Post-print: Alkaloid Biosynthesis Mechanism in Isatis indigotica Under Clubroot Pathogen Infection

Authors: Zhao Shuli, Li Guodong, Zhang Liqin, Zhao Mingzhi, Jianlian Shi, Liu Jiajia

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Abstract

To investigate the effects of clubroot pathogen stress on alkaloids and the expression of key enzyme genes involved in their synthesis in Isatis indigotica, this study performed disease morphological grading, histological observation, physiological and biochemical index determination, as well as transcriptomic and metabolomic analyses on I. indigotica at 0, 7, 14, and 21 days post-infection with the pathogen. The results demonstrated that: (1) At 0, 7, 14, and 21 days post-inoculation, the roots of I. indigotica developed into grade 0, 1, 3, and 5 galls, respectively, with day 7 being the critical time point for cortex invasion. (2) At 14 days post-inoculation with the clubroot pathogen, the contents of soluble protein and malondialdehyde, as well as the activities of superoxide dismutase, peroxidase, polyphenol oxidase, and catalase in I. indigotica leaves were significantly elevated compared with the control group at the same time point, exhibiting an increasing trend with prolonged inoculation duration. (3) Metabolomics analysis detected a total of 161 alkaloids, among which indole alkaloids were predominant; compared with the non-inoculated control, there were 16, 17, and 39 differential metabolites at 7, 14, and 21 days post-inoculation, respectively, and the differential metabolites in each group were primarily enriched in alkaloid and amino acid metabolic pathways. (4) Transcriptome sequencing results revealed that: compared with the non-inoculated control, there were 2,439, 256, and 6,437 differentially expressed genes at 7, 14, and 21 days post-inoculation, respectively, and these three groups were commonly enriched in 11 alkaloid-related metabolic pathways; compared with the non-inoculated control, nine genes (encoding four enzymes: THS, TAT, YUCCA, and ALDH) showed consistently upregulated expression at 7, 14, and 21 days post-inoculation. These findings elucidate the interaction mechanism between Plasmodiophora brassicae and I. indigotica, explore the effects of the clubroot pathogen on indole alkaloid

synthesis and its key enzyme genes, and establish a foundation for future research on clubroot resistance genes and alkaloid secondary metabolic pathways in I. indigotica.

Full Text

Preamble

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Mechanism of Alkaloid Synthesis in *Isatis indigotica* Infected by *Plasmodiophora brassicae*

Shuli Zhao[1], Guodong Li[1], Liqin Zhang[2], Mingzhi Zhao[3], Jianlian Shi[3], Jiajia Liu[1]*

[1] Yunnan University of Chinese Medicine, Kunming 650500, China
[2] Institute of Horticultural Research, Yunnan Academy of Agricultural Sciences, Kunming 650205, China
[3] Haiyuan College, Kunming Medical University, Kunming 650101, China

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Abstract

To investigate the effects of *Plasmodiophora brassicae* stress on alkaloid content and the expression of key biosynthetic enzyme genes in *Isatis indigotica*, we conducted disease severity grading based on morphology, histological observation, physiological and biochemical assays, transcriptomic analysis, and metabolomic analysis at 0, 7, 14, and 21 days post-inoculation. The results demonstrated: (1) Root swelling developed to grades 0, 1, 3, and 5 at 0, 7, 14, and 21 days post-inoculation, respectively, with day 7 representing the critical time point for cortical invasion. (2) At 14 days post-inoculation, soluble protein and malondialdehyde (MDA) contents, as well as superoxide dismutase (SOD), peroxidase (POD), polyphenol oxidase (PPO), and catalase (CAT) activities in leaves increased significantly compared to the control group at the same time point, showing a time-dependent increasing trend. (3) Metabolomic analysis detected 161 alkaloids, with indole alkaloids being the most abundant. Compared to the uninoculated control, 16, 17, and 39 differential metabolites were identified at 7, 14, and 21 days post-inoculation, respectively, with most differential metabolites enriched in alkaloid and amino acid metabolic pathways. (4) Transcriptome sequencing revealed 2,439, 256, and 6,437 differentially expressed genes (DEGs) at 7, 14, and 21 days post-inoculation compared to the control, with 11 alkaloid-related metabolic pathways commonly enriched across all three time points. Notably, nine genes encoding four enzymes (THS, TAT, YUCCA, and ALDH) showed consistently upregulated expression at 7, 14, and 21 days post-inoculation. These findings elucidate the interaction mechanism between *P. brassicae* and *I. indigotica*, reveal the effects of *P. brassicae* on indole alkaloid synthesis and key enzyme gene expression, and provide a foundation for

future research on clubroot resistance genes and alkaloid secondary metabolic pathways in *I. indigotica*.

Keywords: *Isatidis Radix*; *Plasmodiophora brassicae*; antioxidant enzymes; functional genes; indole alkaloids; metabolome

Introduction

Isatis indigotica, a biennial plant belonging to the Brassicaceae family, is widely cultivated throughout China and holds significant economic and medicinal value. Its dried roots and leaves are the source of the renowned traditional Chinese medicines Banlangen (Isatidis Radix) and Daqingye (Isatidis Folium), which are representative drugs for clearing heat and detoxification (Wong et al., 2022). The primary bioactive compounds in *I. indigotica* are alkaloids with antiviral, antibacterial, and immunomodulatory pharmacological activities, among which indole alkaloids have been most frequently reported. Confirmed bioactive indole alkaloids include indigo, indirubin, epigoitrin, 5-hydroxyindole, indole-3-carboxaldehyde, and tryptanthrin, which exhibit antiviral, antitumor, leukocyte inhibitory, and antibacterial activities (Sinha et al., 2008; Chen et al., 2021; Yang et al., 2021). The Chinese Pharmacopoeia specifies epigoitrin and indirubin as important quality control markers for Banlangen and Daqingye preparations (Chinese Pharmacopoeia, 2020).

Clubroot disease in Brassicaceae, commonly known as "big root disease," is caused by the soil-borne obligate parasite *Plasmodiophora brassicae*. This pathogen specifically infects the roots of cruciferous crops including *I. indigotica*, cabbage, rapeseed, radish, and mustard. The infection process typically begins with invasion of root hairs, followed by production of secondary zoospores that invade cortical tissues, causing cortical cell enlargement and deformation. During later infection stages, resting spores form within cells, resulting in tumor-like root swelling, retarded growth, and wilting. Clubroot infection also induces host defense responses, including massive reactive oxygen species (ROS) production that promotes membrane lipid peroxidation and damages cell membrane structure. Defense enzymes such as catalase (CAT), polyphenol oxidase (PPO), superoxide dismutase (SOD), and peroxidase (POD) primarily participate in ROS scavenging and maintenance of cell membrane stability (Qi et al., 2020; Huang et al., 2022; Wang et al., 2022). Qin (2021) reported that CAT, SOD, POD activities and soluble protein content in clubroot-resistant Chinese cabbage DH40R increased significantly at 8 days post-inoculation, conferring strong resistance against *P. brassicae* infection. Zhu et al. (2015) demonstrated that soluble sugars, soluble protein, and MDA serve as primary indicators for screening clubroot-resistant varieties. Guo (2018) found that CAT correlates with disease resistance in rapeseed, while SOD and POD show positive correlations with resistance.

Current research on *P. brassicae* infection in Brassicaceae primarily focuses on

Brassica species, with limited studies on *Arabidopsis*, and virtually no reports on defense mechanisms or metabolic changes in *I. indigotica* upon clubroot infection. Therefore, investigating the interaction mechanisms between *P. brassicae* and *I. indigotica* and the pathogen's effects on accumulation of active compounds is urgently needed. This study examined *I. indigotica* at 0, 7, 14, and 21 days post-inoculation using Illumina high-throughput sequencing and ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Through disease severity grading, histological observation, physiological and biochemical assays, and integrated transcriptomic and metabolomic analyses, we addressed three key questions: (1) the dynamic process of *P. brassicae* infection in *I. indigotica*; (2) the physiological defense mechanisms of *I. indigotica* against clubroot disease; and (3) the effects of *P. brassicae* stress on alkaloid compound biosynthesis in *I. indigotica*. This research establishes a foundation for molecular studies on clubroot resistance genes and secondary metabolite biosynthetic pathways in *I. indigotica*.

Materials and Methods

1.1 Materials

Pathogen and plant material: The *P. brassicae* strain was isolated from clubroot galls on diseased Chinese cabbage collected from Aziying, Panlong District, Kunming, and identified as physiological race 4 using Williams' classification system by Yunnan Academy of Agricultural Sciences. Seeds of small-leaf *I. indigotica* were provided and authenticated by Dr. Liqin Zhang from the Cruciferous Crops Research Group at the Institute of Horticultural Research, Yunnan Academy of Agricultural Sciences.

Reagents and instruments: Trypan blue solution (Sigma), formalin-acetic acid-alcohol fixative (FAA, 50%); bovine serum albumin, riboflavin, guaiacol, 2-thiobarbituric acid (Beijing Solarbio Science & Technology); catechol, trichloroacetic acid, L-methionine (Shanghai Macklin Biochemical); nitrotetrazolium blue chloride (NBT), ethylenediaminetetraacetic acid (EDTA) (German Biofroxx); 30% hydrogen peroxide (Yunnan Jingrui Technology). Instruments included Nikon digital microscope, hemocytometer, beakers, centrifuge tubes, volumetric flasks, measuring cylinders, seedling trays, mortars, pestles, cuvettes, Puxi TU-1810 UV-Vis spectrophotometer, ultra-low temperature refrigerated centrifuge, light incubator, benchtop drying oven, electric constant temperature water bath, 0.001 g precision electronic balance, tray balance, and freezer.

1.2 Experimental Treatment

Frozen clubroot galls were thawed at room temperature, and resting spores of *P. brassicae* were extracted following the method of Yang et al. (2002). Spore

suspension concentration was determined using a hemocytometer and diluted to 5$×10^{7}$ CFU · mL^{-1} for use as pathogen inoculum.

Small-leaf *I. indigotica* was cultivated in 72-cell seedling trays (three seeds per cell). After development of 2–3 true leaf pairs, seedlings were thinned and inoculated with *P. brassicae* (1$×10^{7}$ CFU · mL^{-1} resting spore suspension) using the injection method. A 10 mL syringe was used to slowly inject 2 mL of spore suspension into the root zone of each experimental seedling. Root and leaf tissues were collected from both control (BLG-CK1, BLG-CK2, BLG-CK3, BLG-CK4) and inoculated (BLG-S1, BLG-S2, BLG-S3, BLG-S4) plants at 0, 7, 14, and 21 days post-inoculation, with three biological replicates per group. All samples were flash-frozen in liquid nitrogen and stored at -80°C.

1.3 Analytical Methods

1.3.1 Disease Severity Grading and Histological Observation Disease severity was graded according to standards established by the Ministry of Agriculture's Public Welfare Industry Research Special Project (201003029) (Yang et al., 2014). Root swelling was observed every 24 hours post-inoculation for 21 consecutive days, with histological examination of *I. indigotica* roots. Roots were washed clean, and 1–2 cm sections from the underground portion were fixed in FAA solution for at least 24 h. Fixed tissues were thinly sectioned, stained with trypan blue solution for 2 min, washed 2–3 times with deionized water (ddH_2O) to remove residual stain, and mounted on slides for microscopic observation.

1.3.2 Physiological Index Determination Physiological indices in *I. indigotica* leaves were measured following methods described by Gao (2006) and Wang (2014): (1) MDA content by thiobarbituric acid colorimetry; (2) soluble protein content by Coomassie brilliant blue G-250 staining; (3) POD activity by guaiacol colorimetry; (4) SOD activity by nitroblue tetrazolium photoreduction; (5) PPO activity by catechol method; (6) CAT activity by UV spectrophotometry.

1.3.3 UPLC-MS/MS Analysis Sample preparation: Root and rhizome tissues were vacuum freeze-dried using a Scientz-100F lyophilizer and ground into powder. Fifty milligrams of powder was extracted with 1.2 mL of 70% methanol, vortexed every 30 min (30 s each time, six times total), centrifuged at 12,000 rpm for 3 min, and filtered through a 0.22 m microporous membrane into sample vials for analysis.

Chromatographic and mass spectrometric conditions: Analysis was performed on an ExionLC™ AD ultra-high-performance liquid chromatography system coupled to an Applied Biosystems 4500 QTRAP tandem mass spectrometer. Chromatographic conditions: SB-C18 column (1.8 µm, 2.1 mm × 100 mm, Agilent); mobile phase A: ultrapure water with 0.1% formic acid; mobile phase B: acetonitrile with 0.1% formic acid; flow rate: 0.35 mL · min^{-1};

gradient elution (B%): 0.00 min, 5%; linear increase to 95% within 9.00 min, held for 1 min; 10.00–11.10 min, 95%–5%; 11.10–14 min, 5%; injection volume: 4 L; column temperature: 40°C. Mass spectrometric conditions: electrospray ionization source; voltage: +5,500 / -4,500 V; temperature: 550°C; ion source gas I, gas II, and curtain gas set at 50, 60, and 25 psi, respectively; collision-induced dissociation parameter: high.

Differential metabolites were screened using VIP values from OPLS-DA models combined with fold change values. The screening criteria were VIP ≥ 1, fold change ≥ 1.5, or fold change ≤ 0.67.

1.3.4 RNA Extraction and Library Construction RNA extraction and sequencing were performed by Wuhan Metware Biotechnology Co., Ltd. Total RNA was extracted using the NEBNext® Ultra™ RNA Library Prep Kit from Illumina. RNA integrity and purity were assessed by agarose gel electrophoresis and NanoPhotometer spectrophotometry. Qualified samples were used for cDNA library construction.

1.3.5 Transcriptome Sequencing, Analysis, and Annotation High-throughput sequencing of *I. indigotica* root and rhizome transcriptome libraries was performed using the Illumina platform. Raw image data were converted to raw reads via CASAVA base calling. After quality assessment, filtering, and redundancy removal, high-quality clean reads were obtained. Trinity assembly and hierarchical clustering generated Unigenes. Unigene sequences were annotated against Gene Ontology (GO), Trembl, Kyoto Encyclopedia of Genes and Genomes (KEGG), Swiss-Prot, eukaryotic Ortholog Groups (KOG), NR, and Protein family (Pfam) databases using DIAMOND BLASTX and HMMER software. Differentially expressed genes (DEGs) were identified using the DEGSeq R package, with genes having false discovery rate (FDR) < 0.05 and $|\log_2$ fold change (FC)$| \geq 1$ considered differentially expressed. Functional enrichment analysis was performed on DEGs.

1.3.6 qRT-PCR Validation Plant RNA extraction followed the method described in Section 1.3.4. Thirteen DEGs were selected based on FPKM values from transcriptome data. cDNA was synthesized using a Monad kit, and qRT-PCR was performed using the synthesized cDNA as template with *I. indigotica* Actin gene as internal reference (Qu et al., 2019). Primers were designed using Primer-BLAST (Table 1), and qRT-PCR was conducted on an ABI 7500 real-time PCR system. Amplification program: pre-denaturation at 95°C for 2 min; 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 30 s; melting curve analysis from 65°C to 95°C with fluorescence acquisition every 0.5°C increment. Three technical replicates were performed per sample, and relative expression levels were calculated using the 2^(-ΔΔCT) method. Data were expressed as mean ± standard deviation, and statistical significance was determined by Student's t-test using GraphPad software ($P < 0.05$).

Results

2.1 Disease Severity Grading and Histological Observation

Disease severity grading and histological observation results are shown in Figure 1 [Figure 1: see original paper] and Figure 2 [Figure 2: see original paper]. At 0 days post-inoculation, roots developed normally without tumors (disease index grade 0), with histology showing neatly arranged cells (Figure 1:E, Figure 2:A). At 7 days post-inoculation, roots began to show swelling symptoms (grade 1), with histological evidence of significantly enlarged and deformed cortical cells (Figure 1:F, Figure 2:C). At 14 days post-inoculation, main root gall diameter was less than twice the stem base diameter (grade 3), with secondary zoospores spreading throughout cortical cells and beginning to invade the vascular cylinder (Figure 1:G, Figure 2:E). At 21 days post-inoculation, large tumors formed with diameters 2–3 times that of the stem base (grade 5), with secondary zoospores distributed throughout both cortex and vascular cylinder (Figure 1:H, Figure 2:F). These results demonstrate the complete infection process from macroscopic to cellular levels and validate the feasibility of artificial inoculation.

2.2 Physiological and Biochemical Index Determination

Changes in physiological and biochemical indices between inoculated and control treatments are shown in Figure 3 [Figure 3: see original paper]. Soluble protein content, MDA content, and defense enzyme activities (SOD, POD, PPO, CAT) in inoculated plants showed continuously increasing trends with prolonged infection. CAT and SOD activities in the inoculated group were significantly higher than the corresponding control group from 7–21 days post-inoculation ($P < 0.05$). Soluble protein, MDA, POD, and PPO showed significant differences compared to controls at 14–21 days post-inoculation ($P < 0.05$). These results indicate that *I. indigotica* mounted defense responses to resist *P. brassicae* infection.

2.3.1 Sample Detection and OPLS-DA Analysis

Metabolomic analysis was performed on seven sample groups (BLG-CK1(S1), BLG-CK2, BLG-CK3, BLG-CK4, BLG-S2, BLG-S3, BLG-S4). A total of 161 alkaloids were detected, with "other alkaloids" being most abundant (53% of total alkaloids), followed by indole alkaloids (32%) (Figure 4 [Figure 4: see original paper]). Orthogonal partial least squares-discriminant analysis (OPLS-DA) (Figure 5 [Figure 5: see original paper]) showed clear separation between all sample groups, with the greatest separation between BLG-CK3 and BLG-S3, followed by BLG-CK4 and BLG-S4, indicating that *P. brassicae* exerted the greatest metabolic impact on *I. indigotica* at 14 days post-inoculation.

2.3.2 Screening of Differential Metabolites

Analysis of differential metabolites at 7, 14, and 21 days post-inoculation revealed: 16 differential metabolites (6 upregulated, 10 downregulated) in BLG-CK2-vs-BLG-S2; 17 (9 upregulated, 8 downregulated) in BLG-CK3-vs-BLG-S3; 39 (32 upregulated, 7 downregulated) in BLG-CK4-vs-BLG-S4; 19 (3 upregulated, 16 downregulated) in BLG-S2-vs-BLG-S3; 45 (19 upregulated, 26 downregulated) in BLG-S2-vs-BLG-S4; and 34 (14 upregulated, 20 downregulated) in BLG-S3-vs-BLG-S4 (Table 2). As shown in Figure 6 [Figure 6: see original paper], intersection analysis of BLG-CK2-vs-BLG-S2, BLG-CK3-vs-BLG-S3, and BLG-CK4-vs-BLG-S4 identified five common differential metabolites: cyclobrassinin, Isatindosulfonic acid B, 5,6-dihydroxyindole-5-O-β-glucoside, pantetheine, and p-coumaroyl spermidine, with the first three being indole alkaloids. Intersection of BLG-S2-vs-BLG-S3, BLG-S2-vs-BLG-S4, and BLG-S3-vs-BLG-S4 identified two common differential metabolites: the indole alkaloid Isatisindigoticanine B and the quinoline alkaloid 2-oxo-3,4-dihydro-1H-quinoline-3-carboxylic acid.

2.3.3 KEGG Enrichment Analysis of Differential Metabolites

KEGG pathway enrichment analysis results are shown in Figure 7 [Figure 7: see original paper]. All groups were enriched in metabolic pathways. The three comparison groups BLG-CK2-vs-BLG-S2, BLG-CK3-vs-BLG-S3, and BLG-CK4-vs-BLG-S4 were all enriched in tropane, piperidine, and pyridine alkaloid biosynthesis (ko00960). Except for BLG-CK3-vs-BLG-S3, all groups were enriched in tryptophan metabolism (ko003800), which serves as the precursor pathway for indole alkaloid biosynthesis.

2.4.1 Transcriptome Data Assembly and Quality Analysis

To further investigate alkaloid accumulation mechanisms in *I. indigotica* under *P. brassicae* stress, transcriptome sequencing of 21 samples yielded 1,200,974,786 raw reads and 1,171,808,210 clean reads, totaling 175.77 Gb of valid data. Clean data for each sample exceeded 7 Gb, with Q20 base percentage above 97% and Q30 base percentage above 92%. GC content ranged from 47.0% to 47.69%. Trinity assembly generated 83,775 Unigenes with an average length of 1,708 bp, maximum length of 16,523 bp, minimum length of 201 bp, and N50 of 2,367 bp. The Unigene length distribution (Figure 8 [Figure 8: see original paper]) showed that 51,799 Unigenes (61.83%) exceeded 1,000 bp, and 25,874 (30.89%) exceeded 2,000 bp, indicating high-quality transcriptome data suitable for subsequent analysis.

2.4.2 Unigene Functional Annotation

Unigenes were annotated against seven databases: NR, TrEMBL, GO, Swiss-Prot, KOG, KEGG, and Pfam, with 69,350 (82.78%), 70,281 (83.89%), 60,538 (72.26%), 54,696 (65.29%), 44,620 (53.26%), 55,271 (65.98%), and 55,387

(66.11%) genes successfully annotated, respectively. A total of 72,965 Unigenes (87.1%) were annotated in at least one database. Correlation analysis of gene expression levels between samples demonstrated high consistency within groups, ensuring reliability of subsequent analyses.

Among 60,538 Unigenes with GO annotation, three major categories were identified: in molecular function, catalytic activity (30,758 Unigenes) and binding (36,084 Unigenes) were predominant; in cellular component, cellular anatomical entity was most abundant (51,807 Unigenes); in biological process, cellular process (40,504 Unigenes), response to stimulus (18,474), metabolic process (32,229), and biological regulation (16,606) were highly represented (Figure 9 [Figure 9: see original paper]).

KOG functional classification (Figure 10 [Figure 10: see original paper]) revealed 25 functional categories encompassing most life activities. General function prediction was the largest group (10,102 Unigenes), followed by post-translational modification, protein turnover, and chaperones (4,932 Unigenes), and signal transduction (4,461 Unigenes).

2.4.3 Screening and Functional Analysis of Differentially Expressed Genes

Comparison of inoculated vs. control plants at 7, 14, and 21 days post-inoculation identified 2,439, 256, and 6,437 DEGs, respectively, indicating significant transcriptomic changes upon *P. brassicae* infection. The highest number of DEGs at 21 days suggests dramatic gene expression changes during late infection stages. Seventeen DEGs were common across all three time points, with nine upregulated and eight downregulated. The number of DEGs increased with infection duration, reflecting intensified host responses (Table 3).

GO term enrichment analysis categorized 9,805 DEGs into cellular component, molecular function, and biological process categories. Cellular anatomical entity ranked first in cellular component. In biological process, cellular process, response to stimulus, and metabolic process were most enriched. Binding and catalytic activity were the primary molecular functions (Table 4).

Since alkaloids are the main active components in *I. indigotica* and amino acids serve as alkaloid biosynthesis precursors, we focused on amino acid and alkaloid-related metabolic pathways. KEGG enrichment analysis of DEGs from BLG-CK2-vs-BLG-S2, BLG-CK3-vs-BLG-S3, and BLG-CK4-vs-BLG-S4 identified 11 alkaloid-related metabolic pathways (Table 5), with tryptophan metabolism showing substantial DEG enrichment.

2.5 Mining of Alkaloid Biosynthesis Pathway-Related Genes

We focused on mining indole alkaloid biosynthesis pathways and related genes. As shown in Figure 11 [Figure 11: see original paper], chorismate can be con-

verted to indole via anthranilate synthase (AS) and tryptophan synthase (TSA), ultimately synthesizing tryptophan. Tryptophan decarboxylase (TDC) is a crucial enzyme that catalyzes tryptophan to tryptamine, which can be converted to indole-3-acetate by indole-3-pyruvate monooxygenase (YUCCA) or to 5-hydroxyindole-3-acetic acid by aldehyde dehydrogenase (ALDH).

From all DEGs, we identified 18 genes encoding five key enzymes in the indole alkaloid biosynthesis pathway (AS, TSA, TDC, YUCCA, ALDH) (Figure 12 [Figure 12: see original paper]A), and 18 genes encoding two key enzymes in the isoquinoline alkaloid biosynthesis pathway: thebaine synthase (THS) and tyrosine aminotransferase (TAT) (Figure 12B). Compared to uninoculated controls, nine DEGs showed consistently upregulated expression at 7, 14, and 21 days post-inoculation: three THS-encoding genes (Cluster-24362.0, Cluster-36994.3, Cluster-36129.3), two TAT-encoding genes (Cluster-31730.0, Cluster-28040.6), two YUCCA-encoding genes (Cluster-36192.0, Cluster-36192.1), and two ALDH-encoding genes (Cluster-32381.0, Cluster-28395.0).

2.6 qRT-PCR Validation of Differentially Expressed Genes

To validate transcriptome data accuracy, we selected THS (Cluster-24362.0, Cluster-36994.3, Cluster-36129.3), AUX/IAA (Cluster-34981.0), GH3 (Cluster-16885.9), SAUR (Cluster-27535.0), PYR/PYL (Cluster-37780.0), PP2C (Cluster-35278.2, Cluster-37953.0), ABF (Cluster-37916.2), B-ARR (Cluster-22704.8), TGA (Cluster-22719.0), and AHK (Cluster-28287.0) for qRT-PCR validation (Figure 13 [Figure 13: see original paper]A). Results showed that expression trends of these genes in BLG-CK2-vs-BLG-S2, BLG-CK3-vs-BLG-S3, and BLG-CK4-vs-BLG-S4 were consistent with RNA-seq data ($P < 0.05$) (Figure 13B), confirming the reliability of sequencing results.

Discussion

3.1 Effects of *P. brassicae* Infection on *I. indigotica* Roots

Numerous studies have shown that *P. brassicae*-infected plants develop progressively enlarged root tumors of varying sizes and shapes, with root cells becoming deformed and enlarged due to secondary zoospore invasion (Wei et al., 2021). Root swelling degree typically reflects disease index, while cellular changes mirror the entire infection process. Our 21-day real-time dynamic observation revealed increasing disease indices (grades 0, 1, 3, and 5) at 0, 7, 14, and 21 days post-inoculation. Histological observation showed rapid *P. brassicae* invasion of root cells during 7–14 days post-inoculation, representing the peak infection period, consistent with findings by Xie et al. (2022). Although Wei et al. (2021) reported different infection timing, this discrepancy likely reflects differences in host species and infection conditions. Our combined disease grading and histological approach facilitates comprehensive observation of infection status,

provides a reference for studying other obligate pathogens, and offers theoretical guidance for sampling times in biochemical, transcriptomic, and metabolomic analyses.

3.2 Changes in Physiological and Biochemical Indices in *I. indigotica* Responding to *P. brassicae* Infection

I. indigotica mounts a series of stress responses upon *P. brassicae* infection, consistent with most reported studies (Wang et al., 2020). Our measurements at 0, 7, 14, and 21 days post-inoculation revealed significantly increased soluble protein and MDA contents at 14 and 21 days compared to uninoculated controls, indicating more severe plant damage during late infection stages. Antioxidant enzyme activities (SOD, POD, CAT, PPO) showed continuously increasing trends under pathogen treatment, demonstrating that *I. indigotica* enhances antioxidant capacity to combat *P. brassicae* damage. While CAT and SOD activities differed significantly from 7 days post-inoculation, POD and PPO activities showed significant differences only at 14 days, indicating delayed activation of POD and PPO compared to SOD and CAT, consistent with reports by Zheng et al. (1999).

3.3 Effects of *P. brassicae* Infection on Alkaloid Biosynthesis in *I. indigotica*

As a major medicinal material in China, *I. indigotica* contains the pharmacologically active indole alkaloid epigoitrin. Plants such as *Catharanthus roseus* (Lin et al., 2020), *Rauvolfia serpentina* (Dey et al., 2022), and *Uncaria rhynchophylla* (Liu et al., 2021) have become model species for indole alkaloid biosynthesis research. The indole alkaloid metabolic pathway in *I. indigotica* is complex and diverse, with tryptophan metabolism serving as the precursor pathway (Huang et al., 2016). Currently, few studies have reported on indole alkaloid biosynthesis mechanisms, key enzymes, and gene functions in *I. indigotica*, though environmental stress has been shown to affect alkaloid accumulation (Tang et al., 2016; Jazayeri et al., 2022). Therefore, in-depth investigation of the molecular mechanisms underlying indole alkaloid biosynthesis under clubroot stress is warranted.

Metabolomic OPLS-DA analysis of alkaloids at 7, 14, and 21 days post-inoculation revealed metabolic differences between inoculated and control groups and across time points, with indole alkaloids being the predominant secondary metabolites. KEGG enrichment analysis of differential metabolites showed enrichment in amino acid metabolic pathways, particularly tryptophan metabolism, indicating that *P. brassicae* infection affects alkaloid compound biosynthesis. Further KEGG analysis of DEGs from BLG-CK2-vs-BLG-S2, BLG-CK3-vs-BLG-S3, and BLG-CK4-vs-BLG-S4 identified 11 alkaloid-related metabolic pathways, with numerous DEGs enriched in tryptophan metabolism. Integrated transcriptomic and metabolomic analysis mined 18 DEGs involved in five key upstream enzymes of indole alkaloid biosynthesis (AS, TSA, TDC,

YUCCA, ALDH). Notably, YUCCA and ALDH showed significant upregulation. Compared to uninoculated controls, YUCCA genes (Cluster-36192.0, Cluster-36192.1) showed increased expression, consistent with Cao et al. (2019) regarding biotic stress tolerance. However, YUCCA expression first increased then decreased with prolonged infection, likely representing plant stress responses. ALDH genes (Cluster-32381.0, Cluster-28395.0) were upregulated after inoculation, as ALDH gene families can oxidize toxic aldehydes, reduce lipid peroxidation, and enhance stress tolerance (Du et al., 2022; Zhang et al., 2023). Due to the lack of a complete *I. indigotica* genome under clubroot stress, full annotation of all genes in the indole alkaloid pathway remains challenging, hindering complete downstream pathway analysis. Future whole-genome sequencing and mass spectrometry analysis could address this limitation.

Additionally, we identified 18 DEGs encoding two key enzymes in the isoquinoline alkaloid pathway. THS has been rarely studied but is confirmed as a pathogenesis-related protein PR-10 superfamily member (Ozber et al., 2023). Our results showed that three THS-encoding DEGs (Cluster-24362.0, Cluster-36994.3, Cluster-36129.3) were upregulated after infection, with consistent expression trends in qRT-PCR validation, suggesting THS is closely related to clubroot resistance and warrants functional analysis. Tyrosine serves as an isoquinoline alkaloid precursor, and tyrosine aminotransferase (TAT) catalyzes tyrosine to 4-hydroxyphenylpyruvate. TAT genes have been cloned in *Arabidopsis* (Lopukhina et al., 2001), *Salvia miltiorrhiza* (Huang et al., 2008), and *Perilla frutescens* (Lu et al., 2012), with transcriptional induction by salicylic acid and abscisic acid treatment. Our study also found upregulated TAT-encoding genes (Cluster-31730.0, Cluster-28040.6) under clubroot stress, indicating TAT's important role in stress responses.

qRT-PCR results demonstrated that under clubroot stress, expression of THS, AUX/IAA (Jie et al., 2018), GH3 (Lu et al., 2022), SAUR (Li et al., 2022), PYR/PYL (Kim et al., 2020), PP2C (Yu et al., 2019), ABF (Chen et al., 2021), B-ARR (Falconieri et al., 2022), TGA (Qi et al., 2022), and AHK (Cerbantez-Bueno et al., 2020) were upregulated at all three time points, with expression decreasing over infection duration, accurately reflecting the expression patterns of most genes under clubroot stress.

In summary, transcriptomic and metabolomic analyses of *P. brassicae*-infected *I. indigotica* have substantially enriched our understanding of the plant's biology under clubroot stress, identified key genes involved in indole and isoquinoline alkaloid biosynthesis, and elucidated expression patterns of these genes under stress, laying a solid foundation for future functional studies and mechanistic analysis of alkaloid accumulation in clubroot-stressed *I. indigotica*.

References

Cao X, Yang H, Shang C, et al., 2019. The roles of auxin biosynthesis YUCCA gene family in plants [J]. *Int J Mol Sci*, 20(24), 6343.

Cerbantez-Bueno VE, Zúñiga-Mayo VM, Reyes-Olalde JI, et al., 2020. Redundant and non-redundant AHK cytokinin receptors during gynoecium development and functions [J]. *Front Plant Sci*, 11: 568277.

Chen Q, Lan HY, Peng W, et al., 2021. *Isatis indigotica*: a review of phytochemistry, pharmacological activities and clinical applications [J]. *J Pharm Pharmacol*, 73(9): 1137-1150.

Chen NJ, Zhang GX, Zhang LS, et al., 2021. The role of ABF transcription factors in response to abiotic stress in plant [J]. *J Plant Genet Resour*, 22(4): 930-938.

Dey A, Roy D, Mohture VM, et al., 2022. Biotechnological interventions and indole alkaloid production in *Rauvolfia serpentina* [J]. *Appl Microbiol Biotechnol*, 106(13-16): 4867-4883.

Du HM, Liu C, Jin XW, et al., 2022. Overexpression of the aldehyde dehydrogenase gene ZmALDH confers aluminum tolerance in *Arabidopsis thaliana* [J]. *Int J Mol Sci*, 23(1): 477.

Falconieri GS, Bertini L, Bizzarri E, et al., 2022. Plant defense: ARR11 response regulator as a potential player in *Arabidopsis* [J]. *Front Plant Sci*, 13: 995178.

Gao JF, 2006. *Guidance on Plant Physiology Test* [M]. Beijing: Higher Education Press: 142-218.

Guo Z, 2018. Main Biochemical Indexes and Transcriptome Analysis of Rapeseed Root Disease Resistance [D]. Chongqing: Southwest University.

Huang BB, Yi B, Duan YB, et al., 2008. Characterization and expression profiling of tyrosine aminotransferase gene from *Salvia miltiorrhiza* (Dan-shen) in rosmarinic acid biosynthesis pathway [J]. *Mol Biol Rep*, 35: 601-612.

Huang CP, Liao JL, Huang WJ, et al., 2022. Salicylic acid protects sweet potato seedlings from drought stress by mediating abscisic acid-related gene expression and enhancing the antioxidant defense system [J]. *Int J Mol Sci*, 23(23): 14819.

Huang Y, Yu X, He X, et al., 2016. The biosynthesis and genetic engineering of bioactive indole alkaloids in plants [J]. *J Plant Biol*, 59(3): 203-214.

Jazayeri SM, Pooralinaghi M, Torres-Navarrete Y, et al., 2022. *In silico* alkaloid production and response to natural adverse conditions in *Peganum harmala*: transcriptome analyses [J]. *Bio Technologia (Pozn)*, 103(4): 355-384.

Jie L, Jing JZ, Jin ZZ, 2018. Aux/IAA gene family in plants: molecular structure, regulation, and function [J]. *Int J Mol Sci*, 19(1): 259.

Kim J, Kim D, Cheon CI, et al., 2020. Modeling-based identification of a raptor-binding motif present in *Arabidopsis* ABA receptor PYL1 [J]. *Biochem Biophys Res Commun*, 533(4): 623-628.

Lan M, Li GL, Hu JF, et al., 2019. iTRAQ-based quantitative analysis reveals proteomic changes in Chinese cabbage (*Brassica rapa* L.) in response to *Plasmodiophora brassicae* infection [J]. *Sci Rep*, 9(1): 12058-12058.

Li ZG, Chen HW, Li QT, et al., 2022. Three SAUR proteins SAUR76, SAUR77 and SAUR78 promote plant growth in *Arabidopsis* [J]. *Sci Rep*, 12(1): 1994.

Lin Y, Wang YY, Yu F, 2020. Bioinformatics analysis and prokaryotic expression of jasmonic acid-isoleucine synthase CrJAR1 from *Catharanthus roseus* [J]. *Guihaia*, 40(8): 1181-1187.

Liu Y, Zhang N, Luo J, et al., 2021. Chemical constituents of stems and leaves from *Uncaria rhynchophylla* in Guizhou and their antitumor activities [J]. *Guihaia*, 41(7): 1061-1069.

Lopukhina A, Dettenberg M, Weiler EW, et al., 2001. Cloning and characterization of a coronatine-regulated tyrosine aminotransferase from *Arabidopsis* [J]. *Plant Physiol*, 126: 1678-1687.

Lu S, Wang P, Nai G, et al., 2022. Insight into VvGH3 genes evolutional relationship from monocotyledons and dicotyledons reveals that VvGH3-9 negatively regulates the drought tolerance in transgenic *Arabidopsis* [J]. *Plant Physiol Biochem*, 172: 70-86.

Lu XL, Hao L, Wang F, et al., 2012. Molecular cloning and expression analysis of tyrosine aminotransferase gene fragment in *Perilla frutescens* [J]. *Chin Agr Sci Bull*, 28(24): 207-212.

Ozber N, Yu L, Hagel JM, et al., 2023. Strong feedback inhibition of key enzymes in the morphine biosynthetic pathway from opium poppy detectable in engineered yeast [J]. *ACS Chem Biol*, 18(2): 419-430.

Qi P, Huang M, Hu X, et al., 2022. A *Ralstonia solanacearum* effector targets TGA transcription factors to subvert salicylic acid signaling [J]. *Plant Cell*, 34(5): 1666-1683.

Qi W, Wang F, Ma L, et al., 2020. Physiological and biochemical mechanisms and cytology of cold tolerance in *Brassica napus* [J]. *Front Plant Sci*, 11: 1241.

Qu RJ, Miao YJ, Cui YJ, et al., 2019. Selection of reference genes for the quantitative real-time PCR normalization of gene expression in *Isatis indigotica fortune* [J]. *BMC Mol Biol*, 20(1): 1-11.

Sinha D, Tiwari AK, Singh S, et al., 2008. Synthesis, characterization and biological activity of schiff base analogues of indole-3-carboxaldehyde [J]. *Eur J Med Chem*, 43(1): 160-165.

Tang XQ, Xiao YH, Zhao XL, et al., 2016. Response of biomass and active components in roots and leaves of *Isatis indigotica* Fort. to nitrogen forms [J]. *J Nanjing Agr Univ*, 39(1): 70-77.

Wang D, Sun T, Zhao S, et al., 2020. Physiological change alters endophytic bacteria community in clubroot of tumorous stem mustard infected by *Plasmodiophora brassicae* [J]. *BMC Microbiol*, 20(1): 244.

Wang GD, Chu YX, Xu Z, et al., 2022. Stress physiology of lotus embryo under temperature stress [J]. *Guihaia*, 42(12): 2128-2137.

Wang WL, 2014. *New Plant Physiology Experiment Guide* [M]. Beijing: Xinhua Publishing House: 83-146.

Wei X, Zhang Y, Zhao Y, et al., 2021. Root transcriptome and metabolome profiling reveal key phytohormone-related genes and pathways involved in clubroot resistance in *Brassica rapa* L [J]. *Front Plant Sci*, 12: 759623.

Wong LW, Goh C, Tan J, 2022. A systematic review for ethnopharmacological studies on *Isatis indigotica* Fortune: bioactive compounds and their therapeutic insights [J]. *Amer J Chin Med*, 50(1): 161-207.

Xie Q, Liu XM, Han FQ, et al., 2022. Study on the infection process of *Plasmodiophora brassicae* in broccoli, tomato and capsicum [J]. *Chin Veg*, 398(4): 21-28.

Xue J, 2020. Expression and purification and crystal structure resolution of THS2, a key enzyme in the thebaine synthesis pathway [D]. Tianjin: Tianjin University of Science and Technology.

Yang H, Ren ZH, Huang L, et al., 2014. Identification of main cruciferous cultivars in Hunan province against clubroot [J]. *Hunan Agric Sci*, (2): 5.

Yang LG, Wang Q, Su DNB, et al., 2021. Research progress on chemical constituents and pharmacological effects of *Isatis* [J]. *Chin J Mod Appl Pharm*, 38(16): 2039-2048.

Yang PW, Li JR, Yang QZ, et al., 2002. Extracting and detecting *Plasmodiophora brassicae* resting spores [J]. *J Yunnan Agric Univ*, 17(3): 301-302+306.

Yu XF, Han JP, Wang EF, et al., 2019. Genome-wide identification and homoeologous expression analysis of PP2C genes in wheat (*Triticum aestivum* L.) [J]. *Front Genet*, 10: 561.

Zhang XM, Zhong JW, Cao LA, et al., 2023. Genome-wide characterization of aldehyde dehydrogenase gene family members in groundnut (*Arachis hypogaea*) and the analysis under saline-alkali stress [J]. *Front Plant Sci*, 14: 1097001.

Zheng CM, Teng B, Gao FL, et al., 1999. Studies on the changes of superoxide dismutase, peroxidase and polyphenol oxidase in seed coat of soybeans after infected with soybean mosaic virus [J]. *Sci Agric Sin*, (1): 99-101.

Zhu HF, Li XF, Zhu YY, et al., 2015. Effect of root swelling disease on the growth, physiological and biochemical substances and reactive oxygen species metabolism of Chinese cabbage [J]. *Acta Bot Boreal-Occident Sin*, 35(12): 2469-2476.

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