Inoculation with endophytic *Bacillus megaterium* 1Y31 increases Mn accumulation and induces the growth and energy metabolism-related differentially-expressed proteome in Mn hyperaccumulator hybrid *pennisetum*

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**HIGHLIGHTS**

- Hybrid *pennisetum* was a Mn hyperaccumulator.
- Strain 1Y31 increased the growth and total Mn uptake of hybrid *pennisetum*.
- The proportion of PGPEB was higher in the bacteria-inoculated plants under Mn stress.
- Strain 1Y31 promoted the photosynthesis and energy metabolism of the plants.

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**ABSTRACT**

In this study, a hydroponic culture experiment was conducted in a greenhouse to investigate the molecular and microbial mechanisms involved in the endophytic *Bacillus megaterium* 1Y31-enhanced Mn tolerance and accumulation in Mn hyperaccumulator hybrid *pennisetum*. Strain 1Y31 significantly increased the dry weights (ranging from 28% to 94%) and total Mn uptake (ranging from 23% to 112%) of hybrid *pennisetum* treated with 0, 2, and 10 mM Mn compared to the control. Total 98 leaf differentially expressed proteins were identified between the live and dead bacterial inoculated hybrid *pennisetum*. The major leaf differentially expressed proteins were involved in energy generation, photosynthesis, response to stimulus, metabolisms, and unknown function. Furthermore, most of the energy generation and photosynthesis-related proteins were up-regulated, whereas most of the response to stimulus and metabolism-related proteins were down-regulated under Mn stress. Notably, the proportion of indole-3-acetic acid (IAA)-producing endophytic bacteria was significantly higher in the bacterial inoculated plants under Mn stress. The results suggested that strain 1Y31 increased the growth and Mn uptake of hybrid *pennisetum* through increasing the efficiency of photosynthesis and energy metabolism as well as the proportion of plant growth-promoting endophytic bacteria in the plants.

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1. **Introduction**

Although manganese (Mn) is an essential element for life tissues, excessive Mn concentrations in environments through inputs from natural processes and anthropogenic activities can inhibit plant growth and lead to numerous health problems [1,2]. In fact, high concentrations of Mn in soil and water are a widespread environmental problem and have aroused considerable attention worldwide [3,4]. Recently, phytoremediation approaches have been used for Mn-contaminated soil and water environments [5–7]. Phytoremediation, which mainly uses hyperaccumulators to remove excess heavy metals from contaminated environments, is regarded as a promising cost-effective method [8]. The success of this technique highly depends on the hyperaccumulators. However, most hyperaccumulators are low biomass and grow slowly in the heavy metal-contaminated environments [4,9] and are not suitable for the effective phytoremediation of heavy metal-contaminated environments.

Endophytic bacteria have been reported to promote the growth and metal accumulation of plants growing on heavy
metal-contaminated environments by producing IAA, siderophores, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and arginine decarboxylase [10,11]. However, little is known about the molecular mechanisms involved in endophytic bacteria-assisted phytoremediation of heavy metal-contaminated environments. Proteomics, the comprehensive and quantitative analysis of proteins that are expressed in plant tissues, provides unique insights into biological systems [12]. Using proteomics technology including high-resolution two-dimensional gel electrophoresis (2-DE) and high-sensitivity mass spectrometry (MS), it is possible to gain greater insight into the detailed impact that bacteria have on plants [13]. Proteomics has been used extensively to investigate the protein expression pattern under abiotic or biotic stress [14,15]. Although the proteomic studies of plant–bacterial interactions have been studied [16,17], there are currently few proteomic studies of plants treated with plant growth-promoting endophytic bacteria [13]. Furthermore, no proteomic study has been carried on Mn hyperaccumulator hybrid pennisetum treated with Mn and endophytic bacteria. Hybrid pennisetum belongs to Pennisetum with Gramineae and is a high-stalk perennial grass [18]. It has many advantages, such as rapid growth, large biomass, high resistance to adverse conditions, ease of cultivation and repeated cropping, which make it easy to apply compared with many hyperaccumulators that have been described [18]. Endophytic bacteria may be of particular interest as they have the advantage of being relatively protected from the competitive, high-stress environment of the soil [19]. A better understanding of the molecular mechanisms involved in the endophytic bacteria-enhanced Mn uptake of hybrid pennisetum is needed for the development of efficient phytoremediation systems of Mn-contaminated soils and manganese mine sites [4,6].

In this study, we analyzed the effects of Mn-resistant endophytic Bacillus megaterium Y131 on the growth and Mn accumulation of hybrid pennisetum and then carried out a proteomic analysis of the leaves of hybrid pennisetum subjected to Mn stress and bacterial inoculation to identify proteins, hoping to gain a more thorough understanding of the molecular basis of Mn tolerance and accumulation in this species. Furthermore, the impact of the endophytic bacterium on the relative richness of plant growth-promoting factor-producing endophytic bacteria in the plant tissues under Mn stress was also evaluated in order to understand the microbial mechanism involved in the bacteria-enhanced growth and Mn uptake of hybrid pennisetum.

2. Materials and methods

2.1. Plant and inoculum

Hybrid pennisetum (Pennisetum americanum × P. purpureum) was used in the inoculation experiment. B. megaterium Y131 (accession No. HG316061) was isolated from the leaves of Mn-hyperaccumulator Phytolacca americana grown in Mn-contaminated environment. Strain Y131 can produce indole acetic acid (87.4 ± 3.8 μg mL⁻¹), siderophores, and ACC deaminase and exhibit high Mn (20 mM) resistance [20].

2.2. Pot experimental design

In the pot experiment, a completely randomized design was used to investigate the effects of strain Y131 on the growth, Mn uptake, leaf protein expression profiles and the proportions of IAA- and siderophore-producing endophytic bacteria of hybrid pennisetum grown in Hoagland nutrient solution [21] supplemented with 0, 2, and 10 mM Mn as MnCl₂·4H₂O (pre-experiment showed that the hybrid pennisetum can be resistant to 2–10 mM Mn). Triplicate pots were used for each treatment. The seeds of hybrid pennisetum were surface-sterilized with 10% H₂O₂ for 15 min, washed with sterile water, and germinated for 2 days at 25 °C in the dark. After germination, four uniform seedlings for each treatment were transferred to 2.5-L plastic pots containing Hoagland’s nutrient solution and different concentrations of Mn as described. Pots were placed in a growth chamber (25°C/20°C day/night; 14/10 h, light/dark, and relative humidity of 70–75%).

For inoculation, strain Y131 was grown in Luria–Bertani’s (LB) medium. Cells in the exponential phase were collected by centrifugation at 12,000 rpm for 10 min, washed with sterile saline solution (0.85% NaCl), and recentrifuged. Bacterial inoculum was prepared by resuspending pelleted cells in sterile saline solution to get an inoculum density of 10⁸ cfu mL⁻¹. The roots of the seedlings were immersed in the live bacterial suspensions (5 mL) for 1 h. A dead bacterial (autoclaved at 121 °C for 30 min) inoculated root was prepared as a control. Hoagland’s nutrient solutions were renewed every four days with the volume being restored to its original level. Plants were harvested 45 days after inoculation. Roots and leaves were separated and washed with 0.01 M EDTA and distilled water to remove any nonspecifically bound Mn. The root and leaf samples were divided into three portions. One portion was oven-dried for 30 min at 105 °C, then at 55 °C, until they reached constant weight before determining the tissue dry weight. The oven-dried tissues were ground using a stainless steel mill (FZ102, Tianjing, China) to 0.5 mm for analysis. Subsamples of leaf (200 mg) and root (200 mg) samples were then digested in a mixture of concentrated HNO₃ and HClO₄ (4:1, v/v) [22]. The concentrations of Mn in the samples were determined using an inductively coupled-plasma optical emission spectrometer (ICP-OES) (Optima 2100 DV, Perkin Elmer). The total Mn uptake of roots or leaves = Mn concentration in roots or leaves × dry weight of roots or leaves. The other two portions were used for the analyses of protein expression profiles and endophytic bacteria.

2.3. Protein extraction and solubilization

The proteomic analysis was carried out on the leaves to identify the leaf differentially expressed proteins potentially involved in leaf metal accumulation. This could contribute to the identification of genes responsible for metal tolerance and above-ground hyperaccumulation, considering their potential biotechnological application eventually for phytoremediation purposes [23]. In this study we focused on the differentially expressed proteins on total protein extract without protein fractionation and subcellular localization of the gene product. Total proteins were extracted from the leaves of hybrid pennisetum according to Wang et al. [24,25]. The protocol for protein extraction included leaf tissue powder, TCA/acetone wash, methanol wash, acetone wash, dry, protein extraction and precipitation, wash, and air-dry of the pellet. The proteins were dissolved in SDS buffer [24]. Protein concentration was estimated by Bradford assay (Sigma–Aldrich), following the manufacturer’s instructions.

2.4. Two dimensional gel electrophoresis (2-DE) and staining

The protein precipitate was solubilized in 450 μL of an electro-focusing solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, and 0.5% (v/v) IPG buffer (pH 4–7). The mixture was incubated at room temperature for 60 min. After centrifugation at 10,000 × g for 10 min, the supernatant was applied onto an NL IPG strip (pH NL 4–7, 24 cm, GE Healthcare). Four replica maps were performed. Rehydration loading and IEF were performed as described by Huang et al. [26]. Following the two-step equilibration, the second-dimension separation was carried out on 12.5% SDS-PAGE gel prepared according to Laemmli [27] running
at 20 mA/gel with Protean II xi cell (Bio-Rad, CA). Proteins were detected by Coomassie Brilliant Blue G–250 staining.

2.5. Proteome isolation and differential protein pattern analysis

Total proteins were extracted from the leaves of three biological replicates corresponding to different treatments and separated by 2-DE. MS/MS analysis and protein identification were performed as described [14]. The image analysis of the 2-DE gels (three replicates were performed for each biological replicate and treatment considered) was carried out using PDQuest software (BioRad) version 8.0.1. Each gel was analyzed for spot detection, background subtraction, and protein spot OD intensity quantification. The gel image showing the highest number of spots and the best protein pattern was chosen as a reference template. All spot comparisons and analyses were coordinated through the reference template and its spots were then matched across all gels. Gels were divided into three separate groups (live bacterial inoculation vs dead bacterial inoculation in the presence of 0, 2, and 10 mM Mn, respectively). For each protein spot, the quantity was normalized in each gel by dividing the raw quantity of each spot by the total quantity in the gel of all the spots found in the reference template [28]. For each protein spot, the average spot quantity value and its variance coefficient in each group were determined. Statistical analysis (Student’s t-test) was performed to identify proteins that were significantly (p < 0.05) increased or decreased in the three groups of samples.

2.6. In-gel digestion and MS/MS analysis

Spots of interest were excised from gels and subjected to in-gel trypsin digestion as described [29]. MS and MS/MS analysis of the digested peptide was performed on an ABI 5800 MALDI-TOF/TOF plus mass spectrometer (Applied Biosystems, USA). Data were acquired in a positive MS reflector using a CalMixS standard to calibrate the instrument (ABI5800 Calibration Mixture). Both the MS and MS/MS data were integrated and processed by using the GPS Explorer V3.6 software (Applied Biosystems, USA) with default parameters. Based on combined MS and MS/MS spectra, proteins were successfully identified based on 95% or higher confidence interval of their scores in the MASCOT V2.3 search engine (Matrix Science Ltd., London, U.K.), using the following search parameters: NCBI–Pancieae database; trypsin as the digestion enzyme; one missed cleavage site; fixed modifications of Carbamidomethyl (C); partial modifications of Acetyl (Protein N-term), Deamidated (NQ), Dioxidation (W), Oxidation (M); 100 ppm for precursor ion tolerance and 0.5 Da for fragment ion tolerance.

2.7. Isolation of endophytic bacteria

The endophytic bacteria were isolated from the roots and leaves according to the method of Sun et al. [30]. Briefly, plant tissues were sterilized, washed with sterile water, and ground. Then, plant tissue suspensions were plated onto 1/5-strength LB medium with and without 10 mM of Mn as MnCl₂ to determine Mn-resistant or total culturable endophytic bacteria. Plates were incubated for 7 days at 28 °C. Total six hundreds bacterial isolates (total 12 samples, 50 isolates per sample) were randomly selected and stored on slants. The number of collected bacterial isolates accounted for 91–94% of the total bacterial isolates on the plates from the different samples.

2.8. IAA and siderophore production of the endophytic bacteria

The production of IAA by the endophytic bacteria was determined according to the methods of Gordon and Weber [31] and Sheng et al. [32]. Siderophore production by the bacteria was detected by the method of Schwyn and Neilands [33] using blue agar plates containing the dye Chrome azulé S. Orange halos around the colonies on blue agar were indicative of siderophore excretion.

2.9. Statistical analyses

One-way analysis of variance (ANOVA) and the Fisher’s Least Significant Difference test (Fisher’s LSD) (p < 0.05) were used to compare the averages of dry weights and Mn contents of live bacterial inoculated hybrid Pennisetum and the averages of bacterial counts and proportions of IAA- and siderophore-producing endophytic bacteria isolated from the bacterial inoculated plant with those from the control. Statistical analyses were carried out using SAS 8.2 (Statistical Analysis System, USA).
3. Results

3.1. Plant growth and Mn accumulation

Although high concentration of Mn significantly (p<0.05) inhibited the growth of hybrid pennisetum, the inoculation with strain 1Y31 significantly (p<0.05) increased the growth and Mn accumulation of the plants (Fig. 1). Root and leaf dry weights of hybrid pennisetum inoculated with strain 1Y31 were increased from 28% to 94% and from 29% to 70%, respectively, compared to the control (Fig. 1). Although Mn concentrations in the tissues were not significantly different, the total Mn uptake of the roots and leaves was significantly different between the live and dead bacterial inoculated plants. Inoculation with strain 1Y31 significantly increased the total Mn uptake in the roots (ranging from 23% to 93%) and the leaves (ranging from 30% to 112%) of the plants compared to the control. Furthermore, significantly (p<0.05) higher Mn concentrations were observed in the leaves than in the roots. Notably, more than 13,000 mg kg⁻¹ of Mn was observed in the leaves (ranging from 13,373 mg kg⁻¹ to 13,518 mg kg⁻¹) of the hybrid pennisetum treated with 10 mM Mn.

3.2. Effect of strain 1Y31 on the culturable endophytic bacterial characteristics

Strain 1Y31 showed the different impacts on the culturable endophytic bacterial counts and the proportions of IAA-, and siderophore-producing bacteria isolated from the hybrid pennisetum grown in the different levels of Mn stress (Table 1). The total and Mn-resistant endophytic bacterial counts and the proportions of IAA- and siderophore-producing endophytic bacteria were significantly higher in the roots of strain 1Y31 inoculated plants than that of the control under low, low (2 mM), and high (10 mM) Mn stress. Similarly, the Mn-resistant endophytic bacterial counts and the proportions of IAA- and siderophore-producing endophytic bacteria were significantly higher in the leaves of strain 1Y31 inoculated plants than that of the control under low and high Mn stress. Notably, significantly higher proportions of IAA- and siderophore-producing endophytic bacteria isolated from the roots and leaves were observed in strain 1Y31 inoculated plants than in the control under Mn stress (Table 1).

3.3. Proteomic analysis of leaf differentially expressed proteins

In order to investigate the molecular mechanisms of strain 1Y31-enhanced Mn resistance and accumulation of hybrid pennisetum grown in different levels of Mn stress, the leaf differentially expressed proteins in the live vs dead bacterial inoculated hybrid pennisetum were examined. 2-DE was used to detect the significantly up- or down-regulated proteins (p < 0.05, |ratio| > 3.0) in the leaves of hybrid pennisetum. Similar number of protein spots (ranging from 606 to 673) of the control plants were observed under different levels of Mn stress (Fig. 2). However, significantly more protein spots (ranging from 912 to 1068) in the leaves of strain 1Y31 inoculated plants were found under Mn stress than under no Mn stress (protein spots, 666 ± 72) (Fig. 2). Total 27, 38, and 33 leaf differentially expressed proteins were obtained, among which 22, 20, and 23 proteins were up-regulated at least 3-fold under no, low, and high Mn stress, respectively (Fig. 3, Table S1). Functional annotation showed that the identified proteins were involved in 9 functional categories (Fig. 4, Table S1). However, the differentially-expressed proteins involved in generation of reactive oxygen species, metabolism, signaling, and cellular structure were not observed under no Mn stress (Table S1). In addition, the differentially-expressed proteins involved in energy generation, photosynthesis, response to stimulus, and unclassified were the dominant (>10%) under different levels of Mn stress, while the differentially-expressed proteins involved in metabolism were also the dominant under Mn stress (Fig. 5). Furthermore, the dominant leaf differentially expressed proteins accounted for 81–92% of the total leaf differentially expressed proteins.

3.3.1. Proteins involved in energy generation and metabolism

Under no Mn stress, the highest up-regulated protein (spot 3312) is involved in glycolysis, while the lower down-regulated protein (spot 1503) is responsible for antiporter active transport. The other up-regulated proteins are responsible for the synthesis of carbohydrates for plants (spot 303), the pentose phosphate cycle and the oxidative degradation of glucose (spots 1306, 3612, and 4707 or 5401), the catalytic activation of Rubisco (spots 5606 and 5608), and the oxaloacetate and carbon fixation reaction of C4 plants (spots 7806, 7807, and 7813). Under low Mn stress, the highest up-regulated protein plays a central role in the Calvin cycle. The down-regulated proteins (spots 5704, 6606, and 7404) are involved in the pentose phosphate cycle and the glycolytic pathway. Under high Mn stress, the higher up-regulated proteins (spots 5805 and 6402) are involved in oxidative phosphorylation and photophosphorylation and ATP synthesis. The lower down-regulated protein (spot 1503) can decompose lactose to produce glucose. The other up-regulated proteins (spots 1710 and 3601) are involved in glycolysis and citric acid cycle. Notably, 70%, 73%, and 71% of the specific

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Total bacteria (×10⁶ cfu g⁻¹)</th>
<th>Mn-resistant bacteria (×10⁶ cfu g⁻¹)</th>
<th>The proportion of IAA-producing bacteria (%)</th>
<th>The proportion of siderophore-producing bacteria (%)</th>
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<tr>
<td>Control (no Mn stress)</td>
<td>2.6 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>70 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52 ± 3&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>Root + live bacterium</td>
<td>5.5 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.2 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>96 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>96 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leaf + live bacterium</td>
<td>3.1 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.4 ± 0.06&lt;sup&gt;d&lt;/sup&gt;</td>
<td>78 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>78 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Low Mn stress (2 mM Mn)</td>
<td>2.0 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>62 ± 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>78 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Root + live bacterium</td>
<td>2.1 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.3 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>94 ± 3&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Root + dead bacterium</td>
<td>1.3 ± 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.5 ± 0.03&lt;sup&gt;d&lt;/sup&gt;</td>
<td>64 ± 2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>76 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Leaf + live bacterium</td>
<td>1.6 ± 0.1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.6 ± 0.04&lt;sup&gt;e&lt;/sup&gt;</td>
<td>94 ± 1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>92 ± 2&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>Leaf + dead bacterium</td>
<td>1.5 ± 0.1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.5 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>48 ± 3&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>a, b, c, d, e, f, and g: Means ± standard error (n=3). Data followed by the same letter within a column are not significantly different (p > 0.05) according to Tukey's test.</sup>
differentially expressed proteins involved in energy production were found under no, low, and high Mn stress, respectively.

The differently expressed proteins involved in metabolism were only detected under Mn stress (Table S1). All of the proteins were down-regulated under low Mn stress, while three of the five proteins were up-regulated under high Mn stress. The down-regulated proteins (spots 4203 and 4509) are involved in the degradation of unneeded and damaged cells and the plant defense against biotic and abiotic stress under low Mn stress. Under high Mn stress, the highest up-regulated protein (spot 7010) can degrade damaged proteins. The up-regulated proteins (spots 7509 and 1204) are involved in the metabolism of alanine. In addition, the
differentially expressed proteins involved in metabolism were completely different under low and high Mn stress (Table S1).

3.3.2. Proteins involved in photosynthesis

Under no Mn stress, the highest up-regulated protein (spot 110) is involved in the collection and transmission of light energy. The down-regulated protein (spots 109 and 111) is involved in water cleavage and oxygen release. Furthermore, the up-regulated proteins (spots 8506 and 9503) can catalyze the ribulose 1, 5-bisphosphate (RuBP) carboxylation and oxygenation reactions. Contrary to the oxygen-evolving enhancer protein which was down-regulated under no Mn stress, the protein (spots 1202, 1207, 1208, and 6101) was up-regulated under low Mn stress. The highest up-regulated protein (spot 9605) was ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit under low Mn stress. Under high Mn stress, the highest up-regulated proteins (spots 110, 27504 and 7707) catalyze the ribulose-1,5-bisphosphate (RuBP) carboxylation and oxygenation reactions. Notably, 60%, 50%, and 100% of the specific differentially expressed proteins involved in photosynthesis were found under no, low, and high Mn stress, respectively.

3.3.3. Proteins involved in response to stimulus

All of the differently expressed proteins were up-regulated under no Mn stress but down-regulated under low Mn stress (Table S1). Under high Mn stress, half of the differently expressed proteins were up- or down-regulated. Under no Mn stress, the up-regulated proteins are involved in plant defense against pathogen attack (spots 3005 and 7105) and the major detoxification system for cells against damage and disease (spot 107). Under low Mn stress, the down-regulated proteins included a class of molecular chaperones (spot 7707) and a kind of methylases associated with plant defense (spots 7806, 8802, and 8803). Under high Mn stress, the up-regulated proteins are mainly involved in plant defense (spots 2204 and 5102). Notably, the glutathione S-transferase (spot 7401) which can catalyze the conjugation of the reduced form of glutathione to xenobiotic substrates for the purpose of detoxification was down-regulated under high Mn stress (Fig. 5, Table S1).

4. Discussion

The concentration of Mn (ranging from 1904 to 13518 mg kg⁻¹) in the leaves of the hybrid pennisetum under hydroponics culture was higher than the criterion (1000 mg Mn kg⁻¹) set by Baker and Brooks [34] (Fig. 1). In addition, the hybrid pennisetum was characterized by a Mn translocation factor (ranging from 1.3 to 3.5) greater than one in this study. So the hybrid pennisetum could be identified as a Mn hyperaccumulator [34]. Recently, bacteria-assisted
phytoremediation by hyperaccumulator has been regarded as an effective and promising method [35–37]. Similar results were obtained in this study, where the growth and Mn uptake of hybrid pennisetum were significantly increased after inoculation with strain 1Y31 under different levels of Mn stress. Bacteria having the characteristics of producing IAA, siderophores, ACC deaminase can stimulate plant growth and protect plants against heavy metals toxicity in heavy metal contaminated environments[11,36]. Strain 1Y31 possesses multiple plant growth-promoting characteristics such as IAA, siderophore, ACC deaminase and arginine decarboxylase production ability which may be responsible for the growth promotion and protection of hybrid pennisetum against Mn toxicity (Fig. 1). Notably, strain 1Y31 did not significantly influence the Mn concentrations in the plants compared to the control (Fig. 1), however, strain 1Y31 significantly promoted the growth of hybrid pennisetum, consequently increasing the total Mn uptake of the plants. Dell’Amico et al. [38] also found that Cd-resistant bacteria did not influence Cd concentrations in Brassica napus but increased the plant biomass and consequently the total Cd uptake of the plants.

Interestingly, strain 1Y31 could significantly modulate the relative proportion of IAA and siderophore-producing endophytic bacteria in the roots and leaves of hybrid pennisetum (Table 1). Inoculation with strain 1Y31 was found to significantly increase the relative proportion of siderophore-producing endophytic bacteria of the plants under different levels of Mn stress, however, the increased relative proportion of IAA-producing endophytic bacteria of strain 1Y31 inoculated plants was only observed under Mn
stress (Table 1). In addition, significantly higher relative proportions of IAA and siderophore-producing endophytic bacteria of the plants were observed under Mn stress. These results suggest that IAA and siderophore-producing endophytic bacteria might play an important role in the growth and Mn resistance of the plants and IAA-producing endophytic bacteria might play more important roles in the growth and Mn resistance of the plants under Mn stress condition.

To explore the molecular mechanism of how strain 1Y31 modifies the functioning of hybrid pennisetum under Mn stress, we determined the effect of strain 1Y31 on the leaf protein profiles of the plants under different levels of Mn stress. In this study, enhanced Mn uptake of the plants mediated by strain 1Y31 was due to a stimulatory effect on the plant growth (Fig. 1). Production of the bacterial metabolites (such as indole acetic acid, siderophores, ACC deaminase, and arginine decarboxylase) may modulate the expression of the proteins related to the plant growth and metabolism [11], although molecular mechanisms regulating these plant–microbe interactions are still largely unknown [13]. Interestingly, under different levels of Mn stress, the numbers of leaf proteins were not significantly different in the control plants but significantly different in the bacterial inoculated plants (Fig. 2), suggesting that the difference in the numbers of leaf proteins was mainly affected by the inoculation with strain 1Y31.

In the leaf tissue differentially expressed energy production-related proteins, most of (71–85%) them were up-regulated (Table S1) and the highest up-regulated proteins involved in glycolysis and the synthesis of ATP were found under high Mn stress (Table S1, Fig. 5), suggesting that inoculation with strain 1Y31 increased the efficiency of producing energy in the leaves of the plants and the energy-producing efficiency was higher in the bacterial-inoculated plants under high Mn stress. All of the leaf differentially expressed proteins involved in photosynthesis were up-regulated after the inoculation with strain 1Y31 under Mn stress (Table S1), suggesting that inoculation with strain 1Y31 could increase the biomass of the plants by increasing the efficiency of photosynthesis under no or Mn stress conditions. Farinati et al. [23] also found that the shoot proteome of hyperaccumulator Arabidopsis halleri showed an increased abundance of photosynthesis-related proteins in the presence of bacteria and heavy metals. In the leaf differently expressed proteins involved in response to stimulus, the glutathione S-transferase (GST) involved in the major detoxification system [39] was up-regulated after the inoculation with strain 1Y31 under no Mn stress, suggesting that bacterial inoculation could improve the plant defense systems. Cheng et al. [13] showed that the plant growth-promoting bacteria could increase the expression of plant defense response proteins in the root. Under low Mn stress, the main differently expressed protein related to defense stress for plants was down-regulated. In the presence of salt stress, the defense stress-related protein was reduced corresponding to the increase in plant biomass [40]. It implied that inoculation with strain 1Y31 could relieve low Mn stress, thereby increasing the biomass of the plants (Fig. 1). Notably, the leaf GSTs were lowest down-regulated under high Mn stress. The down-regulation of GSTs by Mn and bacterial inoculation, observed in this study, is therefore unexpected and requires further investigation because GSTs are usually induced under metal stress [41]. Farinati et al. [14] also showed that the expression of GSTF2 was repressed by both heavy metals and microorganisms. A possible explanation could be that the down-regulation of GSTs might be due to the plant-growth-promoting effect that strain 1Y31 provided to the plants [20]. The strain 1Y31 may utilize the photosynthesis-related compounds produced by plants as carbon sources and then promote plant growth and development [20,42]. The study showed that inoculation with strain 1Y31 could alleviate high Mn stress, thereby enhancing Mn accumulation of the plants. Furthermore, the leaf differently expressed proteins involved in metabolism were only observed after the inoculation with strain 1Y31 under Mn stress (Fig. 5). Under low Mn stress, all of the leaf differently expressed proteins involved in metabolism were down-regulated. The leaf differently expressed proteins involved in metabolism included the enzymes which can play roles in the degradation of unneeded and damaged cells and resistance against biotic and abiotic stress. Highest up-regulated protein involved in the degradation of damaged proteins was observed under high Mn stress. Salavati et al. [43] showed that bacterial inoculation could increase the expression of metabolism- and energy production-related proteins in the supernatulating soybean variety (Eb-b0-1). These results suggested that inoculation with strain 1Y31 could relieve the damage of cells caused by Mn stress so as to increase the biomass of the plants. Notably, the proteins that were found to be enhanced after the inoculation with strain 1Y31 included members of different plant energy and photosynthesis categories (Table S1, Fig. 5), suggesting the involvement of different molecular pathways in the energy and photosynthesis among the different levels of Mn stress [44]. As successful plant growth-promoting inoculants, bacteria must be able to rapidly colonize the root system during the growing season [45]. Dilution-plate method using 1/5-strength LB agar containing Mn (20 mM) and kanamycin (50 mg L⁻¹) showed that the tested strain 1Y31 colonized the root and leaf interiors of the hybrid pennisetum. The numbers of the cells in roots and leaves of hybrid pennisetum plants were 10⁶ cfu g⁻¹ of fresh root or leaf at 45 days after inoculation.

5. Conclusion

The results suggested that the plant growth-promoting endophytic strain 1Y31 could significantly increase the biomass, Mn resistance, and Mn accumulation of Mn hyperaccumulator hybrid pennisetum by increasing the expression of photosynthesis- and energy generation-related proteins and decreasing the expression of the response to stimulus-related proteins. Strain 1Y31 might increase the growth and Mn tolerance of the plants by modulating the relative proportion of the hybrid pennisetum-associated plant growth-promoting endophytic bacteria. The results also highlight the importance of plant–microbe interactions in plant protein expression and metal accumulation and emphasize the possibility of exploiting endophytic plant growth-promoting bacteria for the phytoremediation of Mn-contaminated environments. Further understanding of the dynamic changes in the leaf differently expressed proteins induced by the endophytic strain 1Y31 during the plant growth process are needed for the effective endophytic bacteria-enhanced phytoremediation of Mn-contaminated environments by the Mn hyperaccumulator.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2015.07.049

References

Mn$_2^+$ concentrations

pp.

2063–2066.


W. Wang, M. Scali, R. Vignani, A. Spadafora, Protein extraction for 2-D electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds, Electrophoresis 27 (2006) 2782–2786.