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Prunella vulgaris-derived biostimulant enhances copper tolerance in rice by mitigating oxidative damage and regulating ion homeostasis

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ABSTRACT

Overabundance of soil copper may adversely affect natural and agricultural systems. In growing plants, excess copper severely impairs nutrient absorption and metabolic activity in roots. Here, we show that *Prunella vulgaris* (Pv) extract can alleviate copper toxicity in rice seedlings. First, we found that copper stress has inhibitory effects on growth and biomass of rice seedlings. After exposure to high levels of copper, plants exhibited high copper contents, elevated lipid peroxidation biomarker (Malondialdehyde - MDA and ROS) levels, and increased numbers of inviable cells. Interestingly, treatment with 0.25% (w/v) Pv extract robustly rescued the growth of plants exposed to copper stress. Transcriptomic analysis suggested that Pv extract might regulate cell wall properties under copper stress. Furthermore, Pv extract significantly upregulated several genes involved in biosynthesis of proline, deoxymugineic acid, metalloproteins, vacuolar and efflux transporters. These findings suggest that Pv-induced cellular defense responses might help to combat copper-associated oxidative stress. Additionally, LC/MS analysis of Pv extract revealed the presence of diverse bioactive compounds such as caffeic acid, rosmarinic acid, and melatonin. Taken together, these findings reveal mechanisms by which Pv extract attenuates copper toxicity and suggest a means of improving agricultural production.

1. Introduction

Rice is an important crop feeding ever-growing population worldwide. As the crop thriving in flooded fields, its growth and development are dependent on water and soil quality. However, a variety of human activities may lead to copper contamination in soil, which can drastically reshape agricultural productivity and ecological health (Poggere et al., 2023). It is well known that excess copper disturbs plant growth and development. As a redox-active element, elevated copper primarily trigger generation of ROS (oxidative stress) through Haber-weiss and Fenton reaction (Akhtar et al., 2026) leading to lipid peroxidation, protein oxidation, membrane

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destabilization and DNA damage (Sachdev et al., 2021; Thounaojam et al., 2012), ultimately culminating in cell death. In addition, excess copper impairs photosynthetic process in plants. Evidence suggested that exposure to copper toxicity decrease chlorophyll content and photosynthetic efficiency by competing with metal cofactors (Mg, Zn, and Fe) at binding sites in proteins and photosynthetic pigment (E. Xu et al., 2024). Consequently, copper toxicity manifests in chlorotic and wilted leaves, retarded root growth, reduced branching, abnormal dark coloration, and, in severe cases, plant death (Akhtar et al., 2026; Yadav, 2010).

In an agricultural context, it is especially important to manage early stress levels in crops, and there are many new environmentally friendly products that have been introduced for this purpose. Among these newly introduced products, plant-based biostimulants are rich in active compounds, and application of these natural compounds has been reported to enhance plant nutrient uptake and defense activity, promoting plant growth and development (Campobenedetto et al., 2021; Gedeon et al., 2022; Naboulsi et al., 2022; Roussi et al., 2022). Several plant extracts have been shown to act as effective biostimulants. For instance, a study by Ennoury et al. (2023) showed that extract of *Atriplex halimus* positively affects plant metabolism and photosynthesis pigments, leading to improved growth of faba bean. Another study showed that organic extracts from *Casuarina equisetifolia* can stimulate proline production in rice to increase their survival under drought stress (Salsinha et al., 2023). Plant-derived extracts have been widely reported to enhance stress tolerance; however, these studies largely emphasize antioxidant responses or a limited set of candidate genes (Alkuwayti et al., 2020; Gedeon et al., 2022; Pandey et al., 2019; Roussi et al., 2022). As a result, the underlying molecular mechanisms governing plant responses to heavy metal stress remain poorly resolved. Furthermore, a variety of solvents, including water, methanol, and ethanol, as well as extraction techniques such as ultrasonic-assisted and microwave-assisted extraction, have been employed to obtain plant extracts (Elsayed et al., 2023; Kalaivani et al., 2023). Although these approaches can enhance the yield of bioactive compounds, they often involve increased complexity and cost. Therefore, in this study, we aim to develop simplified, cost-effective, and practical extraction methods to facilitate broader application.

In this study, we evaluated an aqueous extract of *Prunella vulgaris* as a potential biostimulant for protecting rice seedlings from copper stress. *P. vulgaris* is a perennial herbaceous plant of the Lamiaceae/Labiatae family, which grows across widespread temperate regions of the world, including in Eurasia, Africa, the Americas and Australia. The plant has been extensively utilized in both European and Chinese traditional medicines (Zholdasbayev et al., 2023). In Europe, fruit spikes of *P. vulgaris* are used as a nutritional supplement or healing agent to treat sore throats, intestinal infections or diarrhea. In Asia, the plant is used as an herbal tea to relieve migraine or fever. Although the aerial parts of *P. vulgaris* are not commonly utilized for medicinal purposes (Pan et al., 2022; Zholdasbayev et al., 2023), previous work showed that these parts are rich in rosmarinic acid, ursolic acid and oleanolic acid (Chen et al., 2012), which exhibit extensive bioactive functions (e.g., anti-hyperglycemia, antifungal, antitumor and anti-inflammatory effects). In the context of plants, it is known that exogenous rosmarinic acid improves thermotolerance in tomatoes (Zhou et al., 2022) and *Arabidopsis thaliana* (Arikan et al., 2022). Additionally, ursolic acid protects from salinity stress by stimulating activities of antioxidant system in rice (Long et al., 2020). Furthermore, compared to known biostimulants such as seaweed-based products, microbial inoculants, and synthetically derived substances requiring advanced production technologies (e.g., amino acids and protein hydrolysates) (Arinaitwe et al., 2025), extracts from the aerial parts of *P. vulgaris* (Pv extracts) may have potential to serve as cost-effective, convenient biostimulants agricultural settings.

In the present study, we sought to investigate whether Pv extracts could mitigate copper-induced damage in rice seedlings. We evaluated the impacts in terms of growth parameters, membrane disruption, copper absorption, and nutrient acquisition. After finding that Pv extracts could indeed protect from the negative effects of copper exposure, we conducted experiments to clarify the molecular mechanisms underlying Pv extract-induced tolerance to copper stress.

2. Materials and methods

2.1. Extract preparation

Aerial parts (leaves and stems) of *P. vulgaris* were used for preparation of aqueous extracts. Dried material was incubated in distilled water at 4 °C at different concentrations: 0.15%, 0.2%, 0.25% (w/v). The range of concentrations (0.15, 0.2, and 0.25%) were selected based on previous studies and preliminary optimization. Initial concentrations (1% and 2%), derived from earlier reports (Desoky et al., 2019; Mona, 2013), were found to be phytotoxic under our experimental conditions, leading to inhibited plant growth. Therefore, the concentration were reduced 10-fold to obtain a lower, non-toxic range suitable for re-evaluating physiological responses of rice seedlings under copper stress condition. After 2 days of incubation, the extracts were filtered with Whatman filter paper.

2.2. Plant culture and treatments

Rice (*Oryza sativa* L. cv. TNG67) seeds were obtained from the Tainan district agricultural research and extension station (Ministry of Agriculture; MOA). The seeds were surface-sterilized with 2.8% (v/v) hypochlorite solution and distilled water. Then, the rice seeds were germinated in water at 37ºC for 2 days. Uniform seeds were collected and grown in plastic petri dishes with moist paper for 3 days at 27ºC.

2.3. Measurement of plant growth, malondialdehyde (MDA), proline level

For the growth assay, 5-day-old uniform seedlings were grown on different hydroponic solutions: distilled water (control), Pv extract (Pv), 25 µM copper(II) chloride (Cu), and Pv extract + 25 µM copper(II) chloride (PvCu). The growth assay was performed for 3 days based on previous study (Lin et al., 2013), which demonstrated the inhibitory effects on root growth in rice seedlings. Growth was measured in terms of length of shoot, root length, and plant biomass after the treatment.

For MDA detection, 5-day-old rice seedlings were used. After 3 days of treatment, 0.05 g of fresh shoots and roots were separately harvested for the analysis. MDA levels were measured following the methodology described in Hodges et al. (1999) with minor modifications. The reaction mixtures contained crude plant extract, 10% (w/v) TCA and 5% (w/v) TBA. Reactions were incubated at 90°C for 15 min. Then, absorbance at 440, 532 and 600 nm was measured to estimate the MDA content.

Proline levels were determined with the assay described by Bates et al. (1973). About 0.05 g of fresh shoots and roots were ground and homogenized with 2% sulfosalicylic acid. Then the homogenates were centrifuged, and the plant crude extracts were added to a reaction mixture containing acetic acid and 140 mM ninhydrin at the ratio 1:1:1. The reactions were incubated in a water bath for 45 mins at 90°C. Then, the reactions were cooled on ice for 20 min. Afterward, 1 mL toluene was added, and the samples were vortexed for 30 s. The concentration of proline was calculated by comparing the absorbance value of the sample at 520 nm to a standard curve.

2.4. Detection of H_2O_2 content and Casparian strip formation

For H_2O_2 detection, roots were incubated in a solution of 1 mg/mL DAB (3 3′-diaminobenzidine) prepared in ddH_2O (pH 3.8). The incubation was conducted for 12 h in the dark. Then, stained roots were washed with ddH_2O and observed under a stereomicroscope.

To observe the formation of Casparian strips, methods described in previous study (Zelko et al., 2012) was applied. Freehand root cross-sections were incubated in berberine 0·01% (w/v) prepared in lactic acid 50% for 1 h, followed by counterstaining in 0.5% aniline blue solution for 30 min. The sections were then rinsed in water and mounted in 50% (v/v) glycerol. Samples were subsequently viewed under fluorescence microscope using blue light (450–490 nm).

2.5. Element analysis

Harvested samples (shoots and roots) from treated groups were dried at 65°C for 2 days. Dried samples were then digested following a previously reported study of Huang et al. (2023). Nutrient elements were analyzed in Pv extract and plant tissues using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (HORIBA Scientific).

2.6. RNA isolation and sequencing

RNA was extracted from roots of 5-day-old rice seedlings grown in four different solutions: distilled water (control), Pv extract (Pv), 5 μM $CuCl_2$ (Cu), Pv extract + 5 μM $CuCl_2$ (PvCu). After 24 h of treatment, roots were harvested and frozen in liquid nitrogen. Then, total RNA from rice roots was isolated using Rneasy Plant mini Kit (Qiagen, Germany) following the manufacturer's procedure. Three biological replicates were analyzed from each treatment.

Isolated RNA was utilized to build sequencing libraries and sequenced according to Illumina HiSeq platform construction. Sequencing data analysis was conducted based on (Pertea et al., 2016). Raw reads were cleaned by Trimmomatic v0.36. Quality reads were mapped to the *Oryza sativa* reference genome (https://ftp.ebi.ac.uk/ensemblgenomes/pub/release-61/plants/fasta/oryza_sativa/dna/Oryza_sativa.IRGSP-1.0.dna.toplevel.fa.gz) using Hisat2 v0.36. Transcripts with FPKM value = 0 were removed before differential expression analysis was performed using the DeSeq2 R package ($|\log2(FC)| \geq 1$ and padj < 0.05). Gene ontology enrichment was carried out using agriGOv2 (https://systemsbiology.cau.edu.cn/agriGOv2/).

2.7. RT-qPCR

The expression of selected genes was validated using GoTaq® qPCR Master Mix (Promega), and relative gene expression was evaluated as previously reported (Schmittgen and Livak, 2008). OsGAPDHC7 (Os08g0126300) was used as reference gene for the experiments. The primer sequences are shown in Table S3.

2.8. Statistical analysis

The data were analyzed using open-source R software. Significant differences among treatments were detected with one-way ANOVA and post hoc Tukey's analysis. Statistical significance was set at $P < 0.05$. Student's *t*-test was used to examine the differences between treatments for proline content and RNA-seq validation experiments.

3. Results

3.1. Chemical composition of P. vulgaris extract

LC-MS analysis revealed that Pv water extract was rich in novel bioactive compounds. Fifty-one compounds were identified, similar to reports from previous studies (Pan et al., 2022; Tang et al., 2022) (Table S1). A mineral nutrient element analysis was also conducted, revealing that the Pv extract contained relatively high levels of calcium (5.09 ppm), potassium (37.76 ppm) and magnesium (5.11 ppm) (Table S2).

3.2. *Effects of copper and Pv extract exposure on TNG67 rice seedling growth*

Exposure of rice seedlings to excessive copper caused severe cell damage in rice roots after 24 h. To show this effect, the root structures of rice seedlings were stained with Evans blue, and cell death was assessed. Compared with the controls, copper-treated rice roots showed intense blue color, indicating the presence of widespread cell death. Interestingly, treatment with different concentrations of Pv extract (0.15, 0.2, and 0.25%) alleviated copper-induced cell death (Fig. S1A) in a dose-dependent manner. The 0.25% Pv extract treatment showed the highest attenuation of copper-induced damage. Thus, this concentration was utilized in subsequent experiments.

Next, we evaluated the effects of Pv extract on growth of rice seedlings under copper stress. As shown in Fig. 1A, Pv extract had growth-promoting effects in non-stressed rice seedlings, as indicated by increases in shoot and root lengths as well as increases in biomass (Fig. 1B) (shoots, 9.6% increase; roots, 22.8% increase; biomass, 47.4% increase). Under copper stress conditions, plant growth was strongly attenuated. Compared with the control group, copper-treated seedlings had significantly reduced length of shoots (22.08% decrease), roots (35.18% decrease), and biomass (38% decrease) (Fig. 1C). However, treatment of Pv extract effectively alleviated copper-induced toxicity in rice seedlings (Fig. 1). Application of the Pv extract restored plant growth, increasing lengths of shoots (36%), roots (105%), and biomass (65.4%), as compared to the copper stress group.

3.3. *Global transcriptome changes induced by Pv extract in copper-stressed rice roots*

To investigate the molecular mechanisms underlying Pv extract-induced stress tolerance to copper in rice seedlings, we conducted transcriptomic analyses. In our RNAseq experiments, we identified a total of 1881 upregulated and 1931 downregulated genes, comparing between the copper stress and control groups. A total of 2149 upregulated and 2878 downregulated genes were identified when comparing between the PvCu and Cu groups. Furthermore, we identified 399 upregulated and 1197 downregulated genes when comparing the Pv extract group with controls (Fig. S2A).

GO enrichment analysis yielded 17 GO terms (biological processes) that were upregulated in the PvCu group as compared to the Cu group. Notably, the identified GO terms were mainly related to defense responses (response to stress, stimulus, oxidative stress) and cell wall biogenesis (glucan metabolism, polysaccharide metabolic process, plant-type cell wall organization) (Fig. S2B).

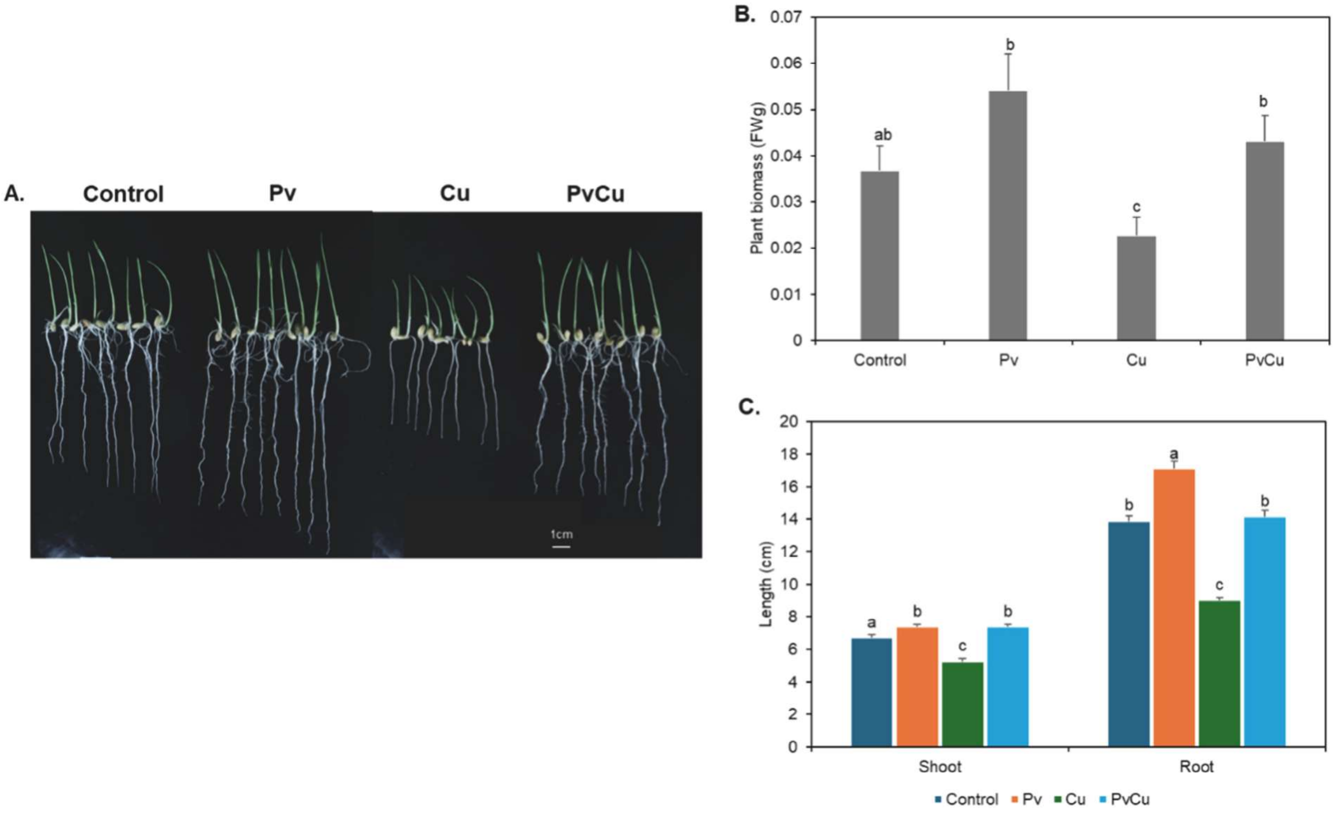

Fig. 1. Pv extract enhances copper tolerance of rice seedlings. **(A)** Phenotypes of 5-day-old rice seedlings grown for 3 days in four different solutions: water (control), Pv extract (Pv), 25 μM $CuCl_2$ (copper), 25 μM $CuCl_2$ + Pv extract (PvCu). **(B)** Plant biomass, **(C)** lengths of shoots and roots. Data are displayed as mean ± SE (n = 3 biological replicates). Different lowercase letters indicate significant difference between treatments, according to Tukey's test ($p \leq 0.05$).

3.4. Pv extract alters metal detoxification pathways in copper-stressed rice seedlings

In our analysis, we identified three DEGs (OsGS1;2, OsP5CS2, and proDH) associated with proline synthesis in Pv-copper-treated rice seedlings (Fig. 2A). Validation experiments were performed with qRT-PCR, confirming the changes in transcript levels of OsP5CR, OsP5CS2, and proDH in the PvCu group as compared to the Cu group (Fig. 2A). Increased proline contents were also observed in PvCu plants (3.1-fold) as compared to seedlings exposed to copper stress alone (Fig. 2B). Detection of oxidative stress indicators, H_2O_2 and MDA, showed that exposure to excessive copper induced ROS accumulation in rice roots. However, H_2O_2 was decreased by 64.6% in roots, and MDA content was reduced by 74.5% in shoots and roots in the PvCu rice seedlings as compared to Cu group (Fig. 2C, D). Taken together, these results suggest that supplementation with Pv extract may enhance tolerance of rice seedlings copper toxicity, with concurrent reductions in oxidative stress.

DMA is a metal chelator that not only benefits plant nutrient acquisition but also prevents interactions between metal and cellular components. Compared to the Cu group, the PvCu seedlings had increased expression levels of several DMA synthesis-related genes, including SAM, NAS and DMAS (Fig. 3A). Metalloprotein-related genes (i.e., HIP, Thi and HMP) also had increased expression levels in PvCu rice roots as compared to the Cu group. Metal sequestration may be upregulated for the purpose of metal detoxification, and we identified high expression of several genes involved in metal sequestration, including vacuolar (OsCAL1 and OsZIP1) and efflux transporter (OsTIP1;2 and OsTIP2;1), in PvCu rice seedlings (Fig. 3B).

3.5. Pv-mediated cell wall organization in rice roots under excess copper

The cell wall is a front-line structure exposed to environmental stimuli. The composition and structure of the plant cell wall are regulated upon encountering heavy metal stress. From our data, we found a total of 150 upregulated genes involved in cell wall biogenesis in PvCu rice seedlings, as compared to the copper-treated group (Fig. 4A). Of these genes, 33 are involved in cellulose and xyloglucan biosynthesis, including UDP-Glc pyrophosphorylases (UGP), UDP-Glc epimerase (UGE), UDP-arabinopyranose mutase (UMA), UDP-Glc dehydrogenase (UGD), cellulose synthase like (CSL), xyloglucan galactosyl transferase (XGT), Xyloglucan fucosyl-transferase (XFT), Xyloglucan xylosyltransferases and galactomannan galactosyltransferases (XXT) and Glucan synthase-like (GSL). In addition to cell wall biosynthetic enzymes, we saw upregulation of genes related to cell wall extension and assembly processes,

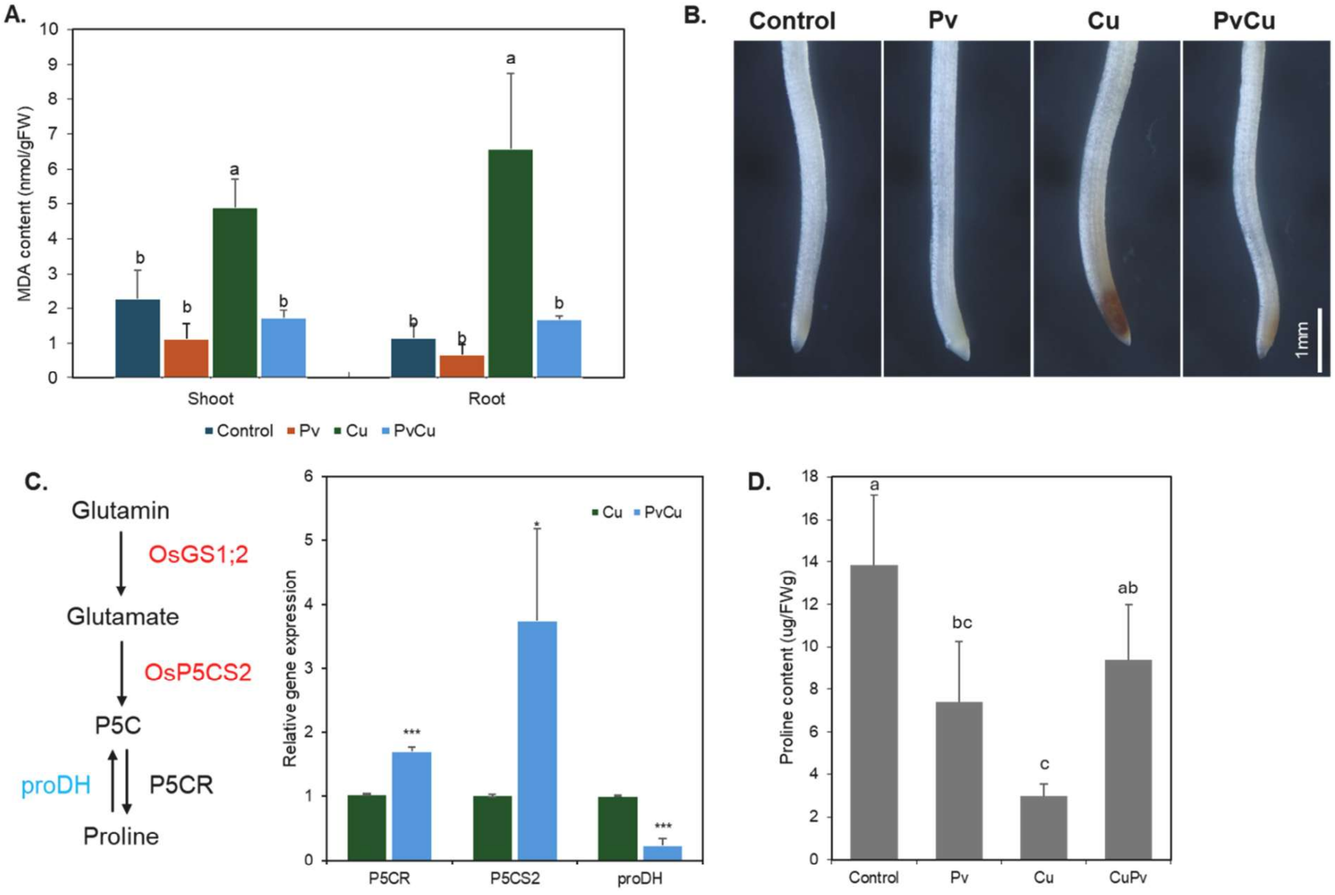

Fig. 2. Pv extract alleviates oxidative stress in rice roots under copper stress. Oxidative stress levels were assessed according to (**A**) MDA content and (**B**) H_2O_2 detection by DAB staining. (**C**) Proline biosynthesis-related genes were upregulated in PvCu-treated plants. Red and blue colors indicate upregulated and downregulated, respectively. (**D**) Proline content. Different letters indicate significant difference between groups ($p < 0.05$, one-way ANOVA, Tukey-HSD).

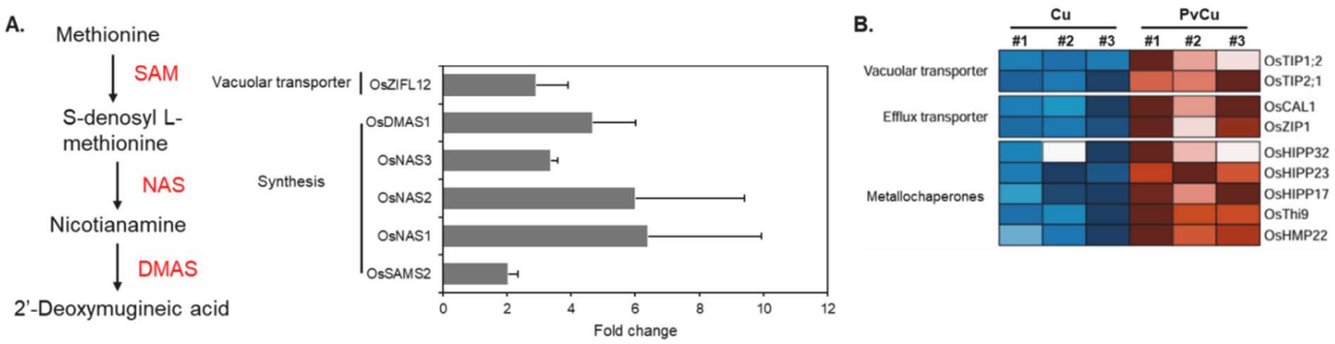

Fig. 3. Pv extract induces metal sequestration mechanisms in rice roots under excess copper conditions. (**A**) Upregulated expression levels of Deoxymugineic acid biosynthesis genes. (**B**) Upregulated expression of genes involved in metal homeostasis. Data are displayed as mean ± SE ($n = 3$ biological replications). The color scale represents the row z-score, with red and blue colors indicating higher and lower expression, respectively.

including loosening (69 upregulated genes) and protein structure (11 upregulated genes) (Fig. 4A). The cell wall loosening genes included members of expansins (EXP: 20 genes), Glycoside hydrolases (GH: 15 genes), Xyloglucan endotransglycosylases/hydrolases (XTH: 12 genes), Beta-galactosidases (BGAL: 1 gene), as well as pectin modification enzymes – Pectin methyl esterases (PME: 5 genes), Rhamnogalacturonan I lyases (PL4: 1 genes) and Pectin acetylesterases (PAE: 2 genes). Upregulated protein structure genes included 5 genes belonging to the Leucine-rich repeat extensins (LRX) and 6 genes belonging to the Arabinogalactan protein (AGP) family.

Fifty-two genes from six families involved in lignin metabolism (4 CL, CSB, CCR, LAC, DIR and Prx) showed high expression levels in PvCu plants (Fig. 4B). We also noted that 4 out of 5 genes associated with Casparian strip formation (CASP) (CASP1: FC 5.3, CASP2: FC 9.3, CASP3: FC 5.9, CASP5: FC 16.2) were highly expressed in the PvCu rice seedlings as compared to the Cu group (Fig. 4C). Of note, our qRT-PCR experiments showed the consistency in expression levels of CASP genes (Fig. 4C), which were further corroborated by enhanced Casparian strip (CS) formation in PvCu-treated roots, as visualized by berberine–aniline blue staining (Fig. 4D).

3.6. Acquisition of nutrients in Pv-treated rice seedlings under copper stress

The contents of different elements (e.g., Ca, Cu, Fe, K, Mg and Na) in treated rice shoots and roots were measured to understand the effects of Pv on acquisition of nutrients in rice roots under copper stress. Differences in nutrient element contents were analyzed in Pv, Cu, and PvCu groups as compared to the control (Table 1). As expected, excess copper led to increased levels of copper in roots as compared to the control. Meanwhile, decreases in K content were observed in the copper-treated rice roots. Interestingly, lower levels of copper were observed in PvCu roots as compared to copper-only group. However, the levels of copper was increased in Pv-treated shoots under stress condition. Furthermore, higher levels of K, Mg were present in the both Pv-treated samples, while Ca levels was only increased in Pv-treated roots, as compared to respective control and stressed groups. These observations suggest that K, Mg and Ca may be involved in Pv-mediated growth promotion of rice seedlings under non-stressed and stressed conditions.

4. Discussion

In this study, we tested the effects of Pv extract on copper-exposed rice seedlings. We first showed that excessive copper exposure impairs rice seedling growth and decreases plant biomass (Fig. 1). Exposure of rice roots to copper stress also results in accumulation of MDA, indicating increased levels of lipid peroxidation (Fig. 2D). Efforts have been made to find strategies to alleviate heavy metal-induced toxicity on plants using plant extracts, hormones or phytochemicals as biostimulants (Altun and Orcan, 2024; Ma et al., 2022; Moravcová et al., 2018; Roussi et al., 2022). An anthocyanin-rich extract derived from red cabbage leaves was shown to alleviate copper-induced oxidative and genotoxic stress in Vicia faba, as reflected by restoration of the mitotic index and a reduction in chromosomal aberrations (Posmyk et al., 2008).

P. vulgaris (also known as self-healing herb) is used in traditional medicines around the world to protect the liver, reduce inflammation, calm sleep disorders, and improve eyesight (Guo et al., 2020; Pan et al., 2022). Extracts of *P. vulgaris* contain myriad bioactive compounds, including flavonoids, terpenoids, organic acids, volatile oils, polysaccharides, phenypropanoids. These compounds may have pharmacological actions related to anti-cancer, antibacterial, antiallergic, anti-inflammatory, and antioxidant functions. In this study, we aimed to investigate the potential of herb extracts to rescue plant growth under copper stress. In our experiments, we showed that supplementation of Pv extract in rice seedlings alleviated copper-induced damage, reduced copper content (Table 1), and mitigated oxidative stress (Fig. 2C, D). Interestingly, the use of Pv extract enhanced shoot and root lengths, and it increased the biomass of rice seedlings under both control and copper-stress conditions (Fig. 1). Thus, we conclude that Pv extract can serve as a biostimulant to promote plant growth. Among the concentrations we tested, 0.25% Pv was the optimal concentration for treatment of rice seedlings (Fig. S1A). Notably, our experiments also showed that copper-induced cell death was lower in plants treated with extracts made from aerial parts rather than the flowering spike (Fig. S1B). These results suggest that the different compounds or different levels of bioactive compounds are present in the distinct parts of plants. Furthermore, investigation of three chemicals identified Pv extracts for their effecs on developing stress tolerance in rice seedlings further confirm the potential role of Pv extract in enhancing rice seedlings survival under copper toxicity (Fig. S1C).

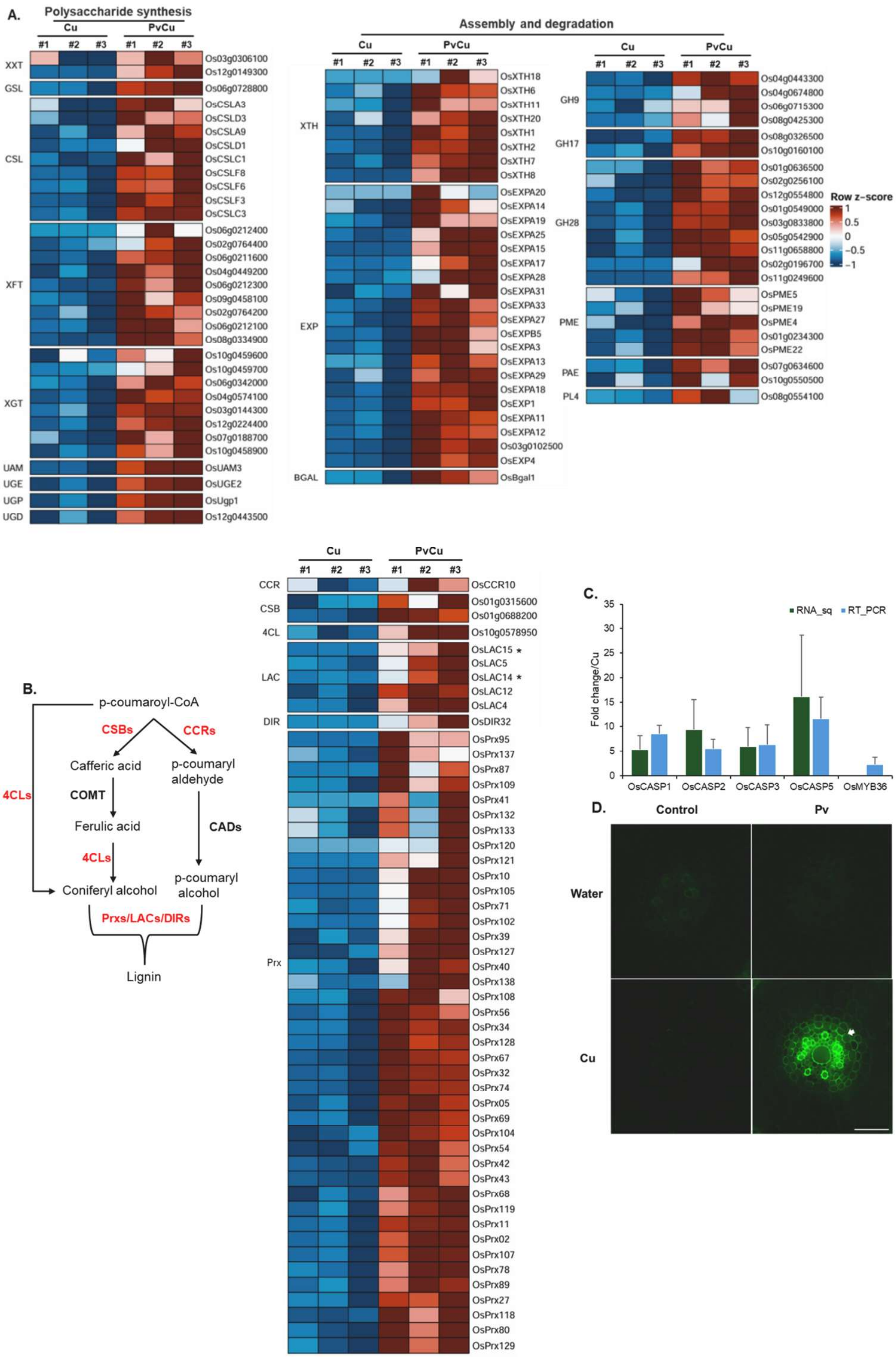

Fig. 4. Gene expression profiles revealed changes in (**A**) cell wall remodeling, (**B**) lignin biosynthesis, and (**C, D**) Casparian strip formation in copper- and PvCu-treated rice roots (white arrow). The color scale represents the row z-score. Red and blue colors indicate higher and lower expression, respectively. Asterisk denotes the altered expression as validated by qRT-PCR. Bar = 50 μm.

Furthermore, the Pv extract contained relatively high levels of Ca, K and Mg (Table S2), and the extract enhanced nutrient accumulation in rice seedlings (Table 1). Some key functions of these elements are to regulate photosynthesis, enzyme activity, stabilization of cell structures and various other physiological processes in plants, which could all contribute to promoting growth of rice seedlings under stress conditions (Ahmed et al., 2023; Y. Li et al., 2022; Mostofa et al., 2022).

Table 1

Element contents in rice shoots and roots grown for 3 days in growth medium containing 0 μM Cu, 0.25% Pv (w/v) extract, 5 μM Cu, and 5 μM Cu + 0.25% Pv (w/v) extract. Data are represented as mean ± SD of three independent measurements. Significant differences between treatments are indicated by different letters ($p < 0.05$, one-way ANOVA followed by Tukey-HSD).

Elements	Shoot				Root			
	Control	Pv	Cu	PvCu	Control	Pv	Cu	PvCu
Ca (mg/g)	1.04 ± 0.10a	1.05 ± 0.10a	1.05 ± 0.11a	1.05 ± 0.09a	0.38 ± 0.02c	0.67 ± 0.03a	0.48 ± 0.06bc	0.59 ± 0.09ab
Cu (mg/kg)	6.98 ± 0.19c	7.04 ± 0.86c	17.61 ± 1.26b	21.33 ± 1.30a	4.63 ± 0.98c	4.36 ± 0.26c	98.22 ± 8.37a	70.73 ± 14.31b
Fe (mg/kg)	30.57 ± 3.15a	28.56 ± 4.04a	28.96 ± 4.33a	26.39 ± 4.21a	35.21 ± 1.73a	34.58 ± 5.54a	38.51 ± 6.10a	33.32 ± 6.12a
K (mg/g)	11.19 ± 1.12b	26.75 ± 0.55a	12.65 ± 0.35b	27.54 ± 1.18a	7.41 ± 2.52ab	10.35 ± 1.11a	4.96 ± 0.36b	8.17 ± 3.28ab
Mg (mg/g)	2.19 ± 0.13b	2.57 ± 0.14a	2.07 ± 0.04b	2.59 ± 0.05a	0.54 ± 0.06b	1.72 ± 0.19a	0.58 ± 0.04b	1.54 ± 0.34a
Na (mg/kg)	0.55 ± 0.07a	0.37 ± 0.04a	0.53 ± 0.11a	0.38 ± 0.07a	1.18 ± 0.36b	2.05 ± 0.21a	1.97 ± 0.07a	1.59 ± 0.43ab

In light of these findings, the application of Pv extracts as biostimulants to mitigate abiotic stress holds notable economic and environmental potential. The usage of plant-derived biostimulants could contributes to reducing reliance on synthetic agrochemicals, thereby lowering input costs and supporting more sustainable agricultural practices (Bulgari et al., 2015). Pv, a widely distributed medicinal herb across diverse precipitations, is primarily harvested for its flowering spikes or seeds, whereas its aerial parts (leaves and stems) are largely underutilized (Zholdasbayev et al., 2023). This underexploited material represents a promising and low-cost resource for value-added applications in crop production. When combined with a simplified extraction method (cold-water extraction), it could provide an additional income stream, a convenient biostimulant application for farmers, and promote environmentally responsible production systems.

4.1. Roles of Pv extract in detoxification of copper in rice seedlings

To alleviate copper toxicity, plants often activate cellular defense strategies, such as heavy metal sequestration and ROS elimination. These defenses may be mediated by increases in heavy metal transporters, antioxidants, and phytochelatins (Noor et al., 2022). Proline is vital for protection from plant stress because of its effects on osmoregulation, antioxidant activity, metal chelation, and protein stability (Hayat et al., 2012; Liang et al., 2013). Under condition of copper stress, proline levels in rice roots were significantly reduced compared to the control, whereas application of Pv extract alleviated this decline (Fig. 2D). Notably, Pv extract did not increase proline levels in non-stressed roots, as compared to the control group, despite the presence of free proline in the extract (Table S1). Although exogenous proline has been reported to elevate endogenous proline levels under non-stress conditions (Yan et al., 2025), the concentration present in the Pv extract may have been insufficient to trigger this response. In addition, transciptional analysis revealed that PvCu-treated roots exhibited increased expression of proline biosynthesis-related genes, including OsGS1;2, OsP5CS2, OsP5CR. OsGS1;2 encoding enzyme glutamine synthetase, participate in production of glutamate, a key precursor for proline synthesis (Yamamoto et al., 2012). Meanwhile, OsP5CS2 and OsP5C, which encode pyrroline-5-carboxylate synthase and reductase, respectively, play key role in catalyzing proline synthesis from glutamate (Y. Zhang et al., 2019). In contrast, the expression of proDH, coding a proline-degrading enzyme (Ingrisano et al., 2023), was reduced in PvCu-treated roots. Collectively, these results indicate that Pv extract enhances porline content via stimulating biosynthesis while suppressing degradation activity. Consistently, the increased content and expression of proline biosynthesis-related genes observed in PvCu-treated roots (Fig. 2C-D) suggests that Pv extract might function as an elicitor, promoting endogenous proline accumulation in response to copper stress.

Moreover, our data also showed upregulated DMA biosynthesis in PvCu roots, as manifested by increased expression of genes, including S-adenosylmethionine synthetase 2 (OsSAMS2), nicotianamine synthase (OsNAS1, 2, 3), and deoxymugineic acid synthase (OsDMAS1) (Fig. 3A). Plants release DMA as phytosiderophores to chelate iron, enabling the iron absorption from rhizosphere (Inoue et al., 2009). At a molecular level, DMA is a metal chelator that binds to metal ions and is transported out of the cytoplasm into the vacuole by specialized DMA transporters (e.g., OsZIFL12) (Che et al., 2019; Schindlegger et al., 2015). Plant-secreted DMA not only benefits nutrient acquisition but also regulates cellular metal homeostasis, as free metals can be toxic to plants (Bashir et al., 2016). Increased accumulation of DMA in PvCu-treated rice roots might could reduce the amount of free copper ion levels in root, hence, reducing copper-induced damage and might facilitating metal sequestration or efflux.

Metallochaperones are proteins containing metal ion-binding structures, and these factors play a key role in regulating metal homeostasis (J. Li et al., 2020). The metallochaperone proteins are divided into two groups, heavy metal-associated plant proteins (HPPs) and heavy metal-associated isoprenylated plant proteins (HIPPs) (De Abreu-Neto et al., 2013). These metallochaperones play important role in copper root-shoot translocation (Deng et al., 2013) and vacuolar trafficking (Ueno et al., 2010). In this study, we identified 5 upregulated genes encoding metallochaperones OsHIPP17, 23, 32, OsThi9, and OsHMP22. Among 5 identified metallochaperones OsHIPP17, 23, 32, OsThi9, and OsHMP22, however, only OsHIPP17 and Osthi9 have been documented to mediate root-to-shoot transport and copper cellular efflux (X. Liu et al., 2023; Shi et al., 2023). Although, the function of remaining metallochaperones remain unclear, they may contribute to copper homeostasis through roles in intracellular distribution, including root-to-shoot translocation or vacuolar sequestration. The cooperative actions of metalloprotein and transport proteins help to maintain the balance of cellular metal ions. Correspondingly, several transporter-related genes, including efflux transporters (OsCAL1, OsZIP1) and vacuolar transporters (OsTIP1;2, OsTIP1;2), were upregulated in PvCu rice seedlings, as compared to copper-treated seedlings. OsTIP1;2 and OsTIP2;1 are known to function in the transit of cellular arsenite and aluminum to root vacuoles, contributing to stress resilience (Karle and Kumar, 2024; W. Zhang et al., 2024). Furthermore, OsZIP1 is an efflux transporter that exports cellular cadmium to the environment (X. S. Liu et al., 2019). Therefore, our transcriptomic data reveal that Pv extract induces molecular alterations that can feasibly contribute to adaptation of rice seedlings to copper stress.

4.2. Potential role of Pv extract-mediated regulation of cell wall in protection from copper stress

The cell wall is a complex matrix of polysaccharides, lignin and structural proteins that serves as a natural barrier to external factors. When plants are exposed to metal stress, the cell wall immediately undergoes structural remodeling, with increased contents of polysaccharides (e.g., cellulose, hemicellulose, glucan, lignin and suberin). Increased levels of these components are associated with thickening of the cell wall to hinder metal penetration. Furthermore, these components contain a abundant functional groups such as -COOH, -OH, SH that bind and fix metal into cell wall, increasing the capacity of the cell wall to prevent entry of metals into the cells (Cheng et al., 2025; Krzesłowska, 2011). Numerous studies have demonstrated that changes in cell wall composition can reduce heavy metal absorption, leading to enhanced plant biomass under stress conditions (Cui et al., 2024; Dang et al., 2023; Zhan et al., 2020). In our study, increased expression levels of several genes associated with synthesis of cellulose, hemicellulose and lignin (> 2-fold increase) were observed in PvCu rice roots as compared to those exposed to copper stress. We also noted an abundance of OsMYB46 transcript levels (3-fold increase), which can lead to activation of cell wall biosynthesis (Ko et al., 2014). In previous work, overexpression of OsMYB46 caused upregulation of several genes related to cellulose, xylan and lignin production (similar to AtMYB46, ZnMYB46 and PvMYB46) (Zhong et al., 2011, 2015). Thus, our results support a potential role of Pv extract in regulating the cell wall (Table 1). Expansin proteins (EXPs, XTHs) can loosen cell wall tension and were upregulated (fold changes 2–159) in PvCu rice roots (Fig. 4A). Intriguingly, auxin has been reported to induce apoplast acidification by stimulating proton pump activity (Majda and Robert, 2018). Auxin-induced acidic apoplasts trigger expansin protein activity, relaxing the connection between structural components of cell wall. Brassinosteroid (BR) is also known to modify the inner architecture of the cell wall by influencing cell wall loosening, cellulose and lignin deposition (Kong et al., 2021; Rao and Dixon, 2017). BR positively mediates cellulose and lignin accumulation via activation of transcription factors brassinazole resistant 1 (BZR1) and BRI1-EMS-Suppresor 1 (BEST1). This activation is initiated by BR-mediated detection of brassinosteroid insensitive 1 (BRI1) and BRI1-associated receptor kinase 1 (BAK1) (Percio et al., 2025). Exogenous BR or activation of BR signaling enhances cellulose deposition in the cell wall (Pramod et al., 2022; Y. Sun et al., 2015). According to Sun et al. (2024), BR treatment increases hemicellulose levels, thickening the cell wall to potentially limit Cd levels in rice roots. This function may lead to improved plant growth under Cd stress. Other studies have shown that extracellular BR can increase the activity of expansion proteins, leading to modified root architecture and ultimately enhanced stress tolerance in Arabidopsis (Abuqamar et al., 2013; P. Xu et al., 2020). Moreover, the interplay of auxin and BR controls cell wall modifications and trigger cell division and elongation (Keuskamp et al., 2011; Xiong et al., 2021). In addition to that, Brs also exerts stimulatory effects on auxin biosynthesis under heavy metal stress, where metals disrupt auxin biosynthesis (Choudhary et al., 2012) and distribution (Peto et al., 2011; Yuan et al., 2013), leading to impaired local auxin gradients and inhibited root elongation. As reported by Choudhary et al. (2012), high level of copper downregulated the expression level of genes involved in auxin synthesis (CYP79B3, YUC1, 3), resulting in reduced levels of free auxin in radish. However, exogenous Brs application reverses this negative effects, restoring auxin content and consequently promoting plant growth. Consistently with these findings, our results revealed the upregulation of genes involved in auxin and BR biosynthesis and signaling in PvCu-treated rice roots (Fig. S3). This suggests that Pv extract induces coordinated phytohormonal crosstalk, which might contribute to regulation of cell wall architecture and enhanced plant tolerance under copper stress.

In rice, the CASP (Casparian strip domain protein) family contains 5 genes (called OsCASP1–5), which play a role in CS formation (Z. Wang et al., 2020). Among these genes, OsCASP1, 2, 3 and 5 were upregulated in PvCu rice roots compared with the copper-only group (Fig. 4C). Expression analysis also showed that expression of OsMYB36 was increased (Fig. 4C). This key transcription factor is known to govern CS formation (Kamiya et al., 2015; Z. Wang et al., 2022). Detecting Casparian strip (CS) formation in roots using berberine–aniline blue staining further confirms the changes in gene expression (Fig. 4D). Overall, our study results are consistent with the report by Wang and colleagues that suggested a potential role of Pv extract in regulating root vascular systems under copper stress (L. Z. Wang et al., 2023).

5. Conclusion

In this study, we evaluated the potential role of Pv extract in facilitating plant defenses against copper stress. Our data suggests that the aqueous extract of Pv aerial parts contains bioactive compounds and nutrients that promote plant tolerance to copper toxicity at least partially by reducing copper uptake in roots. Pv extract also induced transcriptomic changes in expression of genes correlated with cell wall remodeling, CS formation, and the production of metal-binding and metal-exporting proteins. Furthermore, Pv extract may serve as a source of proline and also an inducer of proline synthesis, which contributes to antioxidant activity and attenuates copper-induced damage.

CRediT authorship contribution statement

Thach-Thao Ly: Writing – original draft, Methodology, Investigation, Formal analysis. **I-Fan Wang:** Investigation. **Tri-Phuong Nguyen:** Writing – review & editing. **Hao-Jen Huang:** Writing – review & editing, Validation, Supervision, Conceptualization. **Ying-Lan Chen:** Investigation. **Shih-Feng Fu:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eti.2026.104999.

Data availability

Data will be made available on request.

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