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Mechanisms of Cr(VI) resistance by endophytic *Sphingomonas* sp. LK11 and its Cr(VI) phytotoxic mitigating effects in soybean (*Glycine max* L.)

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ABSTRACT

Chromium Cr(VI) is highly toxic and leads to impaired phenotypic plasticity of economically important crops. The current study assessed an endophytic-bacteria assisted metal bio-remediation strategy to understand stress-alleviating mechanisms in *Glycine max* L (soybean) plants inoculated with *Sphingomonas* sp. LK11 under severe Cr(VI) toxicity. The screening analysis showed that high Cr concentrations (5.0 mM) slightly suppressed LK11 growth and metal uptake by LK11 cells, while significantly enhancing indole-3-acetic acid (IAA) production. Endophytic LK11 significantly upregulated its antioxidant system compared to control by enhancing reduced glutathione (GSH), catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) activities to counteract Cr-induced oxidative stress. Cr toxicity induced cell morphological alteration, as shown by SEM-EDX analysis and triggered significant lipid peroxidation. The interaction between LK11 and soybean in Cr-contaminated soil significantly increased plant growth attributes and down-regulated the synthesis of endogenous defense-related phytohormones, salicylic acid and abscisic acid, by 20% and 37%, respectively, and reduced Cr translocation to the roots, shoot, and leaves. Additionally, Cr-induced oxidative stress was significantly reduced in LK11-inoculated soybean, regulating metal responsive reduced GSH and enzymatic antioxidant CAT. Current findings indicate that LK11 may be a suitable candidate for the bioremediation of Cr-contaminated soil and stimulation of host physiological homeostasis.

1. Introduction

Chromium (Cr) is non-essential metal for plants, and is the second most abundant metal contaminant in the world due to its wide industrial usage (Singh et al., 2013). In agricultural soil, Cr exists stably either in a trivalent Cr (III) or hexavalent Cr (VI) form, which vary in terms of toxicity, mobility, and bioavailability (Dubey et al., 2010). Cr (III) is taken-up by plants through a symplastic pathway via the conversion of Cr (VI) to Cr (III) under reduced conditions, or via the formation of complexes with naturally available nutrients (Dubey et al., 2010; UdDin et al., 2015). Cr (VI) as being highly mobile is considered extremely toxic for both human and plants. Cr(VI) may persist in the soil or sediment for years, especially if the soils are sandy or present low levels of organic matter. It has been demonstrated that Cr (VI) toxicity substantially affects metabolic functions in plants, leading to imbalanced photosynthesis, rapid generation of reactive oxygen species (ROS), ruptured cell membranes, and reduced vital enzymatic processes, which ultimately cause cell death (Emamverdian et al., 2015; Gill et al., 2016; Qing et al., 2015). Continued exposure of Cr (VI) to agricultural fields negatively affect plant physiological and morphological traits, resulting in retarded crop yields (Shahid et al., 2017). Several approaches have attempted to overcome heavy metals contamination including Cr (VI), which including physical and chemical remediating techniques to remove or immobilize metal from the polluted soil (Dhal et al., 2013). Though these methods are quick to perform, but they are not eco-friendly and causing secondary pollutantion (Lotfy and Mostafa, 2014). Alternatively, phytoremediation has gained attention due to its cost-effectiveness, and for being an ecofriendly and efficient remediation method for metal-polluted soil/water (Kamran et al., 2014); however, it still confronts with reduced plant biomass and

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slow growth. Recently, the use of growth-promoting bacteria coupled with phytoremediating plants has been considered as an effective strategy to remove metal contaminants from soil in order to mitigate heavy metal stress in plants and enhance growth and biomass (Fahad et al., 2015; Jing et al., 2014).

Plant growth-promoting rhizobacteria possess metal-remediating potential due to the secretion of various enzymatic and phytochemical products, and can improve plants growth and resistance under adverse metal toxicity (Carlos et al., 2016; Glick, 2014; Luo et al., 2015). However, very few endophytic bacteria with plant growth-promoting potential are known to counteract the adverse effects of metal toxicity, particularly of Cr. Plant growth-promoting endophytic bacteria (PGPEB) colonize roots and help host plants to uptake essential nutrients. They also regulate phytohormonal balance by releasing phytohormones and control plant pathogens by triggering the induce systemic plant response (Ma et al., 2016a). Endophytic bacteria that produce phytohormones [indole-3-acetic acid (IAA), gibberellin, and abscisic acid (ABA)] may help to counteract various biotic and abiotic stresses, including metal stress in plants (Ma et al., 2016a; Nair and Padmavathy, 2014). Previously, endophytic *Bacillus thuringiensis* GDB-1, *Rahnella* sp. JN6, and *Bacillus* sp. E1S2 with phytohormones producing potential are reported for increasing plant biomass and enhance phytoremediation processes under different metals stresses, such as As, Cu, Cd, and Zn (Babu et al., 2013; He et al., 2013). However, the mechanisms involved in managing and tolerating the deleterious effects of metal toxicity, especially those associated with Cr, and the potential of alleviating heavy metal phytotoxicity in plants with PGPEB are not fully understood.

Therefore, the present study was conducted to assess the role of our previously isolated phytohormones producing endophytic *Sphingomonas* sp. LK11 in bioremediating metals and enhancing plant growth and metal tolerance (Khan et al., 2014). Complete genomic sequenced analysis of LK11 revealed the presence of metal-resistance genes and transporters, including *czcA, czcB, czcC copA,* and *ChrA*, which suggests that LK11 is a strong candidate for microbe-assisted phytoremediation strategies (unpublished data). Previously, Khan et al. (2016b) reported the bioaccumulation of metals, such as Cd and Zn, and the translocation ability of LK11 through the regulation of metallothionein expression. However, its metabolic and cellular responses, and its symbiotic role in soybean under metal stress, have yet not been explored. Hence, in the present study, we aimed to determine the hazardous effects of Cr toxicity on LK11 morphology and physiology as well as on its phytohormone (IAA) production capability. Additionally, the effects of LK11 inoculation on soybean growth and stress tolerance were assessed by modulating endogenous phytohormones and stress-related oxidative enzymes under varying levels of Cr stress.

2. Material and methods

2.1. Assessing LK11 resistance and consequent responses to metal toxicity

2.1.1. Assessment of endophytic bacterial growth, IAA production, and metal accumulation under Cr stress

Endophytic *Sphingomonas* sp. LK11 (KF515708) was cultured in Luria-Bertani (LB) broth spiked with various concentrations of Cd, Pb, Al, Cr, and Ni, and grown for 72 h in a shaking incubator at 28 ± 2 °C in order to assess heavy metals resistance and tolerance capability of LK11. The Survival ability of endophytic bacteria under metal stress was determined by analyzing cell biomass production and density at 600 nm. This revealed the ability of LK11 to tolerate Cr(VI) stress. Bacterial cells from Cr-amended broth were collected by centrifugation at 13,000 rpm at 4 °C for 15 min, and Cr was subsequently quantified in bacterial cells via inductively-coupled plasma mass spectrometry (ICP-MS), as reported by Khan et al. (2015). Similarly, the IAA-producing potential of bacteria in Cr-containing medium was analyzed via high-performance liquid chromatography (HPLC), as reported by Khan and Lee (2013).

2.1.2. Scanning electron microscopy- energy dispersive X-ray SEM-EDX analysis

To assess the cell-surface morphological characteristics of LK11 under Cr stress, SEM-EDX analysis was performed. Cr-treated LK11 cells were fixed in 2.5% glutaraldehyde, as reported by Sheng et al. (2016a). Micrographs of LK11 cells were obtained by SEM coupled with EDX analysis.

2.1.3. Determination of antioxidants and related enzymes, and the extent of lipid peroxidation

To assess oxidative stress, LK11 was grown in LB media with various concentrations of Cr (1.0, 2.0 and 5.0 mM). After 3 days, bacterial cells were collected by cold centrifugation (8000 ×*g*, 15 min, 4 °C), washing twice with 50 mM phosphate buffered saline (PBS) (pH 7.0), and were then re-suspended in the PBS. Cells were subjected to ultra-sonication (30 s pulse and 30 s cooling) for 4 min to separate the cytosolic content. Supernatant was obtained by centrifugation (8000 ×*g* for 10 min, 4 °C) and stored at − 80 °C until use.

The extent of malondialdehyde (MDA) production in bacterial cells and plants was determined as described by Zhang et al. (2016) and Bilal et al. (2017). Reduced glutathione (GSH) content in bacteria and plants was assessed as described by Ellman (1959) and Halo et al. (2015), and the concentration was determined by using a standard GSH calibration curve. Similarly, levels of enzymatic antioxidants, catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), in bacterial cells and soybean tissues were determined as described by Halo et al. (2015) and Bilal et al. (2017), respectively. One unit of CAT was defined as the amount of enzyme that caused a reduction in absorbance at 240 nm of 0.01 per minute, while POD activity was defined as the amount of enzyme that caused an increase in absorbance at 470 nm of 0.001 per minute. SOD activity was assessed as described by Zhang et al. (2016). One SOD unit was described as the quantity of enzyme to inhibit 50% photo reduction of NBT, and is expressed as U/mg protein.

2.2. Soybean exposure to chromium stress

2.2.1. Endophytic LK11 and soybean association under Cr stress

Soybean seeds (Taekwang Cultivar) were surface-sterilized with 70% ethanol followed by treatment with 5% Sodium hypochlorite (NaOCl) for 10 min, and were then rinsed with autoclaved double-distilled water. Soybean seeds were grown inside a growth chamber in autoclaved pots containing 500 g horticulture soil composed of cocopeat (68%), perlite (11%), zeolite (8%), as well as micronutrients available as NH^{4+} ~0.09 mg g^{-1}; P_2O_5 ~0.35 mg g^{-1}; NO_3^- ~0.205 mg g^{-1}; and K_2O ~0.1 mg g^{-1}. Then, a suspension of LK11 [2.4×10^8 Colony-forming unit (CFU) mL^{-1}] was applied to the rhizosphere area of 14-day-old soybean seedlings to establish a plant-bacterial symbiotic association. Cr(VI) solution (80 mL) in different concentrations (1.0, 2.0, and 5.0 mM) were applied to 21-day-old soybean seedlings per pot for 10 days. The total Cr (VI) solution applied to each soybean was 41.6, 83.2, 208 mg per pot in the respective concentration (1.0, 2.0, and 5.0 mM) after 10 days application. After 10 days of stress, plants were harvested, frozen in liquid nitrogen, and transferred to −80 °C for biochemical analysis. The growth attributes under each treatment were recorded and chlorophyll content was measured by a chlorophyll meter (SPAD-502 Minolta, Japan). The experiment was replicated three times, and each replicate included 20 plants.

2.2.2. Cr analysis in plant via ICP-MS

To determine the Cr contents in root, stem, and leaves, freeze-dried powdered samples were used for Cr quantification by ICP-MS (Optima 7900DV, Perkin–Elmer, USA) after digesting 0.05 g grinded sample with 7 mL of 65% HNO_3 and 1 mL of 30% H_2O_2 and kept in digestion