

Article

Exogenous Application of an RXLR-Fused CRa Protein Systemically Colonizes Roots and Suppresses Clubroot via Rhizosphere Bacterial Community Remodeling in Tumorous Stem Mustard

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Abstract

Clubroot, caused by the obligate biotrophic protist *Plasmodiophora brassicae*, is a devastating soil-borne disease threatening global cruciferous crop production. To explore non-transgenic control strategies, this study investigated the efficacy of a crude protein extract containing RXLR motif-fused recombinant CRa protein against clubroot in tumorous stem mustard (*Brassica juncea* var. *tumida*) and its regulatory mechanisms in the rhizosphere. Mechanistically, fluorescence tracking confirmed that the CRa protein exploits the xylem stream for systemic translocation, colonizing the stele and vascular tissues within 24 h and significantly reducing the disease index. Ecologically, CRa protein reshaped the rhizosphere microbiome by enhancing α -diversity (Shannon and Simpson indices), increasing network complexity, and specifically enriching beneficial genera such as *Pseudomonas*. Metabolically, CRa protein induced the reprogramming of root exudates, upregulating defensive compounds including jasmonic acid methyl ester, 4-hydroxyglucobrassicin, and (2R)-2-hydroxy-2-phenethylglucosinolate. Overall, exogenous CRa protein activates a coordinated defense response through three key mechanisms: rapid vascular translocation, recruitment of beneficial microbiota, and metabolic reprogramming. This study provides novel insights for developing non-transgenic, microbiome-based green control strategies.

Keywords: clubroot; CRa protein; rhizosphere microbiome; metabolic reprogramming; tumorous stem mustard; *Plasmodiophora brassicae*



Academic Editor: Diego Rubiales

Received: 10 June 2026

Revised: 25 July 2026

Accepted: 27 July 2026

Published: 3 August 2026

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1. Introduction

Clubroot, caused by the obligate biotrophic protist *Plasmodiophora brassicae*, is one of the most destructive soil-borne diseases of cruciferous crops. The pathogen causes severe root hypertrophy and hyperplasia, disrupting water and nutrient uptake and leading to devastating yield losses [1]. With a remarkably broad host range, *P. brassicae* infects 3700 species across 330 genera within the Brassicaceae, encompassing economically vital crops such as rapeseed (*Brassica napus*), Chinese cabbage (*B. rapa* subsp. *pekinensis*), bok choy (*B. rapa* subsp. *chinensis*), kale (*B. oleracea* var. *acephala*), radish (*Raphanus sativus*), cauliflower (*B. oleracea* var. *botrytis*), and tumorous stem mustard (*B. juncea* var. *tumida*) [2]. The disease is especially severe in major producing regions of tumorous stem mustard, such

as Fuling, Chongqing, where reported yield reductions often exceed 60% [1], threatening the sustainability of the local industry.

Genetic resistance remains the most cost-effective and environmentally friendly approach to managing clubroot. Recent systematic syntheses of resistance resources in crucifers have catalogued over 50 major-effect loci distributed across the U-triangle genomes, with the genome harboring the vast majority of cloned resistance genes, including the first characterized clubroot resistance gene *CRA*, as well as *CRb*, *Crr* series, and other widely utilized loci [3–5]. The *CRA* protein provides effective, albeit pathotype-specific, tolerance to *P. brassicae* by preventing the progression of secondary infection and safeguarding the plant's vascular system [6]. However, translating *CRA* into broad-field protection faces persistent challenges. Conventional introgression breeding is time-consuming and hindered by linkage drag and fertility barriers. While transgenic or genome-editing approaches deliver the functional allele directly, they remain entangled in regulatory hurdles, public acceptance issues, and varietal specificity [7]. These constraints motivate the exploration of an alternative strategy: delivering the functional output of a resistance gene as protein rather than DNA.

Advances in NLR (Nucleotide-binding Leucine-rich repeat) protein engineering—such as modifying LRR (Leucine-rich repeat) recognition surfaces or constructing chimeric scaffolds—represent the forefront of next-generation disease resistance breeding [8]. The core challenge lies in the fact that NLRs require intracellular localization to activate immunity. The introduction of the RXLR (Arg-X-Leu-Arg) motif resolves this bottleneck: functioning as a “Trojan horse,” it efficiently delivers the fused *CRA* protein into the cytoplasm, thereby bypassing genetic modification to directly harness the functional output of a native resistance gene [9]. Exogenous activation of intracellular immunity triggers cell-autonomous defenses and reprograms root exudate chemistry. Applications of elicitors such as *flg22*, which shift exudate profiles of flavonoids, glucosinolates, and organic acids [10], serve as key signals for orchestrating rhizosphere microbiome assembly.

The rhizosphere microbiome can suppress pathogens through antagonism or induce systemic resistance [11,12], and its assembly is strongly influenced by root exudate composition [13,14]. Plant resistance genes, particularly those encoding NLR receptors, can modulate root exudate profiles via defense signaling pathways such as salicylic acid and jasmonic acid [15,16], thereby selectively recruiting beneficial microorganisms. Similar microbe-mediated protection has been demonstrated in other systems, such as resistant banana genotypes enriching antifungal taxa to combat *Fusarium* wilt [16], and in rapeseed clubroot, where rhizosphere microbiome structure correlates with disease severity [17].

In the tumorous stem mustard-clubroot pathosystem, however, it remains unknown whether externally applied *CRA* protein can similarly influence root exudation and reshape the rhizosphere microbiome to enhance resistance. We hypothesize that exogenous application of the *CRA*-RXLR fusion protein activates intracellular immune signaling in roots, which reprograms root exudate metabolism, thereby selectively enriching beneficial rhizobacterial taxa that suppress clubroot. In this study, we combined high-throughput amplicon sequencing and metabolomic profiling to characterize changes in rhizosphere bacterial communities and root exudate compositions following *CRA* protein treatment. This integrated analysis aims to clarify the mechanistic link between recombinant *CRA* protein application, rhizosphere metabolic signaling, microbiome assembly, and clubroot resistance, providing a theoretical basis for novel microbiome-oriented disease management strategies.

2. Materials and Methods

2.1. Experimental Materials and Seedling Preparation

The non-resistant tumorous stem mustard cultivar ‘Yong’an Xiaoye’, procured from the Fuling Academy of Agricultural Sciences (Chongqing, China), was employed as the test material. Seeds were surface-sterilized with 75% ethanol for 5 min, rinsed three times with sterile water, and sown evenly on sterile Petri dishes lined with moist filter paper. Each dish was supplemented with 40 mL of half-strength Hoagland nutrient solution containing the following macronutrients: 5 mM KNO₃, 5 mM Ca(NO₃)₂·4H₂O, 2 mM MgSO₄·7H₂O, and 1 mM NH₄H₂PO₄. Micronutrients included 50 µM Fe-EDTA, 25 µM H₃BO₃, 2 µM MnCl₂·4H₂O, 2 µM ZnSO₄·7H₂O, 0.5 µM CuSO₄·5H₂O, and 0.5 µM Na₂MoO₄·2H₂O and incubated at 23 °C under a 16 h light/8 h dark photoperiod for approximately 5 days to obtain healthy, aseptic seedlings with fully expanded cotyledons for subsequent experiments.

2.2. Recombinant Protein Expression and Purification

To investigate the intracellular delivery capability of the RXLR motif, a synthetic gene construct was designed based on the conserved RXLR effector sequences of *Phytophthora sojae Avr1b* [9]. This construct comprised three functional modules: (i) a 42 bp (14 amino acids) core RXLR motif (RXLR-EER domain), which served as a translocation module for cytoplasmic entry; (ii) a 726 bp enhanced green fluorescent protein (eGFP) sequence with the termination codon removed to facilitate real-time tracking; and (iii) the 3975 bp full-length coding sequence (CDS) of the *B. rapa* clubroot resistance gene *CRa* (GenBank AB711028.1). The fusion of these elements generated a continuous open reading frame encoding a predicted 173 kDa fusion protein. The synthetic cassette was commercially synthesized by the Beijing Genomics Institute (Shenzhen, China) and subcloned into the *E. coli* expression vector pET-28a(+), which carries a kanamycin resistance gene (kanR), yielding the pET28a-eGFP-CRa recombinant plasmid (Figure S1). Following transformation into *E. coli* BL21 (DE3) competent cells via heat shock (42 °C, 90 s), positive clones were verified by colony PCR and Sanger sequencing prior to protein expression assays.

A single colony was inoculated into LB liquid medium containing kanamycin (100 mg/L) and cultured at 37 °C with shaking at 200 rpm for 12 h. The culture was then diluted 1:100 into fresh antibiotic-containing LB medium and grown until the OD₆₀₀ reached 0.6–0.8. A 500 µL aliquot was mixed with an equal volume of 30% sterile glycerol, flash-frozen in liquid nitrogen, and stored at −80 °C as a glycerol stock.

For protein expression, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the remaining culture to a final concentration of 1 mM, followed by induction at 16 °C with shaking at 200 rpm overnight (~16 h). Cells were harvested by centrifugation (4 °C, 5000× g, 10 min) and resuspended in pre-cooled PBS buffer. After lysis by sonication on ice, the lysate was centrifuged (12,000× g, 20 min, 4 °C) to separate soluble fractions. Aliquots of the resulting supernatant (crude extract) and pre-induction cell pellets were mixed with SDS-PAGE loading buffer, boiled at 95 °C for 10 min, and separated by 10% SDS-PAGE to verify the expression of the ~173 kDa CRa-eGFP fusion protein. The supernatant containing the soluble recombinant protein was stored on ice for use.

2.3. Root Colonization Assay

The tumorous stem mustard seedlings were vertically inserted into 2 mL centrifuge tubes containing 1.0 mL of the crude protein extract, ensuring that the roots were completely submerged. Holes were made in the tube caps to fix the stems. The plants were maintained at 23 °C under a 16 h photoperiod. Six time gradients were set: 1 h, 3 h, 6 h, 9 h, 12 h, and 24 h. At each time point, seedlings were removed using sterile forceps, placed in sterile Petri dishes, and rinsed five times with sterile water (30 s per rinse). Following

washing, root tips or root hair zones were sampled to prepare temporary aqueous mounts and immediately observed under a fluorescence microscope for green fluorescent signals to evaluate the adhesion and distribution of the recombinant protein in the root tissue [18].

2.4. Preparation of *P. brassicae* Resting Spore Suspension

Resting spores of *P. brassicae* were isolated from clubbed root tissues of field-infected tumorous stem mustard. Diseased roots were washed thoroughly with tap water, soaked in 7.5% (v/v) sodium hypochlorite solution for 10 min, and subsequently rinsed five times with sterile water to remove residual disinfectant. Surface-disinfected gall tissues were minced and homogenized with an appropriate volume of sterile water. The homogenate was filtered through eight layers of sterile cheesecloth, and the filtrate was centrifuged at 4 °C and $5000 \times g$ for 10 min to collect the spore pellet. The pellet was re-suspended in sterile water, and this process was repeated three times to purify the spores. Finally, the spore suspension was adjusted to a concentration of 1.0×10^8 spores/mL using a hemocytometer and stored at 4 °C until use [2]. Spore concentration was determined using a hemocytometer under a light microscope (400 $\times$ magnification), with three technical replicates per sample. To determine the virulence of *P. brassicae*, we employed the sinitic clubroot differential system [19]. Based on reactions on eight differential hosts, the isolate was identified as Pb4. This pathotype is widely distributed across major Brassica-growing regions in China and represents a significant virulence group within the traditional Williams' pathotype 4.

2.5. Pot Inoculation Assay

Four treatment groups were established. CRaP group: seedling roots were immersed in the crude protein extract containing CRa protein for 24 h, transplanted into sterilized substrate for acclimation (3–5 days), and then inoculated with the *P. brassicae* spore suspension (1.0×10^8 spores/mL). CRa group: seedling roots were immersed in the crude protein extract for 24 h and transplanted, followed by inoculation with an equal volume of sterile nutrient solution. PT group: seedlings were immersed in sterile PBS buffer instead of the protein extract for 24 h, transplanted, and inoculated with the spore suspension. CK group: seedlings were immersed in sterile PBS buffer for 24 h, transplanted, and inoculated with an equal volume of sterile nutrient solution. Each treatment comprised 15 biological replicates (pots, 3 plants/pot), thus yielding a total of 60 pots (180 plants) across the experiment.

Five weeks post-inoculation, whole seedlings with intact root systems were carefully harvested. Disease incidence was recorded, and disease indices were calculated following the standard grading scale [20]. Disease severity was graded on a 0–4 scale: 0 = no galls; 1 = 1–25% of root system galled; 2 = 26–50% galled; 3 = 51–75% galled; 4 = 76–100% galled. The disease index (DI) was calculated as: $DI = [\Sigma (\text{number of plants in each grade} \times \text{grade value})] / (\text{total number of plants} \times 4) \times 100$. One-way ANOVA revealed a significant difference in the clubroot disease index between the PT and CRaP treatments. Concurrently, rhizosphere soil tightly adhering to the root surface was collected for subsequent microbial DNA extraction. Thereafter, the harvested roots were thoroughly rinsed with sterile water to collect root exudates.

2.6. Rhizosphere Soil DNA Extraction, Illumina Sequencing, and Analysis

Rhizosphere soil samples were collected from three pooled plants to constitute one biological replicate, with 15 replicates per treatment. Total genomic DNA was extracted from 0.5 g of rhizosphere soil using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, LLC, Irvine, CA, USA) following the manufacturer's protocol. DNA concentration and quality were assessed using a NanoDrop 2000 spectrophotometer and agarose gel electrophoresis. The V3–V4 region of the bacterial 16S rRNA gene was amplified using primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAA T-3').

Purified PCR amplicons were pooled in equimolar ratios and used for library construction following the standard Illumina 16S rRNA gene sequencing protocol. The qualified libraries were then sequenced on an Illumina MiSeq PE300 platform at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

Reads were quality-filtered and trimmed using Trimmomatic (SLIDINGWINDOW:4:20, MINLEN:150), merged using FLASH (min-overlap = 10, max-mismatch = 0.1; mean length ~460 bp), and dereplicated in QIIME2 [21]. Chimeras were subsequently removed using USEARCH prior to taxonomic assignment. Operational taxonomic units (OTUs) were clustered at a 97% similarity level using the UPARSE pipeline [22]. Representative OTU sequences were taxonomically annotated using the RDP Classifier against the SILVA 138 database [23]. Alpha diversity metrics, including Shannon and Simpson indices, were calculated. Statistical differences in Shannon and Simpson indices among treatments were evaluated using the Kruskal–Wallis test followed by Dunn’s post hoc test for pairwise comparisons ($p < 0.05$). Venn diagrams were constructed to visualize the overlap of bacterial taxa at the class and genus levels across different treatments. Relative abundance of bacterial groups at the class and genus levels was calculated. Principal Component Analysis (PCA) based on Bray–Curtis distance was performed. Linear discriminant analysis Effect Size (LEfSe) was employed to identify bacterial genera showing significant differential abundance among groups, based on an LDA score threshold >3.0 and a Kruskal–Wallis test ($p < 0.05$) [24]. Bacterial co-occurrence networks for the four treatments were constructed using the top 100 OTUs and visualized via the Networkx package within the Majorbio I-Sanger Cloud Platform (<https://www.majorbio.com/>) [25].

2.7. Collection of Root Exudates and Non-Targeted Metabolomics Analysis

Root exudates were collected following the method with slight modifications [26]. Intact, washed root systems were individually immersed in 100 mL of sterile Milli-Q water and allowed to exude under light at 25 ± 0.5 °C for 12 h. The collection fluid was filtered through a 0.22 µm membrane to remove impurities, flash-frozen in liquid nitrogen, and stored at -80 °C for metabolomic analysis. For each treatment, five independent biological replicates were established, with each replicate comprising pooled root exudates derived from six individual plants.

Non-targeted metabolomics analysis was performed using liquid chromatography-mass spectrometry [27]. Thawed root exudate samples were vortexed, and aliquots were lyophilized. Samples were reconstituted in 70% methanol containing internal standards, vortexed, centrifuged, and the supernatants were analyzed. Quality control samples were inserted every 10 samples during the experimental run. Metabolites were identified using the MetWare database (<https://www.metware.cn/>) and quantified via multiple reaction monitoring mode on a triple quadrupole mass spectrometer [28].

Venn diagrams were plotted to display shared and unique metabolites among groups. Partial least squares discriminant analysis (PLS-DA) models were constructed, and model validity was evaluated using 200 permutation tests [29]. Differential metabolites were screened based on a combined threshold of $VIP \geq 1.5$, $|\text{fold change}| \geq 2$, $p < 0.05$ (Welch’s *t*-test), and $FDR < 0.05$ (Benjamini–Hochberg), and the results were displayed using volcano plots.

2.8. Correlation Analysis Between Rhizosphere Bacterial Communities and Root Exudates

To elucidate the interactions between rhizosphere bacterial communities and root exudates, Procrustes analysis was performed on PCA of differential bacterial genera and differential metabolites. Mantel tests based on Spearman correlation were used to assess the overall association between bacterial communities and differential metabolites. Further-

more, Spearman correlation analysis was performed to elucidate the relationships between bacterial genera and metabolites. Prior to analysis, genus-level bacterial abundances were CSS-normalized, and metabolite intensities were log₂-transformed to improve normality. Significant correlations were defined based on a combined threshold of Spearman's rank coefficient (ρ) > 0.6 and a two-tailed p -value < 0.05 [30].

3. Results

3.1. Colonization of Recombinant Protein in Tumorous Stem Mustard Roots

Under optimal induction conditions (16 °C with 1 mM IPTG), *E. coli* BL21 (DE3) carrying the pET28a-eGFP-CRa recombinant plasmid produced a distinct, single protein band migrating at ~173 kDa (Figure S2). This molecular weight aligns precisely with the predicted size of the RXLR-eGFP-CRa fusion protein, confirming successful heterologous expression of the full-length fusion construct.

Fluorescence microscopy revealed dynamic temporal progression of protein colonization. One hour post-inoculation (hpi), distinct fluorescent signals were detected in the root cortex (Figure 1A). By 3 hpi, signals expanded laterally within the cortical region (Figure 1B). Initial penetration into ground tissues was observed at 6 hpi (Figure 1C), progressing to widespread distribution throughout inner tissues by 9 hpi (Figure 1D). Characteristic fluorescence appeared in the stele and vascular tissues at 12 hpi (Figure 1E), culminating in complete coverage of root tissues by 24 hpi (Figure 1F). These results demonstrate that the fusion protein systemically colonizes tumorous stem mustard roots within 24 h. Consequently, *P. brassicae* resting spores were inoculated 24 h after protein treatment to ensure optimal protein distribution within host tissues.

3.2. Control Efficacy Against Clubroot

The crude protein extract containing CRa protein significantly suppressed clubroot development, decreasing the disease index from 45.8 to 12.1 (73.6% control efficacy) compared to the PT (Figure S3A). Visual symptoms were also markedly alleviated compared to the heavily diseased PT (Figure S3B), underscoring the suppressive activity of the crude protein extract containing CRa protein against *P. brassicae* proliferation.

3.3. Alpha Diversity and Taxonomic Composition

At the class level, all treatments shared 55 common bacterial taxa. Notably, the CRa group uniquely harbored 9 exclusive taxa, surpassing other treatments (Figure S4A). At the genus level, 450 taxa were shared across groups, with the CRa group again exhibiting the highest number of unique taxa (Figure S4B).

Regarding alpha diversity, the CRa group exhibited the highest Shannon and Simpson indices, followed by the CK and CRaP groups, while the PT group recorded the lowest values (Figure 2A,B). Dominant classes across groups included Gammaproteobacteria, Alphaproteobacteria, Bacteroidia, and Actinobacteria, though their relative abundances varied (Figure 2C). At the genus level, the core taxa comprised *Comamonas*, *Methyloversatilis*, and unclassified Comamonadaceae, with varying relative abundances across groups (Figure 2D).

3.4. Beta Diversity and Indicator Species

PCA revealed non-random, treatment-associated segregation of rhizosphere bacterial communities, with partial overlap among groups (Figure 3A). PC1 explained 51.99% of the total variance, separating the CK group (right quadrant) from the CRaP and PT groups (left quadrant). PC2 explained 18.32% of the variance, distinguishing the CRa group (intermediate position) from other treatments, indicating that treatment type is the predominant, though not absolute, driver of microbiome structure.

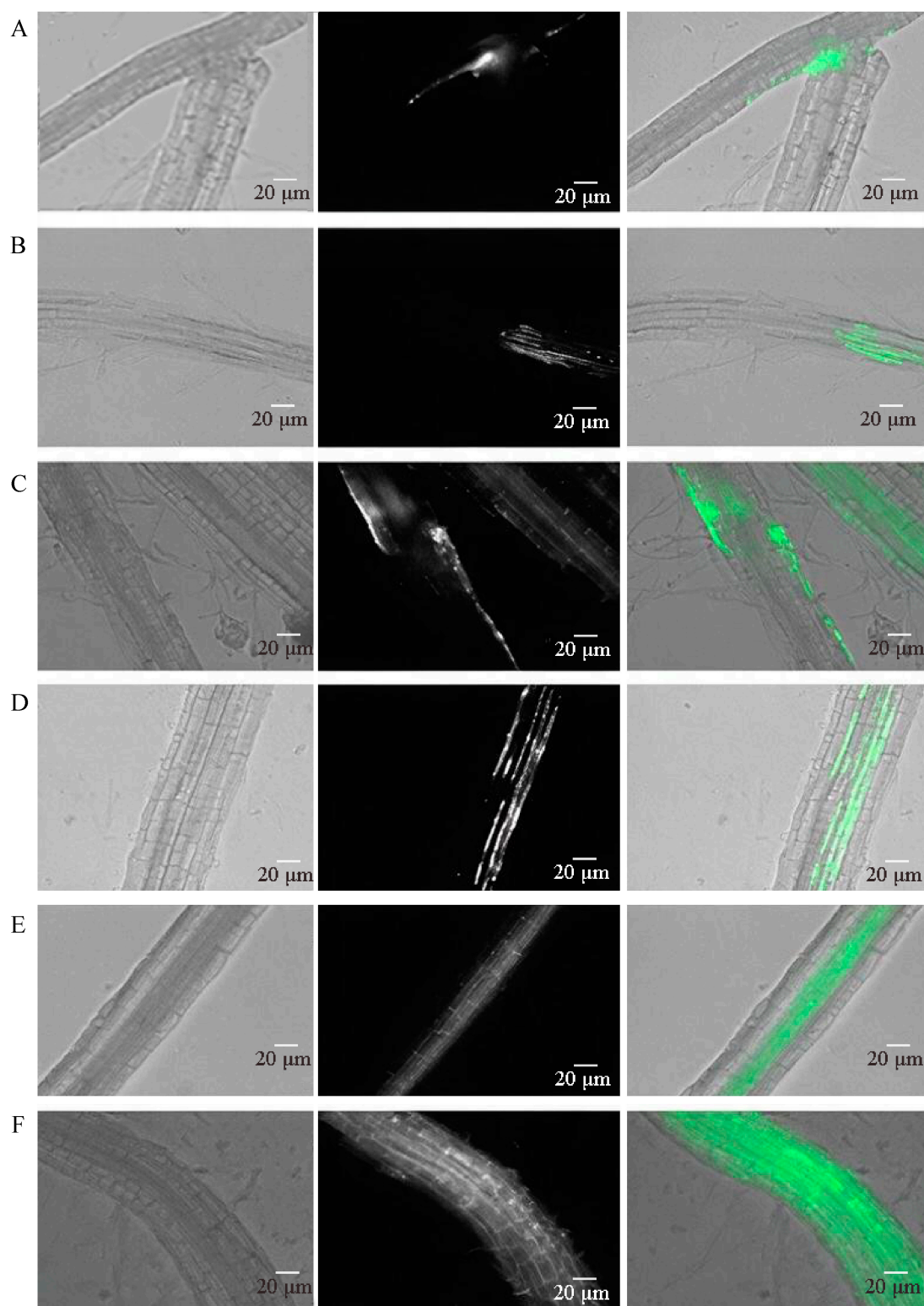


Figure 1. Spatiotemporal dynamics of recombinant CRa protein colonization in tumorous stem mustard roots. **(A)** 1 hpi: Initial detection of fluorescent signals localized in the root cortex. **(B)** 3 hpi: Lateral expansion of fluorescence within the cortical region. **(C)** 6 hpi: Initial penetration of the protein into the ground tissues. **(D)** 9 hpi: Widespread distribution of fluorescence throughout the inner root tissues. **(E)** 12 hpi: Characteristic fluorescence observed in the stele and vascular tissues. **(F)** 24 hpi: Complete coverage of root tissues by the fusion protein, indicating systemic colonization. From left to right, the images represent bright field transmission imaging, fluorescence signal detection, and the merged overlay of both. Scale bars: 20 μm .

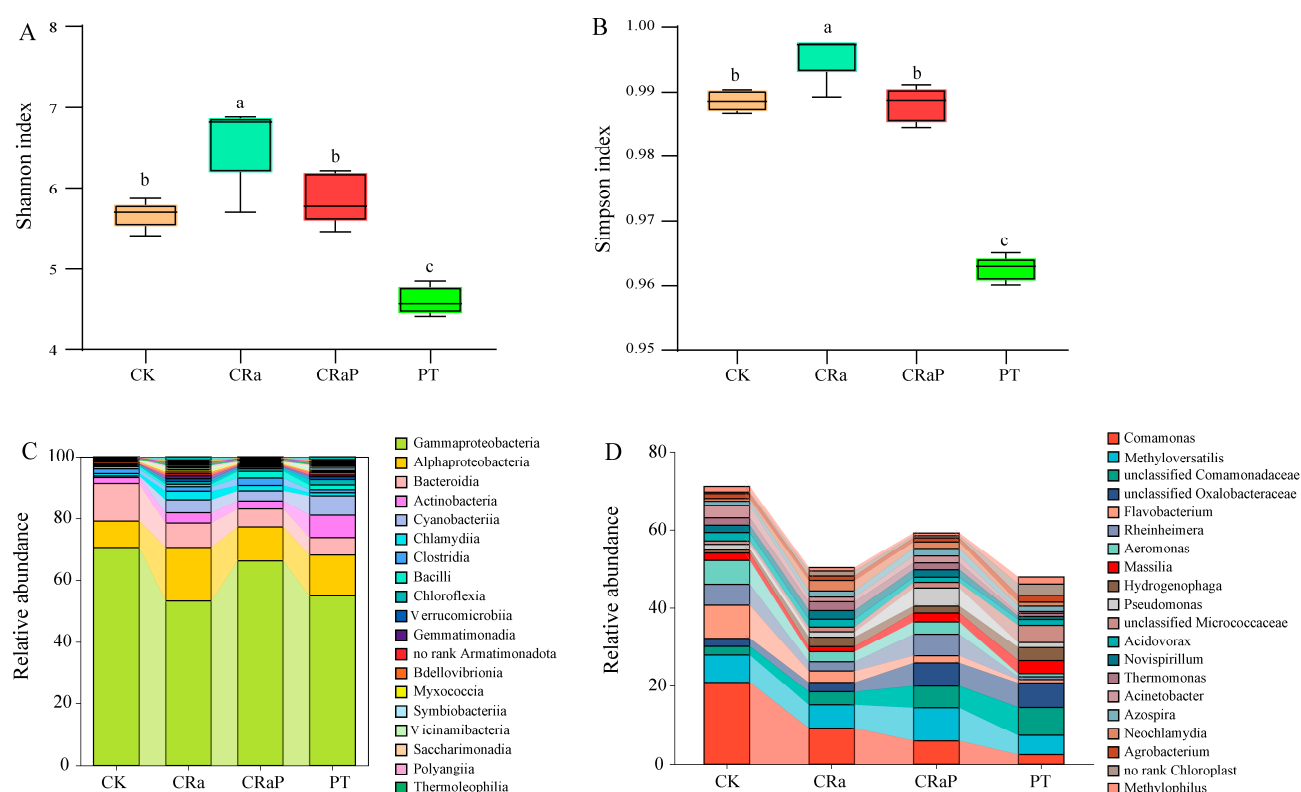


Figure 2. Alpha diversity and taxonomic composition of the rhizosphere microbiome under different treatments. **(A)** Shannon index. **(B)** Simpson index. **(C)** Relative abundance of dominant bacterial classes. **(D)** Relative abundance of core bacterial genera at the genus level. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

Based on Linear discriminant analysis (LDA) scores (>3 indicating significant enrichment), distinct microbial signatures were identified for each group (Figure 3B). CK group was characterized by *Comamonas*, *Acinetobacter*, *Cloacibacterium*, and *Bdellovibrio*. CRa group was marked by *Emticicia*. CRaP group featured *Pseudomonas* and *Azohydromonas*, whereas PT group was dominated by *Ramlibacter*, *Methylibium*, *Limnobacter*, and *Lacibacter*.

3.5. Co-Occurrence Network Analysis

Although the total number of nodes within the co-occurrence networks remained relatively consistent across treatments, the structural complexity varied drastically. Specifically, the CK group exhibited a moderate network architecture, characterized by 715 edges, an average degree of 15.38, a clustering coefficient of 0.493, and a network density of 0.167 (Figure 4A, Table S1). The CRaP group displayed a marginally higher complexity, with 792 edges, an average degree of 16.67, a clustering coefficient of 0.508, and a density of 0.177 (Figure 4B, Table S1). In stark contrast, the CRa group constructed the most intricate network, featuring the highest number of edges (1271), highest average degree (26.76), highest clustering coefficient (0.616), and highest density (0.285), suggesting that the CRa treatment strongly promoted the structural complexity of the rhizosphere microbiome (Figure 4C, Table S1). Conversely, the PT group exhibited a markedly simplified network, with the lowest number of edges (360), lowest average degree (7.35), lowest clustering coefficient (0.318), and lowest network density (0.076) (Figure 4D, Table S1), confirming a severe reduction in network complexity following pathogen infection.

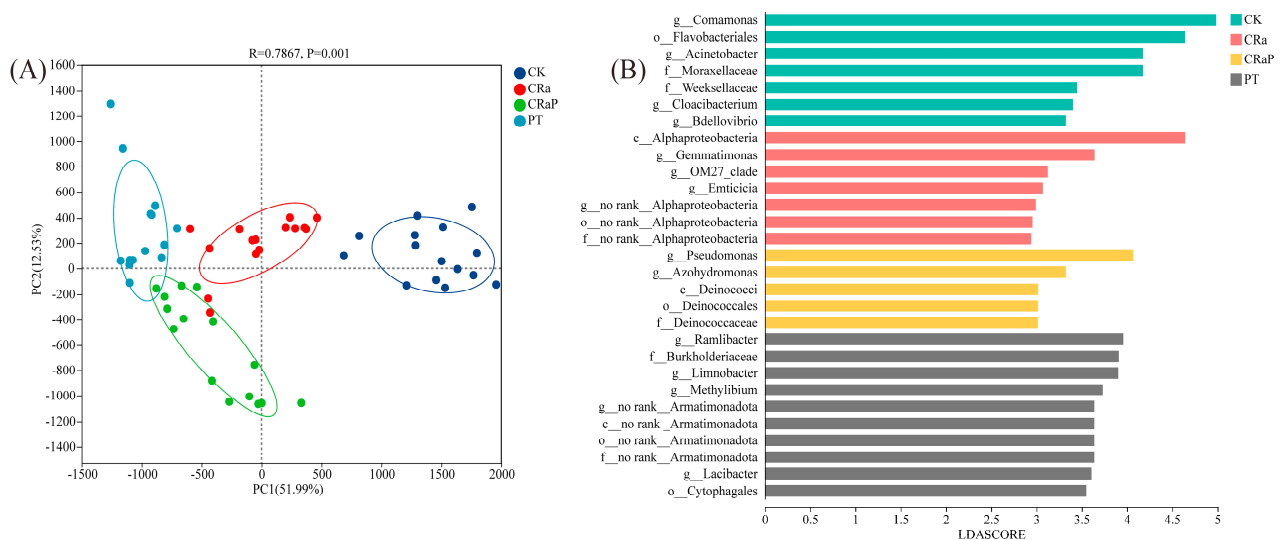


Figure 3. Beta diversity and indicator species analysis rhizosphere bacterial communities. **(A)** Principal Component Analysis. **(B)** Linear Discriminant Analysis Effect Size. Histogram identifying bacterial genera with significant differential abundance among treatments. Only taxa with an LDA score greater than 3.0 are displayed.

The keystone taxon for each treatment was identified based on the highest keystone score: ASV62 (*Lysobacter*) in CK (0.913), ASV185 (*Hydrogenophaga*) in CRaP (0.941), ASV8 (*Novispirillum*) in CRa (0.864), and ASV4 (unclassified *Pseudomonadaceae*) in PT (0.815) (Table S2). Notably, members of the genus *Pseudomonas* were consistently identified as keystone taxa across CK, CRaP, and PT treatments.

3.6. Root Exudate Metabolomics

The identified metabolites spanned diverse chemical classes, including amino acids, organic acids, fatty acids, nucleotides, and lipids (Figure S5A,B). In the root exudate metabolome, lipids and lipid-like molecules were the most abundant (29.12%), followed by organic acids and derivatives (17.94%), organoheterocyclic compounds (16.55%), organic oxygen compounds (14.21%), benzenoids (9.54%), and phenylpropanoids and polyketides (6.95%) (Figure S5C). A total of 1600 metabolites were identified across all treatments (Table S3), of which 1392 (87.00%) were shared among the four groups; the remaining 13% were treatment-specific (Figure S6A).

The PLS-DA score plot shows clear clustering of samples by treatment, with Component 1 and Component 2 explaining 23.3% and 16.9% of the total variance, respectively (Figure S6B) and Permutation testing (200 cycles) returned an R^2 intercept of 0.3502 and a Q^2 intercept of -0.2854 (Figure S6C). Differential metabolite analysis ($VIP > 1.5$, $p < 0.05$) delineated distinct metabolic reprogramming patterns across treatments. Compared to CK, the CRa group selectively elevated levels of the complex glycerolipid MG (0:0/20:3(6,8,11)-OH(5)/0:0) (Figure 5A), whereas the CRaP group triggered a specific enrichment of the defense-related phytoalexin (2R)-2-hydroxy-2-phenethylglucosinolate (Figure 5B). In contrast, plants subjected solely to PT exhibited a marked accumulation of the gibberellin A34 catabolite, indicative of pathogen-induced hormonal disruption (Figure 5C). Direct inter-group comparisons further highlighted the unique biochemical signatures induced by the recombinant protein: the CRaP group uniquely accumulated (2R)-2-hydroxy-2-phenethylglucosinolate relative to the CRa group (Figure 5D), while the PT group showed up-regulation of (2S,3R,4S,5R,6R)-6-ethylxane-2,3,4,5-tetrol compared to the CRa group (Figure 5E). Finally, the CRaP group specifically enhanced jasmonic acid methyl ester levels relative to the PT group (Figure 5F).

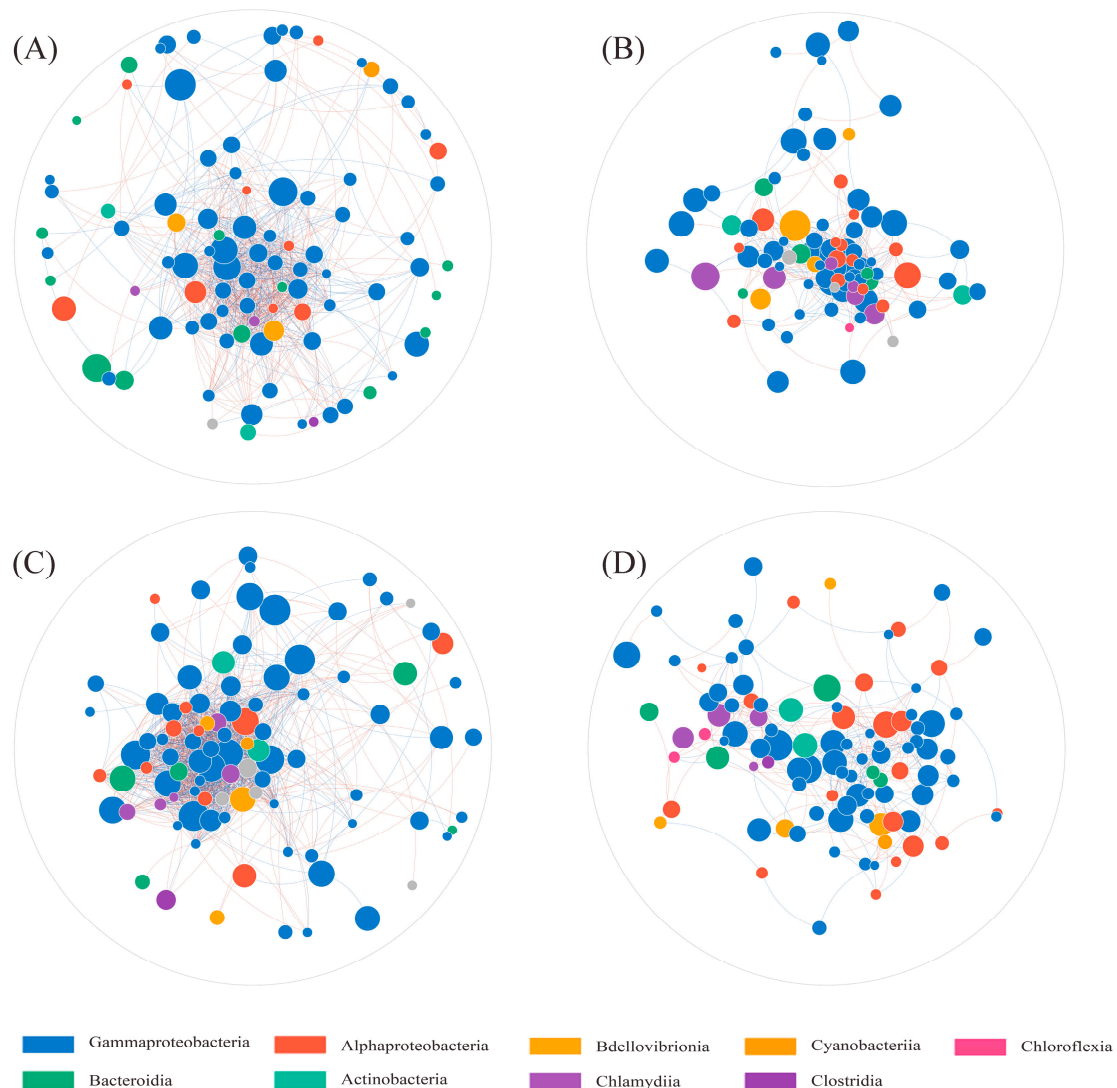


Figure 4. Co-occurrence network analysis of rhizosphere bacterial communities. (A) CK group. (B) CRaP group. (C) CRa group. (D) PT group. Nodes represent bacterial genera, and edges represent significant correlations ($p < 0.05$) between genera. Node size is proportional to the degree of connectivity. While the total number of nodes was similar across all groups, edge density varied significantly, reflecting differences in network complexity and stability.

3.7. Integrated Microbiome-Metabolome Analysis

Procrustes analysis revealed significant congruence between rhizosphere microbiome composition and root exudate profiles ($M^2 = 0.627$, $p = 0.021$; Figure S7). The observed fit demonstrates that specific patterns in root exudation directly correspond to the recruitment and establishment of distinct microbial consortia, confirming a tight coupling between plant metabolism and microbial ecology in the rhizosphere. Mantel tests further demonstrated that CRaP and CRa group exhibited positive correlations with the majority of differential metabolites, including (2R)-2-hydroxy-2-phenethylglucosinolate, jasmonic acid methyl ester, 4-hydroxyglucobrassicin, MG(0:0/20:3(6,8,11)-OH(5)/0:0), and geniposidic acid (Figure 6A). Conversely, PT showed positive associations with the gibberellin A34 catabolite and (2S, 3R, 4S, 5R, 6R)-6-ethylxane-2,3,4,5-tetrol. Spearman's correlation analysis at the genus level revealed distinct interaction networks (Figure 6B). *Pseudomonas* and *Dechloromonas* exhibited significant positive correlations with three defense-associated metabolites: the MG(0:0/20:3(6,8,11)-OH(5)/0:0), the (2R)-2-hydroxy-2-phenethylglucosinolate, and jasmonic acid methyl ester. In contrast, *Methylibium* and

Lacibacter displayed strong positive correlations with the gibberellin A34 catabolite, a phytohormone-derived growth regulator.

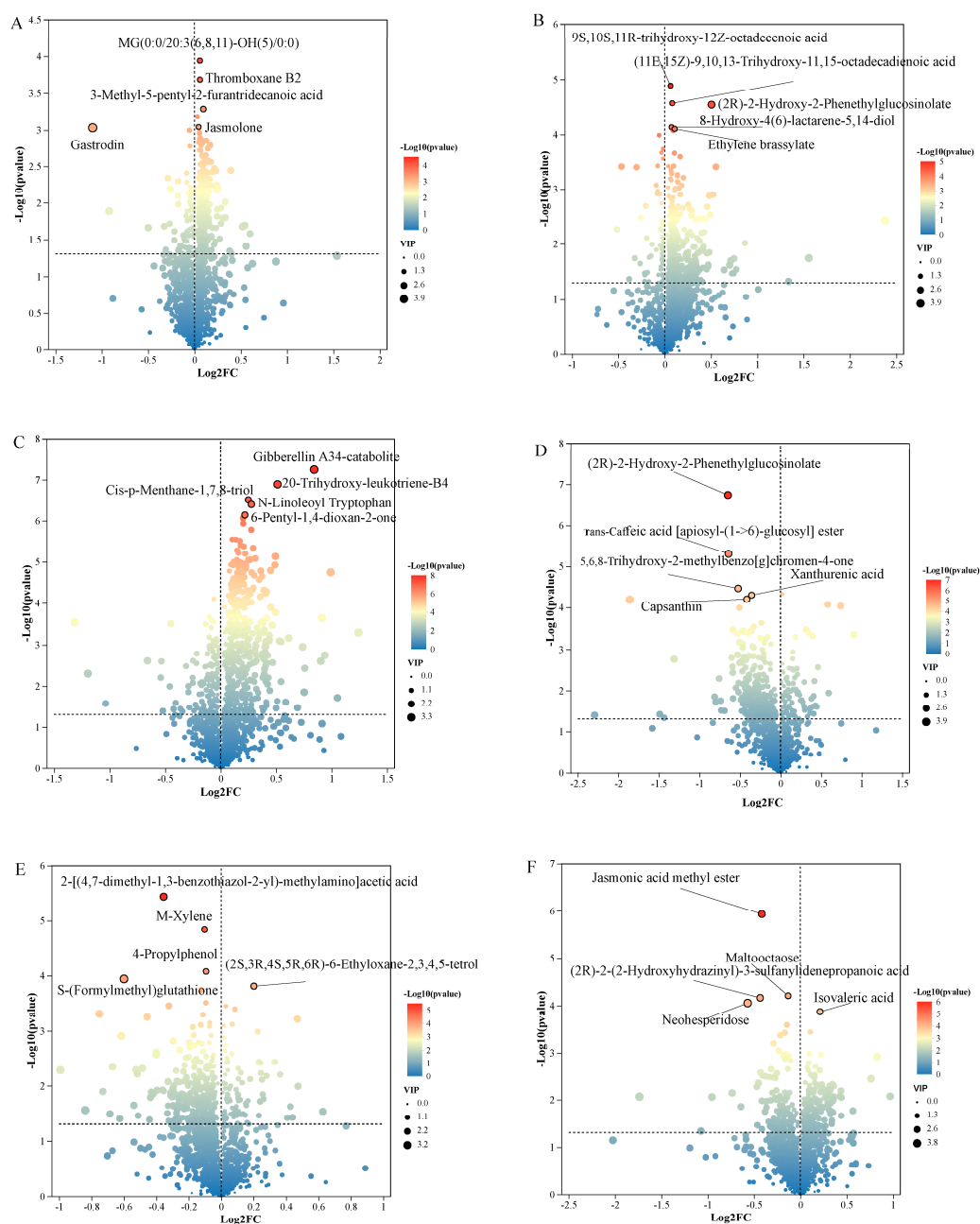


Figure 5. Differential metabolite profiling and metabolic reprogramming under stress and protein treatments. (A) CK vs. CRa. (B) CK vs. CRaP. (C) CK vs. PT. (D) CRaP vs. CRa. (E) PT vs. CRa. (F) CRaP vs. PT. Differential metabolites were screened based on $\text{VIP} \geq 1$ and fold change ≥ 2 or ≤ 0.5 .

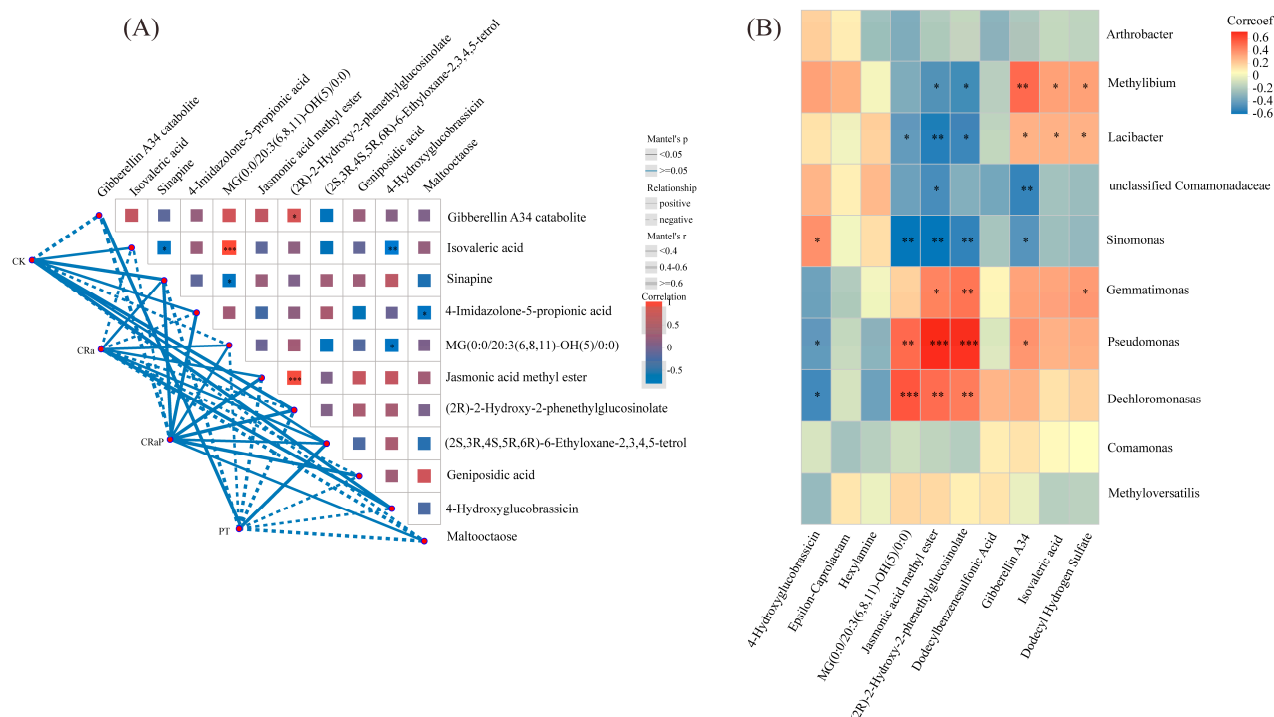


Figure 6. Integrated analysis linking rhizosphere bacterial communities and root exudate metabolite profiles. (A) Mantel test correlations between bacterial community structure and differential metabolite abundance. (B) Spearman correlation hot map of differential genera and key metabolites. * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$.

4. Discussion

This study elucidates how a recombinant protein derived from the *CRA* gene mediates resistance against *P. brassicae*. Through the integration of colonization kinetics, microbiome profiling, and metabolomics, we reveal that the CRa protein functions as a systemic modulator, capable of simultaneously reshaping the rhizosphere microbial consortium and reconfiguring root exudate profiles to establish disease suppression.

4.1. Engineered Systemic Delivery of CRa-RXLR Fusion Protein Activates Immunity

The efficacy of CRa protein is fundamentally rooted in its molecular design. By fusing the *CRa* coding sequence with an RXLR motif—a conserved domain facilitating host cell entry [9]—we enabled the protein to bypass traditional endocytosis and exploit the xylem stream for systemic translocation. Fluorescence microscopy confirmed that this strategy facilitated rapid, deep-tissue colonization within 24 h, reaching the stele and vascular tissues. This preemptive positioning at the invasion front proved highly effective, reducing the disease index from 45.8 to 12.1—an efficacy aligning with the established role of NB-LRR proteins in triggering potent defense responses through their NB-ARC domain-mediated signaling [31].

4.2. CRa Protein Reshapes Rhizosphere Microbiome Structure and Enhances Diversity

Beyond direct defense, CRa protein treatment profoundly restructured the rhizosphere microbiome toward a suppressive state. We observed a clear gradient of ecological maturity: the CRa group fostered the most resilient microbiome, achieving the highest alpha diversity indices (Shannon and Simpson) and harboring the greatest number of unique bacterial taxa. This enhanced diversity confers functional redundancy, increasing the probability of containing taxa capable of antagonizing *P. brassicae* [32].

Co-occurrence network analysis corroborated this transition, revealing that the CRa group harbored the most intricate network, indicative of a highly interconnected community. This complexity aligns with the prevailing notion that disease-resistant cultivars foster more elaborate rhizosphere microbiomes [33]. Conversely, the PT group exhibited a simplified network, suggesting reduced network complexity following pathogen infection. Concomitantly, pathogen infection disrupted root exudation chemistry, directly contributing to the observed reduction in microbial diversity [34].

4.3. Metabolic Reprogramming Drives Defensive Chemistry in Root Exudates

The structural remodeling of the microbiome was driven by CRa protein-induced metabolic reprogramming. Our integrated analysis revealed a specific upregulation of specialized defense metabolites, most prominently (2R)-2-hydroxy-2-phenethylglucosinolate, jasmonic acid methyl ester, and 4-hydroxyglucobrassicin. The accumulation of (2R)-2-hydroxy-2-phenethylglucosinolate is particularly notable. As a hydrolyzable glucosinolate, it plays a dual role upon myrosinase activation: it directly inhibits *P. brassicae* development while releasing volatile compounds that attract beneficial microbes [35]. Likewise, elevated levels of methyl jasmonate indicate activation of the jasmonate pathway, a key regulator of pest and pathogen defenses in Brassicaceae.

These compounds constitute the signature chemical arsenal of the Brassicaceae [36,37]. Their accumulation mirrors the classic defense signature of TIR-NB-LRR activation [38], providing the raw materials for both direct toxicity against plasmodia and the recruitment of beneficial microbiota [39]. Notably, the core differential metabolites identified in this study (geniposidic acid and jasmonic acid methyl ester) are also common defense markers under natural *P. brassicae* infection [40] and avirulent effector protein treatment [41]. This suggests that the CRa protein essentially amplifies the plant's natural metabolic reprogramming program to recruit beneficial microbes more efficiently for disease suppression.

4.4. Synergistic Metabolite-Microbe Interactions Suppress Disease

The convergence of these findings supports a model wherein CRa protein establishes disease suppression through the synergistic interaction of metabolites and microbes. For example, CRaP treatment enriched *Pseudomonas* as a key indicator genus, a hallmark of disease-suppressive soils capable of producing broad-spectrum antimicrobials [42,43]. Moreover, Spearman's correlation revealed that *Pseudomonas* was positively coupled to defensive metabolites, exemplifying the “cry-for-help” hypothesis [41,44]. Here, immune activation via jasmonate signaling reshapes root exudate chemistry—specifically elevating glucosinolates and jasmonates to assemble a health-promoting microbiome [19,45]. This metabolite-microbe synergy aligns with emerging paradigms of microbiome-mediated disease suppression highlighted in recent systematic reviews of clubroot pathogenesis, and complements prior observations that avirulent effectors such as 2565 similarly recruit beneficial rhizobacteria via exudate reprogramming to restrict pathogen proliferation [46].

While these results clearly demonstrate a link between CRa expression and microbiome remodeling, we acknowledge the need to rule out nonspecific protein effects. Although a standalone eGFP control was not included, two points address this concern: (i) the CK and PT controls account for general artifacts caused by protein exposure; and (ii) previous studies confirm that free eGFP does not alter the rhizosphere microbiome or root exudates [47]. Consequently, the observed phenotypes are definitively attributed to the CRa-RXLR fusion.

5. Conclusions

Our study demonstrates that a recombinant CRa protein fused with an RXLR motif confers strong clubroot resistance through three coordinated mechanisms: (i) systemic transport via the xylem; (ii) metabolic reprogramming that boosts defensive compounds, such as glucosinolates and jasmonates; and (iii) restructuring of the rhizosphere microbiome toward a disease-suppressive state enriched with *Pseudomonas* and enhanced microbial networks. This single-dose, non-transgenic approach reverses pathogen-induced dysbiosis while avoiding the fitness costs commonly associated with transgenic overexpression. Since the RXLR-mediated delivery mechanism is conserved across plant families, initial validation in Brassica crops supports its scalability as a strategy for engineering exudate-mediated immunity. Future work should identify the receptors involved in CRa perception and hormone activation, and multi-location field trials are required to translate this concept into a sustainable, microbiome-based disease control strategy.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy16151485/s1>, Figure S1. Construction of the pET28a-RXLR-eGFP-CRa-His6 expression vector. Schematic diagram of the recombinant plasmid pET28a-RXLR-eGFP-CRa-His6. The construct utilizes a T7 promoter to drive the expression of the RXLR-EER secretion signal fused to the eGFP-CRa-His6 coding sequence. The C-terminal 6 × His tag allows for affinity purification. Key backbone elements include the lacI repressor gene, kanamycin resistance gene (KanR), and the pBR322/ColE1 origin of replication. Figure S2: Optimization of CRa-RXLR-eGFP fusion protein expression. (A) Effect of induction temperature. M: Protein ladder (250 kDa); Lanes 1–3: Induction at 16 °C, 25 °C, and 37 °C, respectively. (B) Effect of inducer concentration. M: Protein ladder (250 kDa); Lanes 1–2: Uninduced controls; Lanes 3–5: Induction with 0.5, 1.0, and 1.5 mmol/L IPTG, respectively. Figure S3: Assessment of clubroot disease severity in stem mustard under different treatments. (A) Quantitative comparison of disease indices between the CRaP and PT groups. n = 45 independent plants per group. Error bars represent the standard error of the mean (SEM). Statistical significance was determined via One-way ANOVA; ** indicates a statistically significant difference ($p < 0.01$). (B) Representative phenotypic symptoms of clubroot infection in stem mustard roots. Figure S4: Venn diagrams illustrating the overlap of bacterial taxa. (A) Overlap of bacterial taxa at the class level across the four treatment groups. (B) Overlap of bacterial taxa at the genus level across the four treatment groups. Figure S5: Overview of root exudate metabolic profiles. (A) Classification of identified metabolites based on chemical taxonomy. (B) Classification of metabolites based on their involvement in metabolic pathways. (C) Chemical composition classification of compounds. Figure S6: Overview of root exudate metabolic profiles across treatments. (A) Venn diagram illustrating the overlap of identified metabolites among the four groups. (B) PLS-DA score plot showing distinct clustering of metabolic profiles by treatment. (C) Permutation test results validating the robustness of the PLS-DA model. Figure S7: Procrustes analysis of rhizosphere microbiome composition and root exudate profiles. Table S1. Summary of co-occurrence network topological properties for the four treatments. Table S2. Node-level topological metrics and keystone taxon identification in the co-occurrence networks of the four treatments. Table S3. Annotated metabolomic profiling of tumorous stem mustard reveals CRa-RXLR fusion protein modulates root metabolic reprogramming against clubroot.

Author Contributions: Conceptualization, writing—original draft, data curation, investigation: Q.W.; Methodology, visualization, investigation, formal analysis: J.L.; Software, methodology, investigation and validation: T.C.; Resources, visualization, software, methodology: Z.C. (Zhaoming Cai); Visualization, methodology, investigation and software: Z.C. (Zhiyi Chen); Writing—original draft, writing—review and editing, conceptualization, supervision: X.T.; Writing—original draft, funding acquisition, project administration, writing—review and editing: D.W. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by Chongqing Municipality Natural Science Foundation Innovation and Development Joint Fund (CSTB2023NSCQ-LZX0074), Chongqing Talent Plan “Lump-sum Funding System” Project (CQYC20220302547).

Data Availability Statement: All data supporting this study are included in the manuscript and Supplementary Files. Additional inquiries should be directed to the corresponding authors. The raw 16S rRNA reads have been uploaded to the NCBI Sequence Read Archive (SRA) database with the accession number PRJNA1475733.

Acknowledgments: The authors declare the use of generative artificial intelligence (GenAI) in the preparation of this manuscript. The specific tool employed was DeepSeek-R1 (v1.0), developed by DeepSeek (official website: <https://www.deepseek.com>). The initial prompt provided to the AI was: “Check for spelling and grammar errors”. The final prompt provided was: “Polish the language and correct grammatical errors”.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

eGFP	Enhanced green fluorescent protein
HPI	Hour post-inoculation
LEfSe	Linear Discriminant Analysis Effect Size
NLR	Nucleotide-binding Leucine-rich repeat
OTU	Operational taxonomic units
PCA	Principal component analysis
PLS-DA	Partial least squares discriminant analysis
RXL	Arg-X-Leu-Arg motif
TIR-NB-LRR	Toll/Interleukin-1 receptor-Nucleotide-binding -Leucine-rich repeat
VIP	Variable importance in projection

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