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Expression of Chromium (VI) Reductase Gene of Heavy Metal Reducing Bacteria in Tobacco Plants

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Abstract

A Chromium (VI)[Cr (VI)] reductase gene from heavy metal reducing bacteria *Pseudomonas aeruginosa* HP014 was used to transform tobacco plant cells. A chimeric construct containing the Cr (VI) reductase gene was transfered to tabacco leaf disks using an *Agrobacteriun tumefaciens* binary vector system. From the leaf disks, transformed plantlets were regenerated. Hybridization experiments demonstrated that the Cr (VI) reductase gene was inserted into and expressed in the regenerated plants. The Cr (VI) reduction activity showed that the transgenic plants may be a another possible tool to reduce the pollution of the toxic Cr (VI) in soil.

Introduction

Hexavalent chromium [Cr (VI)] compounds are considered to be extremely toxic, mutagenic, and carcinogenic (Costa, 1991; Gruber and Jennette, 1978; Langaard and Norseth, 1986). The compounds are known to have a strong oxidizing activities and cause various biological damage. The reduction of the hexavalent ion level of the compounds is very important for the protection against the environmental damage. Recently it is reported that some bacteria reduced the Cr (VI) to Cr (III) and resulted in significant reduction of the toxicity (Ajar, 1991; Banks, 1986; Branca, 1990; Costa, 1991; Das and Chandra, 1990; Flora et al., 1985; Gruber and Jennette, 1978; Horitsu et al., 1987; Ishibashi, 1990; Suzuki et al., 1992; Oh and Choi, 1997). A gene conferring the resistance against chromate was cloned from *Pseudomonas aeruginosa* PA01 and characterized (Cervantes et al., 1990). We have considered that plant system may be also used for the reduction the toxic compounds, if the chromium resistant gene is expressed in plants. From this point of view, we have previously cloned a NADH-dependent Cr (VI) reductase gene from *Pseudomonas aeruginosa* HP104 (Jin, 2000). The gene was modified and transformed to *E. coli*. The expression of the cloned gene in *E. coli* showed the higher enzyme activity than the original bacteria. The bacteria strain HP104 was known in the mechanism of aerobic Cr (VI) reduction based on the subcellular location and kinetic parameters of NADH-dependent Cr (VI) reductase activity.

In this study, we report that the Cr (VI) reductase gene was transferred by *Agrobacterium*-mediated binary vector system to tobacco plant cells. The activity of Cr (VI) reduction was detected in the transgenic plants.

Materials and Methods

Plasmids and bacterial strains

Plasmid pBinAR (Hoefgen and Willmitzer, 1990) and derivatives were transformed into *E. coli* strain DH5α (Amersham Co.). For plant transformation the binary vector pBinAR-rtd was transferred by electroporation into *Agrobacterium tumefaciens* LBA4404 (pAL4404) (Hoekema et al., 1983). Plasmid pAL4404 is pTi-C58 with a deletion of the T-DNA.

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Reagents and enzymes

Restriction endonucleases, T_4-DNA ligase and DIG (dioxigenin) for the labelling were purchased by Roche Molecular Biochemicals. Nitrocellulose membranes were supplied from NEN Research Co. The other biochemicals were purchased from Sigma Chemical Co.

Construction of plant transformation vector pBinAR-rtd

Plasmid pBluescript-rtd containing the Cr (VI) reductase gene cloned into *Bam*HI and *Hin*dIII restriction enzyme sites (Jin, 2000) was cleaved with *Hin*dIII and filled with the Klenow fragment of DNA polymerase I. After the digestion of the plasmid with *Bam*HI, the fragment was ligated into *Bam*HI and *Sma*I restriction sites of the binary vector plasmid pBinAR (Hoefgen and Willmitzer, 1990), which contains a CaMV35S promoter and octopin synthase poly (A) sequences. The resulting plasmid pBinAR-rtd (Figure 1) contained the chimeric gene encoding the Cr (VI) reductase under control of the CaMV35S promoter and the termination sequence of the octopin synthase gene.

Transformation of tobacco plants and tissue culture

The vector pBinAR-rtd was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation (Easy Jet System) under the conditions of 2 mm cuvette, 25 μF and 2.5 kV in 500 μL of 10% glycerin solution. For the transformation, tobacco seeds were germinated and grown on germination medium (MS medium, MS vitamin mixture, 3% sucrose, 0.8% Bacto-agar, pH 5.8) under sterile conditions. Small leaf disks of the tobacco plants were infected with transformed *Agrobacterium tumefaciens* strain by the leaf disk method (McCormick et al., 1986). The disks were incubated on the pre-culture medium containing MS-salt (Murashige and Skoog, 1962), 3% sucrose and MS vitamin mixture (pH 5.8). After 2 days, the leaf disks were washed with steriled ddH_2O and placed on the selective medium (MS medium, 3% sucrose, MS vitamin mixture, 2.5 mg/L BA, 0.2 mg/L IAA, 300 mg/L kanamycin, 500 mg/L carbenicillin, pH 5.8). The subsequent culture induced callus and shoots. The shoots were grown on root inducing medium (MS medium, MS vitamin mixture, 3% Sucrose, 300 mg/L Kanamycin, pH 5.8) under the photoperiod of 16/8 light at 25℃. The regenerated plants were subjected to test the southern analysis and enzyme assay of the Cr (VI) reductase.

Southern blot analysis

Isolation of total DNA from tobacco plants was performsed by phenol extraction (Rogers and Bendich, 1988) and the DNA was digested with *Eco*RI and *Hin*dIII. After DNA-separation on a agarose gel, DNA fragments were blotted directly on a nitrocellulose membrane. Southern hybridization was performed according to Sambrook et al. (1989). Labeling of the DNA with DIG (Roche Molecular Biochemicals) was done by random primer hexanucleotide method.

Crude extraction of protein from tobacco plants

The crude plant protein was isolated according to the method of Paul and Robert (1999). Isolation of protein was performed in a cold room at 0~4℃. Leaves were harvested and cooled to 4℃ prior to the addition of liquid nitrogen. The powdered leaf tissues were transferred to prechilled extraction buffer (100 mM Tris, 100 mM ascorbic acid, 20 mM EDTA, 2.5% w/v polyvinyl polypyrrolidone, 150 mM NaCl, 0.1% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride) at 1 mL per gram of leaves. After vigorous shaking, the extract was centrifuged at 4℃ by 10,000 rpm for 10 min. The concentration of protein in the supernatant was measured by Bradford method (Bradford, 1976). Ten microgram of the protein were applied to the enzyme activity assay for the chromium (VI) reduction.

Cr (VI) reduction assay

Cr (VI) reduction activity was assayed colorimetrically by the modified method of Oh and Choi (1997). This procedure measures only hexavalent chromium, and the resulting red-violet color of unknown composition was quantitatively measured spectrometrically at wavelength of 540 nm. The extracted samples were applied to the pre-incubated reaction mixture containing 0.5 mM chromate, 0.4 mM NADH in 2 mL of 50 mM phosphate buffer pH 7.0. After 10 min incubation at 30℃, samples were taken, and the remaining Cr (VI) was measured.

Results

Transformation of tobacco with Cr (VI) reductase gene

The binary vector pBinAR-rtd containing the Cr (VI) reductase gene (Figure 1) was transferred to *A. tumefaciens* strain LBA4404 by electroporation. The transformed *A. tumefaciens* was selected on LB agar plate containing 50

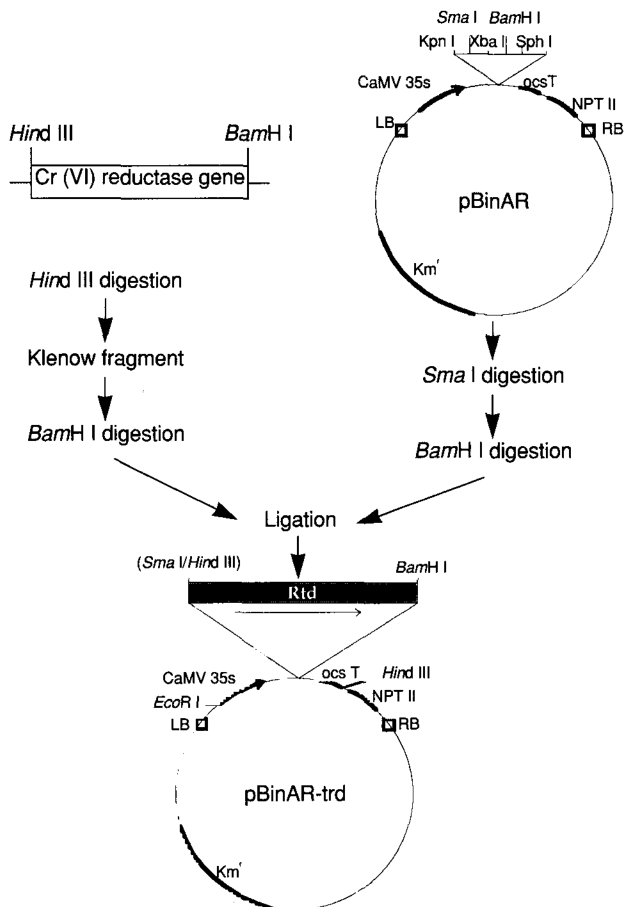

Figure 1. Gene map of the modified chimeric chromium (VI) reductase gene cotained in the plant transformation binary vector pBinAR. The chromium reductase gene was modified for expression in plant. NPT, Neomycin phosphotransferase II; CaMV35s, Cauliflower mosaic virus 35s promoter; LB, Left border; RB, Right border; ocsT, Terminator of octopine synthase gene (The arrow indicates the direction of transcription).

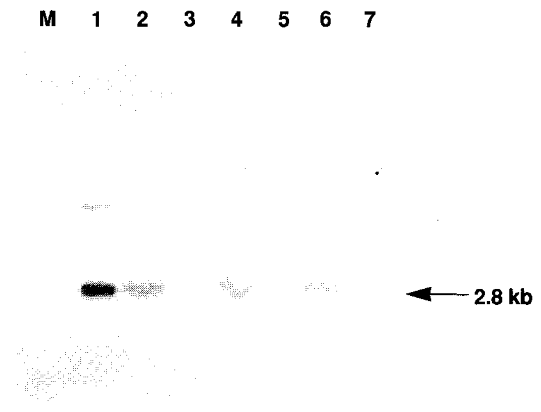

Figure 2. Southern blot analysis of transformed tobacco plants with Cr (VI) reductase gene. Total DNA was isolated from the transformed and wild type tobacco plants and fully digested with *Eco*RI/*Hin*dIII. The fragments were separated on 1% agarose gel. After transfer onto nylon membrane, DNA/DNA hybridization was performed with the DIG labeled *Eco*RI/*Hin*dIII fragment (2.8 kb) of pBinAR-rtd as detection probe. M: λ/*Hin*dIII marker, Lane 1: pBinAR-rtd *Eco*RI/*Hin*dIII digestion, Lane 2: Transgenic tobacco plants 1, Lane 3: Transgenic tobacco plants 2, Lane 4: Transgenic tobacco plants 3, Lane 5: Transgenic tobacco plants 4, Lane 6: Transgenic tobacco plants 5, Lane 7: Non-transformed tobacco plant.

μg/mL kanamycin. The plasmid pBinAR-rtd was transferred *via* the *A. tumefaciens* into tobacco by leaf disc transformation method as described above. One hundreds of the leaf disks were placed on the selective agar medium. The five regenerated shoots were transferred on the root inducing medium for the induction of roots (data not shown). The five whole regenerated plants were applied to further studies.

Detection of chromium (VI) reductase gene

In order to identify the presence of chromium reductase gene in plant, total DNAs was isolated from transformed tobacco plants and analysed by Southern blotting. The total DNA was digested with *Eco*RI/*Hin*dIII completely and separated on a 1% agarose gel. After transfer the DNA onto a nylone membrane, the paper was hybridized with digoxigenin labeled *Eco*RI/*Hin*dIII fragment (2.8 kb) of pBinAR-rtd. A positive bands were detected from three transgenic plants (Figure 2), but not from the other two regenerated tobacco plants and a non-transgenic plants. The positions of bands are identical with that of the 2.8 kb *Eco*RI/*Hin*dIII fragment as marker. This results indicate that the chromium reductase gene was inserted into some transgenic tobacco plants and suggests that the tobacco plants 1, 3, 5 were successfully transformed with chromium reductase gene.

Enzyme activity analysis of Cr (VI) reductase

To detect the expression of the chromium (VI) reductase gene, a crude enzyme extract was obtained from the transgenic tobacco plants 1, 3, and 5 which showed the reductase specific bands in the Southern analysis. The

Table 1. Enzyme Assay of Cr (VI) reductase in the transgenic plants.

Plant Materials	Absorbance of O.D. (540 nm)
P. aeruginosa HP014 (positive control)	-0.249
Untransformed tobacco	0
Transformed tobacco 1	-0.553
Transformed tobacco 3	-0.554
Transformed tobacco 5	-0.557

activity of the chromium (VI) reductase was assayed by measuring the decrease of Cr (VI) as determined as described above. As shown in the table 1, the crude enzymes obtained from the transgenic plants 1, 3, and 5 have the activities of chromium (VI) reduction. The acitivity was not detected from the non-transgenic plant. The enzyme activity test of the reductase indicated that the reductase gene could be expressed in the transgenic plants.

Discussion

The chromium hexavalent compounds are known to have a strong oxidizing activities and cause various biological damage. The reduction of the hexavalent ion level of the compounds is very important for the protection against the environmental damage. The enzyme of Cr (VI) reductase reduces the compounds to the relative stable and non toxic Cr (III) state. The gene encoding the reductase was cloned from *Pseudomonas aeruginosa* HP104 and characterized in our laboratory (Jin, 2000). The bacteria strain was known in the mechanism of aerobic Cr (VI) reduction based on the subcellular location and kinetic parameters of NADH-dependent Cr (VI) reductase activity (Oh and Choi, 1997). It was assumed that the expression of the reductase gene in plants may reduce the toxic compounds of Cr (VI) in the soil. From this point of view, we have transformed tobacco plants with the chromium (VI) reductase gene. The gene of 1.8 kb was successfully transferred and expressed in tobacco plants. The activity of the enzyme expressed was in the level of about 30% lower than that of the host bacteria (data not shown). The reason may be due to that the codon usage of the bacterial gene in plant is different from that in the host bacteria. However, it is assumed that the activity shown in the table 1 may be sufficient to reduce the Cr (VI) in the plants. Plants can, generally, absorb other heavy metal ion from soil. But, it may be more important that the absorbed metal ion in plants should be reduced to non toxic compound. In this point, our results may give a clue to solve soil pollution with toxic heavy metal ion including Cr (VI).

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