JA was also noticed (Lemarié *et al.* 2015). Treatment with exogenous JA could also reduce the root swelling. In the transcriptome analysis, the expression level of gene related to JA pathway has been up-regulated in resistance varieties (Chu *et al.* 2014). JA promoting the synthesis of auxin during *P. brassicae* infection was also involved in pathogenic process of *P. Brassicae* (Grsic *et al.* 1999). Ethylene defective mutants of *Arabidopsis thaliana*, was easily infected by *P. brassicae* than wild type (Knaust & Ludwig-Müller 2013). In transcriptome analysis, the expression of genes related to ethylene pathway was up-regularted in resistant varieties (Chu *et al.* 2014).

In the this study, RNA-Seq was used to reveal DEGs between infected and control plants to investigate clubroot resistance in a highly resistant Chinese cabbage variety (without small spheroid galls (SSG) formation) in response to *P. brassicae* during the early and late stages of infection. Analysis of these DEGs will help elucidate the molecular mechanisms of the interactions between *B.rapa* and *P. brassicae*, especially the defense mechanisms.

Materials and Methods

Experimental materials

Chinese cabbage "Ji Chun Chinese cabbage" (CR-JC, Kexiang Seed, Fuzhou, China) was used as plant material. Previous studies have shown that no small spheroid galls (SSG) were formed on this variety after inoculation. *P. brassicae* was collected from Wang Si Zhen, Dayi County, from root galls (found on the variety DY-W). This strain has been demonstrated ineffective against CR-JC in pot experiments. The physiological races of strain were identified by Williams identification system, and the results were obtained from our laboratory. The clubroot strain used in this paper was the No.4 physiological race.

P. brassicae inoculation

Thawed frozen galls mixed with distilled water were put into a blender. The homogenate was filtered using 8 layers of gauze, adding water as needed, before centrifuging. The centrifugation method was improved based on the study of Peng *et al.* (2016). First, the pretreated preparation was

centrifuged at 500 rpm for 5 min. Then, the supernatant liquid was centrifuged at 3100 rpm for 10 min, and the supernatant was discarded while the precipitate was collected. This step was repeated 3 times. The spores were diluted to a density of 10^8 spores/mL. Inoculated the plant with 1 mL spore suspension in the surrounding soil as described by Peng *et al.* (2016). The inoculated plants were cultured in a 16-h photoperiod chamber at 25ºC, with the soil kept moist. Zoosporangia in a root hair of CR-JC infected with *P. brassicae* were observed by the microscope in 20 times.

Tissue sampling

The roots of CR-JC infected with *P. brassicae* were sampled at 4 and 10 dpi for DEG analysis at the early and late stages of the root hair infection. At the same time, non-inoculated plants at these two time points were sampled as controls. Three independent biological replicates were conducted at each time point. The roots were washed with distilled water and quickly frozen in liquid nitrogen; then they were stored at −80°C.

RNA isolation and transcriptome sequencing

RNA was extracted using the RNAprepPure Plant Kit (Tiangen, Beijing, China). ANanophotometer® spectrophotometer (Implen, CA, USA), 1% formaldehyde gel electrophoresis, and a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) were used to test the purity of RNA.AQubit RNA Assay Kit and Qubit® 2.0 fluorometer (Life Technologies, CA, USA) was used to detect the concentration of RNA. The integrity of RNA was estimated by using an RNA Nano 6000 Assay Kit on the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The RNA from three biological replicates at 4 and 10 dpi had chosen for analysis. Equal quantities of RNA from roots of 3-5 seedlings were pooled for each biological replicate. A 3 µg RNA per sample was used for RNA library preparation. Samples were sent to Nuo he zhi yuan biological Co., Ltd for sequencing.

GO and KEGG enrichment analysis

Three biological replicates of each sample, showing correlation coefficient value of $\geqslant 0.95$ were considered for subsequent gene expression analysis. TopHat was used to map the sequencing reads to reference genome database (http://brassicadb.org/brad/) (Trapnell *et al.* 2009). Gene Ontology (GO) enrichment analysis of DEGs was implemented by the GOseq R package (Ashburner *et al.* 2000). GO terms with corrected P value ≤ 0.05 were considered significantly enriched by DEGs. GO annotation of the unigenes was performed by using the Blast2GO program (Conesa *et al.* 2005). GO functional classification and unigene analysis were performed by using GO sequence and Top GO software. Reference genome was *Brassica rapa* (assembly Brapa_1.0) from NCBI. Unigene sequences were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/) (Kanehisa *et al.* 2004). KOBAS software was used to test the statistical enrichment of DEGs in KEGG pathways. The False Discovery Rate (q value) $\leqslant 0.005$ and the absolute value of log2 Fold Change $\geqslant 1$ was used as the threshold to judge the significance of gene expression differences.

Quantitative RT-PCR analysis

For qPCR, the replicates were done, using independently grown plants at different times. The qRT-PCR was done on the same RNA as the RNASeq. All the seedlings were included independently. qRT-PCR was performed on 30 selected DEGs related to resistance to *P. Brassicae* (including 5 pathogenesis-related (PR) proteins, 1Rgene, 5 auxin associated family proteins, 1 chitinase, 6 peroxidases, 2 beta-1,3-glucanases, trehalose-phosphatase, 1 xyloglucosyltransferase and 1 ethylene-responsive transcription factor) (Table 1 Suppl.). 27 of these DEGs were found at 4dpi.

The SA pretreatment was performed by spraying 0.5 mM SA onto whole plants before *P. brassicae* inoculation. Then, the expression levels of SA-mediated genes and *P. brassicae* gene (EST name: PbrS827; GenBank Acc: JK747456) levels were detected by qRT-PCR analysis at each time point. The specific *Plasmodiophora* gene JK747456 chosen was order to detect the accumulation of *P. brassicae*.

SYBR Green Super Real PreMix Plus (Tiangen) was used on a Real-time PCR system (Bio-Rad, Hercules, CA, USA). The primers are listed in the supplementary material (Table 1 Suppl.). Analysis of gene expression was performed for samples at 4 and 10 dpi. Three independent biological replicates were performed for each sample. *B. rapa 18S rRNA* and *Actin* was *used* as internal controls.

Statistics analysis

All experiments were repeated for three times. Typical results are presented and the standard deviations (n = 3). Student's t test was used for comparison between different treatments. A difference was considered to be statistically significant at $p < 0.05$. Microsoft Excel 2003 was used for data processing, and SPSS 17.0 was used for significant difference analysis.

Results

Symptoms of CR-JC

The differences of gene expression during the infected process of *P. brassicae* were monitored after inoculation. Disease severity was assessed at 4 and 10 dpi after infection with *P. brassicae*. We found that no galls were formed on

roots in CR-JC. Microscopic analysis of roots in CR-JC at 4 and 10 dpi was done. Zoosporangia were observed in a root hair of CR-JC inoculated with *P. brassicae*. Infected root hairs at 10 dpi were more than that at 4 dpi in CR-JC (Fig. 1). The specific *Plasmodiophora* gene JK747456 was chosen in order to detect the accumulation of *P. brassicae* (Fig. 1). The cortical invasion might be delayed. The cell wall was thickened. So the cortical penetration was blocked.

RNA-Seq analysis

RNA-Seq was used to analyse the transcript levels of CR-JC at two stages of infection with *P. brassicae*. A total of 113, 27, 52 reads were generated, constituting 13.82 GB of cDNA sequence. Of these, 27, 139, 852 reads were obtained from B4D-IN, 28, 203, 796 from B10D-IN, 31, 229, 376 from B4D-CK, and 26, 454, 028 from B10D-CK. The GC% values of the sequence data were all >46%, and the Q20 values were all >96%. The quality of the data met the requirements. Table 2 Suppl. shows an overview of the sequencing data. Fragments per kilobase of transcript per million mapped reads (FPKM) density distribution (Fig. 1 Suppl). The percentage of reads mapped to the exons of the genome regions was the highest in all three regions i.e., exon, intergenic and intron (Fig. 2 Suppl.).

Transcriptome analysis

A total of 252 DEGs were found between infected plants and controls at 4 and 10 dpi (Table 3 Suppl.); 76 genes were up-regulated while 77 were down-regulated at 4 dpi. At 10 dpi, only 37 genes were up-regulated and 62 were down-regulated (Fig. 2). The number of genes differentially expressed at 4 dpi was higher than that at 10 dpi (Fig. 2, Tables 3–5 Suppl.).

Functional annotation of DEGs

The sequences of the 252 DEGs were compared to reference databases. The functions of the DEGs were annotated (Table 6 Suppl.): 248 (98.41%) in KEGG and 184 (73.01%) in GO.

The 252 DEGs were distributed into three GO classes: biological process, cellular component, and molecular function. First, we enriched it with genes that were significantly different. Secondly, the hypergeometric distribution test was carried out for GO enrichment, and the significant enrichment still showed $p < 0.05$. There were 97 DEGs in biological process at 4 dpi, as well as many DEGs in carbohydrate metabolic process and heme binding (Fig. 3A). The following subcategories in the biological process class may be related to host defense: “trehalose biosynthetic process”, “trehalose metabolic process”, “response to oxidative stress”, “chitin catabolic process”, “chitin binding”, “peroxidase activity”, “oxidoreductase acting on peroxide as acceptor” and “chitinase activity”. There were 22 DEGs in “single organism metabolic process” and many additional DEGs in the following classes: “oxidation-reduction process”, “oxidoreductase activity”, “transporter activity”, “transmembrane transporter activity”, “ion transmembrane transporter activity”, “peroxidase activity”, “oxidoreductase acting on peroxide as acceptor”, “antioxidant activity”, “heme binding”, and “tetrapyrrole binding” (Fig. 3B). In particular, there was an obvious GO enrichment related to antioxidant enzymes, suggesting that antioxidant enzymes may be related to the defense response of CR-JC.

The sequences of the DEGs were mapped to the reference pathways in the KEGG database to identify the biological pathways activated in *Brassica rapa*. At 4dpi, 248 DEGs were mapped to the KEGG database and assigned to 35 KEGG pathways. More biological pathways containing “Plant hormone pathway”, “biosynthesis of amino acides” and “biosynthesis of secondary metabolites” were actived at 4dpi (Fig. 4A) than at 10dpi (Fig. 4B). The most common term was “metabolic pathways”, which contained 42 DEGs (16.9%), followed by “biosynthesis of secondary metabolites” (14.5%), “phenylalanine metabolism” (4.4%), “plant hormone signal transduction” (4%), “biosynthesis of amino acids” (3.6%), “phenylpropanoid biosynthesis” (3.6%), and “plant–pathogen interaction” (2.8%; Table 1).

DEGs related to plant resistance

To gain a better understanding of the defense mechanisms in *B.rapa* response to *P. brassicae*, 70 DEGs involved in disease resistance were found using keyword searches (Table 7 Suppl.). These genes included13 peroxidase genes, 1 R gene, 2 respiratory burst oxidase genes, 1 respiratory burst oxidase homologue (RBOH), 7 PR proteins, 10 genes related to hormone metabolism (ET [3 genes], auxin [7 genes]), 8 cell wall modification related genes, 4 chitinase family genes, 1 desulfo-glucosinolate sulfotransferase gene, 3 trehalose biosynthetic and metabolic process genes, 1 L-phenylalanine dehydratase gene, and 19 oxidoreductase genes.

The *R*, PR protein, chitinase family, peroxidase, respiratory burst oxidase, oxidoreductase and hormone metabolism genes were obviously up-regulated at 4 dpi, while the peroxidase and cell wall modification related genes were up-regulated at 10 dpi. Among the eight genes related to cell formation found in this study, four were up-regulatedat 10 dpi, including xyloglucanendotransglucosylase-hydrolase 17, lipid binding, pectinesterase and ion transmembrane transporter, with the remaining four genes being down-regulated at 10 dpi, including cell wall–plasma membrane linker protein (Table 7 Suppl.). The GH3 family genes were markedly up-regulated at 4 dpi, and L-phenylalanine dehydratase was also up-regulated.

Gene expression in hormone signaling pathways

In the present study, one NIM1-interaction 2 (NIMIN2)

gene, a component of the SA signal pathway, was up-regulated. Several PR protein genes associated with SA pathway were dramatically up-regulated, especially at 4dpi. Three genes associated with the ET pathway (including ethylene response factor 73 ([ERF073]) and ethylene response factor 104 ([ERF104]) were down-regulated at both time points (Table 7 Suppl.). These results indicated that SA pathway was induced, while the JA/ET pathway was repressed, during the early stage of infection.

Quantitative RT-PCR validation

Thirty DEGs were detected by qRT-PCR to validate the RNA-Seq results, including 21 genes related to clubroot resistance and 19 genes involved in the SA, JA, and ET signal pathways (Table 7 Suppl.). The expression of all 30 DEGs (including 27 DEGs identified in 4dpi) was consistent with the RNA-seq results (Fig. 5). SA-mediated genes were highly enhanced by SA treatment, and the accumulation of *P. brassicae* was markedly reduced (Fig. 6).

Discussion

After four days of inoculation, no protoplast formation was observed. Zoosporangium was seen in the root hair on the 10 day post inoculation. Now specific the infection stage was rephrased. It is very interesting that no small spheroid galls (SSG) is formed on the high resistant cultivar different from typical resistant symptoms. That may be the extreme of the resistance response. Although *P. brassicae* infection is limited in the cortical infection stage in the resistant host, the host's defense system is still rapidly activated. McDonald *et al.* (2014) found that the inoculation with dormancy spore of avirulent strains resulted in no root galls, while the inoculation with secondary zoospore of avirulent strains resulted in the formation of bead-shaped club. Disease index is significantly lower in inoculation with secondary zoospore of virulent strain than with toxic dormant spore inoculation (McDonald *et al.* 2014). This indicates that root hair infection is critical in recognition between the pathogen and host. Host defense may be suppressed in compatibility interaction, while host defense may be activated in incompatibility interaction (McDonald *et al.* 2014).

In this study, 252 DEGs were detected at 4 dpi and 10 dpi. A greater number of DEGs were found at 4dpi. A total of 70 DEGs related to PTI and ETI were found by keyword searches (Table 7 Suppl.), and these DEGs are discussed below.

The synthesis of PR proteins is induced by the invasion of pathogens (Van Loon and Van Strien 1999). Six PR proteins (including 5 PR proteins and 1 lipid transfer protein) were differentially expressed between infected and control plants (Table 7 Suppl.). These 6 PR proteins were markedly up-regulated in CR-JC infected with *P. brassicae*,suggesting that these PR proteins are most likely related to clubroot resistance in CR-JC.

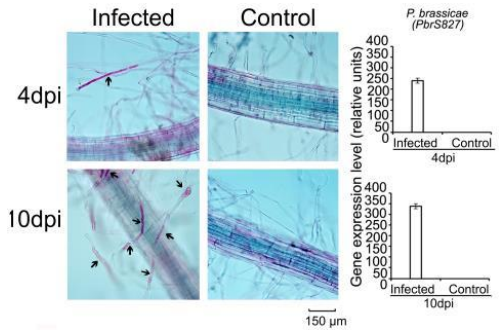

Fig. 1: Zoosporangia in a root hair of CR-JC inoculated with *P. brassicae* and the accumulation of *P. brassicae* in the infected plants and the control at 4 and 10 dpi. Scale bar = 150 µm

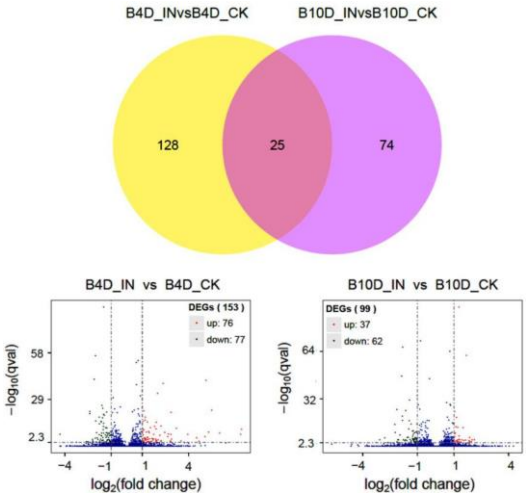

Fig. 2: Comparison of the number and fold change of genes differentially expressed between CR-JC infected with *P. brassicae* (up-regulated and down-regulated, |log2(FoldChange)| > 1 and q value< 0.005) and the control

Peroxidase genes are activated by *P. brassicae* (Shang *et al.* 2011). The superoxide dismutase (SOD) gene was up-regulated at 4 dpi, while the catalase (CAT) gene was down-regulated at 10 dpi. SOD plays a key role in alleviating the peroxide stress induced by pathogens (Shang *et al.* 2010) at the early stage of infection. The down-regulation of CAT would result in reduced tolerance to oxidative stress at the late stage of *P. brassicae* infection.

The results showed that host resistance was regulated mainly by the SA signal pathway in CR-JC, likely because *P. brassicae*, which causes clubrootin *Brassica rapa*, is a soil-borne biotrophic pathogen. Resistance tobiotrophic pathogensis regulated by the SA signal pathway, while resistance to necrotrophic pathogens is regulated by the JA and ET signal pathways (Glazebrook 2005). In the present study, the SA-related genes *NIMIN2*, *PR1*and *PR2* were found to be up-regulated DEGs. The genes mediated by SA are mainly regulated by *NIMIN2* and non-expresser of PR genes 1 (*NPR1*) (Spoel *et al.* 2009 2013; Wang *et al.* 2011b). The SA signal pathway was activated in CR-JC infected by *P.*

Table 1: Top 17 enriched KEGG pathways

Pathway	The number of DEGs	Pathway ID
etabolic pathways	42(16.9%)	ath01100
Biosynthesis of secondary metabolites	36(14.5%)	ath01110
Phenylalanine metabolism	11(4.4%)	ath00360
Plant hormone signal transduction	10(4%)	ath04075
Biosynthesis of amino acids	9(3.6%)	ath01230
Phenylpropanoid biosynthesis	9(3.6%)	ath00940
Plant-pathogen interaction	7(2.8%)	ath04626
Galactose metabolism	5(2%)	ath00052
Glucosinolate biosynthesis	5(2%)	ath00966
2-Oxocarboxylic acid metabolism	5(2%)	ath01210
Starch and sucrose metabolism	5(2%)	ath00500
Peroxisome	4(1.6%)	ath04146
Amino sugar and nucleotide sugar metabolism	4(1.6%)	ath00520
Tropane, piperidine and pyridine alkaloid biosynthesis	4(1.6%)	ath00960
Cysteine and methionine metabolism	4(1.6%)	ath00270
Glyoxylate and dicarboxylate metabolism	4(1.6%)	ath00630
Carbon metabolism	4(1.6%)	ath01200

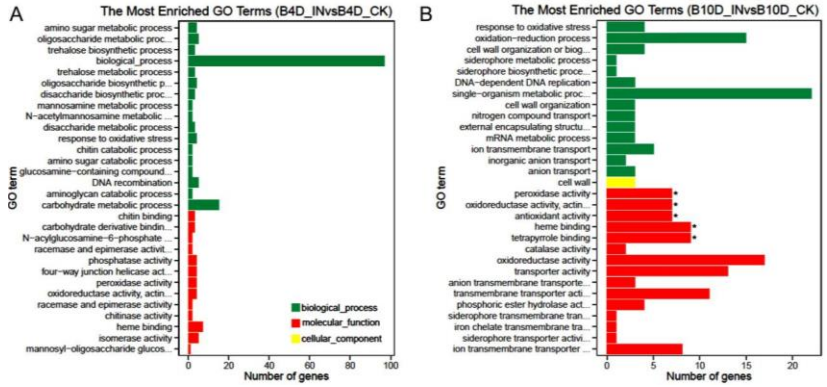

Fig. 3: Comparison of the Gene Ontology (GO) distribution of differentially expressed genes (DEGs) between CR-JC infected with *P. brassicae* and the control at 4 dpi (A) and 10 dpi (B). The * stands for Go terms which were closely related to resistance reaction

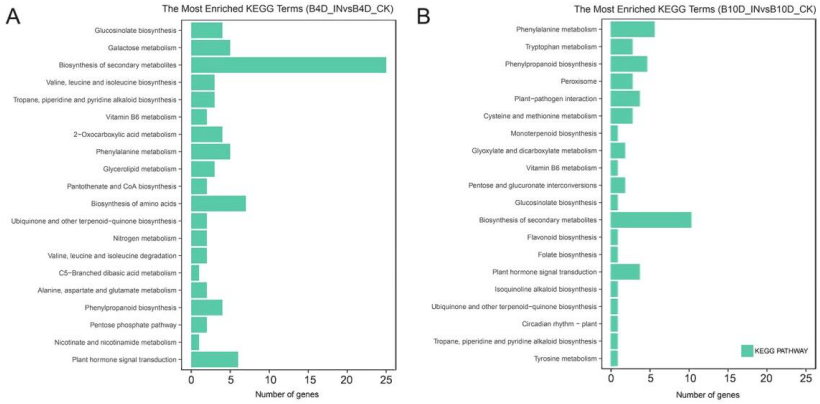

Fig 4: The most enriched KEGG term at (A) 4dpi and (B) 10 dpi

brassicae. Clubroot is suppressed by SA in broccoli (*Brassicaoleracea*var. *italica*) plant (Lovelock *et al.* 2013). At the same time, the accumulation of SA decreased due to a methyl-transferase generated by *P. brassicae* in infected roots (Schwelm *et al.* 2015; Ludwig-Müller *et al.* 2015). Three genes mediated by ET signal were down-regulated in CR-JC, including ethylene-responsive transcription factors (Table 7 Suppl.). Ethylene mediates JA biosynthesis at the early stage of infection in *N. benthamiana* leaves (Yang *et al.* 2017). These results suggested that the JA/ET pathways were repressed. Jubault *et al.* (2013) reported that the SA pathway was induced in *A. thaliana* infected with *P. brassicae*, while

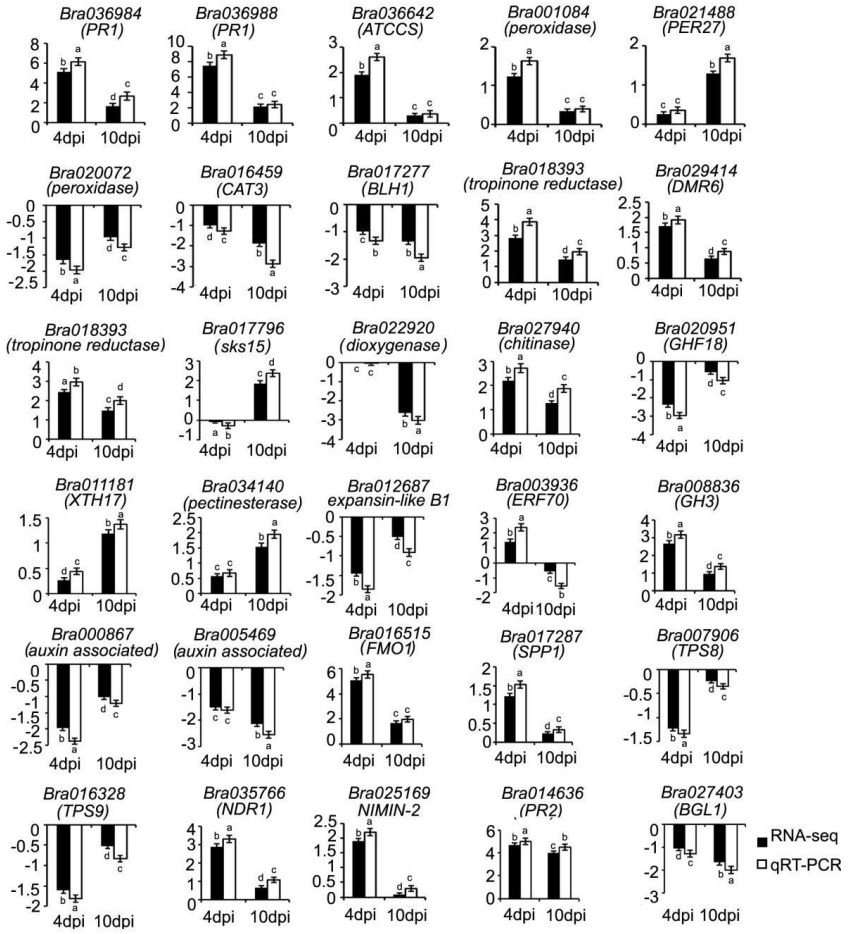

Fig. 5: Validation of RNA-Seq data by qRT-PCR.Thirty DEGs related to clubroot resistance were selected for validation and compared with the RNA-Seq data. The fold change between infected plants and controls is shown on the y-axis, and positive or negative values, respectively, show up- or down-regulation in CR-JC infected with *P. brassicae*. The lower-case letters indicate the significance at P <0.05

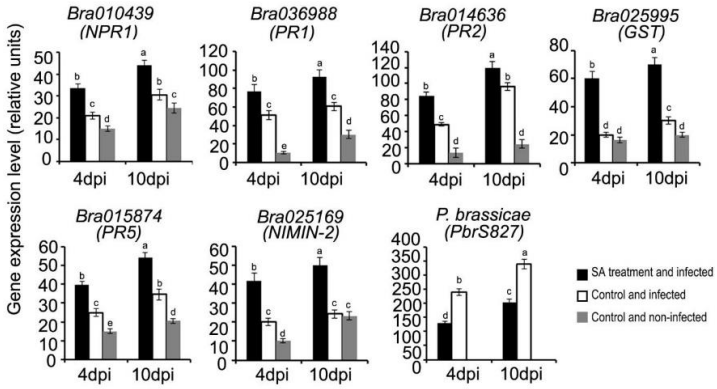

Fig. 6: Detection of the expression of SA-mediated genes by qRT-PCR.Control stands for healthy plants without SA treatment. The lower-case letters indicate the significance at P <0.05

the JA/ET pathways were repressed. The present suggested that SA facilitated the host–*P. brassicae* interaction but that the JA/ET pathways did not.

The cortical invasion stage might be delayed. The cell wall was thickened and the cortical penetration was blocked. 12 DEGs which related to clubroot-induced abnormal cell enlargement and uncontrolled cell division in host roots were identified. The results are consistent with previous

research in *A. Thaliana* (Jubault *et al.* 2013). Many genes related to cell formation were differently expressed at 10 dpi but not at 4dpi. This result indicated that cell division and expansion occurred at the late stage of infection in CR-JC. The abnormal cell division resulted from the destruction of the hormonal balance under aggravated pathogen infection.

In addition, the glucan beta-glucosidase (BGL2) gene, which is also called pathogenesis-related protein 2 (PR2), was notably up-regulated at 4 dpi. Glucan beta-glucosidase plays a role in the degradation of callose, the main component of which is beta-1,3-glucan. Beta-1,3-glucan is not only a defining ingredient of callose but also the main ingredient in many fungal cell walls. Callose deposition can thus protect plants against fungi (Hammond-Kosack and Jones 1996). This result indicated that the defensive functions of the cell wall increased at the early stage of infection and further demonstrated that the SA signal pathway (PR2 is induced by SA) plays an important role in host resistance. SA-mediated gene expression was markedly enhanced while the accumulation of *P. brassicae* declined. It suggested that plant resistance was enhanced by SA treatment, in accordance with the previous conclusion. Host resistance to *P. Brassicae* infection was enhanced by the SA-mediated gene expression induced by exogenous SA.

Four genes related to chitinase that were differentially expressed between the infected plant and the control was identified. Among them, two chitinase-related genes (*Bra037698* and *Bra027940*) were up-regulated at 4 dpi. Chitin is the an important component of the cell wall in *P. brassicae* and is considered a pathogen-associated molecule related to PTI (Latgé and Beavais 2014). Chitin binding proteins in Pb might act to concert chitin to chitinosan and evade PAMP detection mechanisms (Schwelm *et al.* 2015). The induction of chitinases at the early stage of infection might be useful for the degradation of chitin secreted by the pathogen. One chitinase-related gene (*Bra020951*) was down-regulated in infected plants at 4 dpi, and another (*Bra000310*) was down-regulated at 10dpi (Table 7 Suppl.). The roles of chitinase in host–pathogen interaction systems are diverse (Takahashi *et al.* 2005; Marcato *et al.* 2017). The functions of different chitinases in the *Brassica rapa–P. brassicae* interaction system merit further study in the future.

In the root of *A. thaliana* infected by *P. brassicae*, the concentration of trehalose was very high (Brodmann *et al.* 2002). Trehalose exists in both plants and pathogens. Trehalose is both a protective agent of plant stress and a multifunctional sugar of fungi (Gancedo and Flores 2004). It is difficult to determine the effect of trehalose accumulation in infected organs. Studies on trehalose metabolism genes in *A. thaliana* infected by *P. brassicae* showed that trehalose is the toxicity component of the pathogengy (Brodmann *et al.* 2002). Trehalose is accumulated because its synthesis related genes of pathogens are up-regulated. At the same time, trehalose produced by pathogen counteracted the accumulation of trehalose produced during host defense. Trehalose may have a negative effect on specific plant tissues or cells at a certain concentrations.

At both time points, genes involved in growth and the cell cycle, sugar phosphate metabolism (trehalose and glucosinolate) and the SA signal pathway were differentially expressed in infected CR-JC plants. The roles of plant hormones (such as SA, JA/ET and auxin homeostasis) were further proved. Genes related to auxin homeostasis, such as members of the GH3 family, were significantly up-regulated at 4 dpi.This result suggested that auxin plays an important role in the *Brassica rapa–P. brassicae* interaction system at the early stage of infection. The L-phenylalanine metabolic process may also be involved in this host–pathogen interaction.

Conclusion

Transcriptomic responses between the early and late stages of *P. Brassicae* infection in the roots of CR-JC were compared for the first time, and 252 DEGs were found. High expression of SA-mediated genes and PR genes indicated that these genes were very important in the CR-JC response to *P. brassicae* infection. Furthermore, the genes involved in uncontrolled cell division in clubroot were induced at the late stage but not at early stage. The DEG distributions at the early and late time points showed different emphases in CR-JC resistance to *P. brassicae* at different stages. Our research provided a better understanding of the molecular mechanisms of *Brassica rapa* resistance to *P. brassicae*.

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