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Overexpression of a chitinase gene PbChia1 from Plasmodiophora brassicae increases broad spectrum disease resistance

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Abstract

Chitinase plays an important role in plant resistance against chitin containing pathogens by degrading chitin. Clubroot, caused by *Plasmodiophora brassicae,* is a major disease in cruciferous crops worldwide, including vegetable and rapeseed. Chitin is the main component of *P. brassicae* spore cell walls, chitinases can enhance the plant's defense system as they act on chitin. However, the function of chitinases in *P. brassicae* has not reported. Here, we found that chitin is the functional component of *P. brassicae* by wheat germ agglutinin staining and commercial chitinase treatment . Chitin extracted from resting spores of *P. brassicae* was characterized by infrared spectroscopy, X-ray diffraction and thermo gravimetry analysis.The chitinase PbChia1 was identified by chitin pull-down assays combined with LC-MS/MS method. We also showed that PbChia1 was a typical secreted chitinase, could bind to chitin and showed chitinase activity *in vitro*, significantly degrade the number of resting spores of *P. brassicae* and significantly relieve the severity of clubroot symptom, the biocontrol effect was 61.29%. Overexpression of *PbChia1* in *Arabidopsis thaliana* resulted in susceptibility to *P. brassicae*, increase host survival rate and seed yields, and transgenic plants enhanced in chitin-triggered reactive oxygen species burst, MAPK activation and expression of immune-related genes. *PbChia1* transgenic plants showed resistance to other pathogens, such as biotrophic bacteria *Pst* DC3000, necrotrophic fungi *Sclerotinia sclerotiorum* 1980 and *Rhizoctonia solani*. The findings indicate that chitinases *PbChia1* is a candidate gene with potential application for broad spectrum disease resistance in breeding plants to prevent clubroot.

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Abstract

Chitinase plays an important role in plant resistance against chitin containing pathogens by degrading chitin. Clubroot, caused by*Plasmodiophora brassicae,* is a major disease in cruciferous crops worldwide, including

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Key words: *Plasmodiophora brassicae* ; chitin; chitinase; PbChia1; clubroot; disease resistance.

1 | INTRODUCTION

Clubroot is a major disease in the Brassicaceae family, and results in 10%-15% yield reduction on a global scale (Botero et al., 2019). The clubroot pathogen *Plasmodiophora brassicae* is an obligate intracellular biotroph that belongs to the class Phytomyxea within the eukaryote supergroup Rhizaria, one of the least studied groups of eukaryotes (Burki et al., 2010). Disease symptoms of clubroot is formation of galls on the roots of affected plants, which disrupt water and nutrient uptake, leading to wilting, stunting, and, in some instances, death of the infected plant (Hwang et al., 2012). The disease is now found in all brassica-growing areas of the world, and seems to be getting worse everywhere, threat to rapeseed production worldwide. As*P. brassicae* remains uncultivable on synthetic medium, the pathogen has no well-established, easy and accessible transformation system to test gene function. It is challenging to decipher gene functions using reverse genetic tools such as gene knockout and gene silencing, which have been extensively employed to uncover gene functions in flamentous fungi and oomycetes (Kamoun, 2003; Michielse et al., 2005; Nakayashiki et al., 2005). Hence, functions of most of the genes in *P. brassicae* have yet to be elucidated. As the genome of*P. brassicae* is currently available (Rolfe et al., 2016; Schwelm et al., 2015), has provided great opportunities to explore the gene family members, and to uncover their potential functional roles (Chen et al., 2018; Schwelm et al., 2016; Singh et al., 2018). Some *P. brassicae* effectors have been proven to play important roles in the infection s and subsequent disease progression (Bulman et al., 2018; Djavaheri et al., 2019; Pérez-López et al., 2020; Pérez-López et al.,2021; Chen et al., 2021). Understanding the pathogen and resistance in the host plants is important for genetic improvement of plant resistance against clubroot disease. Whole genome investigation of genes of interest along with heterologous expression may offer another avenue for uncovering the biology of this elusive pathogen. *Arabidopsis thaliana* , the model plant in molecular biology, is susceptible to most of the *P. brassicae* pathotypes and its use can facilitate our search for new methodologies and the interpretation of the results.

Chitin, a polymer of β-1,4-linked *N* -acetyl-d-glucosamine, is widely observed in insect carapaces and the cell walls of fungi and*P. brassicae* (Merzendorfer 2011; Thornton et al., 1991). The cell wall determines the shape and strength of the pathogen cells and is a key determinant of cell morphology development. As one of the primary components of the cell wall, chitin plays a very important role in the growth and development of pathogens, as well as the defense against external stress (Kombrink et al., 2011). Chitin is also considered to be a prominent signal to induce the natural immunity of plants for the invasion of pathogens (Pentecost 2013). A previous study suggested that the perception of chitin contributes to enhanced disease resistance in certain crops, such as rice and *Arabidopsis* (Kishimoto et al., 2010; Wan et al., 2012). Chitin compost and broth could suppression of clubroot formation (Jin et al., 2006). *P. brassicae* has two chitin synthases families and chitin-related enzymes of the carbohydrate/chitin binding (CBM18) domain is enriched in the secretome

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(Schwelm et al., 2015). Two candidate secreted effectors CBM18 proteins in *P. brassicae* , PbChiB2 and PbChiB4, suppress chitin-triggered activation of MAPK activation in the host *Brassica napu* s (Muirhead et al., 2022).

Chitinase is an enzyme system that employs chitin as a substrate and hydrolyzes it to N-acetyl oligosaccharide and glucose. As a subgroup of pathogenesis-related (PR) proteins, chitinase is widely present in various organs of higher plants and can be rapidly produced and accumulated when plants are subjected to pathogen infection or abiotic stress sources, such as heavy metals and drought (Bravo et al., 2003; Wang et al., 2015; Li et al., 2018). Therefore, chitinase plays an important role in protecting plants from a variety of pathogens. The induction of pathogens can enhance the activity of chitinase in plants, which subsequently inhibits spore germination and mycelial growth and even directly degrades the chitin of the fungal cell wall (Roby et al., 1988; Ntui et al., 2011) increased tobacco resistance to Fusarium wilt by transferring chitinase genes into tobacco. The same results were also obtained in tomatoes (Jabeen et al., 2015). Similarly, transgenic grapes carrying the wheat chitinase gene exhibit increased resistance to downy mildew (Nookaraju et al., 2012). Marchant et al reported that expression of the chitinase transgene reduced the severity of black spot development by 13–43% in rose (Marchant et al., 1998). Chen et al found that the expression of chitinase reduced the symptoms of clubroot in Chinese cabbage (Chen et al., 2018). Chitinases are believed to function as defense-related genes against pathogens containing chitin (Abeles et al., 1971). The function of chitinase has been analyzed in various plant species, such as tomato (Staehelin et al., 1994), potato (Khan et al., 2017), rice (Zhao et al., 2018) and apple (Fan et al., 2015), the role of the chitinase gene family in the response to clubroot has not been elucidated.

This study aimed to characteristics of chitin extracted from *P. brassicae,* identified and clarified the function of a chitinase gene*PbChia1* in the development of *P. brassicae* . Investigated the effectiveness of the *PbChia1* in improving *P. brassicae*resistance in transgenic plants. This gene will be helpful for the prevention control of *P. brassicae* in breeding for clubroot resistance.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

The *Arabidopsis thaliana* Col-0 and rapeseed cultivar Huashuang 4 were grown in a growth chamber at 22-23 °C, 75% humidity under a 12-h-light/12-h-dark cycle.

2.2 | Pathogen inoculation

For inoculation with *P. brassicae.* Resting spores of *P. brassicae* were extracted from clubroot galls and stored at 4 °C (Asano et al., 1999). 14-days-old *A. thaliana* or rapeseed were inoculated with *P. brassicae* at 1.0×10^6 or 1.0×10^7 spores per plant. Disease severity was assessed using a scoring system of 0–4 modified from Siemens reported (Siemens et al., 2002). A score of 0 indicated no disease; 1, very small galls mainly on lateral roots that did not impair the main root; 2, small galls covering the main root and few lateral roots; 3, medium to large galls, also on the main root; and 4, severe galls on lateral root, main root or rosette, with fine roots completely destroyed. Disease index (DI) was calculated using the five-grade scale according to the formula: $DI = (1n_1 + 2n_2 + 3n_3 + 4n_4) \times 100/4N_t$, where n_1–n_4 is the number of plants in the indicated class and N_t is the total number of plants were tested. The disease severity in rapeseed was assessed using a scoring system of 0–9 modified from the report (Zhao et al., 2022). For inoculation with *Pst* DC300, bacteria was culture in KB medium (Protease peptone 20 g/L, K_2HPO_4 1.5 g/L, $MgSO_4$ 1.5 g/L, 50% Glycerol 20 ml/L) with 50 μg/ml rifampicin at 28 °C to an OD_{600} of 0.8–1.0. Bacteria were collected by centrifugation and resuspended in 2.5 mM $MgCl_2$. Bacterial suspension was further diluted to cell densities of 1×10^5–1×10^6 cfu/ml (OD_{600} = 0.2, approximately 1×10^8cfu/ml) and syringe-infiltrated to the leaves of 4-week-old plants, kept under ambient humidity for 1–2 h for water to evaporate, and after the plant leaves returned to pre-infiltration appearance, plants were kept under high humidity by covering plants with a clear plastic dome for disease to develop. For quantification of Pst-DC3000 bacterial populations, leaf disks ground in 2.5 mM $MgCl_2$,Colony-forming units were determined by serial dilutions and plating on plates containing 50 μg/ml rifampicin and grown at 28 °C for 2 days. Experiments were repeated at least three times. For

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S. sclerotiorum inoculation, six-week-old leaves of *Pbchia1*overexpression transgenic plants were detached and placed in petri dishes (90 mm) lined with sterile wet filter paper. 2mm diameter mycelium block of *S. sclerotiorum* was inoculated on the leaf surface and spray some water to keep high humidity. The plates were incubated in an incubator at 22 °C for disease to develop. 30 hours later, the necrotic lesions were observed and measured the lesion area. Each sample was performed 9 biological replicates. For *R. solani*inoculation, *R. solani* was cultured on PDA plates for 2 days, 10 mycelial blocks with a diameter of 5 mm at the edge of the colony were inoculated to liquid PDB medium and cultured at 22°C with shaking for 7 days, broken mycelium with a homogenizer, and inoculated 1 ml broken mycelium to the roots of 14-day-old *A. thaliana* , cover with a clear plastic dome to keep high humidity for disease to develop, and continue to grow in the growth chamber at 22 °C for 3 days. Disease severity was assessed using a scoring system of 0–4, grade 0: no disease symptoms on the stem; grade 1: the expanded area of the stem disease spot, less than 1/2 of the stem circumference and no disease symptoms at the root; grade 2: the expanded area of the stem disease spot, high in the 1/2 of the stem circumference, the roots have no symptoms; grade 3: the expanded area of the stem lesions is higher than the 1/2 of the stem circumference, and the root is affected; grade 4: the plant is wilting and dying.

2.3 | Measurement of chitin contents in*P. brassicae*

Chitin were crude extracts from *P. brassicae* according to a previously described method with some modifications (Guerriero et al., 2010), Fifteen grams of fresh root galls were ground into power with liquid nitrogen and then homogenized in 5 mL of deionized water. After centrifugation at 13,000 g for 10 min at 4°C, samples were lyophilized in a vacuum overnight using a freeze dryer. 5 g of the dried pellets added 30 mL of 6 M HCl, then the samples were hydrolyzed at 100°C for 4 h, the hydrolysis was neutralized with 10 N NaOH to pH 7.0. The liquid was filtered with filter paper, dried into powder, and the crudely extracted chitin is obtained. The chitin content was determined by measuring the amount of glucosamine released by acid hydrolysis. 0.2 mL of the liquid samples (after NaOH neutralize to pH 7.0) was added to 0.25 mL of 4% acetyl acetone in 1.25 M sodium carbonate, heated for 30 min at 100°C. After cooling down, 2 mL ethanol and 0.25 mL of Ehrlich reagent (1.6 g of N, N-dimethyl-p-aminobenzaldehyde in 60 mL of a 1:1 mixture of ethanol and concentrated HCl) was added to the mixture, heated for 1 h at 60 °C. Centrifuged at 13,000 g for 10 min, and the supernatant was measured for absorbance at 530 nm. The chitin content was calculated based on the standard curve established by measuring the absorbance of known amounts of glucosamine hydrochloride (Sigma, G4875).

2.4 | Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and thermo gravimetry analysis (TGA)

The FTIR, XRD and TGA of crude chitin extracts from *P. brassicae*and commercial chitin (Sigma, CAS No. 1398-61-4) were measured by Shanghai Sci Go Company. The sample was made into a matrix with Potassium bromide (KBr) discs in the ratio of 1:100. The mixture was made into a pellet using hydraulic press and compressed into thin disc. The spectra were collected using Opus Software version-6.5 with the wavenumber region of 4000-400 cm^{-1}. An average spectrum scan of 32 is recorded with the resolution of 4 cm^{-1}. The spectra were recorded in absorbance mode. The XRD data were analyzed using Bruker (D8 advance、Dmax2500、smartlab). The finely powdered chitin sample is smeared over low background sample holder (amorphous silica holder) and fixed on the sample stage in the goniometer. The current and voltage is set to 40 mV and data has been collected in coupled 2ϑ mode. Sample is rotated at a speed of 2°/min during data collection at a fixed wavelength of 1.5418. TGA was carried out using Shimadzu Simultaneous TGA Analyzer was used to study the thermal decomposition behavior of the commercial chitin and *P. brassicae* chitin. 1 mg commercial chitin and *P. brassicae* chitin samples were heated at a heating rate of 10 degC/min from room temperature to 600 degC.

2.5 | Identification of chitinase family genes in*P. brassicae*

The genome of *P. brassicae* was downloaded from DDBJ/ENA/GenBank under the accession number of MCBL01000000 (Bi et al., 2016). Using Blast2GO to systemic investigation chitinase in

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the genome of *P. brassicae* , all data were checked for redundancy by self-BLAST and no alternative splice variants were used to confirm that identified genes were members of the chitinase family. Those proteins containing glycosyl hydrolase domain were defined to belong to the chitinase family. Then, the NCBI CDD (http://blast.ncbi.nlm.nih.gov) and SMART databases (http://smart.embl-heidelberg.de/) were used to further confirm the predicted chitinase genes. The isoelectric point (PI) and molecular weight (MW) of the obtained proteins were predicted using Compute pI/Mw tool online (http://web.expasy.org/compute_pi/). Subcellular localization prediction of each protein was performed by CELLO v.2.5 online (http://cello.life.nctu.edu.tw/) (Yu et al., 2006).

2.6 | Phylogenetic analyses of PbChia1

Phylogenetic analysis was performed based on the full-length protein sequences or conserved domain using the MEGA 6 program by the neighbor-joining method, and a bootstrap test was carried out with 1000 interactions (Saitou and Nei 1987).

2.7 | Colloidal chitin preparation, chitinase screening and chitinase activity assay.

2% colloidal chitin was prepared according to a previously described method (Niu et al., 2016). Chitinase screening and chitinase activity was examined using colloidal chitin as the substrate. To screen the chitinase, 10 g root galls of rapeseed were ground into power under liquid nitrogen and then homogenized in 30 mL of protein lysis buffer at 4 degC for 0.5 h with shaking, centrifugated at 12,000 g for 10 min at 4degC, the supernatant was filtered through a 0.22 mm Millex Syringe Filter Unit (Millipore). Then, 500 uL 2% colloidal chitin was incubated with 30 mL of supernatant in binding buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100) at 4degC for 2 h with shaking. After centrifugation at 1000 g for 3 min at 4degC, the pellet was washed five times with washing buffer (20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.2% Nonidet P-40) and then boiled with SDS PAGE loading buffer. The sediment was analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS, Thermo Fisher Scientific). To test the recombinant protein has chitinase activity, one microgram of recombinant protein was incubated with 0.25mg colloidal chitin at 20degC, 37degC or 50 degC for 1 h in 100mL 50mM sodium acetate buffer (pH 7.0). The reaction was terminated by adding 100 mL 3,5-dinitrosalicylic acid (DNS) by heating at 100degC for 5 min. After cooling, the mixture was diluted with 800 mL H_2O. Centrifugation to remove the undigested chitin. The absorbance of supernatant was monitored at OD_{565} nm. The standard curve was generated by the reaction of GlcNAc and DNS. One unit of chitinase activity was defined as the amount of enzyme required to produce 1 mmol of GlcNAc/h under the above conditions. Using a commercial Chitinase activity assay kit (Beijing Boxbio Science & Technology Co. Ltd, Beijing) to test the chitinase activity of plant. Leaf samples were frozen under liquid nitrogen and proteins extracts were prepared as described by Zhang et al (Zhang et al., 2016). Chitinase activity assays were carried out in 96-well plates following the manufacturer's instructions. Three replicates were performed for each of the treatments.

2.8 | Expression and Purification of Fusion Proteins

The full-length cDNA of PbChia1 without signal peptide was amplified by PCR using PbChia1-eF and PbChia1-eR primers (Table S3) with additional restriction enzyme sites and inserted into pGEX-6P1 for GST fusion. The full-length of PbChia1 coding region without signal peptide was inserted into pET-28a for expression of His-tagged recombinant protein. For protein expression, *Escherichia coli* BL21-Codon Plus (DE3) harboring the plasmids was induced with 1 mM isopropyl1-thio-b-D-galactopyranoside in Luria-Bertani broth for 6 h at 28 degC. GST fusion proteins were purified using glutathione resin (GenScript, Cat. No. L00206). His-tagged proteins were purified using Ni-NTA resin (GenScript, Cat. No. L00250) under native conditions and eluted with a buffer solution. Purified proteins stored at -80 degC.

2.9 | Chitin pull-down assays

The chitin pull-down assay was performed using the method described by Liu et al (Liu et al., 2012). Briefly, insoluble chitin was incubated with purified PbChia1-His or PbChia1-GST in binding buffer (20 mM

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Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100) for 2h with shaking at 4degC. After centrifugation at 1000 g at 4degC for 3 min. The pellet was washed five times with washing buffer (20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.2% Nonidet P-40) and then boiled with SDS-PAGE loading buffer. The chitin-associated PbChia1-His or PbChia1-GST was detected by immunoblotting using antibody.

2.10 | Assay for validation of signal peptide

The yeast invertase assay was used to assess the secretory activity directed by signal peptide (Jacobs et al., 1997). The DNA sequences encoding signal peptide were cloned into pSUC2 plasmid. The recombinant plasmids were transformed into the invertase-negative yeast strain YTK12 (Gietz and Schiestl 2007). Positive clones were replica plated on CMD-W (0.67% yeast N base without amino acids, 0.075% tryptophan dropout supplement, 2% sucrose, 0.1% glucose, and 2% agar) or YPRAA (1% yeast extract, 2% peptone, 2% raffinose, and 2 μg/mL antimycin A) plates and incubated at 30 °C for 3-5 days. Invertase enzymatic activity was detected as previously described (Lyda et al., 2015). Yeast cells were applied with 1 mL of buffered sucrose (25 mM sucrose in 0.2 M NaAc, pH 5.0), incubated at 37 °C for 30 min, then added with an equal volume of 2,3,5-triphenyltetrazolium chloride (TTC, 0.2% TTC in 4% NaOH). If invertase can be secreted, it will enable conversion of sucrose into glucose and fructose, reduce alkaline TTC to insoluble, red-colored 1,3,5-triphenylformazan (TPF) at room temperature for 5 min.

2.11 | Chitinase treat with clubroot

Rapeseed cultivar HuaShuang 4 was surface sterilized and germinated on a sterilized wet-filter paper in a Petri dish for 7 days, then transformed to 10 mL tubes with 1/2 Hoagland nutrient solution. The plants were grown in a plant growth chamber maintained at 70% humidity and 23 with a 16/8 h day/night cycle. Each plant was inoculated with 1 mL resting spores of *P. brassicae* ($1x10^7$spores/mL), commercial chitinase (Sigma C6137, chitinase extract from*Streptomyces griseus*) or purified PbChia1 protein added to the tube to check resistance to clubroot. The concentration of commercial chitinase or purified PbChia1 protein is 0.005 U/ml. We filled some 1/2 Hoagland nutrient solution to the tubes every day and completely changed Hoagland nutrient solution each 7 days post inoculation to avoid contamination. To test the chitinase affect the resting spores of*P. brassicae* germination, commercial chitinase or purified PbChia1 protein incubated with resting spores of *P. brassicae* in the root exudates of rapeseed for 3 or 6 days. Used 4'-6-diamidino-2-phenylindole-dihydrochloride (DAPI) staining to check the resting spores of *P. brassicae* germination (Chen et al., 2018).

2.12 | RNA isolation, DNA isolation and Quantitative PCR

Plant materials or root galls washed with sterilized water, finely ground in liquid nitrogen, total RNA was isolated using TRIZOL reagent (Chen et al., 2016). RNA samples were treated with DNase I to remove potential contaminating genomic DNA, followed by extraction with phenol: chloroform. First-strand cDNA was prepared using oligo (dT)18 primer. cDNA as templates for quantitative PCR (qPCR). qPCR was performed on a CFX96 real time PCR system (Bio Rad), using iTaq Universal SYBR Green super mix (Bio Rad). The following cycling conditions were used: 95for 2min30 s, 95 for 15 s, 57 for 15 s, and 72 for 15 s. The reaction was performed for 40 cycles, followed by a step at 72 for 5 s. Arabiodopsis actin gene (AT3G18780) as internal control gene. Experimental treatment with three technical replicates and a minimum of three biological replicates. For quantification of *P. brassicae* DNA content in infected roots, DNA was extracted from root samples using the cetyl trimethyl ammonium bromide (CTAB) method (Chen et al., 2016). The relative amounts of *P. brassicae* and *A. thalian* a DNA were determined by qPCR, employing specific primers for *P. brassicae*target actin gene AY452179.1 and Arabidopsis actin gene AT3G18780, respectively. Primer listed in Table S3.

2.13 | Generation of transgenic Arabidopsis plants

The full-length PbChia1 cDNA was amplified by PCR using PbChia1-XbaI-F and PbChia1-BamHI-R (Table S3) and cloned into XbaI-BamHI of pCNF3 to generated pCNF3-PbChia1-FLAG, the recombinant plasmid

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was transformed into *Agrobacterium tumefaciens* GV3101, *A. tumefaciens* were transformed to 5- or 6-week-old Col-0 plants by floral dip method (Clough and Bent 1998). T0 seeds were screened in Murashige and Skoog (MS) media containing kanamycin (50 mg/ml). Homozygous plants were identified by PCR and western blot. Plants of the T3 generation were used for subsequent studies.

2.14 | Microscopic Analysis

For trypan bule staining, roots were treated with 0.05% trypan bule solution for overnight, and excess dye was removed after brief rinsing in H_2O (Liesche et al., 2015). The root samples were subsequently observed using Nikon light microscopy (Nikon Eclipse 90i or Olympus BX63, Japan). For WGA488 staining, wheat germ agglutinin (WGA)-Alexa Fluor 488 (Invitrogen, Cat. No.: W11261) with final concentration of 10 μg/ml was added with roots infected by *P. brassicae* or resting spores of *P. brassicae* for 10 min prior to microscopy (Hogekamp et al. 2011). A confocal microscope (Leica SP8) was used to detect the WGA-Alexa Fluor 488 signal. For DAPI staining, the resting spores with nucleus were counted under UV excitation. For electron microscopy analysis, the roots were fixed in 2.5% glutaraldehyde for 4 h, and were subsequently postfixed in 1% osmium tetroxide for 3 h, washed, dehydrated through an ethanol series, and embedded in London resin white. Ultrathin sections were examined through TEM (HITACHI, H-7000, Japan). For subcellular localization, the YFP fluorescent signal of epidermal cell layers was examined using a confocal laser scanning microscope (Leica SP8), a laser with a 488 nm wavelength was used, and emissions were detected between 490 nm and 560 nm.

2.15 | ROS assay

ROS burst was evaluated with a luminol-based assay. Leaves from 4-week-old Arabidopsis plants of each genotype were excised into leaf discs of 0.25 cm^2 and incubated overnight in 96-well plates with 100 μL of H_2O to eliminate the wounding effect. H_2O was replaced with 100 μL reaction solution containing 50 μM luminol and 10 μg/mL horseradish peroxidase (Sigma, USA) with 100 nM flg22 or chitin supplementation. Measurement was completed with a luminometer (Perkin Elmer, 2030 Multilabel Reader, Victor X3) immediately after adding the solution with 1.5 min interval reading times for a period of 30 min ROS production values from 20 leaf discs per treatment are expressed as the mean relative light units.

2.16 | MAPK assays

Ten-day-old seedlings germinated on half-strength MS plates were transferred to 2 mL H_2O in a 12-well plate to recover overnight and then treated with 100 nM flg22 or chitin for 5, 15, 30 and 45 min, respectively. Seedlings were ground in IP buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, and 1% Triton X-100), and supernatants were collected after centrifugation. The cleared lysate was incubated with SDS sample buffer and loaded onto 12% SDS-PAGE gels. Activated MAPKs were measured by immunoblotting with an α-pErk1/2 antibody (Cell Signaling, product number 9101, USA) and a secondary antibody, goat anti-rabbit IgG-HRP (Santa Cruz, product number sc-2004).

2.17 | Statistical analysis

Two-tailed Student's t-test and One-way ANOVA followed with Kruskal-Wallis test for multiple comparisons were conducted using Prism 8. All experiments were repeated independently two or three times.

3 | RESULTS

3.1 | Chitin extraction and characterization from *P. brassicae*

The resting spores of *P. brassicae* contain chitin in their cell walls (Moxham et al., 1983). Through WGA488 staining, not only the resting spores, but also the primary plasmodia, zoosporagia and secondary plasmodia contain chitin (Figure 1a and Figure S1), the results indicated that different lifecycle stages of *P. brassicae*rich in chitin. Crudely extracted chitin was subjected to physicochemical evaluation based on FTIR spectrum, XRD and TGA techniques (Figure 1b, c). Figure 1b shows the FTIR spectrum of chitin of *P. brassicae* and commercial chitin (α-chitin), the two bands of around 1662 cm^{-1} and 1631 cm^{-1}indicated the presences of amide I and amide II groups, which conformed that the *P. brassicae* chitin was in α-form (Jang et al., 2004).

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Figure1C visualizes the XRD spectra from *P. brassicae* , there was a main diffraction peak of chitin around 19.2°, and a main diffraction peak around 26.3° was also a small weak diffraction peak, which was consistent with the crystal structure of chitin. However, unlike commercial α-chitin, the chitin diffraction peaks in the crudely extracted of *P. brassicae* were less pronounced, which may be related to the different sources of chitin. TGA analysis as shown in Figure 1d, the first and second weight loss results for different chitin were very similar. However, in the third stage, the weight loss results of chitin were different. The weight-loss rate of chitin of *P. brassicae* was very slow, and the reason was not clear. It is speculated that the two sources of chitin may be different, or that the chitin of*P. brassicae* is a crude extraction, and it contained other impurities that could not be decomposed. We next used chitinase (from*Streptomyces griseus*) to examine the *P. brassicae* contain chitin, treated resting spores of *P. brassicae* with chitinase for 3 days, the number of resting spores were significantly decreased of 85.90% (Figure 1e). These results suggested that chitin is the functional component of *P. brassicae.*

3.2 | Identification and functional analysis of chitinase PbChia1 from *P. brassicae* genome ZJ-1

Chitinases are believed to function as a guardian against chitin containing pathogens. By using Blast2GO to systemically investigate chitinases in the genome of *P. brassicae* ZJ-1, we identified 17 genes encoding putative chitinases (Table S1). These chitinase genes were predicted to encode 275 to 1968 amino acids in length, with putative molecular weights ranging from 29.3 kDa to 209.5 kDa and pIs ranging from 4.66 to 8.89. The subcellular localization was predicated to be in the mitochondria, cytoplasma or secretory. 16 chitinases belonged to GH18 family, and 1 chitinase belonged to the GH19 family. Transcriptome analysis of 17 chitinase genes showed different expression patterns (Figure 2a), suggested that they may have functional differentiation. Phylogenetic tree analysis showed that 13 chitinases were closely related to plant-derived chitinases and only 2 chitinases are closely related to fungal-derived chitinases (Figure S2). Using LC-MS/MS method to screened functional chitinase from *P. brassicae,* 15 chitinases were screened from clubroot galls of rapeseed (Table S2). Only one chitinase was derived from *P. brassicae* , gene number was PlasB_10400 and named *PbChia1* (Table S2). The full-length cDNA of *PbChia1* contained an open reading frame of 1203 nucleotides encoding a peptide of 400 amino acid residues with the predicated molecular mass of 44.3 kD. The protein contains a signal peptide and typical GH18_chitinase domain (Figure 2b). Yeast invertase assay showed that PbChia1 harbors a functional N-terminal signal peptide (Figure 2c). We next examined the subcellular localization of PbChia1-YFP fusion proteins with (PbChia1-YFP) and without signal peptide (PbChia1$^{\Delta\Sigma\Pi}$-YFP) in plant cells. PbChia1-YFP and PbChia1$^{\Delta\Sigma\Pi}$-YFP were transiently expressed in leaves of*N. benthamiana* . In cells expressing PbChia1-YFP, the yellow fluorescence signal was observed in the plasma membrane, cytoplasm and nucleus, while in cells expressing PbChia1$^{\Delta\Sigma\Pi}$-YFP, the yellow fluorescence signal was mainly concentrated in the nucleus (Figure S3a). Then, apoplast washing fluid was extracted, and Western blotting indicated that PbChia1 but not PbChia1$^{\Delta\Sigma\Pi}$ was detected in the apoplast washing fluid (Figure S3b), suggesting that PbChia1 is a secreted protein. qPCR shows*PbChia1* constitutive expression level in different lifecycle stages, and these results consistent with transcriptome data (Figure S3c and Figure 2a). We next expressed PbChia1 protein and tested whether it had chitinase activity. The sequences encoding without signal peptide of PbChia1 were fused with His and GST, respectively. Two recombinant proteins were produced in *Escherichia coli.* PbChia1-His and PbChia1-GST were purified and then tested for their chitinase ability by examining its enzymatic activity using colloidal chitin as the substrate, PbChia1-His and PbChia1-GST had ability to bind chitin, respectively (Figure 2d, e). Besides, they could effectively hydrolyze chitin into monomeric GlcNAc (Figure 2f, g), both of them shown highest enzyme activity at 37 °C and decreased rapidly at 20°C or 50°C. These results indicated that PbChia1 is a functional chitinase.

3.3 | PbChia1 inhibited *P. brassicae* resting spore germination and reduced pathogenicity of *P. brassicae.*

To examine the inhibitory effect of chitinase, resting spores of*P. brassicae* incubated with purified PbChia1 protein for 3 days. The number of resting spores was significant decreased (Figure 3a), on the third day, the level reduced by 71.20% (Figure 3a). We further checked germination of *P. brassicae* resting spores, nuclear

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status was examined with DAPI staining at 3 days (Figure S4 and Figure 3b). The germination rate of *P. brassicae* resting spores (without a nucleus) with PbChia1 and chitinase treatment was 46.30% and 42.00%, respectively. While the germination rate was 68.20% in the control group. The germination inhibition rate of PbChia1 was 32.10% (Figure 3b), indicating that PbChia1 protein can significantly reduce the germination of *P. brassicae* resting spores *in vitro* . In order to explore the effect of PbChia1 protein on the development and pathogenic process of clubroot, host plants were grown in liquid nutrient solution and co-inoculated with resting spores of *P. brassicae* and PbChia1 protein. Rapeseed inoculated with resting spores only and with GST protein shown galls on the roots, while co-inoculated with resting spores and PbChia1 or chitinase protein did not form obvious galls on the roots (Figure 3c). The disease index of inoculated with resting spores only and with GST protein was 73 and 72.32, respectively (Figure 3d), while of co-inoculated with resting spores and PbChia1 or chitinase was 28.1 and 33.3, respectively (Figure 3d). The biocontrol effect of PbChia1 was 61.29%, suggesting that the use of PbChia1 protein in vitro alleviates the symptoms of clubroot. The root slices and content of *P. brassicae* in root cells shown PbChia1 protein significantly reduce the pathogens inside the hosts (Figure S5). qPCR indicated that relative accumulation amount of *P. brassicae* in the roots of PbChia1 protein treatment was lower than that of GST treatment (Figure 3e). Taken together, PbChia1 could degrade the concentration of resting spores of *P. brassicae,* reduce the germination rate of the resting spores of *P. brassicae* , reduce the content of pathogen and relieve the clubroot symptom *in vitro* . The results indicated that chitin may play important roles during clubroot development.

3.4 | Overexpression of *PbChia1* in Arabidopsis increase resistance to *P. brassicae*

To characterize PbChia1 biological functions, we generated stable transgenic *A. thaliana* lines constitutively expressing the full length of *PbChia1* under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter. The phenotypes of 3 independent homozygous*A. thaliana* lines (L2, L3 and L5) and Col-0 plants was similar, such as morphology of leaves, root hair growth and seeds yield (Figure S6). Next, we analyzed whether the over expression of*PbChia1* is accompanied by changes in susceptibility to *P. brassicae* . We observed that Col-0 and empty vector *A. thaliana*plants infected with *P. brassicae* for 21 days formed club-shaped galls on the roots ((Figure 4a), transgenic *A. thaliana* lines had no obvious symptoms of swelling (Figure 4a). The disease index of*PbChia1* transgenic plant was much lower than Col-0 and empty vector plants (Figure 4b), qPCR conformed that the density of *P. brassicae* in the infected roots of Col-0 and empty vector was less than that in *PbChia1* transgenic lines (Figure 4c). Young plants may be killed by the clubroot disease within a short time after infection, we examined the survival rate of the infected plants at 30 days and 55 days after inoculation. The average survival rate of transgenic plant was 3.13 and 3.96 folds than that of Col-0 plants at 30 days and 55 days after inoculation, respectively (Figure 4d, e). The seeds of surviving plants were harvested and weighed. The weight per plant of transgenic plant was about 2.15 folds than that of Col-0 plants (Figure 4f, g). Taken together, these results suggested that *PbChia1* transgenic plants strong enhance the disease resistance to *P. brassicae,* it can relieve the severity of clubroot symptoms, increase the survival rate of infected plants and seeds yields.

The life cycle of *P. brassicae* consists of three stages: survival in soil, root hair infection, and cortical infection (Schwelm et al., 2015). To investigate the potential involvement of the transgenic lines in the *P. brassicae* infection process, we compared the root hair infection rate (form primary plasmodia and zoosporangia) and cortical infections rate (form secondary plasmodia) between the Col-0, EV and transgenic *PbChia1* plants (Figure 5a and 5b). The results suggested that both root hair infection and cortical infection rate decreased significantly compare Col-0 with transgenic *PbChia1* plants (Figure 5b). We further to observe the*P. brassicae* development state in the galls of the infected roots, the results of TEM shown that many early mature resting spores with typical nuclei and nucleoli were found in infected Col-0 and EV root cells (Figure 5c). While the secondary zoosporangia were observed on the infected roots of transgenic L3 plants (Figure 5c). These observations suggested that transgenic *PbChia1* plants may inhibited development of *P. brassicae* . In order to explore the disease resistance mechanism of transgenic PbChia1 plants, we measured the chitinase activity of Col-0 and transgenic *PbChia1* plants, the relative chitinase activity of*PbChia1* transgenic plants was 1.91 folds higher than that of Col-0 (Figure 5d). Besides, in *PbChia1* transgenic lines, plants with relatively high expression level of PbChia1 had mild symptoms (Figure S6). We hypothesis that *PbChia1* transgenic plants

increased chitinase activity to resistance to *P. brassicae* .

3.5 | PbChia1 alters certain host defense responses and resistance both biotrophic and necrotrophic pathogens

P. brassicae is obligatory and biotrophs that need to evade the plant immune response for an extended duration to complete their life cycle. As a first measure of detecting potential roles in defense modulation of PbChia1. Flagellin 22 (flg22) or chitin is a well characterized PAMP and has been known to induce PTI in plants when administered (Sun et al., 2015). We assessed transcript levels of a panel of known defense-related plant genes in the *PbChia1*transgenic lines. Early immune-related genes *FRK1, WRKY30, PP2C, NHL10, PHI1* and later immune-related gene *PR1* was selected for detection, all of them were significantly up-regulated compare Col-0 and*PbChia1* lines after flg22 treatment (Figure 6b). These findings confirm that PbChia1 is likely to alter certain defense responses. The expression level of these defense genes changes the resistance to*P. brassicae.* We next asked if PbChia1 activate or suppress general defense responses. To assess this possibility, we activated immune responses using flg22 and monitored reactive oxygen species (ROS) accumulation and activation of mitogen-activated protein kinase cascades (MAPKs) between Col-0 and *PbChia1* transgenic lines (Figure 6a, c). Our comparative analyses shown flg22 induced significantly increased ROS accumulation in the *PbChia1* transgenic lines compared to Col-0 (Figure 6a). The phosphorylation level of MAPKs was increased in the *PbChia1* transgenic lines compared to Col-0 (Figure 6c). Besides, we checked chitin trigged plant immunity, same with flg22 treatment, ROS accumulation, immune-related genes and MAPKs activation all increased in Col-0 than that of *PbChia1* transgenic lines (Figure S7). All these results suggested that overexpression of*PbChia1* in plants would enhance PAMP-triggered immunity.

The *PbChia1* transgenic plants had obvious resistance to the biotrophic pathogen *P. brassicae* . We further examined the disease resistance of *PbChia1* transgenic plants to other pathogens, including the bacterial pathogen *Pseudomonas syringae* pv. tomato (*Pst*) DC3000, the necrotrophic pathogen *Sclerotinia sclerotiorum* 1980 and *Rhizoctonia solani.* The disease symptoms were attenuated and *Pst* DC3000 multiplied much less in the transgenic lines than in Col-0 plants by *Pst-DC3000* infection (Figure 7a and 7b). Quantitative analysis of *B. cinerea* symptoms on transgenic *PbChia1* and non-transformed Col-0 leaves, transgenic plants formed smaller lesion than that of Col-0 (Figure 7c and 7d). The disease index of Col-0 inoculated with *R. solani* was 93.75, while that of L3 and L5 transgenic plants were 58.33 and 50.00, respectively (Figure 7e, f), indicating that *PbChia1* transgenic in Arabidopsis could enhance disease resistance to *R. solani* . The data presented above show that while PbChia1 alters host defense pathways, enhance plant immunity on a global scale, suggested that chitinase PbChia1 has a broad application prospect.

4 | DISCUSSION

4.1 | PbChia1 as a potential gene for clubroot breeding

Rapeseed is a major crop species that provides abundant amounts of oil to humans. Clubroot caused by the soil-living, obligate biotrophic protist *P. brassicae* is the most devastating diseases of cruciferous crops worldwide. The resting spores of *P. brassicae*can persist more than 17 years in an infested field without a host (Wallenhammar 1996). There is no effective method to eradication of*P. brassicae* from an infested field, but is controlled most effectively by the use of resistant cultivars. Advances in genetics, molecular biology techniques and 'omics' research have helped to identify and choose several major loci, QTL and resistance genes from the *Brassica* genomes for use in clubroot resistance breeding (Hasan et al., 2021). However, field population of *P. brassicae*generally exhibits diversity for virulence (Holtz et al., 2018; Sedaghatkish et al., 2018), Strelkov et al. (2018) reported a total of 17 pathotypes from canola fields in Canada. Clubroot resistance in some of the resistant germplasm and reported race-specific (Hirani et al., 2018), and population structure of this pathogen can also change in the infested field in a short time (Hasan et al., 2021). Repeated cultivation of cultivars in the clubroot-infested fields can result in resistance breakdown due to the emergence of new virulent pathotypes, rare pathotypes to multiply rapidly and pathogen population structure where the rare pathotypes have become predominant (Strelkov et al., 2016; Sedaghatkish et al., 2018; Cao et al., 2020). Choose resistance genes has broad-spectrum resistance to *P.* brassicae pathotypes is need for the development

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of clubroot resistant cultivars. Chitinases, a subgroup of pathogenesis-related (PR) proteins (Legrand et al., 1987), can catalyze the hydrolysis of chitin, the main component of the pathogen cell wall, thereby inhibiting fungal growth and improving plant's defense against fungi (Abeles et al., 1971; Zhang et al., 2016). The resting spore cell wall of *P. brassicae* had been reported to contain chitin (Moxham and Buczacki 1983). Here, we found that the chitin could be detected in the whole life cycle of *P. brassicae* through WGA488 staining (Figure 1a and Figure S1), indicating that the metabolism of chitin is very important in the growth and development of *P. brassicae* . The crudely extracted chitin from the resting spores was detected by FTIR, XRD and TGA, and the chitin of *P. brassicae* conformed to the characteristics of ∝-chitin (Figure 1b, c, d). Treatment with commercial chitinase could significantly reduce the number of *P. brassicae*(Figure 1e). These results suggested that chitin and metabolism-related enzymes can be used as a target for clubroot control.

Through genome-wide analysis, 17 chitinase genes were screened in the genome of *P. brassicae* ZJ-1 (Table S1, Figure 1e and Figure S2). Using LC-MS/MS technology, 15 chitinases were screened from the total protein of rapeseed roots with severe disease, and only one chitinase named PbChia1 was derived from *P. brassicae* (Table S2). We first characterization chitinase *PbChia1* from a protist, the biotrophic soilborne plant pathogen *P. brassicae* . PbChia1 was a secreted chitinase (Figure 2 and Figure S3), and expressed in all developmental stages of *P. brassicae* (Figure 2a and Figure S3c). Purified PbChia1 protein from *Escherichia coli* inhibit development of clubroot (Figure 3). Overexpressing the gene PbChia1 in *A. thaliana* exhibited significantly increased resistance to *P. brassicae* (Figure 4), inhibited the rate of root hair infection and cortical infection, suppressed the development of *P. brassicae*(Figure 5a, b), consistent with enhanced resistance to the pathogen, the chitinase activity of transgenic plants was significantly increased compared to the control plants, suggesting the antimicrobial property of the expressed chitinase in plants against the pathogen (Figure 5c). Similar results were also reported for antifungal activity of chitinase in enhancing resistance against other fungal and bacterial pathogens (Dana et al., 2006; Cletus et al., 2013; Zhang et al., 2016; Yang et al., 2020). We also observed that higher expression level of PbChia1 shown more resistance to *P. brassicae* and chitinases were not toxic to plant hosts (Figure S6 and S7). These results suggested that PbChia1 as a potential gene for clubroot breeding.

4.2 | Regulation of chitinase PbChai1 in *P. brassicae*

PbChia1 transgenic plants could significantly improve plant resistance to *Pst* DC3000, the necrotrophic fungi *S. sclerotiorum* 1980 and *R. solani* (Figure 7). These results indicated that the *PbChia1* transgenic plants had acquired a wide range of disease resistance. In order to further explore the disease resistance mechanism of *PbChia1* transgenic plants, the plants were treated with the PAMPs, flg22 and chitin, which can induce innate immune responses in plants. *PbChia1* transgenic plants could enhance the burst of ROS, activation of MAPKs and expression levels of marker genes related to immunity (Figure 6 and Figure S8). These results demonstrate that *PbChia1* overexpression increased the accumulation of ROS and triggered disease defense-related mechanisms in plants. The results are consistent with the findings of Yang et al., who observed transgenic plants expressing chitinase in soybean (Yang et al., 2020). The chitinases play a dual role, both by inhibiting fungal growth by cell wall digestion and by releasing pathogen-borne elicitors that induce further defense reactions in the host (Dana et al., 2006). In protist *P. brassicae,* chitin is the functional component, degradation of chitin maybe physiologically crucial for normal growth and development, but it is not yet possible to knock out *PbChia1*to verify its function because of the parasitic nature. We hypothesis is that PbChia1 degrade chitin surfaces to soluble N-acetyl glucosamine oligosaccharides that can be utilized as a carbon source, decompose chitin or N-acetyl glucosamine oligosaccharides is very likely accompanied by the triggering of other defense-related mechanisms, that why *PbChia1* transgenic plants enhanced resistance to *P. brassicae* , *Pst* DC3000, *S. sclerotiorum* and *R. solani.* It's not clear these defense-related mechanisms are activated is directly or indirectly.

4.3 | How did *P. brassicae* suppress chitin-triggered immunity

P. brassicae is able to infection susceptible hosts form galls suppressing pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). Chitinase cleave the chitin randomly and hence generates different

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chitin oligosaccharides, are recognized by cell surface LysM receptors LYK5 and CERK1, activating PTI (Cao et al., 2014; Sánchez-Vallet et al., 2015). To escape chitin-triggered immunity, fungal pathogens have developed several strategies. *Magnaporthe oryzae* secretes a LysM-containing effector protein, to compete with OsCEBiP for chitin binding, thereby preventing the activation of PTI for rice blast disease (Mentlak et al., 2012). Effector protein Ecp6 in*Cladosporium fulvum* sequesters fungal chitin oligomers to avoid chitin triggering immunity in the host plant (deJonge et al., 2010). The pathogen secreted of chitin binding effectors to escape chitin-triggered immunity, not only capture free chitin oligosaccharides but also act as a shield to protect pathogens from plant chitinases (Marshall et al., 2011). A recent reported that two CBM18 proteins of *P. brassicae*PbChiB2 and PbChiB4 act as effectors for protecting the clubroot pathogen and for suppressing chitin-triggered immunity during infection (Muirhead et al., 2022). In soil-borne fungus *Verticillium dahliae,* utilize an enzymatically active polysaccharide deacetylase, effectively converts chitin oligomers into chitosan, to prevent the activation of the chitin-triggered immune response during root infection (Gao et al., 2019). How did protist *P. brassicae* overcome host immunity to successful colonize the root, by avoiding the activation of, or directly suppressing PTI? Therefore, we plan to study the effectors functions in future work to answer this question.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure legends

FIGURE 1 Characteristics of chitin extracted from *P. brassica* . (a) Cross section of galls on the roots of rapeseed, stained with WGA488 and take images on confocal microscopy. Bar =10 μm. (b) FTIR spectrum of commercially available alpha chitin and chitin extract from *P. brassicae* . (c) XRD spectrum of commercial alpha chitin and chitin extract from *P. brassicae* . (d) TGA thermograms of commercial alpha chitin and chitin extract from *P. brassicae* . (e) Chitinase treated with of *P. brassicae* resting spores of *P. brassicae* . The resting spores of *P. brassicae* were treated with commercial chitinase for 3 days at room temperature,

the number of resting spores of *P. brassicae* was counted. Statistical analysis was performed by Student's t-test (two-tailed). Data are mean ± s.d. n = 3 biological replicates. Experiments were repeated three times with similar results.

FIGURE 2 PbChia1 is a chitinase. (a) Heatmap visualization of 17 predicted chitinase genes in different life stage of *P. brassicae*. A colored bar indicating normalized reads per log10 (FPKM) accompanies in the expression profile during three life stages. IN indicated for cortical infection, PZ indicated for primary zoospores and RS indicated for resting spores. Genes and samples were clustered according to their expression profiles. (b) Schematic illustrations of PbChia1. (c) PbChia1 contains a functional signal peptide. Using a yeast signal-sequence trap system, culture filtrate of yeast expressing the plasmid pSUC2-SPPbChia1, which expresses the PbChia1 signal peptide under control of the pSUC2 promoter, produces a red color similar to that of the positive control of yeast expressing pSUC2-SPAvr1b, which contains the signal peptide from the *Phytophthora sojae* effector Avr1b. Yeast expressing the empty pSUC2 vector was used as a negative control. (d) and (e) Chitinase activity of PbChia1. PbChia1 was expressed as a His tag fusion protein in pET28a (d), was also expressed as a GST fusion protein in vector pGEX-6P1, GST alone served as a control (e). The chitinase enzyme activity of affinity-purified His-PbChia1, GST- PbChia1 and GST protein was analyzed, using chitin as substrate. The reaction of DNS (3,5-dinitrosalicylic acid) and GlcNAc was monitored at OD 565 nm. One unit of chitinase activity was defined as the amount of enzyme required to release 1 mmol of N-acetyl-D-glucosamine per hour. Data are mean ± s.d. (f) and (g) Affinity-purified His-PbChia1 (f) and GST- PbChia1 (g) protein have ability to bind chitin. Purified recombinant proteins were incubated with colloid chitin for 1 h with constant shaking, and the chitin-associated His-PbChia1 (f) and GST- PbChia1 (g) was detected using immunoblotting.

FIGURE 3 Purified PbChia1 protein treatment of *P. brassicae* . (a) The concentration of resting spores of *P. brassicae* were treated with 0.3 mg/mL PbChia1 protein for 3 days, resting spores of *P. brassicae* was counted by a hemocytometer. (b) Germination rate of resting spores of *P. brassicae* after incubation with PbChia1 treatment for 3 days. Resting spores of *P. brassicae* stained with DAPI and the nucleus were observed under UV excitation. (c) and (d) Gall phenotype of rapeseed infected with *P. brassicae* for 30 days in different treatments. c shows the phenotype of the whole plants, bar = 1cm. d shows the root phenotype, bar = 1mm. (e) Phytopathological analysis of rapeseed inoculated with *P. brassicae* and PbChia1 protein. Percentages of plants in the individual disease classes are shown. For each treatment, 24 to 28 rapeseed plants were analyzed. (f) Pathogen DNA content quantification by qPCR, rapeseed actin gene as reference gene, and the actin gene of *P. brassicae* is used as the index of the content of *P. brassicae* . In a, b and f, One-way ANOVA with Kruskal-Wallis test. Data are mean ± s.d. n = 3 biological replicates, different letters indicate significant differences ($P< 0.05$). All experiments in this figure were repeated three times with similar results.

FIGURE 4 Overexpression of *PbChia1* in Arabidopsis increase resistance to *P. brassicae* . (a) Galls phenotype of overexpression*PbChia1* transgenic plants inoculated with *P. brassicae* for 21 days, scale bar=1 cm. (b) Phytopathological analysis of Col-0 and transgenic plants. The percentages of plants in the individual disease classes are shown. For each treatment, 40 Arabidopsis plants were analyzed. (c) qPCR examined pathogen content of galls of Col-0 and transgenic plants infected by *P. brassicae* . Statistical analysis was performed by one-way ANOVA with Tukey's test (significance set at$P$ [?] 0.05). Different letters (a, b and c) are significantly different. n = 4 biological replicates; data are shown as mean +- s.d. (d) Phenotype of Col-0 and transgenic plants inoculation with *P. brassicae* for 30 days, each flat grown 40 plants, the seeds of survival plants were harvested (e). (f) Survival rate of Col-0 and PbChia1 transgenic plants infected with *P. brassicae* for 30 days and 55 days. 40 plants were analyzed. (g) The weight of seeds of Col-0 and transgenic plants inoculation with *P. brassicae* . Statistical analysis was performed by one-way ANOVA with Tukey's test (significance set at P [?] 0.05). Different letters are significantly different. n = 3 biological replicates; data are shown as mean +- s.d. All experiments in this figure were repeated three times with similar results.

FIGURE 5 Overexpression of *PbChia1* in Arabidopsis inhibited*P. brassicae* development. (a) The images show the key steps of*P. brassicae* infection in *PbChia1* transgenic lines and control plants, including root hair

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and cortical infections stages. Primary plasmodia of root hair infection were observed in the roots stained with trypan blue for 3 days post inoculation. Zoosporangia of root hair infection and secondary plasmodia of cortical infection were observed in the roots stained with trypan blue for12 days post inoculation. The pictures were taken under light microscopy. Scale bar=10 μm. (b) Colonization rate (100%) of primary plasmodia, zoosporangia and secondary plasmodia per root of *PbChia1* transgenic lines and control plants. The roots of more than 15 plants were selected and sliced into 1–2 cm segments. A total of approximately 100 root segments per sample were observed and counted to determine the presence of infection. Statistical analysis was performed by one-way ANOVA with Tukey's test (significance set at P [?] 0.05). Colonization rate indicated by different letters are significantly different. n = 3 biological replicates; data are shown as mean ± s.d. (c) Transgenic plants and control plants were inoculated with *P. brassicae* for 21 days, and the developmental status of *P. brassicae* was observed by transmission electron microscope. CW = plant cell wall; Nu = nucleus; N = nucleus without nucleolus; scale bar=2 μm. (d) Determination of relative chitinase activity of *PbChia1* transgenic lines and control plants, statistical analysis was performed by one-way ANOVA with Kruskal-Wallis test (significance set at P[?] 0.05), different letters shown significantly different, n = 3 biological replicates; data are shown as mean ± s.d.

FIGURE 6 Flg22-induced immune response in *PbChai1* transgenic lines. (a) Flg22-induced ROS burst is increased in *PbChai1* overexpression lines. 10-day-old seedings grown in MS plates were treated with 100 nM flg22 and the values represent means ± s.d (n=24 biological replicates). (b)-(f) Immune response related genes were induction of in *PbChai1* transgenic lines. 10-day-old seedings grown in MS plates were treated with 20 μg/mL chitin for 0.5h-2h. Data were normalized to *Arabiodopsis* actin gene (AT3G18780) expression in qPCR analysis. Statistical analysis in b to f were performed by one-way ANOVA with Kruskal-Wallis test (significance set at P [?] 0.05). Different letters (a, b and c) are significantly different. n = 3 biological replicates; data are shown as mean ± s.d. All experiments were repeated three times with similar results. (g) Activation of MAPK triggered by flg22. 10-day-old seedings grown in MS plates were treated with 100 nM flg22 for 0 min, 5 min, 15 min and 45 min, respectively. Experiments were repeated two times with similar results.

FIGURE 7 *PbChia1* transgenic lines increased disease resistance to *Pst* DC3000, *S. sclerotiorum* and *R. solani*. (a) Phenotype of *PbChia1* transgenic lines and control plants inoculated with *Pst* DC3000 at 1×10^6 cfu ml^{-1} for 4 days. (b) Bacterial populations in *PbChia1* transgenic lines and control plants on day 0 and day 5 after leaf infiltration with $1x10^6$ cfu ml^{-1}. Statistical analysis was performed by one-way ANOVA with Tukey's test (significance set at P[?] 0.05). Bacterial populations indicated by different letters are significantly different. n = 4 biological replicates, data are shown as mean +- s.d. (c) Phenotype of *PbChia1* transgenic lines and control plants inoculated with *S. sclerotiorum* 1980 for 30 h. (d) Statistics of lesion area of *PbChia1* transgenic lines inoculated with *S. sclerotiorum* 1980 for 30 h. n = 10 biological replicates, data are shown as mean +- s.d. One-way ANOVA with Tukey's test were analyzed differences, same letters show no significant difference, P [?] 0.05. (e) Phenotype of *PbChia1* transgenic lines and control plants inoculated with *R. solani* for 3 days. (f) Disease index statistics of *PbChia1* transgenic lines and control plants with *R. solani* for 3 days, n = 12 biological replicates, data are shown as mean +- s.d. One-way ANOVA with Kruskal-Wallis test were analyzed differences, same letters show no significant difference, P [?] 0.05.

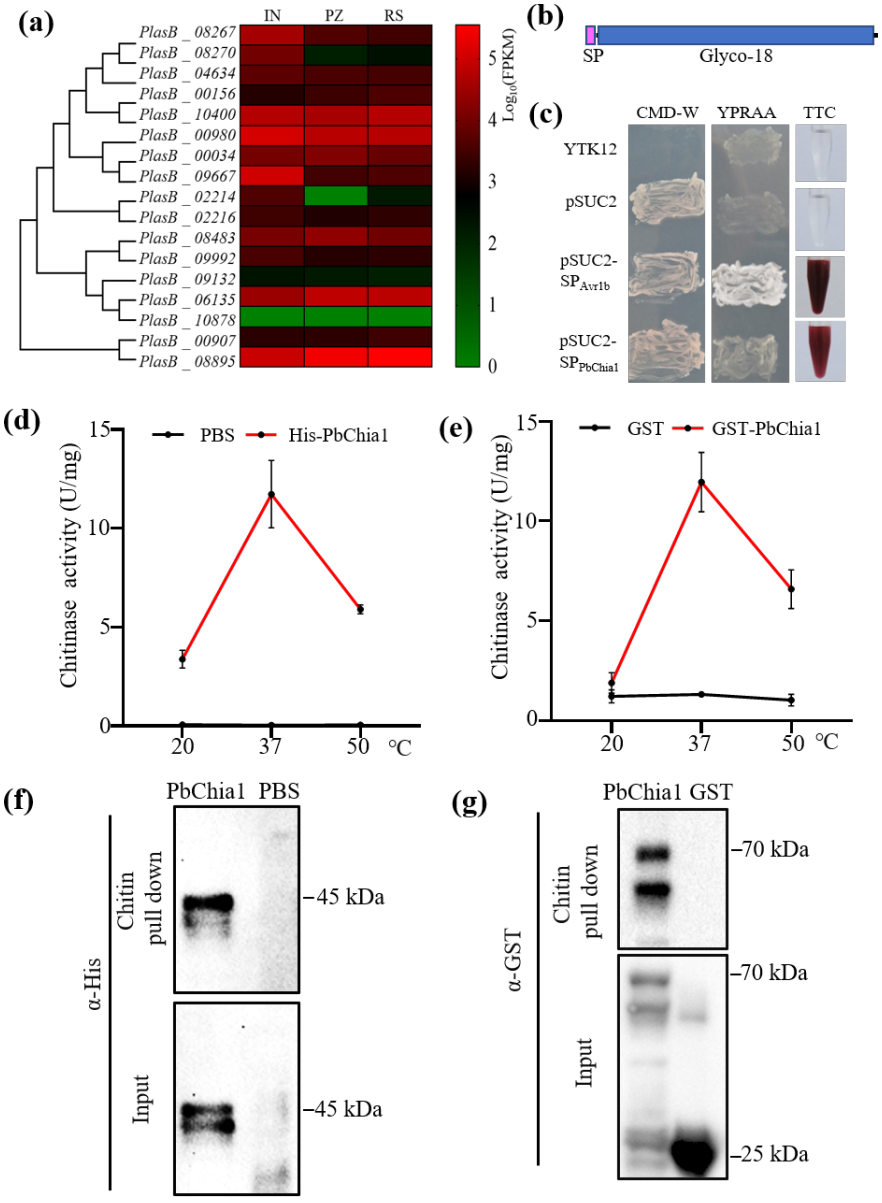
Supplement information:

Figure S1. *P. brassicae* were stained by WGA488. (a) and (b) Two-week-old Col-0 inoculated with *P. brassicae* for 2 days, roots were stained with WGA488, primary zoospores of *P. brassicae* penetrated the cell wall and forms primary plasmodia in the root hairs (a), scale bar, 20 μm. Primary zoospores reached the surface of root and root hairs (b), scale bar, 100 μm. (c), Two-week-old Col-0 inoculated with *P. brassicae* for 12 days, roots were stained with WGA488, primary zoospores or secondary zoospores penetrated root cortex cells, in which the pathogen develops into secondary plasmodia, scale bar, 50 μm.

Figure S2. Phylogenetic analysis of the relationships among chitinases from different species. The phylogenetic tree was constructed using the neighbor-joining method (MEGA6.0) and Poisson method was used to perform phylogenetic analysis with 1,000 bootstrap replicates. All chitinase sequences were obtained from

GenBank, and the accession numbers of chitinase were listed in the form of "CAF1252575". The star indicates PbChia1. Sequences from fungi are indicated in purple; bacterial sequences are black; sequences from insects are pink; oomycete sequences are light blue; plant sequences are indicated in green; and *P. brassicae* sequences are red.

Figure S3. Subcellular localization and gene expression pattern of PbChia1. (a) Subcellular localization of the YFP-tagged PbChia1 and PbChia1^{SP} in *N. benthamiana* leaves. PbChia1 and PbChia1^{SP} was expressed as a fusion protein with YFP under the control of the 35S, respectively. The pla



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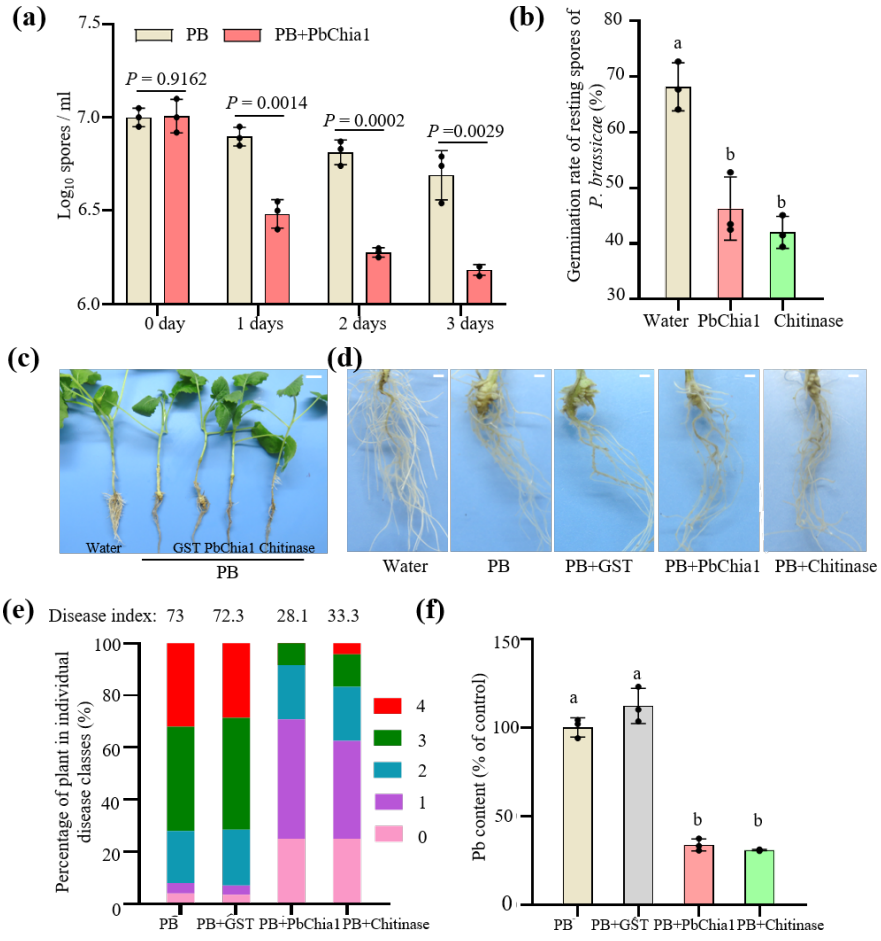

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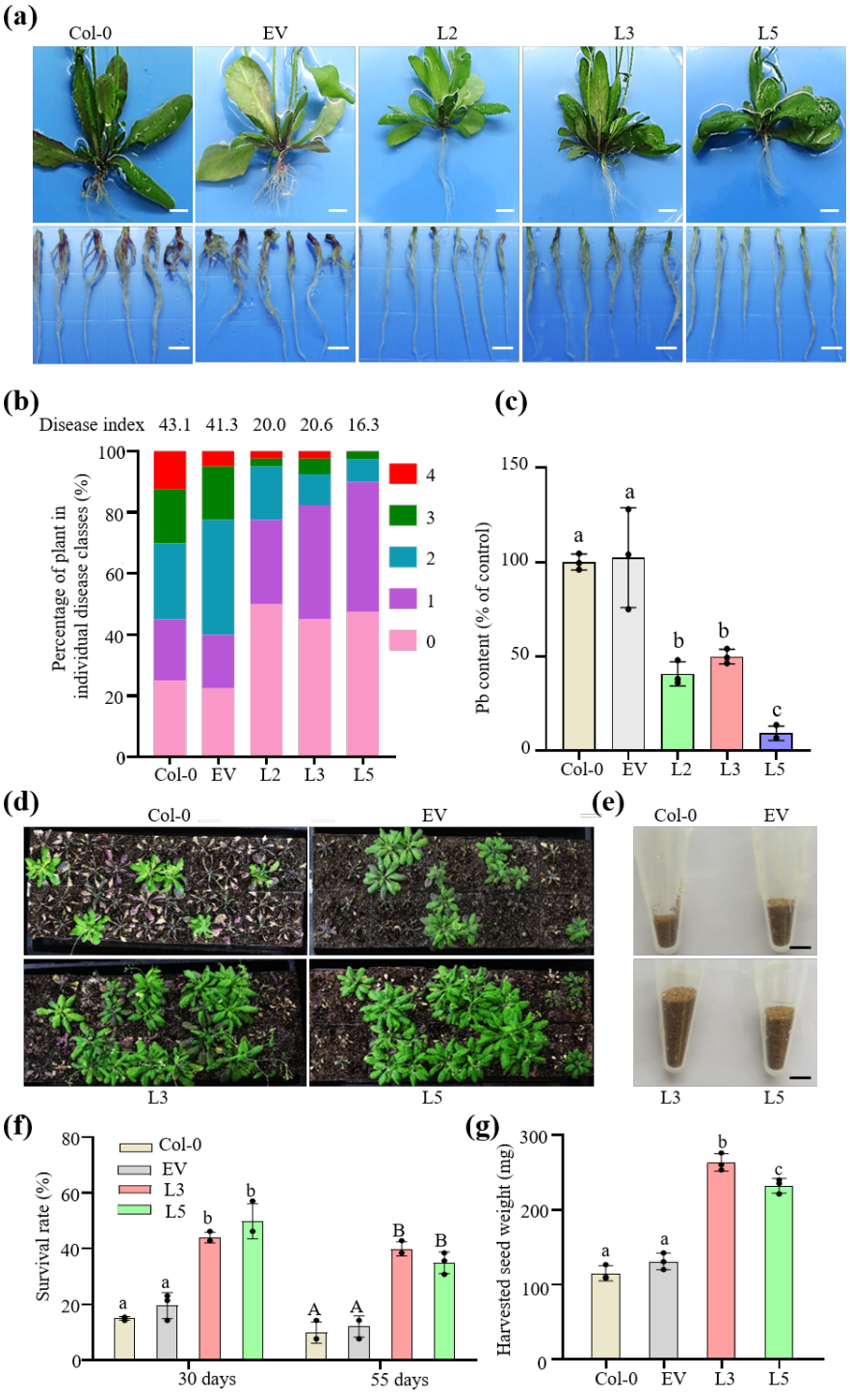

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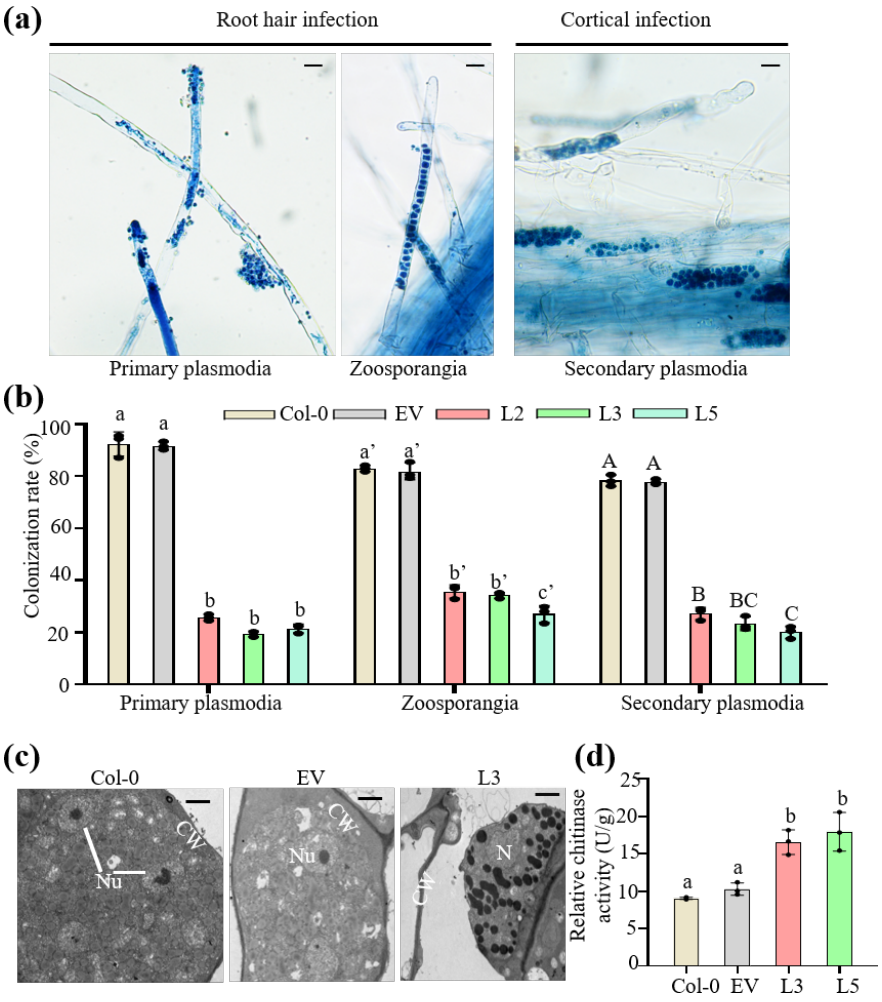

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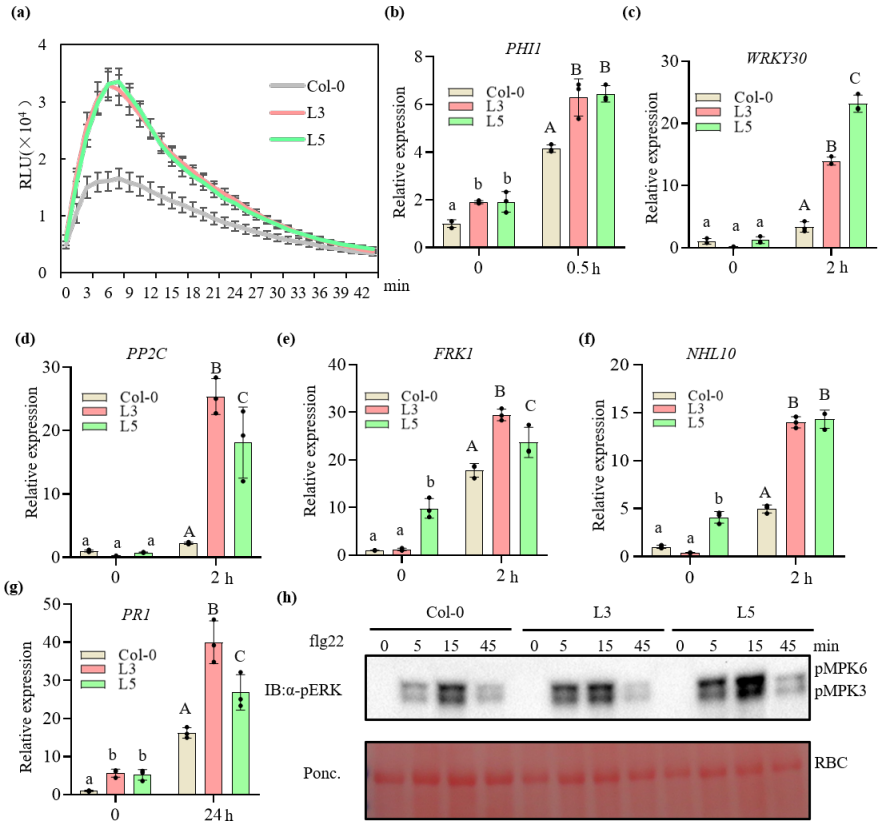

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