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Enhancement of heavy metal accumulation by tissue specific co-expression of *iaaM* and ACC deaminase genes in plants

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ABSTRACT

1-Aminocyclopropane deaminase (ACC) and tryptophan monooxygenase are two enzymes involved in plant senescence-inhibiting and growth-promoting regulation, respectively. In this study, two binary vectors were constructed in which the *Agrobacterium iaaM* gene was under the transcriptional control of a xylem-specific glycine-rich protein promoter alone, or co-expressed with the bacterial ACC deaminase gene, which was driven by the constitutive CaMV *35S* promoter. Transgenic petunia shoots co-expressing both genes were able to root on medium supplemented with 7.5 mg l^{-1} $CoCl_2$. When T_1 transgenic tobacco plants were grown in sand supplemented with Cu^{2+} and Co^{2+}, tissue specific co-expression of both *iaaM* and ACC deaminase genes showed faster growth with larger biomass with a more extensive root system, and accumulated a greater amount of heavy metals than the empty vector control plants. When T_1 transgenic tobacco plants were grown in soil watered with different concentrations of $CuSO_4$, xylem specific expression of the *iaaM* gene caused the accumulation of more Cu^{2+} than the empty vector control at lower $CuSO_4$ concentrations, but showed severe toxic symptoms at concentration of 100 mg l^{-1} $CuSO_4$. T_1 transgenic plants co-expressing both genes accumulated more heavy metals into the plant shoots and can tolerate $CuSO_4$ at 150 mg l^{-1}. In addition, plants co-expressing these two genes can grow well in a complex contaminated soil containing both inorganic and organic pollutants, while the growth of the control plants was greatly inhibited.

1. Introduction

Heavy metal pollution of soils and waters is a major environmental problem. Heavy metals, unlike organic pollutants, cannot be chemically degraded or biodegraded by microorganisms. Commonly, decontamination of metal-contaminated soils requires the removal of toxic metals, and the development of plant-based clean-up methods of heavy metal contaminated environments has generated considerable interest in recent years (review by Kramer, 2005). Using plants to extract, sequester, degradate and/or detoxify contaminated environments is termed phytoremediation (Meagher, 2000), and has emerged as a cost-effective, environment-friendly cleanup alternative in recent years.

Plant properties important for efficient phytoremediation are tolerance and accumulation of pollutants, which are determined by uptake, root–shoot translocation, intracellular sequestration, chemical modification and degradation, and general stress resistance to various contaminants (Hall, 2002; Pilon-Smits and Pilon, 2002). Finding hyperaccumulating plants from wild habitats offers one option, but these plants tend to be slow growing and produce little biomass under contaminated conditions (Brewer et al., 1999). Early studies to produce plant mutants or hybrid plants via somatic hybridization capable of accumulating large amounts of heavy metals have been reported (Brewer et al., 1999; Schulman et al., 1999), but this type of works has not been commercialized. Phytoremediation of contaminated environments via transgenic plants offers many advantages over hyperaccumulating plants from wild habitats, and considerable progress has been made with this approach in recent years (Cherian and Oliveira, 2005). With the use of genetic engineering approaches, it is feasible to manipulate a plant's capacity to tolerate, accumulate and/or metabolize pollutants with the production of large biomass, thus to create ideal plants for environmental cleanup (Kramer and Chardonnens, 2001; Pilon-Smits and Pilon, 2002). Enhancement of heavy metal tolerance and accumulation in plants has been achieved by overproducing various metal chelating molecules, e.g., phytochelatins (Pomponi et al., 2006) and metal transporters (Song et al., 2003; Yazaki et al., 2006). For example, Martinez et al. (2006) transformed *Nicotiana glauca* with the wheat (*Triticum aestivum*) phytochelatin synthase (*TaPCS1*) gene, the transgenic plants confers up to 9 and 36 times more Cd and Pb accumulation in the shoots

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under hydroponic conditions, and a 3- and 6-fold increase in mining soils with a biomass 100 times more than *Thlaspi caerulescens*.

Many studies have demonstrated that bacteria containing 1-aminocyclopropane (ACC) deaminase activity modulate stress-induced production of ethylene in plants, and thus might cause an enhanced uptake of inorganic contaminants through modification of root architecture and of the root uptake system of the plant (review by Arshad et al., 2007). The use of ACC deaminase bacteria exhibiting resistance to heavy metals was primarily to test their potential to facilitate plant growth under stress conditions created by heavy metals. For example, Burd et al. (2000) reported the use of an ACC deaminase bacterium to protect canola (*Brassica napus*) and tomato (*Lycopersicon esculentum*) seeds from the toxicity of high concentrations of $NiCl_2$. They attributed this effect to the ability of the bacterium to lower the level of ethylene induced by $NiCl_2$. The potential of ACC deaminase bacteria to promote root and shoot growth of the seedlings of Indian mustard and rape grown in the presence of $CdCl_2$ in the nutrient solution was also documented (Belimov et al., 2001). They concluded that ACC deaminase bacteria offer a great potential to be used for improvement of plant growth, particularly for hyperaccumulators under unfavorable environmental conditions. In subsequent reports, inoculation of sunflower with rhizobacterium *Pseudomonas putida* caused a marked decrease in cadmium phytotoxicity and increased metal accumulation in the plant root by up to 40% (Wu et al., 2006). ACC deaminase from *Pseudomonas fluorescens* mediated plant resistance to saline stress, resulted in increased yield when compared with strains not having ACC deaminase activity (Mayak et al., 2004).

Studies have been conducted to assess the potential of transgenic bacteria and plants expressing ACC deaminase genes for hyperaccumulation of heavy metals. Reed and Glick (2005) compared the efficiency of engineered bacteria that carry ACC deaminase with control bacteria in promoting canola seed germination and root elongation under stress conditions caused by copper or polycyclic aromatic hydrocarbons in contaminated soils. They reported that both control and transformed bacteria were equally useful in the promotion of seed germination and root elongation under contaminated soils. This could be due to the fact that efficiency of inoculated transgenic bacterial strains is determined by biotic and abiotic factors such as soil pH, temperature, moisture content (Reed and Glick, 2005). In another report, bacterial ACC deaminase was expressed in tomato under the control of two tandem cauliflower mosaic virus (CaMV) *35S* promoters, the *rolD* promoter (root-specific expression) from *Agrobacterium rhizogenes*, or the pathogenesis-related *prb-1b* promoter from tobacco. The growth of transgenic tomato plants in the presence of a number of metals was monitored. Transgenic tomato plants driven by different promoters showed enhanced tolerance to and were able to acquire higher metal levels than untransformed plants, and transgenic plants controlled by the *prb-1b* promoter accumulated larger amounts of metals within the plant tissues. Moreover, transgenic plants in which the ACC deaminase gene was under the control of the *rolD* promoter acquired higher levels of biomass than any of the other plants (Grichko et al., 2000). Transgenic canola plants expressing ACC deaminase gene were used to test their potential to grow in the presence of high levels of arsenate in the soil for metal accumulation in plant tissues. The ability of the plant growth-promoting bacterium to facilitate the growth of both non-transformed and ACC deaminase expressing canola plants was also tested. In all cases, transgenic canola expressing ACC deaminase genes accumulated larger amounts of arsenate from the contaminated soil than non-transformed plants (Nie et al., 2002).

Phytoremediation is a complex process, including solubilization and mobilization of pollutants in the soil, uptake and sequestration into the plant, vascular transport and tissue distribution in leaf tissue, and trafficking and sequestration in different organelle of the plant (Clemens et al., 2002; Meagher, 2000). To increase phytoremediation efficiency, the gene expression patterns may only be needed to target certain cellular compartments and organ types such as chloroplast (Ruiz et al., 2003), vacuole (Song et al., 2003), leaf (Peterson and Oliver, 2006) and root (Kim et al., 2005) by means of different promoters and targeting signals, or under specific responsive promoters in inducible conditions. It has also been shown that more than one gene will need to be transformed into plants in order to substantially enhance phytoremediation capacity (Dhankher et al., 2002).

In this study, two binary vectors expressing the *iaaM* gene alone or in combination with the ACC deaminase gene were constructed under different promoters and used for petunia and tobacco transformation. The *iaaM* gene was from *Agrobacterium* and encodes a tryptophan monooxygenase (*iaaM*) that catalyzes the synthesis of indole-3-acetamide, a precursor of indole-3-acetic acid, and was driven by the vascular specific glycine-rich protein (GRP) promoter (Keller et al., 1989). The ACC deaminase gene was from the plant growth promoting rhizobacterium *P. putida* UW4 and was under the control of the CaMV *35S* constitutive promoter. Comparison of these two constructs on petunia and tobacco growth and heavy metal accumulation were studied.

2. Materials and methods

2.1. Binary vectors construction

Two plasmids named pUC-*iaaM* and pUC-*iaaM*/ACC were kindly provided by Dr. John Ke of University of Minnesota at St. Paul, USA. In these two plasmids, the ACC deaminase gene was driven by the CaMV *35S* promoter and the *iaaM* gene was under the control of the GRP promoter. The *iaaM* gene is from *Agrobacterium tumefaciens* and ACC deaminase gene is from *Pseudomonas putida*. A *Sal*I/*Eco*RI restricted fragment containing the *iaaM* gene from pUC-*iaaM* was purified with a Qiagen plasmid kit (Qiagen, Valencia, CA), and subcloned in the pBI101 binary vector (Clontech), which was also digested with the two restriction enzymes, the resulting plasmid was named pBI-*iaaM*. A *Sal*I/*Eco*RI digested fragment from zpUC-*iaaM*/ACC containing both the ACC deaminase gene and the *iaaM* gene was purified and subcloned in pBI101, which was also digested with *Sal*I/*Eco*RI, this plasmid was named p*iaaM*/ACC. pBI101, pBI-*iaaM* and pBI-*iaaM*/ACC plasmids were mobilized into *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993) by the method of Chung and Miller (1993) and used for plant transformation.

2.2. Plant transformation

Petunia (*Petunia hybrida* Vilm.) seeds were purchased from PanAmerica Seed via a retail distributor in Dalian, China. The seeds were surface sterilized by soaking for 10 min in 1.5% sodium hypochlorite and then thoroughly rinsed with sterile distilled water and germinated on Murashige and Skoog (MS) basal agar medium (Murashige and Skoog, 1962). Leaf tissues from young seedlings were used for transformation following the protocol of van der Meer (1999) with *A. tumefaciens* harboring pBI101, pBI-*iaaM* or pBI-*iaaM*/ACC. The leaf disks were transferred onto MS agar medium containing 30 g l^{-1} sucrose, 0.2 mg l^{-1} 6-benzylaminopurine and 100 mg l^{-1} kanamycin, and transferred onto the same medium every 2 wk. The resistant shoots were rooted on MS basal medium supplemented with 30 g l^{-1} sucrose, 0.1 mg l^{-1} NAA (α-naphthaleneacetic acid) and 50 mg l^{-1} kanamycin.

Tobacco (*Nicotiana tabacum* L. cv. Samsun) axenic plants were grown *in vitro* in MS basal agar medium containing 30 g l^{-1} sucrose. Leaf disks were dipped in *A. tumefaciens* harboring either