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ORIGINAL ARTICLE

Transcriptome analysis of *Brassica juncea* var. *tumida* Tsen responses to *Plasmodiophora brassicae* primed by the biocontrol strain *Zhihengliuella aestuarii*

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Abstract

Mustard clubroot, caused by *Plasmodiophora brassicae*, is a serious disease that affects *Brassica juncea* var. *tumida* Tsen, a mustard plant that is the raw material for a traditional fermented food manufactured in Chongqing, China. In our laboratory, we screened the antagonistic bacteria *Zhihengliuella aestuarii* against *P. brassicae*. To better understand the biocontrol mechanism, three transcriptome analyses of *B. juncea* var. *tumida* Tsen were conducted using Illumina HiSeq 4000, one from *B. juncea* only inoculated with *P. brassicae* (P), one inoculated with *P. brassica* and the biocontrol agent *Z. aestuarii* at the same time (P + B), and the other was the control (H), in which *P. brassicae* was replaced by sterile water. A total of 19.94 Gb was generated by Illumina HiSeq sequencing. The sequence data were de novo assembled, and 107,617 unigenes were obtained. In total, 5629 differentially expressed genes between biocontrol-treated (P + B) and infected (P) samples were assigned to 126 KEGG pathways. Using multiple testing corrections, 20 pathways were significantly enriched with Qvalue ≤ 0.05. The resistance-related genes, involved in the production of pathogenesis-related proteins, pathogen-associated molecular pattern-triggered immunity, and effector-triggered immunity signaling pathways, calcium influx, salicylic acid pathway, reactive oxygen intermediates, and mitogen-activated protein kinase cascades, and cell wall modification, were obtained. The various defense responses induced by the biocontrol strain combatted the *P. brassicae* infection. The genes and pathways involved in plant resistance were induced by a biocontrol strain. The transcriptome data explained the molecular mechanism of the potential biocontrol strain against *P. brassicae*. The data will also serve as an important public information platform to study *B. juncea* var. *tumida* Tsen and will be useful for breeding mustard plants resistant to *P. brassicae*.

Keywords *Brassica juncea* var. *tumida* Tsen · Transcriptome · Differentially expressed genes · *Plasmodiophora brassicae* · Resistant genes · *Zhihengliuella aestuarii*

Introduction

Brassica juncea var. *tumida* Tsen (Brassicaceae) is a feature vegetable in Fuling, Chongqing and is the raw material for "Fuling mustard" pickles, which is a famous fermented food. This crop's production is severely affected by clubroot disease caused by *Plasmodiophora brassicae* (Ludwig-Muller and Schuller 2008), and this has caused significant economic losses. Owing to cell elongation and cell division in infected roots and hypocotyls, the symptoms caused by *P. brassicae* are typical hypertrophied cells (root galls) (Ludwig-Muller and Schuller 2008; Kobelt et al. 2000). The vasculature of the infected plant was destroyed, making the uptake of nutrition and water difficult and resulting in foliar wilting when

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under slight soil moisture stress. Galls constitute strong metabolic sinks that redirect assimilates and other nutrients from shoots to roots to meet the pathogen's own needs (Paesold et al. 2011), leading to yield decreases or plant death.

P. brassicae is an obligate biotrophic pathogen that can exist in soil for over 20 years (Donald and Porter 2009). The disease is difficult to control using only conventional chemical or cultural means (Donald and Porter 2009). Although the breeding of resistant cultivars is an effective measure to control the disease, resistant varieties are very scarce and the variation in the pathogen's virulence is great (Hirai 2006). Biological control has received more attention recently because it is environmentally friendly, cost-effective, and sustainable (Jaschke et al. 2010). The antagonism mechanisms of biocontrol strains include the competition for nutrients and space, mycoparasitism, the production of antifungal metabolites, plant growth promotion, and the induction of defense responses in plants (Howell 2003).

The lifecycle of *P. brassicae* consisted of two phases: a primary phase that takes place mainly in root hairs and occasionally in epidermal cells and a secondary phase that occurs predominantly in the root cortex (Ludwig-Muller and Schuller 2008). The primary phase of infection can still occur in resistant hosts, but not the secondary phase (Donald et al. 2008).

Plants combat pathogen attacks through two innate immune techniques: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006). When PAMPs are detected by pattern recognition receptor (PRR) proteins on the surface of the host cell, the PTI pathway is triggered (Jones and Dangl 2006). The pathogen can avoid PTI by secreting effectors into host cells. When the effectors are recognized by specific resistance (R) genes in the host, ETI is triggered (Jones and Dangl 2006).

The analysis of global gene expressions is a powerful tool to study the interactions between pathogens or insect and hosts, especially the resistance mechanisms and resistant genes. Wang et al. (2016) study the differential gene expression profiles between the *Ectropis oblique* damage-induced tea plants and undamaged control using RNA sequencing (RNA-Seq) and indicate that plant secondary metabolites and the signaling pathways may play an important role in defense against insects. So far, some work has been conducted to examine the interaction between host and *P. brassicae*. Using the Arabidopsis Affymetrix ATH1 full-genome chip, Siemens and his colleagues identified differently expressed genes between infected and control roots, including genes associated with growth and cell cycle, sugar phosphate metabolism, and defense (Siemens et al. 2006). The protein profile changes at the primary infection stage in *Arabidopsis thaliana* and Canola showed that most of the differentially regulated proteins were involved in metabolism, cell defense, and the detoxification of reactive oxygen species (ROS) (Devos et al. 2006; Cao et al. 2008). In *A. thaliana*, at 4 days after inoculation (DAI), the expression levels of genes that took part in the pathogen's recognition and signal transduction were highly induced (Agarwal et al. 2011). A transcriptome analysis of *Brassia rapa* using two near-isogenic lines carrying clubroot-resistant and clubroot-susceptible alleles showed that the differently expressed genes were mainly associated with signal transduction, defense, transport, and metabolism (Chen et al. 2016).

In our laboratory, we screened an antagonistic bacteria *Zhihengliuella aestuarii* B18 against *P. brassicae*, which was obtained from the rhizospheric soil of *B. juncea* var. *tumida* grown in Fuling, Chongqing. The biocontrol effects of the antagonistic strain against clubroot on *B. juncea* var. *tumida* reached 63.4 and 49.7% in the greenhouse experiment and field trial, respectively (Luo et al. 2017). To better understand the biocontrol mechanism, a transcriptome analysis of *B. juncea* var. *tumida* Tsen infected by *P. brassicae* was conducted after the biocontrol agent *Z. aestuarii* was applied. We constructed three cDNA libraries using Illumina HiSeq 4000, one from *B. juncea* only inoculated with *P. brassicae* (P), one inoculated with *P. brassica* and the biocontrol agent *Z. aestuarii* (deposit number CICC11045s in the China Center of Industrial Culture Collection) at the same time (P + B), and the other was the control (H), in which *P. brassicae* was replaced by sterile water. A total of 107,617 unigenes were obtained and these unigenes' functional annotations were determined. A differential expression analysis was performed among the biocontrol-treated (P + B), the infected (P), and the control (H) samples, and resistance-related genes were identified among the differentially expressed genes. The transcriptome results were validated by reverse transcription quantitative PCR (qRT-PCR). The data will serve as an important public information platform to study *B. juncea* var. *tumida* Tsen and will be useful for breeding mustard plants resistant to *P. brassicae*.

Materials and methods

Plant material

Seeds of *B. juncea* var. *tumida* were sown in a stainless steel tray (35 cm × 20 cm) that was almost full of perlite (Thermal Insulation Material Factory, Chongqing, China) supplemented with 600 mL of Murashige and Skoog nutrient solution (Murashige and Skoog 1962). The mustard seedlings were maintained in an illuminated incubator at 25 °C under a 11-h/13-h (light/dark) photoperiod with light supplied by GXZ-300B lamps at an intensity of 12,000 lx.

Preparation of resting spore suspensions

Naturally infected diseased plant roots of *B. juncea* var. *tumida* were derived from fields in the Fuling District of Chongqing. The diseased roots were stored at − 20 °C until required. The resting spores were extracted by homogenizing mature clubroot galls of *B. juncea* var. *tumida* in sterile water before filtering the homogenate through gauze (25-μm pore) and clarifying the filtrate by two centrifugation steps at 1475×*g* (Xiao and Guo 2002). The final precipitate of resting spores was re-suspended in sterile water and adjusted to a concentration of 1.5×10^8 spores mL^{-1}. Three independent experiments were conducted to produce the dataset.

Liquid cultures of bacteria

The bacterial isolates were cultured in LB liquid medium and grown on rotary shakers at 180 rpm at 30 °C for 2 days. The bacterial liquid was collected and adjusted to 10^8 colony forming units mL^{-1} using LB medium.

P. brassicae inoculation and root tissue sampling

Seedlings with four leaves were inoculated by adding 400 mL of spore suspension and 200 mL of bacterial culture to each tray (P + B), while the other group was inoculated with 400 mL of spore suspension and 200 mL of sterile water (P) as a positive control. The seedlings inoculated with only 600 mL sterile water served as negative controls (H). Two independent experiments were conducted to produce the dataset. On the 6th and 13th day after inoculation (DAI), seedlings were inoculated with 200 mL of bacterial culture per tray, while the positive and the negative control groups were inoculated with 200 mL of sterile water. The root tissues were harvested at 15 days after the first inoculation and washed with water to remove the perlite. The dried roots were immediately frozen in liquid nitrogen and stored at − 80 °C before further analysis. Every treatment was repeated three times, and every sample consisted of 20 plants per treatment. The disease symptoms were recorded 28 DAI.

RNA extraction

Total RNA was isolated using TRIzol according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA; Cat. no. 15596-026). The extracted RNA was treated with DNase I (Fermentas, Hanover, MD, USA; Cat. no. EN0531) to remove potential genomic DNA contamination. The purity of the RNA was confirmed using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

cDNA library construction for transcriptome analysis

The cDNA library construction and sequencing were performed by Beijing Genomics Institute (Wuhan, China) using NEBNext® Ultra™ RNA library Prep kit for Illumina® (NEB, USA; Cat. no. E7530L). The mRNA was isolated from total RNA using beads with Oligo(dT) primers. Under an elevated temperature, mRNA was fragmented into small pieces after the fragmentation buffer was added. Using the mRNA fragments as templates, the first-strand cDNA was synthesized with random primers. Then, the second-strand cDNA was obtained using DNA polymerase I and RNase H. The synthetic cDNAs were end-repaired by polymerase and ligated with Solexa adapters. Suitable fragments were chosen as templates for the AMPure™ XP system (Beckman Coulter, Beverly, MA, USA; Cat. no. A63882), and PCR amplification was performed to enrich the product. The three libraries (P + B, P, and H) were sequenced using an Illumina HiSeq 4000 (Table 1).

Sequencing reads filtering, assembly, and unigene functional annotations

To yield clean reads, raw reads of low-quality were removed using the Soap software package (http://soap.genomics.org.cn/), including reads with adaptors, reads in which unknown bases were more than 1%, and reads having a percentage of bases of quality lower than 10 were greater than 20%. The clean reads were stored in FASTQ format. Then, the clean reads were de novo assembled using the Trinity program (https://github.com/trinityrnaseq/trinityrnaseq/wiki) to determine the unigenes (Grabherr et al. 2011). To form longer fragments without unknown sequences, Trinity was used to combine the reads overlapping in certain sequences to obtain contigs. These contigs were processed to form longer sequences (unigenes) using Trinity. Then, the unigenes from different samples were spliced, and redundancy was removed by the clustering software Tgicl (http://www.jcvi.org/cms/research/projects/tdb/overview/) to produce the longest possible non-redundant unigenes (all-unigenes) (Pertea et al. 2003). The generated unigenes were compared with the non-redundant protein (NR, ftp://ftp.ncbi.nlm.nih.gov/blast/db), Swiss-prot (http://ftp.ebi.ac.uk/pub/databases/swissprot), the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg), and the cluster of Orthologous Groups (COG) databases (http://www.ncbi.nlm.nih.gov/COG) using the BLASTX algorithm (E-value < 10^{-5}, http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the unigenes' direction was determined based on the best aligning result. Using the NR annotation, the Blast2GO program (https://www.blast2go.com) acquired GO annotations for the unigenes. Then, WEGO (http://wego.genomics.org.cn) and Top GO software (http://bioconductor.uib.no/2.7/bioc/html/topGO.html)

Table 1 Summary of sequencing results

Samples	Total reads (Mb)	Total nucleotides	Q20 (%)	GC (%)	N50	Total contigs	Mean length of contigs	Total unigenes	Mean length of unigenes
P + B	67.23	79,922	96.86	44.72	1317	139,977	756	79,922	827
P	65.11	80,772	96.89	44.46	1295	138,318	742	80,772	817
H	67.11	85,606	96.96	44.42	1317	148,971	759	85,606	832

B represents biocontrol strain *Z. aestuarii*, P represents *P. brassicae*, and H represents the negative control

programs were used to carry out GO functional classifications and enrichment analyses to understand the distributions of the gene functions. The KEGG pathway annotations and enrichment analyses were performed using BLASTALL software against KEGG (http://www.genome.jp/kegg/) databases.

Differentially expressed gene analysis

The genes' expression levels were evaluated by counting the read density as reads per kilo base per million mapped reads (Mortazavi et al. 2008). The correlation of the detected sequence in two parallel libraries was assessed statistically by Pearson's correlation coefficient (P value ≤ 0.001) using DEGseq2 software (http://bioinfo.au.tsinghua.edu.cn/software/degseq). P values were corrected using the Benjamini–Hochberg program to determine the false discovery rate (Thissen et al. 2002). Genes were regarded as significantly differentially expressed if the log2 fold change of the reads per kilo base per million mapped reads was > 2 and the false discovery rate was ≤ 0.001, based on published literature (Chen et al. 2016).

qRT-PCR

To validate the transcriptome results, qRT-PCR was performed using the iCyclerTM Thermal Cycler system (Bio-RAD, Hercules, CA, USA) with the specific primers for the selected 22 genes that were related to resistance (Table 2) according to our bioinformatics analysis and previous reports (Siemens et al. 2006; Devos et al. 2006; Cao et al. 2008; Agarwal et al. 2011; Chen et al. 2016; Chu et al. 2014). Total RNA was extracted three times from (B + P, P) and H roots at 15 days post-infection and treated with DNase I (Fermentas) prior to reverse transcription. Three RNA sets were used for cDNA synthesis using both oligo (dT)18 primers and random hexamer primers provided as part of the RevertAidTM First Strand cDNA Synthesis kit (Fermentas, Pittsburg, PA, USA; Cat. no.K1622) following the manufacturer's instructions. The PCR was performed with three cDNA sets at each point as templates using the SYBR® Premix Ex TaqTM II (Takara, Dalian, Liaoning, China; Cat. no. RR820A). Conditions used for amplification were 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 61 °C (57 °C for reference genes) for 20 s, and 72 °C for 30 s. The ACT and UBE cDNAs (encoding the actin and ubiquitin-conjugating enzymes, respectively) were used as internal controls to standardize transcript levels. The qPCR results were analyzed using ANOVA (analysis of variance).

Results

Disease symptoms

The disease indices of the biocontrol-treated and infected samples were 35.4 and 96.8, respectively, 28 DAI (Luo et al. 2017). The morphology of the biocontrol-treated and infected samplesis shown in Fig. 1.

Sequencing reads filtering and assembly

To evaluate the molecular mechanisms of the potential biocontrol strain against *P. brassicae*, three cDNA libraries were constructed. The transcriptome samples were P + B, P, and H. A total of 19.94 Gb was generated after Illumina HiSeq sequencing. The ratio of the Q20 was greater than 96%, which indicated that the sequencing contained most of the expressed genes. The details of the sequencing are listed in Table 1. The total lengths and the average lengths of the transcripts from the B + P sample were 105,901,898 and 756 bp, respectively, while those of the transcript from the P sample were 102,641,447 and 742 bp, respectively. The total lengths and the average lengths of the transcripts from the H sample were 113,116,391 and 759 bp, respectively.

The sequence data were de novo assembled, and 107,617 unigenes were obtained. The total lengths and the average lengths of the unigenes were 104,094,386 and 967 bp, respectively. The GC percentage of the unigenes was 44.54%, and the N50 was 1488 bp.

Differentially expressed genes among three transcriptome sample

A total of 13,226 differentially expressed genes were identified between the P + B and H samples. Among these, 5708

Table 2 The primers designed for the qRT-PCR analysis

Gene	Accession number	Primer name	Sequence
Lipid transfer proteins	CL4183.contig1	LTP-F	GCACAGTGGGAAGTAGCTTG
		LTP-R	GGCTAGAGGCTAGACTTGGG
Thaumatin	CL7115.contig3	Tha-F	GCACCGTCTTTACCATCGTG
		Tha-R	TTAAGTCGACGGAAGCTCCA
BAK1	Unigene23458	BAK1-F	TAAGCGCCATTCTGACTCCT
		BAK1-R	TGGTTTCGCAATGCTTTCCA
CERK1	CL3604.contig1	CERK1-F	ATCTGGCGGTGAAGTGTTTG
		CERK1-R	CTGGAGCAGTTACACCAACG
RPS4	CL3351.contig1	RPS4-F	ACTGGTGTCATCGTCATGGT
		RPS4-R	CGAGTGGTGAACTCGTTGTC
RPS5	Unigene41313	RPS5-F	ATTCAGAAAGGCCCAATGCC
		RPS5-R	AGATCCTTCTCTGGCACCAC
MAPK7	CL1005.Contig8	MAPK7-F	TGGCGAATTCCCAAATCCAC
		MAPK7-R	TTGGTTTCAGTTGCCGATGG
Calmodulin-like protein	CL6475.contig2	CLP-F	GGGAAACACTTCCTCGATGC
		CLP-R	CCCTTTGCGTTGCGATCTAA
Calmodulin-binding protein	CL8316.Contig8	CBP-F	CTTCCAGCTTTGGCAAGTGT
		CBP-R	TAAGGCACGTTCCACCTCTT
arabinogalactan protein	Unigene40439	ARA-F	GCGGTGGCATCTTCTATTCC
		ARA-R	CGGTTGTACAATGGCGAGTT
PR1	CL12690.Contig2	PR1-F	GGGCCTTACGGAGAGAACTT
		PR1-R	GTGTAGTGACCGCAAACTCC
SAUR	Unigene19142	SAUR-F	GCCGTCAAGAACTGTGACTC
		SAUR-R	GCTCCGGTCCAACATAAACC
GH3 family protein	CL2550.Contig2	GH3-F	ACAACTCCTTGTGGTTTGCC
		GH3-R	GCACCGACACTTACAACCTC
Glutathione S-transferase	CL6386.Contig3	Glu s-tra-F	AAGAGCAGGCACCTTGTTTG
		Glu s-tra-R	CCTCAAGGGTCTGCAAGAGA
heat shock proteins	CL11002.Contig2	HSP-F	GCCTCACTTGCATGACGATT
		HSP-R	GAGCCACCCAGGGTAAATCT
CDPK	CL8426.contig1	CDPK-F	CAACGACGTTCTTCCTGGAC
		CDPK-R	ACCAGCAGCAACTTCTCTCT
JAZ	CL5009.Contig1	JAZ-F	CCGATCACTGTGAAGGAGGA
		JAZ-R	GTCTCCGTAAGAGACGCTGA
NPR1	Unigene9371	NPR1-F	AGCTTCACGTCTTCCCACTT
		NPR1-R	CGTCGGTGTAGTCGCAATTT
MEKK1	CL6418.Contig7	MEKK1-F	CTCTCCTCCGATTCCGTCAA
		MEKK1-R	TCGGAAACGGAACGAGTTTG
MKK4	Unigene45908	MKK4-F	AGACGACTCGTTCTCCATCC
		MKK4-R	AGAAGGACGGCTCTTAACCG
WRKY29	CL3.Contig1	WRKY29-F	ACTCGAGCCAAATCTTCCCA
		WRKY29-R	TCTTCGGTTGCTTGGTAGGT
WRKY22	CL4233.Contig2	WRKY22-F	TGATGGCCTCTTGGTGAACT
		WRKY22-R	TGAAGAAGAGCGACAACCCT
Actin		ACT-F	CCGACCGTATGAGCAAGGAAA
		ACT-R	TTCCTGTGGACAATGGATGGA
UBE		UBE-F	ATCGCTCGTGGTCGTTTA
		UBE-R	ACCTTCCCAATCAGTCCC

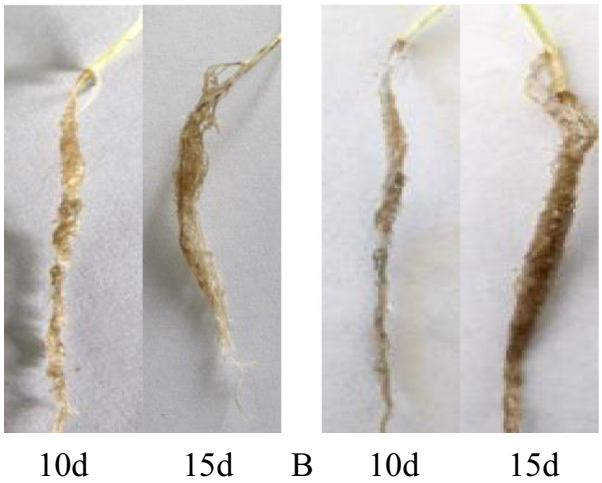


Fig. 1 Root development in biocontrol-treated and the infected samples: **a** roots from the biocontrol-treated group, **b** roots from the infected group. 10 and 15d means 10 and 15 days post-infection

genes were up-regulated, while 7518 genes were down-regulated. There were only 2524 genes up-regulated and 13,137 genes down-regulated in P compared with H. In P + B, there were 8161 genes up-regulated and 1643 genes down-regulated when compared with P (Fig. 2).

Unigene functional annotation

The generated unigenes were compared with the database NT, NR, Swissprot, KEGG, COG, GO databases, and the unigenes' functional annotations were determined based on the best aligning result. In the NT database, 93,536 (86.92%) sequences had similarities to known sequences. In the NR and Swissprot databases, 91,646 (85.16%) and 63,035 (58.57%) sequences, respectively, were functionally annotated.

The unigenes were aligned with the COG database, and 33,432 sequences (31.07%) were distributed into 25 COG categories. Among the 25 COG categories, "General function prediction only" (11,842, 16.14%) was the largest group, followed by "Transcription" (7299, 9.95%), "Translation, ribosomal structure and biogenesis" (5711, 7.785%), and "Replication, recombination and repair" (5601, 7.63%). The smallest groups were "Nuclear structure" (10, 0.0136%) and "Extracellular structures" (35, 0.0477%). The functional distribution of the COG annotation is shown in Supplementary Fig. 1.

The unigenes were then compared with the GO database, and 75,926 sequences were grouped to the three categories: biological process, cellular component, and molecular function. The GO functional analysis was also applied to the differentially expressed genes between biocontrol-treated (P + B) and infected (P) samples. A total of 6797 sequences were divided into 54 groups. Among the 54 groups, "cell" (6440, 5.2%) and "cell part" (6440, 5.2%) were the largest groups, followed by "organelle" (5106, 4.14%), "cellular process" (4790, 3.9%), and "metabolic process" (4670, 3.8%) (Supplementary Fig. 2).

The unigenes were also aligned with the KEGG database, and 52,352 sequences were distributed into 5 categories and 20 functional groups. The five categories were as follows: "cellular processes," "environmental information processing," "genetic information processing," "metabolism," and "organismal systems." Among the 20 functional groups, "global map" was the largest group, followed by "translation," "carbohydrate metabolism," "folding, sorting and degradation," "signal transduction," "environmental adaptation," and "signal transduction." The KEGG functional analysis was also applied to the differentially expressed genes between P + B and P sample. In total, 5629 differentially expressed genes were assigned to 126 KEGG pathways. Of these, 20 pathways were significantly enriched. The most represented pathways were as follows: metabolic pathways (1614 members, 28.67%), biosynthesis of secondary metabolites (807 members, 14.34%), plant hormone signal transduction (422 members, 7.5%), endocytosis (342 members, 6.08%), RNA transport (332 members, 5.9%), glycerophospholipid metabolism (331 members, 5.88%),

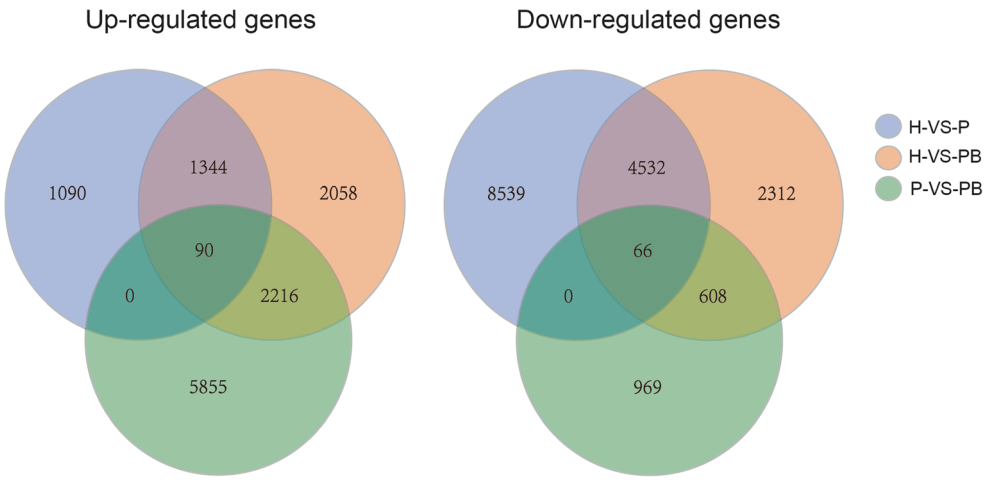


Fig. 2 Venn diagram of differentially expressed genes among biocontrol-treated (P + B), infected (P), the control (H) samples

and plant–pathogen interaction (311 members, 5.52%) (Supplementary Fig. 3). The transcriptome data were deposited in NCBI's Sequence Read Archive under the project ID PRJNA339019 with the accession number SRR4034946.

Identification of genes involved in resistance to *P. brassicae*

To determine the molecular mechanisms of the potential biocontrol strain against *P. brassicae*, the resistance-related genes were obtained by combining those in the literature with those expressed in enriched KEGG pathways (Fig. 3). These genes include the following: PR proteins, PRRs involved in PTI, R proteins involved in ETI, calcium influx genes, hormone regulation genes, respiratory burst oxidase and mitogen-activated protein kinase (MAPK) cascades genes, cell wall modification genes, and cytokinin synthase gene, which were all up-regulated in BP compared with P, except cytokinin synthase gene (Table 3).

qRT-PCR validation

To validate the transcriptome results, 22 genes related to resistance were analyzed using qRT-PCR, including lipid transfer proteins (CL4183.contig1), thaumatin (CL7115.contig3), BAK1 (Unigene23458), CERK1 (CL3604.contig1), RPS4 (CL3351.contig1), RPS5 (Unigene41313), calmodulin-binding protein (CL8316.Contig8), calmodulin-like protein (CL6475.contig2), CDPK (CL8426.contig1), PR1 (CL12690.Contig2), NPR1 (Unigene9371), SAUR (Unigene19142), GH3 family protein (CL2550.Contig2), JAZ (CL5009.Contig1), MAPK7 (CL1005.Contig8), MEKK1 (CL6418.Contig7), MKK4 (Unigene45908), WRKY29 (CL3.Contig1), WRKY22 (CL4233.Contig2), glutathione S-transferase (CL6386.Contig3), heat shock protein (CL11002.Contig2), and arabinogalactan protein (Unigene40439). For all 22 genes, the expression tendencies indicated by the qRT-PCR were almost the same as in the transcriptome analyses (Fig. 4, supplementary Fig. 4), except CL6475.contig2, CL11002.Contig2, and CL3.Contig1.

The expression level of CL6475.contig2 and CL3.Contig1 in BP was slightly less than H, and CL11002.Contig2 in H was slightly higher than P, but the difference between two samples was much smaller. Thus, the results indicated that our transcriptome data was reliable.

Discussion

RNA-sequencing technology is a powerful tool for studying gene expression variation and can be used in non-modal organisms without known genome sequences. RNA-sequencing is more comprehensive and accurate than expressed sequence tag libraries and is more widely used to obtain differently expressed genes and their annotations under various conditions. Using Illumina paired-end RNA-sequencing, Tang et al. (2017) investigated the transcriptome changes during barley grain development from two barley landraces with the differential seed starch synthesis traits. Janská et al. (2011) reported gene expression in the leaf and crown of the winter barley cultivar Luxor, following the exposure of young plants to various periods of low (above and below zero) temperatures. Szeszko et al. (2016) identified differentially expressed genes of porcine luteal ovarian cells in response to adiponectin treatment. In our study, 19.94 Gb was generated by Illumina HiSeq sequencing, and 107,617 unigenes were obtained with a mean length of 967 bp. Compared with H, there were more up-regulated genes and less down-regulated genes in P + B than in P. Compared with H, the genes with opposite expression patterns in B + P and P were most likely related to resistance against *P. brassicae* (Fig. 5).

Up-regulated genes expressed in the P + B sample but not in the P sample

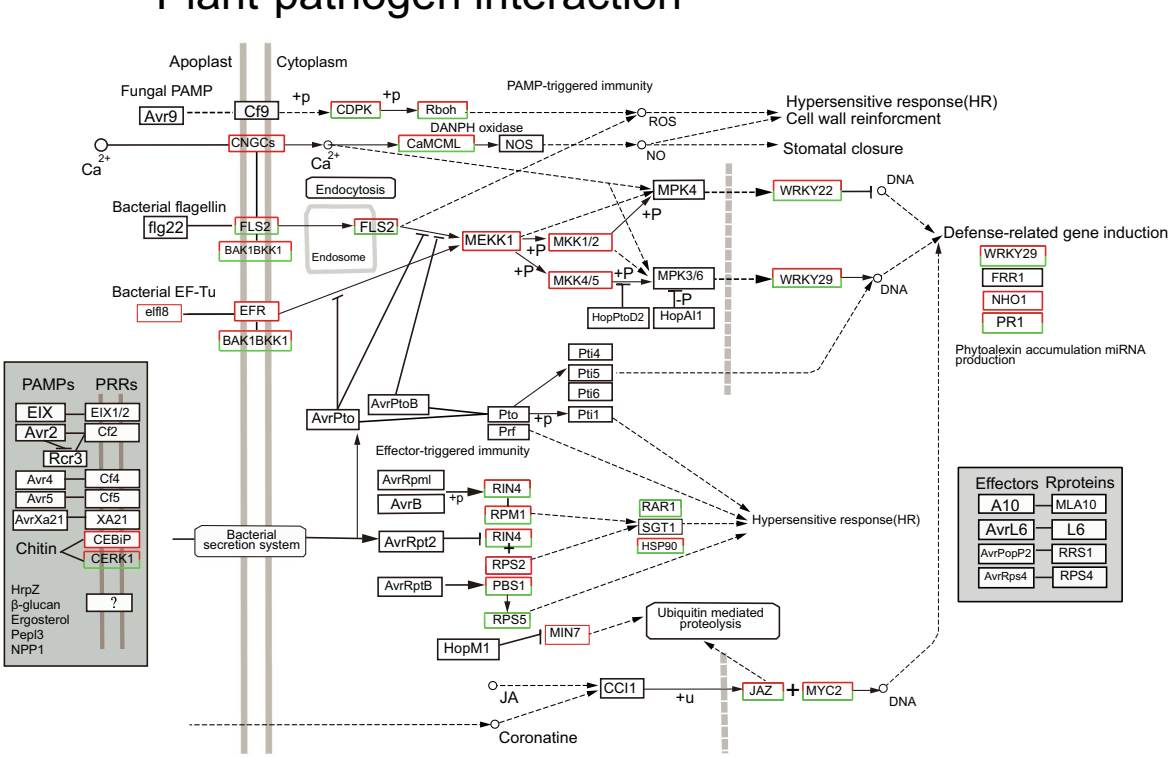
PR proteins

Plants defend against pathogen invasion in various ways, including the production of PR proteins that are induced specifically under pathological or other related conditions (Sels et al. 2008). Among these PR proteins, thaumatin family proteins and lipid transfer proteins belonged to PR-5 and PR-14, respectively (Liu et al. 2010; De Oliveira Carvalho and Gomes 2007). A transcriptome analysis of *B. rapa* CR BJN3-2 carrying clubroot-resistance genes showed that five thaumatin family proteins and six lipid transfer proteins were up-regulated following inoculation with *P. brassicae* compared with the susceptible allele BJN3-2 (Chen et al. 2016). In our experiment, compared with H, six PR proteins (five thaumatin family proteins and one lipid transfer proteins) were up-regulated in P + B, while they were down-regulated in P. This means that the expression levels of thaumatin family proteins and lipid transfer proteins were suppressed by the pathogen and induced by the biocontrol strain *Z. aestuarii*. Thus, the PR proteins may be involved in resistance to *P. brassicae* in *B juncea* var. *tumida* Tsen induced by *Z. aestuarii*.

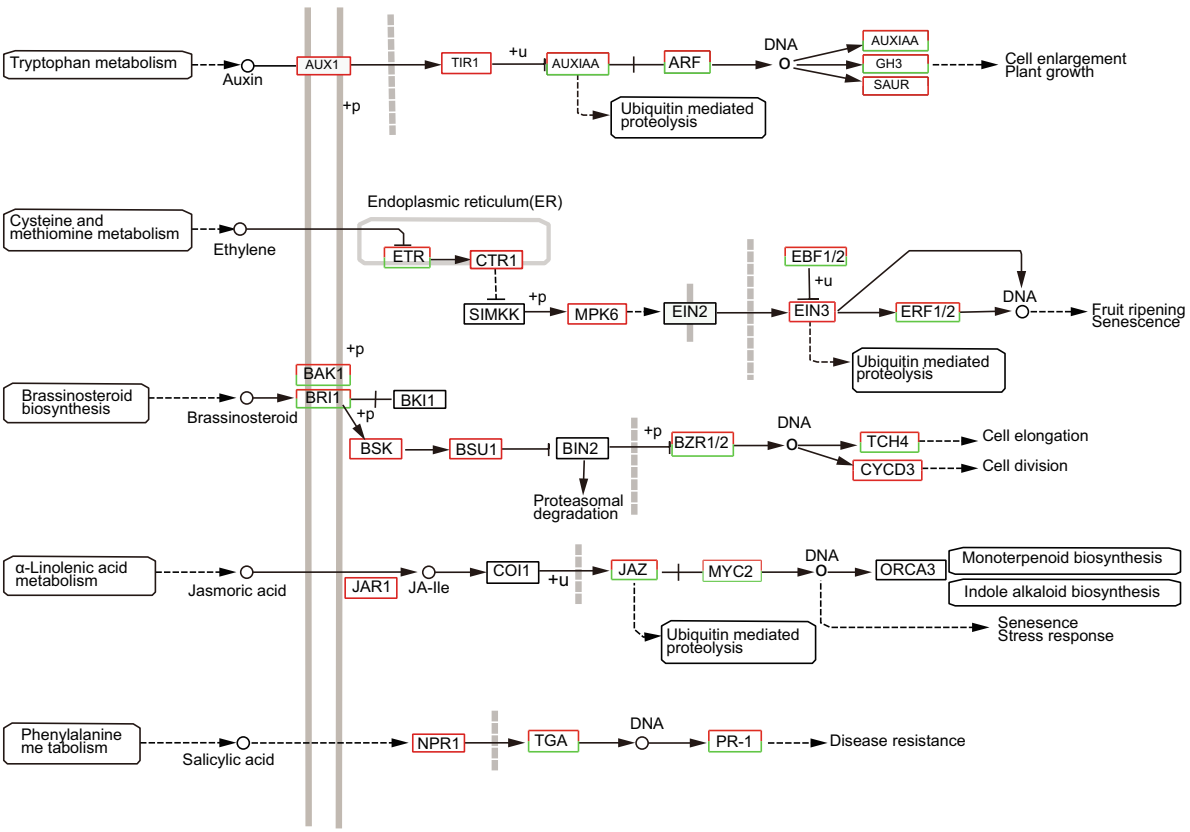
PRRs involved in PTI

Plants first combat pathogens through PTI pathways, which occur when PAMPs are detected by PRR proteins on the surface of the host cell (Jones and Dangl 2006). Some important *PRR* genes triggered by PAMPs, such as brassinosteroid insensitive 1-associated kinase 1 (BAK1), flagellin sensing 2 (FLS2), chitin elicitor receptor kinase (CERK), and chitin elicitor-

A


Plant-pathogen interaction
Apoplast
Cytoplasm
Fungal PAMP
Avr9
Cf9
CDPK
Rboh
PAMP-triggered immunity
Hypersensitive response(HR)
Cell wall reinforcment
ROS
DANPH oxidase
CNGCs
CaMCML
NOS
NO
Stomatal closure
Ca2+
Endocytosis
Bacterial flagellin
flg22
FLS2
BAK1BKK1
Endosome
MEKK1
MKK1/2
MKK4/5
MPK4
MPK3/6
WRKY22
WRKY29
DNA
Defense-related gene induction
WRKY29
FRR1
NHO1
PR1
Phytoalexin accumulation miRNA production
Bacterial EF-Tu
elfi8
EFR
HopPtoD2
HopAI1
PAMPs
PRRs
EIX
EIX1/2
Avr2
Cf2
Rcr3
Avr4
Cf4
Avr5
Cf5
AvrXa21
XA21
Chitin
CEBiP
CERK1
HrpZ
β-glucan
Ergosterol
Pepl3
NPP1
?
AvrPto
AvrPtoB
Pto
Prf
Pti4
Pti5
Pti6
Pti1
Effector-triggered immunity
AvrRpml
AvrB
RIN4
RPM1
RAR1
SGT1
HSP90
Hypersensitive response(HR)
Bacterial secretion system
AvrRpt2
RPS2
AvrRptB
PBS1
RPS5
HopM1
MIN7
Ubiquitin mediated proteolysis
JA
CCI1
JAZ
MYC2
Coronatine
Effectors
Rproteins
A10
MLA10
AvrL6
L6
AvrPopP2
RRS1
AvrRps4
RPS4

B


Plant hormone signal transduction
Tryptophan metabolism
Auxin
AUX1
TIR1
AUXIAA
ARF
DNA
AUXIAA
GH3
SAUR
Cell enlargement
Plant growth
Ubiquitin mediated proteolysis
Endoplasmic reticulum(ER)
Cysteine and methiomine metabolism
Ethylene
ETR
CTR1
SIMKK
MPK6
EIN2
EIN3
EBF1/2
ERF1/2
Fruit ripening
Senescence
Brassinosteroid biosynthesis
Brassinosteroid
BAK1
BRI1
BKI1
BSK
BSU1
BIN2
BZR1/2
TCH4
CYCD3
Cell elongation
Cell division
Proteasomal degradation
α-Linolenic acid metabolism
Jasmoric acid
JAR1
JA-Ile
COI1
JAZ
MYC2
ORCA3
Monoterpenoid biosynthesis
Indole alkaloid biosynthesis
Senescence
Stress response
Phenylalanine me tabolism
Salicylic acid
NPR1
TGA
PR-1
Disease resistance

◀ **Fig. 3** The enriched KEGG pathways of the resistance mechanism of the *B. juncea* var. *tumida* Tsen de novo transcriptome affected by biocontrol strain *Z. aestuarii*. **a** Plant hormone signal transduction pathways. **b** Plant–pathogen interaction pathways. The red and green frames represent genes and enriched functions that were up- and down- regulated in biocontrol-treated and the infected samples

binding protein (CEBiP), can transduce signals that trigger PTI (Dodds and Rathjen 2010). In our study, three CERKs were induced in P + B and suppressed in P compared with H. The expression levels of four *BAK1* genes were greatest in H and lowest in P. The *BAK1* genes were marginally induced by the biocontrol agent. In previous reports, BAK1, CEBiP, and CERK1 were up-regulated in a resistant banana line infected with *Fusarium oxysporum* (Li et al. 2012). Our results were in agreement with theirs. Thus, we hypothesized that the PTI pathways play roles in the resistance to *P. brassicae.*

R protein involved in ETI

Pathogens secrete effectors into host cells to avoid PTI. When the effectors are recognized by specific resistance (R) genes in the host, the ETI pathways are triggered to combat the pathogens. RPS4, containing the avirulence gene AvrRPS4, was specifically resistant to P. syringae pv. tomato (Wirthmueller et al. 2007), and RPS5, carrying the avirulence gene AvrPphB, was specifically resistant to P. syringae strains (Ade et al. 2007). RPS4 and RPS5 were up-regulated in clubroot-resistant *B. rapa* "CR BJN3-2" compared with in clubroot-susceptible allele lines at 0, 12, 72, and 96 h after inoculation (Chen et al. 2016). Consistent with these reports, two RPS4 and two RPS5 proteins were up-regulated after the root of *Brassica juncea* var. tumida Tsen inoculated with *P. brassicae* was drenched with the biocontrol strain. In our experiment, not only PTI, but also ETI, pathways were triggered after the addition of biocontrol strain and took part in the resistance to *P. brassicae.*

Calcium influx

Calcium is an important secondary messenger involved in signal transduction pathways that is regulated in many plant cell processes, such as responses to abiotic and biotic stress (Zipfel 2009). Calcium cannot only reduce root-hair infections but also inhibits the production of differentiated and dehisced

Table 3 Functional classification of *B. juncea* var. *tumida* Tsen transcripts that were differentially regulated between biocontrol-treated (BP) and the infected (P) root tissues at 15 days post-inoculation

Unigene	Annotation	Log2 (fold change (B + P/P))	*P* value	FDR	Up/down
CL7115.contig3	thaumatin family proteins	7.924813	0.000381	0.000899	Up
CL4183.contig1	lipid transfer proteins	1.592176	1.48E-16	9.07E-16	Up
Unigene23458	brassinosteroid insensitive 1-associated receptor kinase1 (BAK1)	5.643856	2.92E-09	1.13E-08	Up
CL3604.contig1	CERK1	2.134301	0.000133	0.000332	Up
CL3351.contig1	RESISTANT TO *PSEUDOMONASSYRINGAE* 4 (RPS4)	3.158429	9.70E-11	4.17E-10	Up
Unigene41313	RESISTANT TO *P. SYRINGAE* 4 (RPS5)	3.556948	1.72E-05	4.76E-05	Up
CL8316.Contig8	calmodulin-binding protein	7.882643	7.53E-88	2.61E-86	Up
CL6475.contig2	calmodulin-like genes	1.876425	1.38E-08	5.06E-08	Up
CL8426.Contig1	CDPK	6.357552	2.12E-07	7.03E-07	Up
CL12690.Contig2	pathogenesis-related protein 1(PR1)	7.498251	2.29E-84	7.59E-83	Up
Unigene19142	SAUR (small auxin up RNA) family protein	7.139551	4.48E-05	0.000119	Up
CL2550.Contig2	GH3 (Gretchen hagen 3) family proteins	6.918863	5.01E-19	3.47E-18	Up
CL5009.contig1	JAZ	7.813781	4.27E-18	2.82E-17	Up
Unigene9371	NPR1	2.375867	0.000224	0.000546	Up
CL1005.Contig8	mitogen-activated protein kinase (MAPK)	6.894818	2.63E-15	1.50E-14	Up
CL6418.contig7	MEKK1	6.189825	6.54E-14	3.44E-13	Up
Unigene45908	MKK4	8.366322	8.52E-09	3.18E-08	Up
CL3.contig1	WRKY29	7.61471	3.09E-16	1.86E-15	Up
CL4233.contig2	WRKY22	8.543032	2.26E-34	2.79E-33	Up
CL6386.Contig3	glutathione S-transferases	9.157347	6.29E-47	1.08E-45	Up
CL11002.contig2	HSP	4.958421	2.05E-14	1.11E-13	Up
Unigene40439	arabinogalactan protein	8.174926	1.00E-09	4.01E-09	Up
CL7115.contig3	thaumatin family proteins	7.924813	0.000381	0.000899	Up
Unigene13014	cytokinin synthase gene	−4.21005	4.24E-67	1.08E-65	Down

sporangia of *P. brassicae* in infected Chinese cabbage root hairs (Donald and Porter 2009). In the field, calcium nitrate has been widely used to control clubroot (Donald and Porter 2009). Calcium-mediated signaling processes appeared necessary for the increase in phenylalanine ammonia-lyase activity induced by *P. brassicae* (Takahashi et al. 2002). In our study, some genes involved in calcium influx were up-regulated in the biocontrol-treated sample compared with P, including CDPK, calmodulin-like genes, and the calmodulin-binding protein, while the calmodulin-like and *CDPK* genes were expressed less in the P + B sample than the H. Due to the *P. brassicae* infection, the expression level of calmodulin-like and *CDPK* genes were down-regulated and could be compensated partly by the biocontrol strain. In susceptible *Arabidopsis* infected by *P. brassicae*, the *CDPK* gene was up-regulated at 4 DAI when pathogen recognition and signal transduction occurred to trigger the early stages of plant defense responses (Agarwal et al. 2011). At 4 DAI, but not 7 or 10 DAI, some resistance genes, including *CDPK*, which also existed in other resistant host-pathogen interactions, were induced in susceptible *Arabidopsis* (Agarwal et al. 2011; Kasukabe et al. 2004; Kuznetsov et al. 2006). Compared with susceptible BJN 3-2, calmodulin-like genes were induced at 0 h after inoculation with *P. brassicae* in resistant Chinese cabbage "CR BJN 3-2" (Chen et al. 2016). Our data was aligned with these genes, and the calcium-influx signaling pathway may be involved in the resistance to *P. brassicae.*

Hormone regulation

In the current study, the genes related to auxin regulation, such as the SAUR and GH3 family proteins (Wu et al. 2012; Kant et al. 2009; Ding et al. 2008; Fu et al. 2011; Zhang et al. 2007; Wang et al. 2012; Glawischnig 2007), were induced in the B + P sample compared with the P sample. The expression level of GH3 was much higher in the B + P sample than the other two samples, while the SAUR protein was comparable with H. Thus, the suppression effect of the pathogen may be counteracted by the biocontrol strain. The GH3 family protein is linked to intensive immunity in rice by suppressing auxin accumulation (Ding et al. 2008; Fu et al. 2011), and the overexpression of GH3.5 in *Arabidopsis* resulted in the enhanced biosynthesis of camalexin, which is a major phytoalexin resistant to pathogens (Zhang et al. 2007; Wang et al. 2012). The biosynthesis of camalexin may lead to the suppression of auxin synthesis owing to the common precursor, tryptophan (Glawischnig 2007). The SAUR protein takes part in auxin biosynthesis and signaling pathway (Wu et al. 2012) and acts as negative regulator of auxin synthesis and transport in rice (Kant et al. 2009). In previous reports, the SAUR and GH3 family proteins were induced in resistant canola compared with sensitive plants (Chu et al. 2014). Our results were in accordance with these studies. In clubroot disease, auxin is involved in pathogenicity (Ludwig-Muller and Schuller 2008). The up-regulation of the SAUR and GH3 family

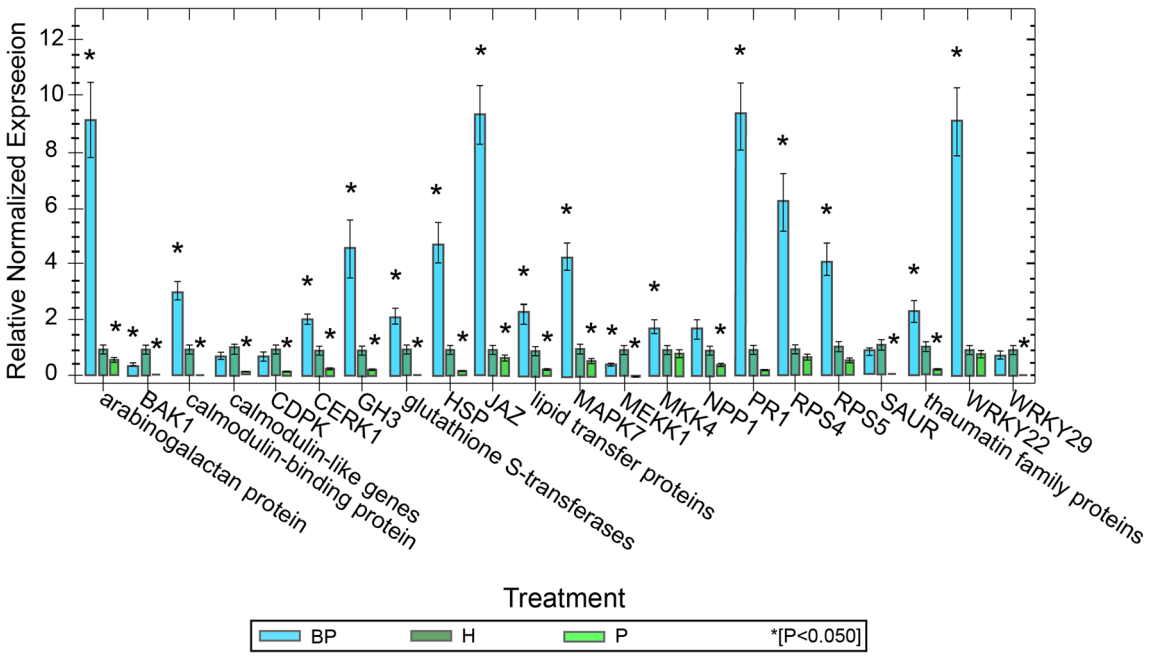


Fig. 4 qRT-PCR validation. The relative expression was analyzed at 15 days post-inoculation with ACT and UBE as standards. BP, P, and H represent biocontrol-treated, infected, and control samples, respectively. Asterisks indicate significant differences (p value < 0.05) compared with H. Error bars represent ±SD of three independent PCR amplifications and quantifications.

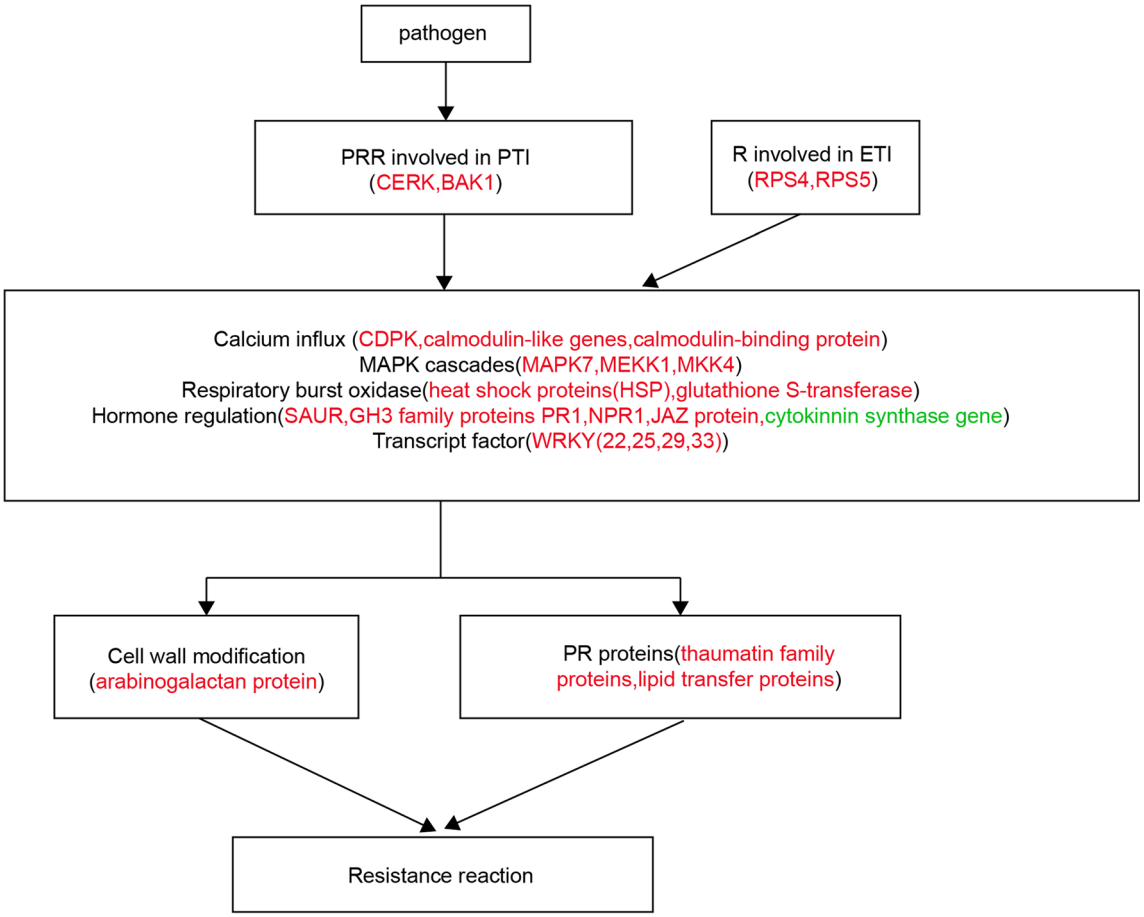


Fig. 5 Schematic diagram illustrating the defense mechanisms induced by *P. brassicae.* Red and green represent up- and down-regulated genes, respectively

proteins results in a reduction of auxin and is related with the resistance to *P. brassicae*.

In our study, the *PR1* and *NPR1* genes, which are involved in salicylic acid (SA) signaling, were also up-regulated in the B + P sample (Spoel et al. 2003). As shown in Fig. 4, the expression level of PR1 and NPR1 in the B + P sample was much higher than the P and H samples. The *NPR1* and *PR1* genes were localized downstream of the SA pathway and were linked with disease resistance (Fig. 3). Plant resistance to biotrophic pathogens is mainly controlled by the SA pathway, and host resistance to necrotrophic pathogen is regulated by the ethylene and jasmonic acid signaling pathway (Glazebrook 2005). Lovelock et al. (2013) demonstrated that exogenous applications of SA can suppress clubroot in broccoli. The SA signaling pathway was down-regulated in susceptible *A. thaliana* (Agarwal et al. 2011), and the *PR1* and *NPR1* genes involved in the SA pathway were up-regulated in clubroot-resistant near-isogenic lines of Chinese cabbage (Chen et al. 2016). Thus, the SA-signaling pathway was related with resistance to *P. brassicae*, which was induced by the biocontrol strain. In our experiment, the JAZ protein, which was related to JA biosynthesis and signaling (Fig. 3), was also up-regulated in the BP sample, and the expression level was much higher than the P and H samples (Fig. 4). In other reports, the SA pathway was induced, and the JA pathway was suppressed in resistant plants (Chen et al. 2016; Jubault et al. 2013). Our result conflicted with those of previous studies. Lemarie et al. (2015) demonstrated that both the SA and JA pathways contribute to resistance against *P. brassicae* in *Arabidopsis*. Consistent with these results, in our experiment, both the SA and JA pathways were involved in resistance to *P. brassicae* after the biocontrol strain was applied.

Respiratory burst oxidase and MAPK cascades

In resistant plants, early defense responses occur within minutes of infection, including the activation of MAPK cascades and burst of reactive oxygen intermediates (Torres and Dangl 2005; Zhao et al. 2005; Jones and Dangl 2006). In our study, 2 *MAPK7*, 20 heat shock proteins, 9 glutathione S-transferase, 2 *MEKK1*, 2 *MKK4*, and 11 *WRKY* (22, 29) genes were up-regulated in the B + P sample compared with the P sample and H, except the *MEKK1* and *WRKY 29*, which was lower in the B + P sample than H. MEKK1 is required for basal

defenses (Zhang et al. 2012) and was suppressed by *P. brassicae*. After the addition of *Z. aestuarii*, the expression level of *MEKK1* was only slightly up-regulated. The *MEKK1* gene may not be involved in resistance to *P. brassicae*. The heat shock proteins and glutathione S-transferase are involved in the oxidative burst, which functions as antimicrobial compounds and signaling molecules in the plant defense reaction (Wan et al. 2002; Sagi and Fluhr 2001; Agarwal et al. 2011). The oxidative burst was repressed at 4 days after infection in the compatible *A. thaliana* infected by *P. brassicae* (Agarwal et al. 2011) and the ROS level was higher in resistant *B. rapa* inoculated with *P. brassicae* (Chen et al. 2016). Our result contradicted that of a compatible phenotype but was in accordance with the resistant phenotype. The WRKY proteins belong to a superfamily of transcription factors, which respond to wounding and other stresses and act in a complex defense-response network in plant immunity (Dellagi et al. 2000; Pandey and Somssich 2009). In our experiment, WRKYs 22 was highly up-regulated in the B + P sample compared with P and H, while the expression level of WRKY 29 in the B + P was slightly lower than H. The WRKY 22 and WRKY 29 gene may be involved in resistance to *P. brassicae*. Thus, the MAPK cascades and reactive oxygen intermediates may work together to combat *P. brassicae*.

Cell wall modification

In our study, arabinogalactan protein, which is related to cell wall modification, was largely induced by the addition of the biocontrol strain and was much higher than in the P and H samples. The fasciclin-like arabinogalactan protein is an important component of plant cell walls (Estevez et al. 2006) and is involved in cell adhesion and the plant's detection of invading pathogens (Mellersh and Heath 2001; Hardham et al. 2007). In susceptible *Arabidopsis* infected by *P. brassicae* at 4 DAI, the arabinogalactan protein was down-regulated (Agarwal et al. 2011). In contrast, the arabinogalactan protein was up-regulated in the B + P sample and may strengthen cell walls and inhibit the invasion of *P. brassicae*.

Down-regulated genes

The gene encoding cytokinin synthase (Unigene13014) was down-regulated in the B + P sample compared with the P sample. Compared with H, the cytokinin synthase gene was up-regulated in the P sample, while it was down-regulated in the B + P sample. The cytokinin synthase gene plays a role at late time points during root-gall formation (Ludwig-Muller and Schuller 2008). The down-regulated expression of the cytokinin synthase gene results in the alleviation of the clubroot disease symptoms by biocontrol strain.

Conclusions

In our study, the genes and pathways involved in plant resistance were induced by the biocontrol strain. The transcriptome data explained the molecular mechanism of the potential biocontrol strain against *P. brassicae*. The data will also serve as an important public information platform to study *B. juncea* var. *tumida* Tsen and will be useful for breeding mustard plants resistant to *P. brassicae*.

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Authors' contributions CYZ and YLL conceived and designed the study. YLL, DWD, YS, XYW, YMP, and JP collected samples and performed the experiment. YLL carried out the data analysis. YLL and CYZ contributed to the writing of the manuscript. All of the authors read and approved the final manuscript.

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