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Evaluation of transgenic tobacco plants expressing a bacterial Co–Ni transporter for acquisition of cobalt

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ABSTRACT

Phytoremediation is a viable strategy for management of toxic wastes in a large area/volume with low concentrations of toxic elemental pollutants. With increased industrial use of cobalt and its alloys, it has become a major metal contaminant in soils and water bodies surrounding these industries and mining sites with adverse effects on the biota. A bacterial Co–Ni permease was cloned from *Rhodopseudomonas palustris* and introduced into *Nicotiana tabacum* to explore its potential for phytoremediation and was found to be specific for cobalt and nickel. The transgenic plants accumulated more cobalt and nickel as compared to control, whereas no significant difference in accumulation of other divalent ions was observed. The transgenic plants were evaluated for cobalt content and showed increased acquisition of cobalt (up to 5 times) as compared to control. The plants were also assessed for accumulation of nickel and found to accumulate up to 2 times more nickel than control. At the same initial concentration of cobalt and nickel, transgenic plant preferentially accumulated cobalt as compared to nickel. The present study is perhaps the first attempt to develop transgenic plants expressing heterologous Co transporter with an improved capacity to uptake cobalt.

1. Introduction

Extensive use of cobalt (Co), nickel (Ni) and their alloys in electroplating and petrochemical industries has resulted in contamination of these metals in soil and water bodies near these industries and mining sites in many countries including India (Dantu, 2009; Kiran et al., 2006; Krishna and Govil, 2007; Soco and Kalembkiewicz, 2007). These metals can enter food chain and cause toxicity (Denkhaus and Salnikow, 2002; Gál et al., 2008). Animal studies indicate various adverse effects of Co on health with increased exposure and route of entry (Haga et al., 1996; Lison et al., 2001; Migliori et al., 1994). In plants, high Co concentrations cause leaf fall, inhibition of greening, premature leaf stomata closure and reduced shoot weight (Palit et al., 1994). Chemical methods used for the removal of Co and Ni from the effluents (Jacobs and Walter, 2005) are expensive and impractical for large areas with low contamination. In addition, inorganic pollutants being non-degradable, are more difficult to clean up from the contaminated soils (Pilon-Smits, 2005). Therefore, there is a need to develop alternative strategies for waste management along with the existing methods to effectively contain the contamination levels. Biotechnological approaches like biosorption have been used for remediation of Co from the environment (Egila et al., 2010; Pal et al., 2006). Phytoremediation or the use of plants to remove toxic elemental pollutants is a low cost "green technology" and is favored in large areas with low concentrations of heavy metals (Eapen and D'Souza, 2005). Higher plants absorb Co from soil by passive transport. The transport of Co through cortical cells is by both active uptake and passive diffusion. The xylem transport of Co is through transpirational flow. Low mobility of Co restricts its transport to leaves from stems (Palit et al., 1994). Some plants growing in serpentine soil can hyper accumulate cobalt (Reeves and Baker, 2000). However, these plants are slow growing with low biomass and restricted in geographical distribution. In the recent years, creation and the use of genetically modified plants for the purpose of phytoremediation of toxic metals and xenobiotics have gained a lot of attention as a cost effective and environment friendly technology (Doty, 2008; Eapen and D'Souza, 2005; Eapen et al., 2007; Kotrba et al., 2009).

Bacterial Co–Ni transporters are well characterized as compared to that in plants. Co and Ni uptake in bacteria are mediated by at least two ATP binding cassette systems (Eitinger and

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Mandrand-Berthelot, 2000; Eitinger et al., 2005). Secondary transporters of NiCoT family in several species are also able to take up either or both Ni and Co (Hebbeln and Eitinger, 2004).

In this study a secondary Ni–Co transporter (NiCoT, TC 2.A.52) from *Rhodopseudomonas palustris* was cloned into plant expression vector and introduced into *Nicotiana tabacum*. The potential of these transgenic plants to remediate cobalt was evaluated. Our results show that the heterologous expression of the transporter results in increased accumulation of Co and Ni by the transgenic plants, without any non-specific transport of other divalent ions like Fe, Cd, Zn and Cu.

2. Materials and methods

2.1. Construction of plant expression vector

The genomic fragment coding for a nickel/cobalt permease (*NiCoT*) from *Rhodopseudomonas palustris* CGA009 (pCH675-RP, a kind gift from Prof. T. Eitinger) (Hebbeln and Eitinger, 2004), was subcloned into a plant binary expression vector pCAMBIA1301 (CAMBIA, Australia) (supplementary material, S1). Plasmid pNiCoT-RP was introduced into *Agrobacterium tumefaciens* EHA105 cells by electroporation (Eppendorf, Germany).

2.2. Plant transformation and generation of transgenic plants

Transgenic *Nicotiana tabacum* L. cv Havana 425 plants were developed using *Agrobacterium* mediated co-cultivation method as reported previously (Dixit et al., 2010). In brief, the explants after co-cultivation of two days were transferred to regeneration medium [Murashige and Skoog's (MS) medium with 2 mg l^{-1} benzyl adenine (BA) and 0.1 mg l^{-1} Indole acetic acid (IAA)] supplemented with Hygromycin B (25 mg l^{-1}) and cefotaxim (500 mg l^{-1}). Regenerated multiple shoots were further sub-cultured on rooting medium (1/2 MS) with Hyg B (25 mg l^{-1}) for 2 weeks. Complete plants thus obtained, were transferred to Hoagland's hydroponic medium (pH 5.6) (Hoagland and Arnon, 1938) and/or soilrite (mixture of 75% Irish peatmoss and 25% horticulture grade expanded perlite; Keltech Energies Ltd., Bangalore, India) for 2 weeks before actual experiments. Seeds from T_0 plants were germinated *in vitro* in presence of 25 mg l^{-1} of hygromycin to select hyg^R T_1 lines. Transgenic shoots were multiplied by micropropagation along with control for replicate analysis. Histochemical assay for reporter gene (*uidA*) product was conducted after two days of co-cultivation as described by Jefferson (1987).

2.3. Segregation analysis of transgene in T_1 generation

Segregation analysis of transgene in T_1 generation was done for hygromycin-resistant trait as described previously (Dixit et al., 2010). Data was statistically analyzed using χ^2 test to study the segregation of *hptII* gene. Hygromycin resistant plants were randomly selected and tested for the presence of *NiCoT* using PCR as described below.

2.4. Molecular analysis of transgenic plants

Total genomic DNA from non-transformed control and 20 putatively transformed T_0 was isolated using method previously described (Joshi-Saha and Gopalakrishna, 2007). *NiCoT* gene specific oligos(Table S1) were used to check the presence of transgene by PCR amplification.

Stable integration of *NiCoT* in the genome of transgenic plants was confirmed by Southern hybridization using genomic DNA of untransformed control and putative transgenic plants as described previously (Singh et al., 2011). Probe was prepared with PCR product of *NiCoT* gene using DIG labeling kit (Roche Biochemicals, Germany).

To confirm the expression of transgene, total RNA from control and transformed plants was isolated using TRI reagent (Sigma). cDNA was synthesized using Affinity Script Multiple Temperature cDNA synthesis kit (Stratagene, USA). Reverse-Transcription PCR (RT PCR) was carried out to confirm the transcription of *NiCoT* gene. PCR of actin gene was done as control.

2.5. ^{60}Co uptake by transgenic plants

Well rooted 1 month old plants of five confirmed transgenic lines (in triplicates) and control plants were transferred to 1 l Hoagland's Medium devoid of Co and grown for 10 days under controlled conditions. Plants were then transferred to Hoagland's medium spiked with 2.96×10^5 Bq ^{60}Co as $^{60}CoCl_2$ (specific activity, 6.179×10^{10} Bq g^{-1}). After 15 days, plants were harvested and separated into roots and shoots, dried to constant weight at 70 °C and used for estimation of γ activity of ^{60}Co. Samples were analyzed using gamma spectrometer with 8K multichannel analyzer (MCA) and P-type high purity germanium detector having resolution 2 keV with respect to gamma energy of ^{60}Co at 1332 keV. Photo-peaks at gamma energy of 1172 keV and 1332 KeV were identified peak for ^{60}Co estimation. MCA unit was standardized and efficiency was determined using known gamma energies standard source 154Eu prior to analysis.

2.6. Uptake of Co at different concentrations

Well rooted, 1 month old control and transgenic plants (T_0 and T_1) in triplicates, were grown in hydroponics in Hoagland's medium spiked with different concentrations of Co [0.424 μM (1×), 4.24 μM (10×), 21.2 μM (50×), 42.4 μM (100×), 212 μM (500×), 424 μM (1000×)] supplied as $CoCl_2 \cdot 6H_2O$. The concentrations selected were environmentally relevant and chosen appropriately to expose plants from low to moderate to high levels of Co (Gál et al., 2008; Collins and Kinsela, 2010). Plants were initially grown in Hoagland's medium devoid of any Co for 10 days under controlled conditions and then exposed to the respective concentrations of Co for a period of 30 days (unless stated otherwise) in growth room (24 ± 2 °C) under white fluorescent light (12.2 μM photon m^{-2} s^{-1}) at a photoperiod of 14 h light/10 h dark. At the end of 30 days, plants were harvested, washed thoroughly with ion free double distilled water thrice, blotted gently and analyzed for metal content as described previously (Singh et al., 2011). In brief, roots and shoots were separated manually and dried in an oven at 70 °C for 1 week. Dried plant tissues were ground and digested in an acid mixture of HNO_3:$HClO_4$ (5:1, v/v) at 80 °C. Cobalt or Ni content was estimated by GBC 932 B+ Atomic Absorption Spectrophotometer (GBC, Melbourne, Australia) using air-acetylene flame.

2.7. Specificity of metals

Control and transgenic (T_0) plants (in triplicates) were grown for 30 days in Hoagland's medium spiked with 21.2 μM of either Co ($CoCl_2 \cdot 6H_2O$) or equimolar concentration of Ni ($NiCl_2 \cdot 6H_2O$), Fe ($FeCl_2 \cdot H_2O$), Cd ($CdCl_2$), Zn ($ZnCl_2$) or Cu ($CuCl_2 \cdot 2H_2O$). At the end of experiment, plants were harvested and analyzed for all the metal ions in roots and shoots using Atomic Absorption Spectrophotometer as described in Section 2.6.