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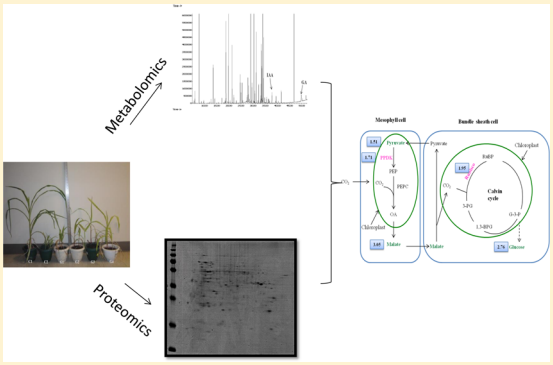
Integrated Metabolomic and Proteomic Approaches Dissect the Effect of Metal-Resistant Bacteria on Maize Biomass and Copper Uptake

Kefeng Li, Venkataramana R. Pidatala, Rafi Shaik, Rupali Datta, and Wusirika Ramakrishna*

Department of Biological Sciences, Michigan Technological University, Houghton, Michigan 49931, United States

Supporting Information

ABSTRACT: Marginal soils arise due to various industrial and agricultural practices reducing crop productivity. *Pseudomonas* sp. TLC 6-6.5-4 is a free-living multiple-metal-resistant plant-growth-promoting bacteria (PGPB) isolated from Torch Lake sediment that promotes maize growth and nutrient uptake. In this study, we examined both PGPB−soil and PGPB−plant interactions. PGPB inoculation resulted in significant increase in maize biomass. Soil inoculation before sowing seeds and coating seeds with the PGPB resulted in higher copper uptake by maize compared to other methods. The PGPB−soil interaction improved phosphorus uptake by maize and led to significant decrease in organic bound copper in marginal soil and a notable increase in exchangeable copper. PGPB improved soil health based on soil enzyme activities. Metabolomic analysis of maize revealed that PGPB inoculation upregulated photosynthesis, hormone biosynthesis, and tricarboxylic acid cycle metabolites. Proteomic analysis identified upregulation of proteins related to plant development and stress response. Further, the activity of antioxidant enzymes and total phenolics decreased in plants grown in marginal soil suggesting alleviation of metal stress in presence of PGPB. The ability of PGPB to modulate interconnected biochemical pathways could be exploited to increase crop productivity in marginal soils, phytoremediation of metal contaminated soils, and organic agriculture.



INTRODUCTION

Deficiency of nitrogen, phosphorus, and other macro- and micronutrients in soils results in reduced plant biomass and significant loss of plant productivity.[1] Marginal soils are poor soils with deficiency of nutrients and/or high concentration of heavy metals and low levels of microbial activity. To meet increasing food and energy needs, development of novel technologies that enable cultivation of crops with sustainable productivity on marginal lands is essential.

Copper is an essential micronutrient for all living organisms. The natural concentration of copper in soils ranges from 5 to 70 mg/kg.[2] However, high levels of copper have been found in many industrial sites as well as agricultural soils due to various anthropogenic practices such as mining, application of copper fungicides, and composts.[3] Exposure to excess copper can lead to severe cell damage due to the generation of free radical species and the inactivation of enzymes.[4] Copper mine tailings, known as stamp sand in Upper Peninsula, Michigan, is a typical marginal land with high levels of copper and low phosphorus and microbial activities. Phytoremediation has been proven to be a reliable and sustainable approach for cleanup of heavy metals and restoration of soil quality. Recent studies have identified some metal-resistant plant-growth-promoting bacteria (PGPB) that have the potential to facilitate phytoremediation of metal-contaminated soil.[5,6]

One area that has received little attention in bacteria-assisted phytoremediation is the bacterial inoculation method. The majority of previous studies for bacteria-assisted phytoremediation introduced PGPB by soaking the roots of plant seedlings in bacterial suspension before transferring the plants into contaminated soil.[7−9] However, this approach is not practical for field-scale and commercial use. The success of bacteria-assisted phytoremediation was mainly evaluated based on metal removal, and very little emphasis was placed on soil quality and health.

Modern "omics" techniques with integrated bioinformatics open novel avenues to investigate plant−microbe interaction in an unbiased manner. However, the focus has been on endophytic nitrogen-fixing PGPB such as *Rhizobium* in the nodules of leguminous plants rather than free-living phytohormone and siderophore-producing PGPB.[10−12] In our previous study, we have identified a free-living multiple-metal-resistant bacteria *Pseudomonas* TLC 6-6.5-4 from Torch Lake sediment contaminated by stamp sand.[13] This strain promotes growth and copper uptake in maize by production of the plant hormone indole-3-acetic acid (IAA) and siderophores and

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solubilization of phosphate and metals. Here, we investigated the influence of different modes of PGPB application on PGPB−maize−soil interactions and their effect on plant biomass, metal uptake, and soil health. Proteomic and metabolomic analysis identified candidate proteins and metabolites, which are likely to play a role in regulating biochemical, molecular, and physiological responses of maize grown in marginal soils with PGPB. We correlated changes in the maize metabolome to the alterations in the proteome through integration of our metabolomics and proteomics data into the maize metabolic network. Further, we analyzed the activity of antioxidant enzymes, chlorophyll content, and total phenolics for cross-validation of the metabolomic and proteomic results.

MATERIALS AND METHODS

Soil Analysis. Copper mine tailings, called "stamp sand", was collected from the Gay stamp sand site in Upper Peninsula, Michigan. Soil physicochemical properties including soil pH, organic matter, texture, total copper, and total and plant available phosphate were measured according to U.S. EPA and USDA protocols.[14]

Inoculation of PGPB Using Different Methods. *Pseudomonas* sp. TLC 6-6.5-4 was grown at 30 °C in LB broth with shaking until an optical density (OD_{600}) of 0.7 was obtained. The bacterial culture was centrifuged, and the pellet was resuspended in sterile saline buffer (0.85% NaCl) to a final concentration of 10^8 CFU/mL. Three control groups were set up: first containing maize inbred line B73 (*Zea mays* subsp. *mays*) grown in normal potting mix (C1); second, maize in stamp sand inoculated with saline buffer (C2); and third, stamp sand alone without plants (C3). Treatment plants used for testing four bacterial inoculation methods were categorized into four groups. In the first group (G1), soil was inoculated with bacterial culture five days before sowing maize seeds. A 100 mL amount of bacterial suspension was sprayed on the soil surface while the same volume of saline buffer was sprayed in the control group. After five days, maize seeds were sown. In the second group (G2), maize seeds were soaked in 100 mL of bacterial suspension for 1 h to allow the bacterial cells to adhere to seeds. The control group was soaked in saline buffer. In the third group (G3), 100 mL of bacterial culture was sprayed on the soil surface 15 days after sowing the seeds. The same amount of saline buffer was sprayed in the control group. In the fourth group (G4), maize seedlings were grown in normal soil for 15 days. The plants were uprooted for immersion in 100 mL of bacterial suspension culture or saline buffer (C2) for 2 h for colonization followed by transplanting in stamp sand. Each treatment was performed in triplicate pots, and 4−6 biological replicates were set up for each group. The group information is summarized in Table S1a (Supporting Information). Plants were grown in a greenhouse for 45 days at 25 °C with a photoperiod of 16 h day/8 h dark.

Estimation of Plant Biomass and Soil Enzyme Activities. Both soil and plant samples were collected after 45 days. Plants were dried at 60 °C. Biomass (dry weight) was recorded. Copper concentration in plant materials was measured using atomic absorption spectroscopy (AAS) after digestion with HNO_3 (65% w/w) followed by H_2O_2 (30% w/w). Soil enzyme activities were analyzed within 48 h after collection. Soil dehydrogenase activity was measured using 2,3,5-triphenyltetrazolium chloride (TTC) as the substrate.[15] Soil catalase activity was determined by back-titrating residual H_2O_2 with $KMnO_4$.[16] Soil invertase activity was estimated based on glucose production per gram of soil (dry weight) per 16 h.[17]

Plant Total Soluble Protein and Antioxidant Enzyme Assays. Plant total soluble protein was extracted twice using ice-cold phosphate buffer (50 mM, pH 7.8). Protein concentration was quantified by bicinchoninic acid (BCA) assay. The activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) were measured as described by Sunkar.[18] One unit of SOD enzyme activity was defined as the amount of enzyme required for 50% reduction of nitrobluetetrazollum (NBT) absorbance. The POD activity was expressed as ΔA470 $min^{-1}\cdot mg^{-1}$ protein. The APX activity was expressed as ΔA290 $min^{-1}\cdot mg^{-1}$ protein. The CAT activity was measured as the decrease in absorbance at 240 nm (ΔA240 $min^{-1}\cdot mg^{-1}$ protein).

Estimation of Total Phenolics and Chlorophyll Content. Total phenolics in maize samples were extracted in ice-old 95% methanol and determined using the Folin−Ciocalteu method with gallic acid as the standard.[19] The total phenolic values were reported in milligrams of gallic acid equivalents per gram of fresh weight (mg/g FW). The pigments were extracted with 80% acetone, and the absorbance was measured at 663 and 645 nm. The chlorophyll content (chlorophyll a, b, and total chlorophyll) was calculated using the reported equation.[18]

Estimation of Total Heterotrophic Bacteria, Bacterial Inoculum, Plant Phosphorus Uptake, and Copper Speciation. To investigate the effect of PGPB on plant nutrition and metal uptake, metal mobility, and soil microbial diversity, another pot experiment was set up based on the optimal bacterial application method (presowing soil inoculation and seed coating methods). Three pots in each group were harvested every 15 days. The control groups include maize grown in stamp sand without PGPB, PGPB grown in stamp sand without maize, and pots with stamp sand alone. Bacterial population dynamics was evaluated by the plate dilution technique. For counting PGPB inoculants in soil and plant rhizosphere, 100 μL of soil suspension was spread on basal salt medium (BSM) with two intrinsic selective markers including carbenicillin (100 μg/mL) and Cu^{2+} (3 mM). Glycerol (1%) was used as a carbon source. The presence of *Pseudomonas* sp. TLC 6-6.5-4 was also confirmed by amplification of 16S rDNA and sequencing. For total heterotrophic bacteria, about 0.3 g of soil was mixed with 1 mL of sterile 0.85% NaCl solution and vortexed for 5 min. Soil suspension was serially diluted and spread on nutrition agar. Tessier's sequential extraction procedure was performed for speciation analysis of copper in stamp sand.[20] The copper concentration in each fraction was determined using AAS. The phosphorus content in plant tissues was determined by the ammonium molybdate−ascorbic acid colorimetric method.[14]

Proteomic, Metabolomic, and Western Blot Analysis. The effect of PGPB inoculation on the maize proteome and metabolome in both normal and marginal soils was investigated using the optimal PGPB delivery method (presowing soil inoculation and seed coating methods). A total of four groups were set up with 4−6 biological replicates in each group including maize in normal soil (M+N), maize in normal soil with bacteria (M+N+PGPB), maize in stamp sand (M+S), and maize in stamp sand with bacteria (M+S+PGPB) (Table S1b, Supporting Information). Total protein was extracted using the ReadyPrep protein extraction kit (Bio-Rad, CA) and separated

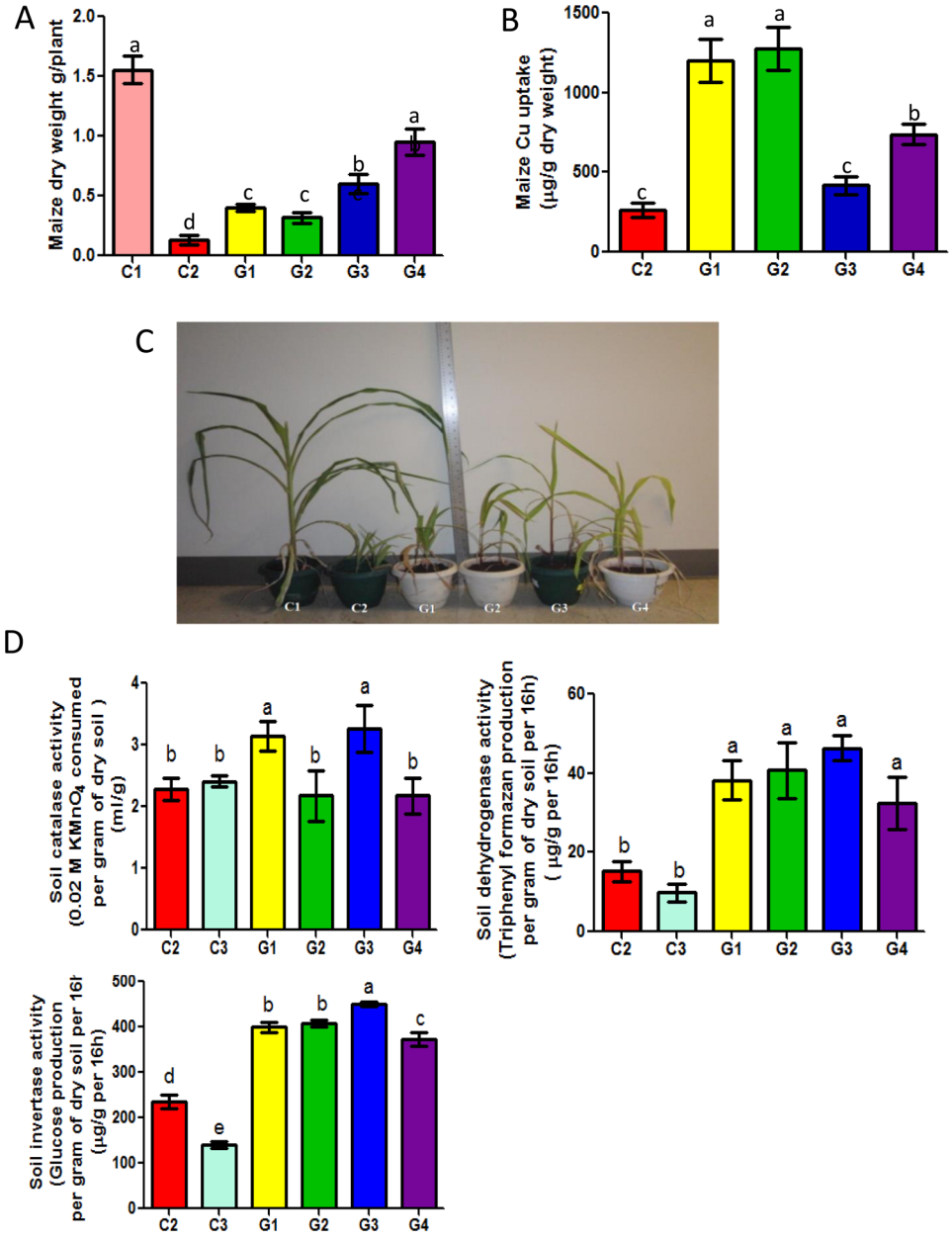

Figure 1. Different PGPB delivery methods influence maize growth, copper accumulation, and soil enzyme activities. (A) Maize average dry weight. (B) Maize plants grown for 45 days. (C) Copper accumulation of maize grown for 45 days in stamp sand with four different PGPB inoculation methods. (D) Soil enzyme activities. Data is mean ± SD (n = 6). Data indexed by different letters are significantly different according to Tukey's test (p < 0.05). C1: maize grown in normal soil; C2: maize grown in stamp sand; C3: stamp sand alone. Four groups based on the PGPB inoculation method are as follows: G1: presowing soil inoculation; G2: seed coating method; G3: postgermination inoculation; G4: root immersion method.

by two-dimensional (2-D) gel electrophoresis according to the manufacturer's instructions. In the first dimension, 180 μg of protein was loaded on 11 cm immobilized pH gradient strips (IPG strips, pH 3−10, nonlinear, Bio-Rad, CA). Second dimension electrophoresis was performed on 8−16% precast polyacrylamide gels. Gels were stained by Coomassie Blue G-250, and the images were captured using a GS-800TM densitometer (Bio-Rad, CA). Differentially expressed proteins were analyzed using ImageMaster 2D platinum (version 7.0, GE Healthcare, WI). Protein spots with a fold change greater than or equal to 2 (p < 0.05) were considered to be differentially expressed. The protein spots were excised from the gels, washed in water, destained, and digested with trypsin as described previously.[21] The peptides were analyzed by a Microflex matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker, MA) in positive ion reflectron mode with automated postsource decay (PSD) function. The top three most intense peptides detected for each spot in the mass spectrometry (MS) mode were automatically selected for PSD analysis to obtain MS/MS information. Proteins were identified using MS-Fit (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msfitstandard). Functional annotation of the identified proteins was carried out according to MaizeCyc and Uniprot databases (http://pathway.gramene.org/gramene/maizecyc.shtml; http://www.uniprot.org).

For gas chromatography−mass spectrometry (GC-MS), the extraction of metabolites from maize tissues and the

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derivatization procedures were performed according to Shevchenko et al.[21] Whole maize plants were ground into powder in liquid nitrogen, and 100 mg was weighed for metabolites extraction. Maize metabolites were extracted with 80% prechilled methanol for 10 min by sonication on ice. Ribitol (0.2 mg/mL) was added as the internal standard prior to extraction. The metabolites were derivatized with methoxyamine hydrochloride and *N*-methyl-*N*-trimethylsilyltrifluoracetamide. An Agilent 6890 gas chromatograph coupled with a 5973 quadrupole mass spectrometric detector was used for the analysis. The mass spectrum was deconvoluted using the automated mass spectral deconvolution and identification system (AMDIS). The peaks were identified by the National Institute of Standards and Technology (NIST) library and Golm metabolome database (http://gmd.mpimp-golm.mpg.de).[22] Prior to statistical analysis, the peak area of the metabolites was normalized by the peak area of the internal standard (ribitol) and plant fresh weight. Hierarchical cluster analysis with Pearson correlation as the distance measure and student's *t* test was performed with TIGR MeV4.7.4. Principle component analysis and pathway analysis were performed using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca).

Western blot analysis was performed using primary antibody, antigamma glutamylcysteine synthetase, and the corresponding horseradish peroxidase conjugated secondary antibodies. Proteins were visualized by using West Pico Chemiluminescent Substrate (Pierce). Immunoreactive protein bands were acquired using a LAS 4000 mini luminescent image analyzer. Band intensity was quantified using Quantity One (Bio-Rad).

RESULTS

PGPB Inoculation Influences Maize Growth, Copper Uptake, and Soil Enzyme Activities. The effect of PGPB inoculation modes on the growth and metal uptake of the host plant and the soil enzyme activities was investigated in maize grown on stamp sand, which is a marginal soil. Analysis of the basic physiochemical properties and metal concentrations of the stamp sand found them to be sandy loam with the total copper content of 2966.88 ± 74.59 mg/kg (Table S2, Supporting Information). The total phosphorus and plant available phosphorus were estimated to be 46.47 ± 10.53 and 6.1 ± 0.28 mg/kg, respectively. *Pseudomonas* sp. TLC 6-6.5-4 bacteria[13] inoculated using all four methods, presowing soil inoculation (G1), seed coating method (G2), postgermination inoculation (G3), and root immersion method (G4) exhibited significant improvement in biomass (2.5−7-fold) (Figure 1A, C) in comparison with the control plants grown in marginal soil without bacterial inoculation. Among the four methods, the root immersion method resulted in the highest increase of biomass. Maximum copper uptake (4.4-fold higher than control 2) was observed in G1 and 2 (Figure 1B). Application of PGPB in all four methods led to significantly higher soil invertase and dehydrogenase activities compared with marginal soil alone (control 3) and marginal soil with maize (control 2) (Figure 1D). No significant differences were found in soil dehydrogenase activities among the four inoculation methods. With respect to soil catalase, the highest enzyme activity was achieved in soil inoculation methods 1 and 3.

Presowing soil inoculation (G1) and seed coating (G2) methods were combined for further experiments because of higher copper uptake and feasibility for field-scale use compared to other inoculation methods.

Increase of Maize Biomass and Copper Uptake Associated with PGPB−Soil Interactions. We next investigated whether the enhancement of maize biomass and copper accumulation was associated with PGPB−soil interactions. The population dynamics of total heterotrophic bacteria (THB) and PGPB inoculants in both rhizospheric and nonrhizospheric soil was evaluated. Both the number and the diversity of the culturable heterotrophic bacterial population in stamp sand were very low before planting maize. The mean density was only $4.8 \times 10^3 \pm 1.7 \times 10^2$ CFU/g dry weight with the bacterial genera identified belonging to *Bacillus* and *Staphylococcus*. As expected, both maize growth and PGPB inoculation improved the THB population in stamp sand (Figure S1A, B, Supporting Information). Total heterotrophic bacteria in nonrhizospheric soil reached a steady state with no significant differences in the number of THB among the groups after 30 days (Figure S1A, Supporting Information). Bacteria in plant rhizosphere grew faster, and the density of THB in the rhizospheric soil was much higher than that of the nonrhizospheric soil (Figure S1B, Supporting Information). Inoculation of PGPB significantly improved the number of THB present in maize rhizosphere compared with uninoculated control group. The theoretical inoculum of PGPB was about $1.6 \times 10^6 \pm 4.2 \times 10^4$ CFU/g dry weight. The TLC 6-6.5-4 population in all the groups was less than the theoretical inoculum after 15 days suggesting a loss of PGPB after inoculation (Figure S1C, D, Supporting Information). Significant change in the PGPB population was not observed in soil without the host plant (Figure S1C, Supporting Information). In contrast, the number of PGPB increased in both rhizospheric and nonrhizospheric soil in the presence of maize. The density of PGPB in maize rhizosphere reached $9 \times 10^5 \pm 2.5 \times 10^4$ CFU/g after 45 days, which is close to the theoretical inoculum ($1.6 \times 10^6 \pm 4.2 \times 10^4$ CFU/g dry weight).

Phosphorus is one of the limiting factors for plant growth in marginal soil as the total phosphate and plant available phosphorus is very low (Table S2, Supporting Information). Our previous study demonstrated that *Pseudomonas* sp. TLC 6-6.5-4 is able to solubilize rock phosphate in bacterial medium.[13] Hence, we determined maize phosphate assimilation from soil along with maize growth. We found that inoculation of *Pseudomonas* sp. TLC 6-6.5-4 resulted in significant improvement of phosphorus uptake from marginal soil by maize in both roots and shoots in 45 days (Figure S2A, B, Supporting Information). Pearson's correlation analysis identified root and shoot biomass to be positively correlated with phosphorus uptake by roots ($r = 0.9857$, $p < 0.05$) and shoots ($r = 0.8287$, $p < 0.05$), respectively.

Copper accumulation in maize roots was found to be higher than in shoots that was further enhanced by the inoculation of PGPB (Figure S2C, D, Supporting Information). PGPB also increased the copper translocation factor (copper concentration in shoots/roots) from 0.38 to 0.46. Further, the geochemical partitioning of copper between five operationally defined forms was examined to investigate the effect of PGPB on soil copper mobility. The main copper species in stamp sand before adding PGPB was carbonate associated and Fe−Mn oxide copper, which accounted for about 84.2% of the total copper in the stamp sand (Table S3, Supporting Information). The exchangeable copper was very low. The highest exchangeable copper concentration found in maize grown with PGPB was 3-fold compared to maize grown without PGPB and 7-fold

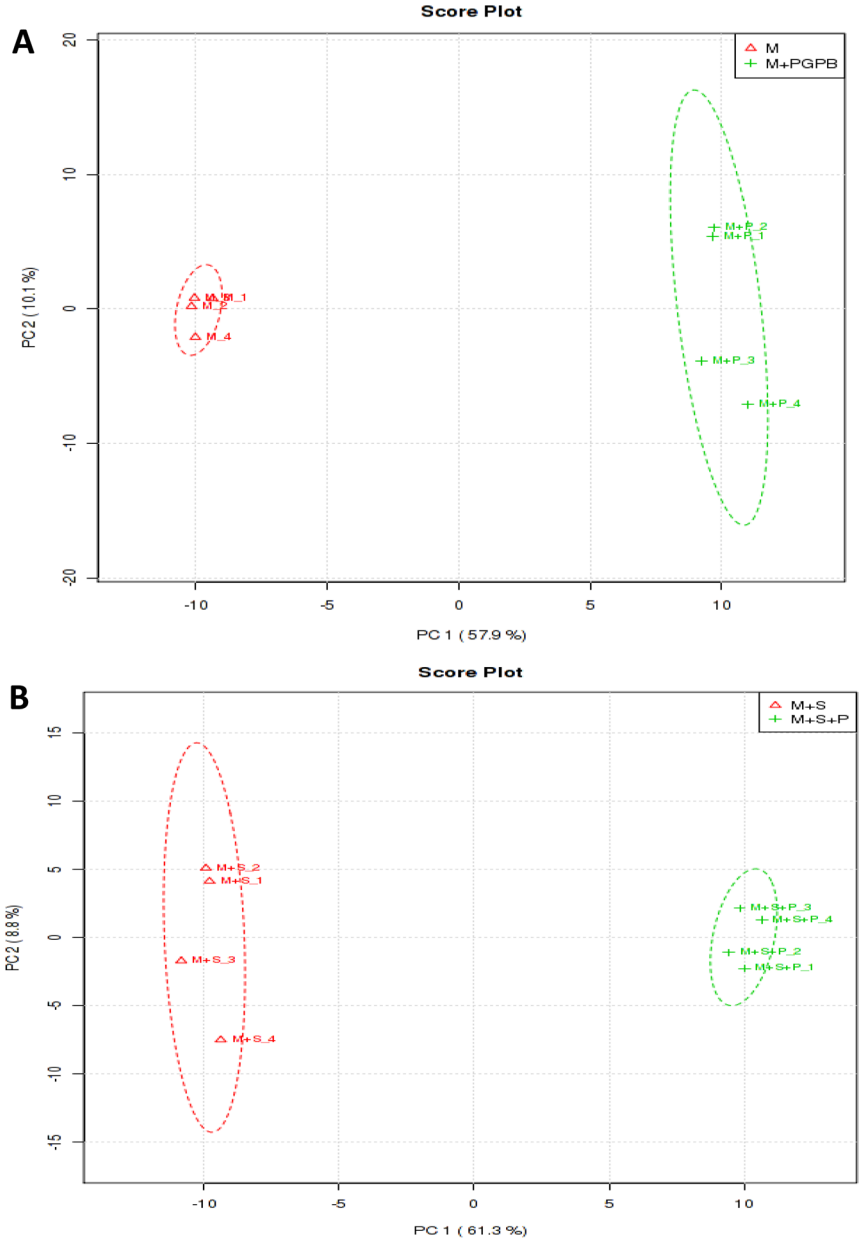

Figure 2. Principle component analysis (PCA) score plot showing distinct grouping of metabolites from maize with and without PGPB. (A) Maize grown in normal soil. (B) Maize grown in stamp sand. M: maize alone in normal soil; M+PGPB: maize in normal soil with PGPB; M+S: maize in stamp sand; M+S+PGPB: maize in stamp sand with PGPB. Each symbol on the score plot represents data from all the differentially expressed metabolites in one biological replicate reduced to the first and second principle components (PCs). Replicate samples from each group clustered together.

compared to initial stamp sand. This group also showed maximum reduction of organic bound copper (Table S3, Supporting Information). The total copper in maize and soil (Cu_{maize} + Cu_{soil}) was 9035.5 ± 414 mg/pot after 45 days with maize and PGPB that was mass balanced to the initial stamp sand (8898 ± 223.6 mg/pot). The copper concentration in maize roots was negatively correlated with the concentration of organic matter bound copper in soil ($r = -0.824$, $p < 0.01$) and positively correlated with the concentration of exchangeable copper in soil ($r = 0.848$, $p < 0.05$). Similarly, the copper concentration in maize shoots was also highly correlated with the decrease of organic matter bound copper and the increase of exchangeable copper with the coefficient factors of −0.818 and 0.87 ($p < 0.05$), respectively (Table S3, Supporting Information).

PGPB Display Differential Impact on Regulatory and Metabolic Proteins of Maize Grown in Normal and Marginal Soils. The total soluble protein content of maize grown with PGPB increased by 19% and 20% in normal and marginal soils, respectively (Figure S3, Supporting Information). To determine the proteins up- or downregulated in the maize proteome under normal and marginal soils, we performed proteomic profiling of 45 day old maize plants grown in normal and marginal soils with or without PGPB. The representative gels are show in Figure S4A, B (Supporting Information). In normal soil, on an average, 510 spots were detected per gel, and principle component analysis (PCA)

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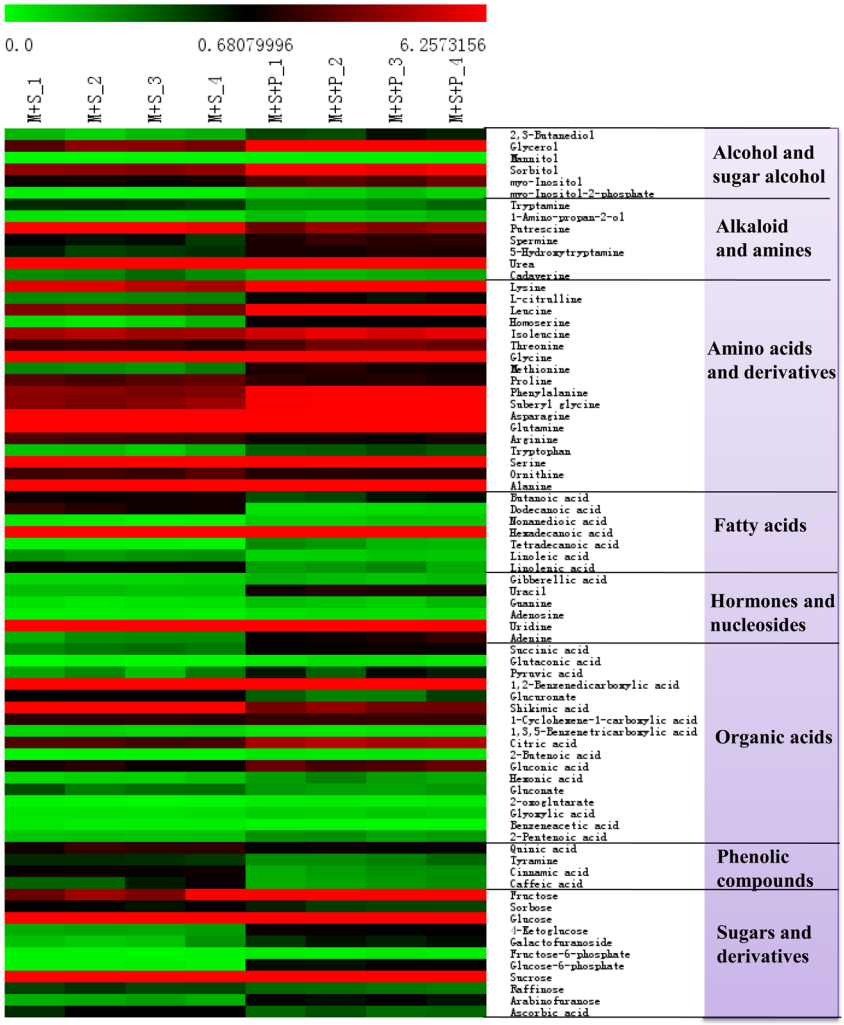

Figure 3. Relative metabolite levels of major metabolite groups in maize grown in stamp sand with PGPB (M+S+P) or without PGPB (M+S). 1−4 are four replicates. Metabolite concentrations were normalized to maize fresh weight and the internal standard ribitol. Color bar represents the relative concentration of the metabolites from lowest (green) to highest (red). All the metabolites listed are significantly different between two groups according to the *t* test ($p < 0.05$).

showed the complete separation of maize with PGPB from maize without PGPB (Figure S5A, Supporting Information). Eighty five maize proteins showed significant changes in response to PGPB inoculation (Fold change ≥ 2 and $p < 0.05$), and 17 proteins were successfully identified by MALDI-TOF/TOF (Table S4 and Figure S4A, B, Supporting Information). Functional annotation analysis revealed that the main host proteins altered by PGPB inoculation are related to plant development and photosynthesis (33%) in normal soil (Figure S6A, Supporting Information). The upregulation of photosynthetic proteins was supported by an increase in chlorophyll a and total chlorophyll content of maize leaves (Figure S7, Supporting Information). Other maize proteins that have a role in regulation and signal transduction (22%), cellular metabolism (17%), and protein folding and degradation (11%) also changed in response to PGPB.

An average of 498 spots was detected on 2-D gels of maize grown in stamp sand (Figure S4C, D, Supporting Information), and PCA showed the distinct proteome between maize in stamp sand with and without PGPB inoculation (Figure S5B, Supporting Information). The major proteins altered in maize grown in marginal soil with PGPB compared with maize without PGPB belong to cellular metabolism and stress response (47%) (Figure S6B and Table S5, Supporting Information). Proteins involved in DNA repair, methionine biosynthesis, malate metabolic process, photosynthesis, and carbon fixation were upregulated (Table S5, Supporting Information). In contrast, the activity of major antioxidant enzymes in maize grown in marginal soil was significantly reduced as a result of PGPB inoculation. These include glutathione S-transferase (GST), CAT, POD, SOD, and APX (Table S5 and Figure S8, Supporting Information).

Plant phytochelatins play an important role in plant development and metal detoxification.[23] Western blot analysis showed that γ-glutamylcysteine synthetase (GCL), a key enzyme for phytochelatin synthesis, was significantly upregulated in marginal soil compared with maize grown in normal soil ($p < 0.05$, Figure S9, Supporting Information). GCL expression was downregulated in the presence of PGPB in marginal soil and upregulated in the presence of PGPB in normal soil, which is in agreement with the proteomic data. These results indicate that PGPB alleviates the stress of host plants caused by high concentration of copper in stamp sand.

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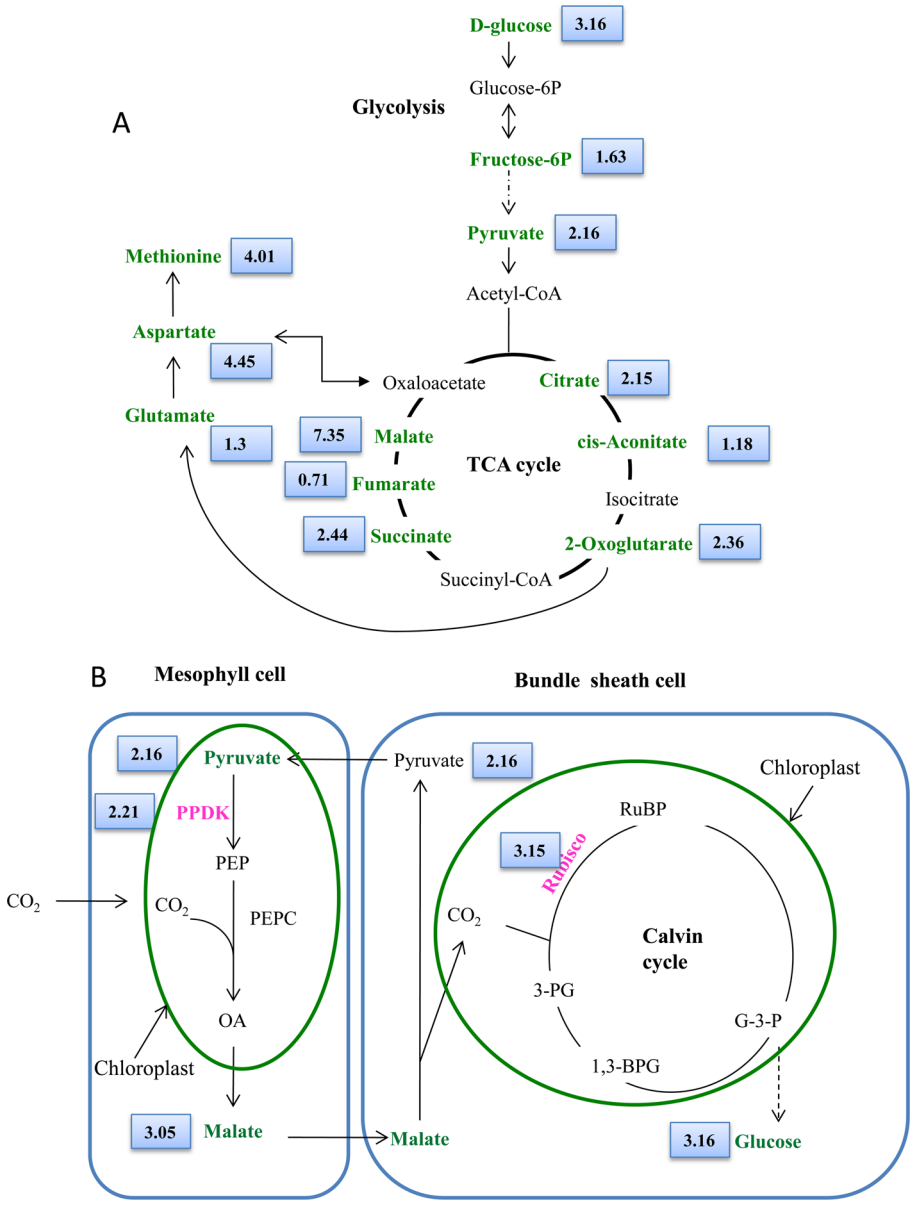

Figure 4. Changes in the levels of metabolites and enzymes in biochemical pathways contributing to biomass and nutrients in maize grown in stamp sand with PGPB. (A) Tricarboxylic acid (TCA) cycle and related pathways. (B) Carbon fixation and Calvin cycle. Green color indicates identified metabolites. Blue boxes represent the average fold increase in levels of metabolites or proteins (maize + PGPB/maize). Pink color indicates the enzymes identified in proteomic analysis. The other metabolites and enzymes involved in the pathways were not identified in this study. 3PG: 3-phosphoglycerate; 1,3-BPG: 1,3-bisphosphoglycerate; G3P: glycerate 3-phosphate; PEP: phosphoenol pyruvate; OAA: oxaloacetate; Ru5P: ribulose-5-phosphate. PPDK: pyruvate phosphate dikinase; PEPC: phosphoenolpyruvate carboxylase; Rubisco: ribose-1,5-bisphosphate carboxylase oxygenase.

PGPB Regulate Common and Unique Metabolic Pathways of Maize in Normal and Marginal Soils. PGPB inoculation led to a 170% increase in maize total phenolics in normal soil compared to maize grown without PGPB (Figure S10, Supporting Information). Total phenolics in maize grown in stamp sand were about 5-fold higher than maize grown in normal soil, probably due to the high levels of heavy metals. However, addition of PGPB to stamp sand significantly reduced the total phenolics ($p < 0.05$, Figure S10, Supporting Informationa). The difference in phenolic compounds in maize in normal and marginal soils induced by PGPB suggests that PGPB might have an effect on the host metabolome. To investigate this possibility, we performed untargeted profiling of maize metabolites in response to PGPB in normal and marginal soils. We identified a total of 204 compounds in maize grown in normal soil with or without PGPB that included amino acids, organic acids, sugar and sugar alcohols, nucleosides, hormones, lipids, and sterols. PCA resulted in a first principal component (PC1) separating maize with PGPB from maize without PGPB inoculation (Figure 2). The score plot reflects the distinct metabolic profiles of each group. Together, the first two components of PCA captured 68% (PC1) and 70.1% (PC2) of variance within the maize samples from normal soil and marginal soil, respectively. A total of 73 metabolites showed statistically significant changes in response to PGPB in normal soil ($p < 0.05$, Figure S11, Supporting Information). We integrated our metabolomics data into the maize global metabolome through

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MaizeCyc. Pathway analysis identified the upregulation of maize carbon fixation during photosynthesis due to the inoculation of PGPB (Figure S12, Supporting Information). The upregulation of carbon fixation was consistent with the increase of chlorophyll content and the upregulation of photosynthetic related proteins (Figure S7 and Table S3, Supporting Information). Similarly, tricarboxylic acid (TCA) cycle metabolites including citrate, succinate, fumarate, and malate increased to 1.8−5.3-fold (Figure S12A, Supporting Information). Interestingly, the elevated level of gibberellins (GAs) and the upregulation of maize IAA biosynthesis were also observed in maize grown in normal soil with PGPB (Figure S13A−C, Supporting Information). This is consistent with the upregulation of maize growth-regulator-like protein, an auxin binding peptide identified by our proteomic analysis (Table S3, Supporting Information). In contrast, gluconic acid, galacturonic acid, and iduronic acid, which are the building blocks of maize cell wall polysaccharides, decreased significantly following the inoculation of PGPB.

PCA clearly showed separation of metabolites in maize grown in marginal soil with and without PGPB (Figure 2). The relative levels of 77 maize metabolites showed significant changes due to PGPB inoculation ($p < 0.05$, Figure 3). Pathway analysis identified glutathione, proline, and ascorbate metabolism, aminoacyl-tRNA biosynthesis, TCA cycle, galactose metabolism, and carbon fixation as the major metabolic pathways that were modulated by PGPB in stamp sand. Application of PGPB led to the downregulation of maize glutathione and proline metabolism compared with non-inoculated control in marginal soil (Figure S14, Supporting Information). This was supported by significant decrease in antioxidant enzyme activities and downregulation of γ-glutamylcysteine synthase and glutathione S-transferase in maize grown in marginal soil with PGPB compared with maize alone (Figures S8 and S9 and Table S5, Supporting Information). Spermine, which enhances plant growth and abiotic stress tolerance, was upregulated in marginal soil with PGPB (Figure S14, Supporting Information). Metabolites that are part of photosynthesis in maize grown in marginal soil with PGPB were significantly upregulated (Figure 4). This correlated well with an increase in the levels of three enzymes in photosynthesis, oxygen-evolving enhancer protein, pyruvate phosphate dikinase 1, and Rubisco, in the proteomic analysis (Table S5, Supporting Information). The levels of TCA cycle metabolites citrate, succinate, 2-oxoglutarate, and malate increased to 2.15−7.35-fold in maize grown in marginal soil with PGPB compared to maize grown without PGPB.

DISCUSSION

Our study showed that the influence of PGPB on plant growth and metal uptake is dependent not only on the concentration of heavy metals and quality of the soil but also on the bacterial delivery modes (Figure 1). This might be due to a change in PGPB colonization efficiency caused by different inoculation methods. Postgermination root adhesion resulted in the highest increase of biomass. However, it is not practical for field application. The highest copper accumulation was achieved using pregermination soil colonization and seed coating methods. Taking into account the higher plant biomass, metal uptake, soil enzyme activity, and the feasibility of field-level use, we propose that the combination of inoculation of metal-resistant bacteria by the presowing and seed coating methods are better for bacterial-assisted phytoremediation.

We found that the total population of culturable bacteria in stamp sand is extremely low. A similar phenomenon was also observed in the mine tailings collected from a state superfund site in Arizona.[24] After inoculation of PGPB, the density of bacterial inoculants declined and re-expanded progressively being dominant in both rhizospheric and nonrhizospheric soil, which suggests that the soil bacterial community has improved by PGPB inoculation (Figure S1, Supporting Information). The correlation of maize growth with an increase in PGPB suggests that they successfully established a mutualistic relationship with the host plant. However, the potential role of nonculturable bacteria in PGPB−maize−stamp sand interactions was not studied.

Pseudomonas TLC 6-6.5-4 solubilizes phosphate.[13] Previous studies tested phosphate solubilizing bacteria using an agar assay in vitro, which is not directly associated with phosphate uptake by plants in soil.[25,26] Our study provides a direct evidence for higher phosphate assimilation by maize from soil after PGPB inoculation (Figure S2A, D, Supporting Information).

We found that the increase of copper uptake in maize with PGPB inoculation has a strong correlation with the decrease of copper bound with organic matter and the increase of exchangeable copper in stamp sand. This could be due to the degradation of organic matter by the PGPB, leading to the release of soluble and exchangeable metals.

The symbiosis between plants and PGPB in plant rhizosphere is one of the world's most prevalent mutualism. The biochemical basis of the interaction between plants and free-living PGPB remains largely unknown compared with the symbiotic interaction between leguminous plants and N-fixing PGPB in nodules.[27] Our study revealed that *Pseudomonas* TLC 6-6.5-4 triggers the plant's response in a similar manner to other beneficial microbes and plant pathogens in some aspects. The plant cell wall and cell membrane are the first barriers for plant−microbe interactions.[28] In our study, we found that PGPB inoculation led to the downregulation of profilin-1, a protein for cytoskeletons structure and uronic derivatives of glucose that are building blocks of cell wall polysaccharides (Table S4, Supporting Information).[29] The decrease of cytoskeleton and cell wall organization related genes and metabolites was also observed in potato during bacterial pathogen infection as well as in *Medicago truncatula* during the development of symbiosis with arbuscular mycorrhizae.[30,31] Therefore, profilin-1 and gluconic acid could be used as biomarkers for plant−microbe interaction.

Plant hormones have profound effect on plant growth and development. Our study demonstrated that *Pseudomonas* TLC 6-6.5-4 has the ability to not only produce plant auxin IAA themselves but also directly impact the biosynthesis of maize plant hormones IAA and GAs (Figure S13, Supporting Information).[13] Interestingly, disturbing auxin homeostasis is also a common virulence mechanism for some pathogens such as *Agrobacterium tumefaciens*, which uses auxin and other hormones to induce cell growth and division of the host plants and thus the formation of galls.[32] In addition, rhizobia activate auxin responsive promoters in legume plants to facilitate the formation of nodule meristem.[33] Although *Pseudomonas* TLC 6-6.5-4 shares some common manners with plant pathogens during plant−microbe interaction, it bypasses the induction of salicylic acid (SA)-mediated systemic acquired resistance (SAR) normally used by bacterial pathogens. The carboxylic acid azelaic acid was reported to prime plants to accumulate SA and

therefore induce the whole plant immunity.[34] Our GC-MS analysis showed that there was no significant difference in the level of azelaic acid in maize with and without PGPB inoculation ($p < 0.05$) (data not shown). In addition, there was no increase in the major antioxidant enzymes activities in maize grown in normal soil with PGPB except APX (Figure S8, Supporting Information). Besides the N-fixation rhizobia, the endophytic plant growth promoting bacteria *P. fluorescens* FPT9601-T5 upregulates nodulin-like genes in *Arabidopsis* even though no nodules are formed.[35] However, in this study, we did not find any upregulation of nodulin-like proteins in maize.

Our results demonstrated that PGPB help plants in a dynamic manner in different soil environments. Proteins and metabolic pathways altered by PGPB in maize grown in normal soil were related to growth and development such as the upregulation of growth-regulator-like protein and photosynthesis. The PGPB helped maize grown in stamp sand to cope with heavy metal stress by regulating a number of proteins and metabolic pathways. TCA cycle metabolites upregulated in maize in this study can enter other metabolic pathways, and they can be generated by other metabolic pathways.[36] For example, 2-oxoglutarate and oxaloacetate are used to generate organic compounds involved in nitrogen assimilation (Figure S15, Supporting Information). Oxaloacetic acid, malate, and succinate can be generated from phosphoenolpyruvate, glyoxylate, and γ-aminobutyric acid, respectively. Similarly, fumarate can be generated from arginine and purine. The TCA cycle can operate in a noncyclic manner to achieve these functions and along with glycolysis generate ATP for higher biomass production. Carboxylic acids including citrate and malate have several cellular functions in addition to serving as a carbon source for microbes.[37] Carboxylates in root exudates can bind metal ions to form complexes, which are nontoxic. Interestingly, our previous studies showed that citrate is also involved in the sequestration of copper in *Pseudomonas* TLC 6-6.5-4 itself.[38] The downregulation of antioxidant enzymes activities, glutathione metabolic pathway, and the decrease of nonenzymatic antioxidants (proline and phenolic compounds) indicate that PGPB was alleviating copper stress to maize plant.

Plant phenolic compounds are aromatic secondary metabolites, and many of the phenolic root exudates serve as chemoattractants and carbon sources for soil microorganisms as well as effective antioxidants.[39,40] The inoculation of PGPB in normal soil enhanced maize secondary metabolites including phenolic compounds probably due to the exogenous plant hormone IAA secreted by PGPB (Figure S10, Supporting Information). Similarly, the increase of maize secondary metabolites was also observed in the interaction between maize and IAA-producing PGPB *Azospirillum*.[41] It was proposed that plants and their symbiotic partner mycorrhizal fungi maintain a fair, two-way transfer of resources in plant rhizosphere.[42] It is likely that the PGPB–maize interaction follows a similar mechanism. PGPB promote plant growth by production of IAA and other compounds, and maize plants in turn release more phenolic compounds in root rhizosphere that serve as the carbon source for PGPB. In stamp sand, a significant increase in total phenolics was observed in maize (Figure S10, Supporting Information). It is likely that the accumulation of total phenolics in maize grown in stamp sand was to scavenge the excess free oxygen species induced by copper. PGPB inoculation reduced the levels of total phenolics of plants grown in stamp sand, which most likely buffered the typical copper induced stress response.

Our study investigated the role of PGPB in enhancing maize biomass and nutrient uptake employing a multifaceted approach. It is likely that this is accomplished by regulation and dynamic interaction of plant proteins and metabolites as well as soil properties. PGPB characterized in this study has the potential to be used to produce biofuels from plants grown in marginal soils, remediating soils contaminated with heavy metals, and organic agriculture.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental materials and methods; tables of the experimental setup, the physicochemical properties of stamp sand, the effect of bacterial inoculation on copper mobility in stamp sand, and maize proteins altered in response to PGPB in normal soil and stamp sand; and figures showing total heterotrophic bacteria and PGPB population dynamics in stamp sand, the effect of PGBP–soil interactions on maize growth, the total soluble protein in maize growth in normal soil and stamp sand with or without PGPB inoculation, representative 2-D gel images of proteins extracted from maize, PCA score plots, classification of differentially expressed proteins, chlorophyll content of maize grown in normal soil and stamp sand with or without PGPB inoculation, antioxidant enzyme activities of maize grown in normal soil or stamp sand with or without PGPB, western blot analysis of GCL expression in maize grown in normal soil and stamp sand with or without PGPB, total phenolics in maize grown in normal soil and stamp sand with or without PGPB inoculation, relative metabolite levels of major metabolite groups in maize grown in normal soil with and without PGPB, maize TCA and carbon fixation metabolites were upregulated in the presence of PGPB in normal soil, PGPB enhances the levels of plant hormones IAA and GA, the glutathione metabolic pathway was downregulated in maize grown in stamp sand with PGPB, and the involvement of TCA metabolites in ammonium assimilation. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (906) 487-3068; fax: (906) 487-3167; e-mail: wusirika@mtu.edu.

Notes

The authors declare no competing financial interest.

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