Genes for defense response to *Plasmodiophora brassicae* during late infection in small spheroid galls of *Brassica rapa*

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Abstract

Plasmodiophora brassicae is a biotrophic pathogen causing clubroots of cruciferous crops. The *Brassica rapa* accession T1-145 has an ability to produce small spheroid galls (SSGs), which represent neither a fully compatible interaction nor a complete resistance. To explore the defense response in SSGs induced by *P. brassicae* infection, global transcriptome profiling SSGs was performed at different time points. By comparing gene expression patterns, we identified many defense related genes. The first group included genes encoding receptor-like protein/kinases, such as *cysteine-rich receptor-like protein kinases*, *receptor-like proteins*, *phloem intercalated with xylem/tracheary element differentiation inhibitory factor receptor* (*PXY/TDR*), *PXY-correlated 1*, *wall-associated kinases*, *nuclear shuttle protein-interacting kinase*, *lectin receptor-like kinases*, and *flagelin-sensitive 2*, which might activate a basal defense. The second group involved robust effector-triggered immunity response genes such as *resistance to leptosphaeria maculans 1B*, *constitutive shade-avoidance 1*, *target of avirulence B operation 1*, *ribosomal protein of the small subunit 6*, *resistance to Pseudomonas maculicola 1-interacting protein 4*, *enhanced disease resistance 2L*, and *recognition of Peronospora parasitica 13-like protein 4*. The third group included genes encoding secondary cell wall formation related protein/s, a nodulin-like protein, a germin-like protein, a jacalin-related lectin, a defensin-like protein, tumor inhibitors, and sugars will eventually be exported transporter, which might contribute to quantitative resistance against *P. brassicae*. The gene expressions were the highest at the late stage of infection. To our knowledge, it is the first report on exploring defense response genes during SSG occurrence by a transcriptome analysis. Our data would provide useful information to further explore molecular mechanisms of the incomplete resistance.

Additional key words: biotrophic pathogen, effector-triggered immunity, gene expression patterns, receptor-like protein/kinases.

Introduction

The clubroot disease in cruciferous crops is caused by *Plasmodiophora brassicae,* a soil borne and obligate biotrophic pathogen (Crisp *et al.* 1989). The disease is spreading rapidly in Asia and has become an emerging problem in many countries. Clubroot disease is difficult to be controlled by either chemicals or cultural practices. The development of a resistant cultivar is believed to be the most effective and environmentally friendly option. Previous reports pointed out that clubroot resistance (CR) is controlled by a single resistance gene in *B. rapa* (Piao *et al.* 2009). Quantitative trait loci involved in *B. rapa* CR were also studied (Suwabe *et al.* 2006). The effectiveness of *CR*

genes has been tested against some different pathotypes. For example, CRa, CRb, CRk, Crr1, Crr3, and Crr4 were resistant to pathotype 2 in *B. rapa* (Suwabe *et al.* 2006). *P. brassicae* populations in the fields are often a mixture of different pathotypes (Xue *et al.* 2008). *P. brassicae* is a highly variable pathogen and its genotyping revealed the presence of distinct populations (Holtz *et al.* 2018). Due to the continuous cropping of a single resistant cultivar, the virulent pathotypes are likely to be selected and then compromise the resistance (Tanaka *et al.* 2013). Therefore, it is necessary to generate a cultivar resistant to the broad spectrum of *P. brassicae* races.

The life cycle of *P. brassicae* has three phases: survival in the soil, root hair infection, and cortical infection phase (Kageyama and Asano 2009). The cortical infection stage is crucial to gall development in roots, which creates a strong metabolite sink to attract assimilates from other tissues (Ludwig-Müller 2014). Small spheroid galls (SSGs) are different in size and form from the typical spindle galls of clubroots. The SSGs are generally regarded as a resistance form, however, the resting spores can be also observed in SSGs; they represent neither complete resistance, nor full susceptibility (Osaki *et al.* 2008, Rennie *et al.* 2013, Peng *et al.* 2016).

A previous report demonstrated the difference between symptomless roots and gall tissue in *Brassica oleracea* by transcriptome analysis (Ciaghi *et al.* 2019). Jubault *et al.* (2013) also pointed out that the partial resistance to clubroot in *Arabidopsis* is due to a reduction or delay in the host primary metabolism and induction of an earlier or stronger plant defense response. However, the transcriptome analyses were mainly focused on the early infection phase (Jubault *et al.* 2013, Chen *et al.* 2016, Zhao *et al.* 2017). In this study, we focused on the different infection periods, including root hair infection phase (primary stage), cortical infection phase (secondary stage), and the symptom development phase, to discover which stage was the key one and which defense pathways might be involved in the restricted development of galls in *B. rapa* genotype T1-145, using a transcriptome analysis. We also wanted to explore the main regulatory pathways on which the specific incomplete resistance in SSG depends. This would provide more references for revealing the resistance mechanism of the host against *P. brassicae.*

Materials and methods

Preparation of resting spore suspension: For preparation of plant inoculations, *Plasmodiophora brassicae* resting spores were prepared as previously described (Ji *et al.* 2014). Root galls were homogenized using 10 % (m/v) sucrose in a blender. The slurry was filtered through 8 layers of gauze and the suspension was clarified by centrifugation at 3 000 *g* for 9 min. The pellet was suspended in sterile water and transferred into a new tube containing 40 cm^3 of water. After centrifugation at 3 000 *g* for 12 min, the resting spores precipitate was resuspended in sterile water, and adjusted to a concentration of 1×10^7 (spores) cm^{-3}, and stored at 4 °C. For pathotype determination

according to the Williams, 30 seedlings from differential hosts were inoculated and three replicates were taken for the pathotype determination. The Williams identification hosts Jersey Queen and Badger shipper cultivars of *Brassica oleracea* var. *capitata*, and Laurentian and Wilhelmsburger cultivars of *Brassica napobrassica* were inoculated with *P. brassicae* resting spores. The judgment standard of pathotype was set accorded to previous report (Williams 1996).

***Plasmodiophora brassicae* inoculation:** Seeds of Chinese cabbage (*Brassica rapa* L.) genotype T1-145 were sterilized with 75 % (v/v) ethanol for 5 min and then repeatedly washed with sterile water. After seedlings grew for one week, or cotyledons were fully expanded, 0.5 cm^3 of spore suspension of the Xichang (XC) isolate (from Xichang in Sichuan Province, China) or the Shifang (SF) isolate (from Shifang in Sichuan Province, China) was inoculated at the base of each seedling. Resting spores were diluted to a density of 10^7 spores cm^{-3} in sterile distilled water. The XC isolate and SF isolate were both pathotype 4 based on Walliams identification system. The inoculated plants were maintained in a growth chamber under a 16-h photoperiod, an irradiance of 120 µmol m^{-2} s^{-1}, and a temperature of 25 °C, and the soil was kept moist during the treatment period. The roots were harvested at 4, 10, 16, and 45 d post inoculation (dpi) and were washed thoroughly with distilled water to remove the spores adsorbed onto the surface. They were then frozen in liquid nitrogen and stored at -70 °C. Total RNA of the clubroot was extracted and sent for sequencing.

Analysis of RNA sequence (RNA-seq): The RNA was extracted from two biologically different root samples using *TRIzol*® reagent (*Life Technologies*, Carlsbad, CA, USA) following the manufacturer's protocol. The RNA quantity and quality were determined using a *2100 BioAnalyzer* (*Agilent Technologies*, Santa Clara, USA). The RNA libraries were constructed with 2 µg of total RNA and subjected to high-through-put sequencing by a *HiSeq-4000* sequencer (*Illumina*, San Diego, CA, USA). Approximately 10 Gbp of reads for each sample were obtained. The filter software *Ngqc* was used to analyze the quality of the raw reads, after filtering the raw reads by removing adapter and reads with unknown base 'N' content greater than 5 %.. Sequencing reads were compared to the reference database for the *B. rapa* genome (http://brassicadb.org/brad/) using *Cufflinks* software and the length statistics of reference transcriptome sequences were performed with *perl* scripts (Trapnell *et al.* 2009). Nonemapping sequences were regarded as novel genes.

The fragments per kilobase million method was used to calculate the normalized expression data of each gene (Mortazavi *et al.* 2008). The *R language* software was used for the generation of heatmap (Audic and Claverie *et al.* 1997).

Differentially expressed genes (DEGs) were selected by the *DEseq2* software, with the criteria of absolute Log2 (fold change) ≥ 2 and false discovery rate ≤ 0.01 between any two samples (Wang *et al.* 2010). The fragments per

kilobase million of genes were log-converted. The value of Log2 (fragments per kilobase million +1) for each transcript was received.

The *Gene ontology* (*GO*) database was used for gene ontology analysis (https://en.wikipedia.org/ wiki/ Hypergeometric_distribution) and *GO* enrichment analysis was performed using *GOseq* (Young *et al*. 2010). Unigene sequences were compared with the nonredundant protein database and *GO* (Ashburner *et al*. 2000). To elucidate the metabolic pathways of these predicted genes, the *Kyoto encyclopedia of genes and genomes* (*KEGG*) pathways of DEGs were compared (Wu *et al*. 2006).

Reverse transcription quantitative PCR analysis: Root samples were collected at 4, 10, 16, and 45 dpi. The cDNA was synthesized from 0.5 µg of total RNA using *ReverTraAce qPCR RT Master Mix* with gDNA remover (*Toyobo*, Osaka, Japan). A two-step real-time PCR reaction was performed using a *QuantiFast™ SYBR®* qPCR kit (*Qiagen*, Hilden, Germany) with 100 ng template DNA and 10 nM of each primer in a final volume of 0.01 cm^3, according to the following protocol: polymerase activation at 95 °C for 5 min followed by denaturation at 95 °C for 10 s, and concurrent annealing and extension at 65 °C for 30 s. The *B. rapa* actin (*ACT2*) gene was used as an internal control. Primer sequences are listed in Table 1 Suppl.

Results and discussion

Comparative analysis of *P. brassicae* infection in *B. rapa*: The T1-145 genotype was susceptible to XC isolate and developed the typical spindle galls. A special interaction (T1-145 inoculated with SF isolate) consistently resulted in SSG production (Fig. 1). The plasmodia in the root hair and cortical tissue had no difference between SF and XC infections at 4 and 10 dpi. Many sporangia were obviously empty in the sample of SF infection at 16 dpi, but the resting spores could be observed in the SSGs and spheroid galls at 45 dpi. However, by observation of the ultrastructure of bare-handed section, we could observe that the resting spores of XC isolates were more concentrated and so the number of XC spores in a cell was higher than in the case of SF isolate (Fig. 1 Suppl.).

The RNA-seq analysis: The samples of T1-145 after SF isolate and XC isolate infection at 4, 10, 16, and 45 dpi were compared by a transcriptome analysis. Clean reads were in the range from 62 860 626 to 85 058 774, and clean bases ranged from 9 to 12 Gbp. Data from the two biological replicates were highly consistent according to principal components analysis, which indicated that no obvious bias in the cDNA library construction between the two biological replicates. The guanine and cytosine (GC) values of sequence data from the 24 libraries ranged from 45 to 50 %, the quality of clean reads Q30% were all > 89 %, and the quality of clean reads Q20% were all > 95 %. Therefore, the quality of the sequencing data was sufficient for further analysis. An overview of the sequencing data is shown in Table 2 Suppl.

Functional annotation of DEGs: A total of 809 *GO* terms in *B. rapa* by comparing SF isolate with XC isolate were assigned to three classes (corrected *P* value < 0.05): 'biological process', 'cellular component', and 'molecular function'. The top ten significantly enriched *GO* terms at each time point were listed on Table 3 Suppl. For instance, in the 'molecular function' class, *GO* terms 'cofactor binding' and 'aldehyde-lyase activity' at 4 dpi, 'NAD binding' at 10 dpi, 'microtubule motor activity', 'microtubule binding', 'tubulin binding', 'motor activity' and 'cytoskeletal protein binding' at 45 dpi were specially enriched.

To identify the biological pathways involved in defense against *P. brassicae* in *B. rapa*, we analyzed the differentially expressed unigenes in *KEGG*, the top 25 enriched *KEGG* pathways are listed in Table 1 (corrected *P* value < 0.05). 'Ribosome' (813, 22.66 %) was the most common term, followed by 'starch and sucrose metabolism' (167, 4.65 %), 'amino sugar and nucleotide sugar metabolism' (158, 4.40 %). Previous reports indicated that *P. brassicae* infection caused significant increase of glucose and fructose content, also suggesting that sugar translocation and *P. brassicae* growth are closely related (Li *et al*. 2018). The *KEGG* of 'plant hormone signal transduction' (285, 7.95 %) was also obviously enriched. The clubroot disease pointed to an involvement of plant hormones in clubroot symptom development, such as cytokinins and auxin. Cytokinin oxidase/dehydrogenase overexpressing plants were disease resistant (Devos *et al*. 2006, Siemens *et al*. 2006). These pathways might be partly contribute to the SSG development.

The number of responsive genes is different between SF and XC isolate inoculations in *B. rapa* T1-145: To identify the genes showing the most differential response to SF and XC isolate infections, we selected the differentially expressed genes (DEGs) at variations of absolute Log2 ≥ 2 and false discovery rate ≤ 0.01 at every time point. Totally 6 359 DEGs were identified. Comparing the sample of SF infection with the control and the sample of XC infection with the control, there were more up-regulated genes at 4, 16, and 45 dpi than at 10 dpi (Fig. 2*A*,*B*). Next we compared the number of DEGs between the samples of SF and XC isolate infections. The numbers of up-regulated genes were 469, 162, 44, and 1706 at 4, 10, 16, and 45 dpi,
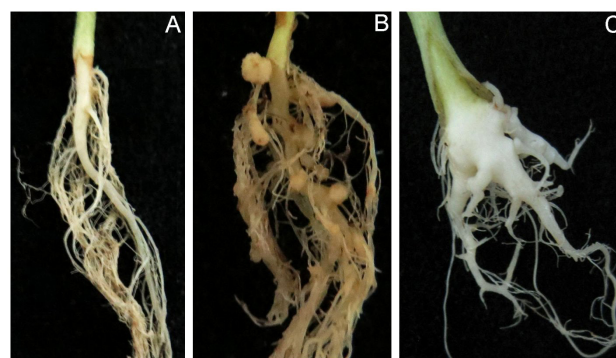

Fig. 1. Morphology differences of Chinese cabbage plants. The healthy root (*A*), the root with small spheroid galls (*B*), the root with spindle galls (*C*).

Table 1. Top 25 enriched *Kyoto encyclopedia of genes and genomes* pathways. DEGs - differentially expressed genes, SF - Shifang isolate, XC - Xichang isolate.

Pathway	The number of DEGs	Pathway ID	SF *vs.* XC
Ribosome	813 (22.66%)	ath03010	up
Starch and sucrose metabolism	167 (4.65%)	ath00500	up
Amino sugar and nucleotide sugar metabolism	158 (4.40%)	ath00520	up
Plant hormone signal transduction	285 (7.95%)	ath04075	up/down
Plant-pathogen interaction	141 (3.93%)	ath04626	up/down
Oxidative phosphorylation	110 (3.07%)	ath00190	up
Carbon fixation in photosynthetic organisms	89 (2.48%)	ath00710	up
Carbon metabolism	84 (2.34%)	ath01200	up
Glyoxylate and dicarboxylate metabolism	63 (1.76%)	ath00630	up/down
Photosynthesis	50 (1.39%)	ath00195	up
Proteasome	45 (1.25%)	ath03050	up
Glycine, serine, and threonine metabolism	42 (1.17%)	ath00260	up
Alanine, aspartate, and glutamate metabolism	30 (0.84%)	ath00250	up
Fatty acid metabolism	29 (0.81%)	ath01212	up
Fatty acid biosynthesis	22 (0.61%)	ath00061	up
Biosynthesis of secondary metabolites	231 (6.44%)	ath01110	down
Protein processing in endoplasmic reticulum	192 (5.35%)	ath04141	down
Phenylpropanoid biosynthesis	163 (4.55%)	ath00940	down
Biosynthesis of amino acids	161 (4.49%)	ath01230	down
Phenylalanine metabolism	127 (3.54%)	ath00360	down
RNA transport	117 (3.26%)	ath03013	down
Endocytosis	101 (2.82%)	ath04144	down
Phenylalanine, tyrosine, and tryptophan biosynthesis	40 (1.12%)	ath00400	down
Glycerolipid metabolism	33 (0.92%)	ath00561	down
α-Linolenic acid metabolism	33 (0.92%)	ath00592	down

respectively. The numbers of down-regulated genes were 142, 655, 245, and 791 at 4, 10, 16, and 45 dpi, respectively (Fig. 2*C*). Obviously, there were more up-regulated genes at 45 dpi than the other time points, which indicated that the late infection period might be critical for maintaining the defense against *P. brassicae*.

To identify which genes were most likely associated with the resistance, we classified the DEGs into tree types. The first type: the gene expressions were up-regulated in both sample of SF isolate infection [Log2 (SF *vs.* control) ≥ 2] and XC isolate infection [Log2 (XC *vs.* control) ≥ 2] at a time point by comparing with control. Due to the up-regulation of gene expression under the infection of *P. brassicae*, we suggested that most of these genes
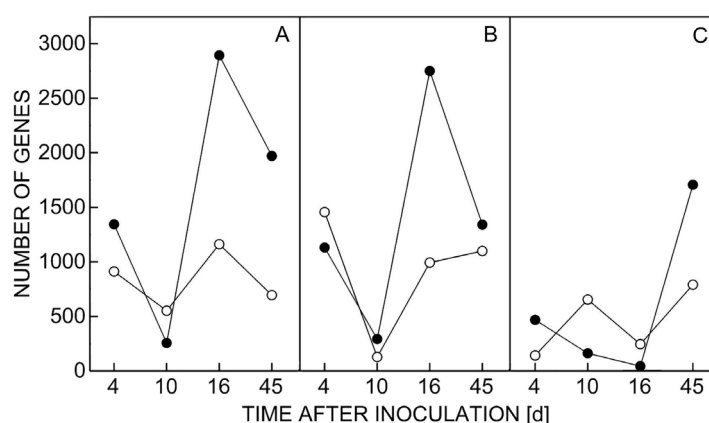
Fig. 2. The number of differentially expressed genes. Numbers of up-regulated (*closed circles*) and down-regulated (*open circles*) genes by comparing samples of Shifang (SF) isolate infection with a control at 4, 10, 16, and 45 days post inoculation (dpi, *A*), by comparing samples of Xichang (XC) isolate infection with a control at 4, 10, 16, and 45 dpi (*B*), and by comparing samples of SF infection with XC infection at 4, 10, 16, and 45 dpi (*C*).

might be associated with the basic resistance of host against *P. brassicae* or contributing to sustained growth of *P. brassicae*.

The second type: the gene expressions were lower in the sample of SF isolate infection than XC isolate infection. *1*) 569 DEGs were selected at variations of absolute Log2 (SF *vs.* XC) ≤ -2 at a time point and -1 ≤ Log2 (SF vs. XC) ≤ 1 at the other time points. *2*) 440 DEGs were selected at variations of absolute Log2 (SF *vs.* XC) ≤ -2 at a time point and Log2 (SF *vs.* XC) ≤ -1 at the other time points. Due to the high-expression of DEGs with XC isolate infection, the second type of DEGs might be beneficial to the development of the typical spindle galls of clubroots.

The third type: The gene expressions were obviously higher in the sample of SF isolate infection than XC isolate infection and 2 232 DEGs were finally obtained (Log2 (SF *vs.* XC) ≥ 2) at a time point. The third type of DEGs might be important for the host defense against the *P. brassicae* SF isolate.

Expression patterns over the time course of infection classified genes contributing to the defense: We suggest that the third type genes might be associated with the host defense. To be more accurate in demonstrating the post-invasive defense, we further performed analysis on the expression patterns of 2 232 DEGs. We classified the third type of DEGs into four groups: Group A including 355 genes fits the absolute Log2 (SF *vs.* XC) ≥ 2 only during the root hair infection period (at 4 dpi). Group B including 151 genes fits the absolute Log2 (SF *vs.* XC) ≥ 2 only during the cortical infection period (at 10 and 16 dpi). Group C including 1 580 genes fits the absolute Log2 (SF *vs.* XC) ≥ 2 only during the symptomatic period (at 45 dpi). Group D including 146 genes fits the absolute Log2 (SF *vs.* XC) ≥ 2 during root hair, cortical infection or symptomatic period.

To confirm the evaluation of RNA-seq data, we repeated the time course experiment and collected samples for the reverse transcription quantitative PCR on six representative genes (Fig. 3).

Differentially expressed genes involved in the resistance to *P. brassicae*: The third type DEGs were used to analyze the defense related genes. All detailed data are listed on the website https://share.weiyun.com/U3b0imIV, password: 6utmqg, and some representative data are shown in Fig. 4. The resistance proteins are crucial to detect pathogen effectors and trigger the subsequent defense responses. Previous work has shown that the *Arabidopsis* target of avirulence B operation 1 (TAO1) is a toll/interleukin 1 receptor-nucleotide binding leucine-rich repeat receptor-like kinase protein, which contributes to disease resistance induced by the *Pseudomonas syringae* effector AvrB (Eitas *et al.* 2008). Ribosomal protein of the small subunit 6 (RPS6) was found conferring resistance to wheat stripe rust in barley (Li *et al.* 2016). *Arabidopsis* resistance to *Pseudomonas syringae* pv. maculicola-interacting protein 4 (RIN4) regulates plant immune responses to pathogen-associated molecular patterns and type III effectors (Sun *et al.* 2014). The disease resistance genes *resistance to leptosphaeria maculans 1B*, *constitutive shade-avoidance 1*, *RPS6*, *RIN4*, *recognition of Peronospora parasitica 13*, *nonrace-specific disease resistance 1*, and *TAO1* were all induced, which might be involved in the defense response against *P. brassicae*.

Receptor-like protein/kinase: Cysteine-rich receptor-like protein kinases (CRKs) are one large subfamily of receptor-like protein kinases (RLKs) in *Arabidopsis*, which play an important role in response to abiotic and biotic stresses. Receptor-like proteins (RLPs) assume an important role for pathogen-associated molecular pattern-triggered immunity (Pitino *et al.* 2015). Here, seven *CRKs* and eight *RLPs* were induced after SF and XC isolate infections. For example, *CRK10* was induced at 4 and 45 dpi, the expression in the SF infected sample was 3-fold higher than that of
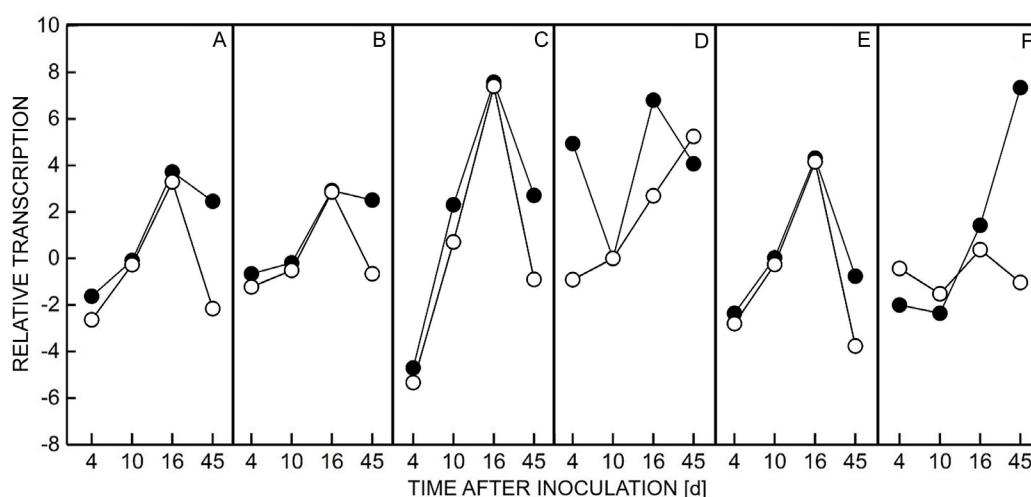
Fig. 3. Validation of data of RNA sequence by reverse transcription quantitative PCR. Relative transcriptions of *phloem intercalated with xylem-correlated 1* (*A*), *tractheary element differentiation inhibitory factor receptor* (*B*), *sugars will eventually be exported transporter* (*C*), *chitinase* (*D*), *defensin-like proteins* (*E*), and *defensin-like proteins* (*F*) genes. Comparison of the Shifang isolate (*closed circles*) or the Xichang isolate (*open circles*) infection with a control.

XC infected one at 45 dpi. The *CRK11*, *12*, *18*, *40*, and *41*, *RLP12* (*Novel00805, BraA08001180, BraA09002935, Novel00953, BraA01003449, BraA04000761*), and *RLP2* (*BraA08003352*) were induced at 16 dpi and maintained high at 45 dpi after SF infection.

Lectin receptor-like kinases (LecRKs) belong to a specific pattern recognition receptors family and are important players in plant innate immunity (Luo *et al*. 2017). Here, LRKS7, LRKS6, LRK16, and *LecRK44* were up-regulated at 16 dpi, and their expressions were 2-fold higher upon SF infection than upon XC infection at 45 dpi.

Secondary vascular development requires contributions from a number of leucine-rich repeat receptor like kinases. *Arabidopsis leucine-rich repeat receptor like kinase* genes (*phloem intercalated with xylem-correlated* (*PXC*) *1, 2,* and *3*) strongly correlate with several key regulators of vascular development, phloem intercalated with xylem/ tracheary element differentiation inhibitory factor receptor (PXY/TDR), which are involved in the secondary cell wall formation in xylem fibers (Wang *et al*. 2013). Here, the leucine-rich repeat receptor-like tyrosine-protein kinases *PXC1* (*BraA03002010*), *PXC2* (*BraA02000006*), *PXC3* (*BraA05004219* and *BraA04002945*), and TDR (*BraA03004735*) were up-regulated at 16 dpi and the expression was still up-regulated at 45 dpi upon SF isolate

infection. The *wuschel homeobox related* (*WOX*) *4*, *11*, and *13* expressions were higher after SF infection than after XC infection at 45 dpi. Therefore, the PXC1 - phloem intercalated with xylem / TDR - WOX4 signaling pathway probably regulates the maturation of interfascicular fiber cells and the initiation of secondary cell wall deposition to affect *P. brassicae* extension.

Wall-associated kinases (WAKs) have been shown to be positive regulators of fungal disease resistance in several plant species (Delteil *et al*. 2016). Here, *WAK5* (*BraA08003146* and *BraA06001040*) and *WAK-like 16* (*BraA02002703*) were up-regulated at 16 dpi; their expressions were higher after SF infection than after XC infection at 45 dpi. The *WAK2* (*BraA06001041*), *WAK4* (*BraA06001042*), and *WAK-like 9* (*BraA08003575*) expressions were higher upon SF infection than upon XC infection at 4 and 45 dpi.

Incompatible strubbelig receptor family 3 SRF3 alleles are required for an enhanced early immune response to pathogens (Alcázar *et al*. 2014). Here, *strubbelig* (*BraA09000500, BraA06002540*, and *BraA09000534*), *SRF8* (*BraA08001525*) and *SRF6* (*BraA06001833*) were up-regulated at 16 dpi after SF infection but down-regulated after XC infection at 45 dpi.

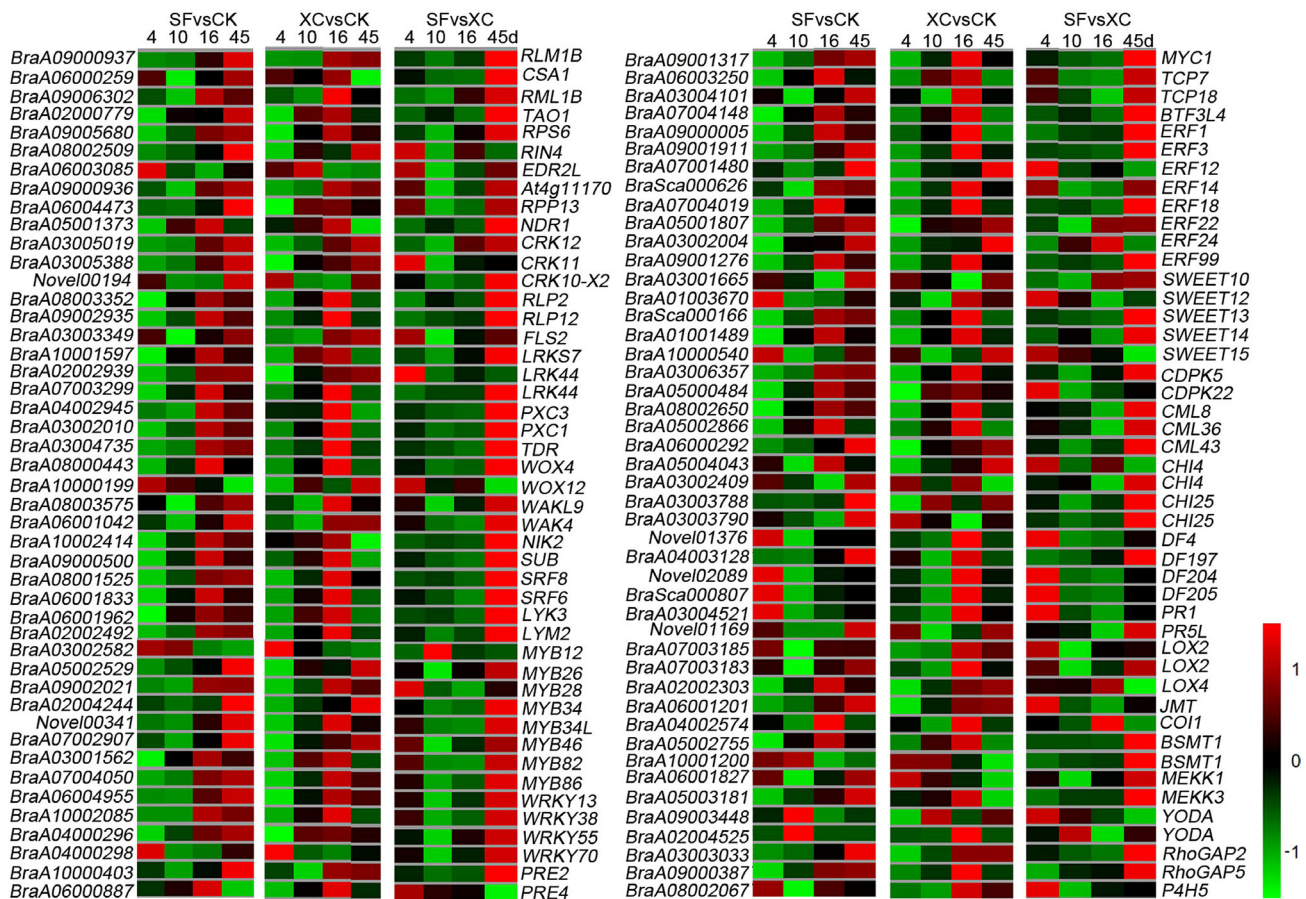The nuclear shuttle protein-interacting kinase (NIK)

Fig. 4. Heat maps of differentially expressed genes (DEGs) for putative candidate genes assigned to clubroot-resistant genes in *Brassica rapa* determined at 4, 40, 16, and 45 days after inoculation. *Green* indicates down-regulated DEGs, and *red* indicates up-regulated DEGs when compared Shifang (SF) with control (CK), Xichang (XC) with CK, and SF with XC.

is a leucine-rich repeats-containing receptor-like serine/threonine kinase and involved in plant development and defense (Santos *et al*. 2010). The *NIK1* (*BraA10000339*), *NIK2* (*BraA06004385* and *BraA10002414*), and *NIK3* (*BraA01002380*) were significantly induced already at 16 dpi and still up-regulated at 45 dpi upon SF infection. We suggest that these protein kinases might have an important role against the SF isolate infection.

Transcription factors related defense: In plants, transcriptional regulation is an important way for modulating defense responses. Genes with expressions more than 2-fold higher by SF *vs*. XC infection were selected including 19 myeloblastosis (*MYB*), 22 *WRKY*, 2 *pathogen-responsive elements*, 1 *myelocytomatosis 1*, 2 *teosinte branched1/cycloidea/pcf* (*TCP*), 1 *basic transcription factor 3*.

The WRKY transcription factors have been reported to regulate defense positively or negatively (Timmermann *et al*. 2017). We detected 22 *WRKY* transcription factors, *i.e*., *WRKY13*, *WRKY38*, *WRKY42*, *WRKY46*, *WRKY54*, and *WRKY55*, whose expressions were 2-fold higher after SF infection than after XC infection. It is still unclear whether WRKYs act positively or negatively in response to the presence of *P. brassicae*, and thus the role of WRKY in clubroot resistance needs further study.

One function of TCP proteins is to regulate defense gene expression (Kim *et al*. 2014). We found that *TCP7*, *15*, and *18* expressions were 2-fold higher upon SF *vs*. XC infection at 45 dpi. Therefore, these transcription factors with high expressions upon SF infection might play an important role against *P. brassicae*.

Cell wall modification: The cell wall biogenesis, especially the secondary cell wall formation and deposition, usually confers broad-spectrum resistance to pathogens. For example, *Arabidopsis* the walls are thin 1 confers broad-spectrum resistance against vascular pathogens (Denancé *et al*. 2013). Over-expression of *GhLac1*, which functions as a lignin polymerization enzyme in *Gossypium hirsutum* can confer an enhanced resistance against pathogens (Hu *et al*. 2013).

Our analysis showed that 97 cell wall formation and deposition related genes up-regulated after the infections. The *xyloglucan endotransglucosylase/hydrolase 5*, *7*, *8*, *9*, *16*, *17*, *22*, *25*, *31*, *32*, and *33*, *fasciclin like arabinogalactan proteins 1*, *8*, *10*, *11*, and *17*, *cellulose synthase A*, *B4* and *D5*, *COBRA-like protein 6*, *pectin methylesterases 1*, *53*, and *68*, and *pectin methylesterase inhibitors 3*, *6*, *16*, *18*, *44*, *51*, *61*, and *64*, *subtilisin-like protease 1.2, 1.6, 1.7, 2.5, 3.3, 3.5*, *walls are thin 1 related protein*, *galacturonosyltransferase 1*, *cinnamyl alcohol dehydrogenase 6* and *7*, *laccase 4*, *10*, *11*, *12*, and *22*, and *irregular xylem 9*, *10*, *14H*, and *15*. The *3-ketoacyl-CoA synthase 6*, required for synthesis of epicuticular waxes, at 4 dpi, *trichome birefringence like 6*, *18*, *33*, *36*, *37*, and *39* at 16 and 45 dpi, *callose synthase 3* and *9*, and *plasmodesmata callose-binding protein 1, 3*, *4*, and *5* at 45 dpi had higher expressions upon SF infection than XC infection.

In this analysis, cell wall formation and deposition related genes were mainly up-regulated at 16 and 45 dpi, but not at the early infection stages, indicating the cell wall modification enhanced the resistance to SF isolate during the late infection periods.

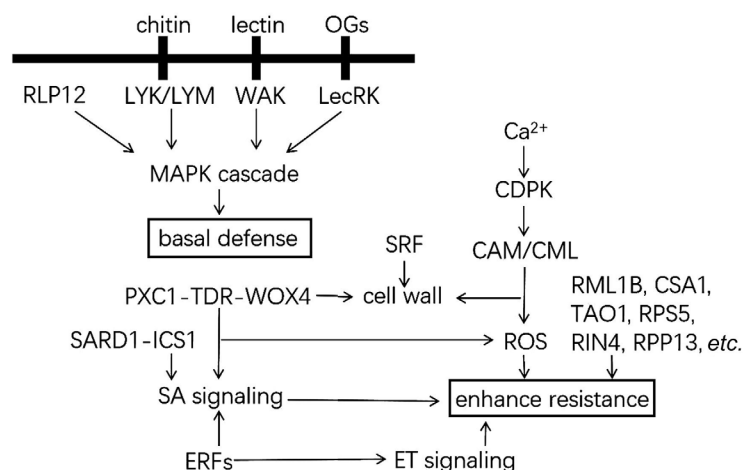**Reactive oxygen species scavenging** related genes exhibited higher expressions after SF infection compared

Fig. 5. A hypothetic working model for activation of the defense of the *Plasmodiophora brassicae* Shifang isolate in *Brassica rapa*. CAM/CML - calmodulin/calmodulin-like, CDPK - calcium-dependent protein kinase, CSA1 - constitutive shade-avoidance 1, ET - ethylene, ERFs - ethylene responsive factors, ICS1 - isochorismate synthase 1, LecRK - lectin receptor kinase, LYK/LYM - lysin motif receptor kinase / lysin motif, MAPK - mitogen-activated protein kinase, OGs – Oligogalacturonides, PXC1 - phloem intercalated with xylem-correlated 1, RIN4 - resistance to *Pseudomonas syringae* pv. *maculicola* interacting protein 4, RLP12 - receptor-like protein, RML1B - resistance to *Leptosphaeria maculans* 1B, ROS - reactive oxygen species, RPP13 - recognition of *Peronospora parasitica* 13, RPS5 - ribosomal protein of the small subunit 5, SARD1 - systemic acquired resistance deficient 1, SA - salicylic acid, SRF - strubbelig receptor family, TAO1 - target of AvrB operation 1, TDR - tracheary element differentiation inhibitory factor receptor, WAK - wall-associated kinase; WOX4 - wuschel homeobox related 4.

with XC infection. *Glutaredoxin, L-ascorbate oxidase, peroxidase, catalase*, and *respiratory burst oxidase homolog protein E* were induced at 4 and 45 dpi.

Sugar transporters: Sugars will eventually be exported transporter (SWEET) proteins, a novel family of sugar transporters, act as important players in sucrose phloem loading. Due to the disruption of sugar efflux and changes in sugar distribution, SWEET can enhance the resistance to biotic and abiotic stresses (Li *et al.* 2017). In our data, we identified 12 *SWEET* genes, including 3 genes (*BraA10000540, BraA01003670*, and *BraA06000647*) at 4 dpi, 3 genes (*BraA02000503, BraA06004193*, and *BraA09004936*) at 10 dpi, 5 genes (*BraA08002029, BraA01003908, BraSca000166, BraA06002331*, and *BraA01001489*) at 45 dpi, and 1 gene (*BraA03001665*) at 16 and 45 dpi, whose expressions were 2-fold higher upon SF infection than XC infection. The disruption of sugar efflux and changes in sugar distribution in plant roots might influence *P. brassicae* infection and extension.

Calcium and calcium-dependent protein kinases: Calcium is an essential second messenger and calcium-dependent protein kinases (CDPKs) and CDPK-related kinases (CRKs) regulate plant responses to abiotic and biotic stresses (Wang *et al.* 2016). Here we found that the expressions of three *mitochondrial calcium uniporter*, three *calmodulin-like*, and three *calcium binding* genes were more than 2-fold higher at 45 dpi when comparing SF infection with XC infection. The CDPK15 was up-regulated at 4 dpi. The CDPK-related kinase 4 and 7 were induced upon SF infection at 16 dpi; CDPK5 and CDPK22 at 16 and 45 dpi.

Chitinase response to *P. brassicae* infection: Most chitinases can degrade chitin, which is the main component of cell walls in *P. brassicae* (Schwelm *et al.* 2015). Six *endochitinases* were identified. The *BraA05004043* was highly expressed over the time course of SF infection. The *BraA03003790* and *BraA03002409* were up-regulated after SF infection at 4 and 45 dpi; the expression was about 4 to 7-fold higher after SF *vs.* XC infection at 45 dpi.

Pathogenesis-related (PR) proteins and defensin-like proteins: One of the features of plant defense responses is the production of PR proteins (Van Loon *et al.* 1999). Pathogenesis-related proteins *PR1* and *PR5*, and *defensin-like proteins 4, 37, 196, 197, 204*, and *205* were induced. The expressions of *PR1* (*BraA03004521*) was 11-fold higher, *PR5* (*BraA07004010*) was 4-fold higher at 4 dpi, *PR1* (*BraA03006209, BraA03006206*, and *BraA08001998*) and *PR5* (*Novel01169*) were 2 to 3-fold higher at 45 dpi after SF *vs.* XC infection. These PR proteins are most likely involved in *B. rapa* resistance to *P. brassicae.*

Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signaling pathways: *P. brassicae* is an obligate biotrophic protist. Plant resistance to biotrophic pathogens is believed to be controlled by the SA-mediated signaling pathway. In this study, *non-inducible immunity 1,*

phytoalexin deficient 4, isochorismate synthase 1, and *systemic acquired resistance deficient 1* were induced. The ethylene responsive factors (ERFs) can regulate the SA and JA/ET signaling pathways, and they are responsible for generating tolerance to biotic stresses in plants. We identified *41 ERFs* including *ERF 1, 3, 12, 13, 14, 16, 18, 19, 20, 21, 22, 24, 25, 34, 35, 38, 43, 54, 95, 107, 115,* and *122, aintegumenta, aintegumenta-like, cytokinin response factor, related to apetala 2*, and *wax inducer 1*. Thus, numerous ERFs were involved in plant responses to *P. brassicae* during the late infection stages and could be considered as potential candidate genes for further functional validations.

Secondary metabolites associated with plant defense: Secondary metabolites are involved in diverse stress-induced responses. Previous studies have reported that nodulin proteins are associated with plant defense in *Medicago truncatula* (Gamas *et al.* 1998). Serine carboxypeptidase-like protein is required for disease resistance in oat (Mugford *et al.* 2009). Trehalose-6-phosphate synthase/phosphatase is involved in resistance to *Botrytis cinerea* and *Pseudomonas syringae* pv. *tomato* DC3000 in tomato (Zhang *et al.* 2016). Thaumatin-like protein, germin-like protein and patatin-like protein 2 (PLP2) can enhance resistance to some pathogens (La Camera *et al.* 2009, Liu *et al.* 2016). Here, we found that eight *early nodulin-like proteins*, four *serine carboxypeptidase*, five *thaumatin-like proteins*, two *patatin-like proteins* with higher expressions at 16 dpi after both SF and XC infections, and still maintained higher at 45 dpi after SF infection. The secondary metabolism might play roles for resistance against SF infection extension.

Conclusions

In this study, we identified a number of genes that might contribute to defense against *P. brassicae* through transcriptome analysis on a time course of infection. Most of the genes up-regulated in cortical stage of infection by SF and XC isolates were down-regulated at 4 dpi, which indicated that the defense response activated during the cortical infection period were suppressed during the root hair infection period. This may explain that the root hair infection can occur in various plants including resistant, susceptible, or non-hosts plants, and the cortical infection only occurs in the susceptible plants. Based on the continuous defense against *P. brassicae*, such as the restriction of *P. brassicae* proliferation, extension, and secondary tissue development during the cortical infection period, the symptom finally developed into two different types.

Here, we compared the data from SGGs sample of SF infection with spindle galls sample of XC infection, and proposed a working hypothesis for the defense against SF infection (Fig. 5). Firstly, there are a few receptors, such as CDPK, lysin motif receptor kinase / lysin motif, WAK, and LecRK that recognize Ca^{2+}, chitin, oligogalacturonides, and lectin. Other pattern recognition receptors *RLP12*, and

flagelin-sensitive 2 are highly induced upon SF infection, so a series of defense pathways may be activated. Subsequently, genes involved in MAPK cascades are also up-regulated, which in turn presumably leads to activation of the basal defense. Secondly, there is a effector-triggered immunity response in *B. rapa* T1-145 to SF infection, which is mediated by NB-LRR disease resistance proteins resistance to *leptosphaeria maculans* 1B, constitutive shade-avoidance 1, TAO1, RPS6, RIN4, *enhanced disease resistance 2L*, and recognition of *Peronospora parasitica* 13 like protein 4. In addition, a batch of genes including *SRF*, *MYB*, and *PXC1-TDR-WOX* are up-regulated, which probably lead to the secondary cell wall formation. Many *ERFs*, *WRKY*, and *MYB* transcript factors are induced, most of which can act as transcriptional activators or repressors of SA, JA, and ET signaling, reactive oxygen species, cell wall integrity, *etc*. According to this model, these genes could be the candidates for engineering disease resistance by transgenic approaches.

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