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Transgenic alfalfa plants co-expressing glutathione S-transferase (GST) and human CYP2E1 show enhanced resistance to mixed contaminates of heavy metals and organic pollutants

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ABSTRACT

Transgenic alfalfa plants simultaneously expressing human CYP2E1 and glutathione S-transferase (GST) were generated from hypocotyl segments by the use of an *Agrobacterium* transformation system for the phytoremediation of the mixed contaminated soil with heavy metals and organic pollutants. The transgenic alfalfa plants were screened by a combination of kanamycin resistance, PCR, GST and CYP2E1 activity and Western blot analysis. The capabilities of mixed contaminants (heavy metals-organic compounds) resistance of pKHCG transgenic alfalfa plants became markedly increased compared with the transgenic alfalfa plants expressing single gene (GST or CYP2E1) and the non-transgenic control plants. The pKHCG alfalfa plants exhibited strong resistance towards the mixtures of cadmium (Cd) and trichloroethylene (TCE) that were metabolized by the introduced GST and CYP2E1 in combination. Our results show that the pKHCG transgenic alfalfa plants have good potential for phytoremediation because they have cross-tolerance towards the complex contaminants of heavy metals and organic pollutants. Therefore, these transgenic alfalfa plants co-expressing GST and human P450 CDNAs may have a great potential for phytoremediation of mixed environmental contaminants.

1. Introduction

Soils are continuously polluted by anthropogenic activities such as industrial development, fossil fuel burning, mining, intensive agriculture and livestock production. Prevalent contaminants include petroleum hydrocarbons (PHC), polycyclic aromatic hydrocarbons (PAHs), halogenated hydrocarbons, pesticides, solvents, metals, and salt. A number of plants can degrade various organic chemicals or accumulate high amounts of heavy metals absorbed from soil. The use of plants to remediate polluted soils, sediments, and groundwater is called phytoremediation. Phytoremediation of land contaminated with inorganic and/or organic pollutants has now emerged as a promising strategy, and attracted much attention and research over the last decades [1–6]. Although bioremediation using plants has acquired the status of a proven technology for the treatment of heavy metal [7] and organic-contaminated soils [8,9], field trials have suggested that these plants often accumulate a specific element, grow slowly with low biomass and agronomic characteristics, and breeding potential [10]. Moreover, unlike bacteria and mammals, plants are autotrophic organisms that lack the enzymatic machinery necessary for efficiently metabolizing organic compounds, consequently the rate of contaminant removal using conventional plants is inadequate and slow [11,12]. Therefore, an ideal plant for rehabilitation would be a high biomass producing crop that can tolerate and accumulate pollutants, in this case, and well-adapted to the harsh environment of the contaminated soil. The use of transgenic plants can overcome many of the obstacles of traditional phytoremediation. There is great promise that transgenic plants expressing bacterial or mammalian genes involved in xenobiotic metabolism will improve the efficiency and safety of phytoremediation, leading to a wider application in the field [13].

γ-glutamylcysteine synthetase (γ-ECS, E.C. 3.2.3.3) is the rate-limiting regulatory enzyme in the biosynthesis of the ubiquitous tripeptide thiol compound glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine). The increased production of GSH contributes to the antioxidative protection of plant cells against oxidative stress caused by various environmental factors [14,15]. The glutathione S-transferases (GSTs, E.C. 2.5.1.18.), which catalyze conjugation reactions between GSH and a number of xenobiotics, play crucial roles in the degradation of toxic substances. Many studies of transgenic plants expressing GSTs in *Aspergillus nidulans*, Indian mustard (*Blassica jucea*), tobacco and poplar trees (*Populus trichocarpa*), etc., have shown increased resistance to a variety of environmental stressors including heavy metals such as cadmium and nickel [16], herbicides such as atrazine and chloroacetanilide [17–19], and

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other organic pollutants including 1-chloro-2,4-dinitrobenzene (CDNB), metolachlor, prechirachlor, cinnamic acid and phenanthrene [18,20,21].

In addition to glutathione conjugation, cytochrome P450 (P450 or CYP) oxidation is an important mechanism for xenobiotic detoxification. P450s encompass the largest family of plant proteins, however, molecular information on plant P450s related to xenobiotic metabolism is limited. Only some herbicide-metabolizing P450 genes have been cloned and characterized, such as CYP73A1 and CYP76B1 from Jerusalem artichoke (*Helianthus tuberosus*)[22,23], CYP71A11 from tobacco (*Nicotiana tabacum*)[24], and CYP71A10 from soybean (*Glycine max*)[25]. In contrast, the enzymatic functions of mammalian P450s have been well studied. In humans, 11 P450 proteins catalyze 90% of reactions with xenobiotics [26]. Isoform P450 2E1 (CYP2E1) has been identified in mammalian systems as important in the metabolism of several xenobiotics contaminants. Tobacco plants (*N. tabacum* cv. Xanthii) were transformed to express human CYP2E1 under the Mac promoter resulting in a marked increase in metabolism of trichloroethylene (TCE) and ethylene dibromide compared to vector controls in hydroponic reactors [27]. The preliminary results showed that transgenic tobaccos metabolized TCE 640-fold faster than the wild type. Besides, transgenic plants expressing CYP2E1 gene were also demonstrated to result in the enhanced metabolism of volatile hydrocarbons [28], and hexahydro-1, 3, 5-trinitro-1,3,5-triazine (RDX) [29].

The optimization of phytoremediation using genetic modifications may require the introduction of several genes for transport, multistep metabolic pathways, and sequestration. Transgene stacking, in which multiple traits are conferred to plants by the expression of two or more foreign genes, has been used to develop plants for agricultural applications, though problems, such as silencing, have been encountered [30]. Inui and Ohkawa [31] accomplished to co-express three human P450 species, CYP1A1, CYP2B6 and CYP2C9 in transgenic potato plants. These transgenic potato plants exhibited remarkable cross-tolerance towards sulfonylurea and other herbicides. Kawahigashi et al. [32] also reported the phytoremediation of herbicide atrazine and metolachlor by transgenic rice plants expressing human CYP1A1, CYP2B6 and CYP2C19. Transgenic tobacco (*N. tabacum* cv. LA Burley 21) lines simultaneously expressing three genes encoding enzymes: serine acetyltransferase, γ-glutamylcysteine synthetase and phytochelatin synthase, showed the greatest effects (about 8-fold elevation of thiols) [33]. These results indicated that a multitransgene strategy will contribute to a strong increase in the levels of pollutants in transgenic plants and an increased phytoremediation capacity.

Alfalfa (*Medicago sativa* L.), as a deep-rooted perennial plant with a highly productive biomass and drought tolerance, is fast growing, is available in large amount during several months of the year, does not have any reported environmental hazards [34]. In this sense, it is an ideal natural resource for the remediation of contaminated soils [35,36]. There are reports on the use of this plant for phytoremediation of soils contaminated with heavy metals [37] and organic pollutants including polychlorinated biphenyls (PCBs) [38], phenanthrene and pyrene [39]. These studies showed that alfalfa has strong potential for use in the remediation of organic and/or heavy-metal contaminants. However, compared with contaminated soil with heavy metals or organic contaminants alone, the mixed contaminated soil with heavy metals and organic pollutants are more common. Most importantly, it has been shown that alfalfa has the capacity to decontaminate heavy metal and organic pollutants-contaminated soil [40], e.g., the copper and benzo [α] pyrene (B[α]P) contaminated soil [41].Transgenic alfalfa expressing the *Pseudomonas aeruginosa* citrate synthase (CS) exhibited enhanced tolerance to aluminum (Al) compared with the non-transgenic control [42]. Wang et al. [43] found that transgenic alfalfa expressing a modified bacterial atrazine chlorohydrolase (AtzA) grew over a wide range of atrazine concentrations. To date, however, few studies have addressed the production of transgenic alfalfa plants designed for the phytoremediation of the mixed contaminated soil with heavy metals and organic pollutants. In the present study, we attempted to construct transgenic alfalfa plants simultaneously expressing human CYP2E1 and GST. The transgenic plants tolerance towards the mixed contaminated soil of heavy metals and organic pollutants were investigated.

2. Materials and methods

2.1. Construction of expression plasmid

Expression plasmid pKHCG harboring GST and human CYP2E1cDNAs in tandem was constructed as follows: human CYP2E1 cDNA (1.69 kb) was cloned according to previously described methods by Lu et al. [44]. GST cDNA (665 bp) was provided by Dr. Sung-Jong HONG (Chung-Ang University, Korea). The β-glucronidase (GUS) expression units in pBI121 and pKH200 were each replaced with the GST and human CYP2E1 gene, to yield the plasmid pIGST and pKE1, respectively. The expression unit 35S-GST-Nos-T from pIGST was inserted into plasmid pKE1 derived from pKH200 to construct the recombinant plasmid pKHCG for co-expression of GST and human CYP2E1cDNAs (Fig. 1). It contains the GST and human CYP2E1 genes both driven by the cauliflower mosaic virus 35S promoter with a Ω-enhancer sequence. The construct also contains the nptII gene, which confers kanamycin resistance. Matrix attachment regions of tobacco (MARs) was also contained in this construct.

2.2. Plant materials and transformation

Alfalfa (*M. sativa* L.) was used for transformation. Seeds of alfalfa (*M. sativa* L. cultivar) were purchased from the Academy of Agricultural Sciences, Jiangsu Province, China. The seeds were surface-sterilized by soaking in 70% ethanol (5 min), 10% sodium hypochlorite (10 min) and rinsed five times in sterile distillled water before use. Alfalfa plants were grown on a 1/2 MS medium in a plant growth chamber under fluorescent light at 25 °C during a 14 h photoperiod and a 10 h dark period for 5–7 days. Aseptic seedlings with a length of 2–3 cm were harvested for co-cultivation. Hypocotyl sections were taken from the aseptic seedlings, cutting into 5 mm in length and used to induce the callus.

Agrobacterium-mediated transformation of alfalfa plants was carried out as the following procedure. *Agrobacterium tumefaciens* strain LBA4404 harboring the expression plasmid pKHCG was infected to alfalfa callus derived from hypocotyl. Hypocotyl sections 5 mm in length were immersed in a culture of *Agrobacterium* (OD600 = 0.25) for 3 min at 26 °C, blotted on filter paper and placed on solid MS media containing 0.5 mg/L KT and 0.05 mg/L naphthaleneacetic acid (NAA). After four days of co-cultivation in the dark at 26 °C, the hypocotyl sections were washed by soaking in sterile distilled water containing 200 mg/L ampicillin (Amp) for 20 min, MS liquid medium containing 200 mg/L Amp for 20 min, rinsed three times in sterile distilled water, blotted on filter paper and placed on solid MS media containing 0.5 mg/L KT, 0.05 mg/LNAA and 250 mg/L Amp in a plant growth chamber under fluorescent light at 26 °C. After 7 days of sterilization cultivation, to induce callus the tissue were placed on solid MS media containing 0.5 mg/L KT, 0.05 mg/LNAA, 100 mg/L Amp and 50 mg/L Km under fluorescent light at 26 °C. After 3–4 times of subculture (once every 7 days), the callus were induced to form embryos. When they reached 2–5 mm in length, the embryos were transferred directly to MS

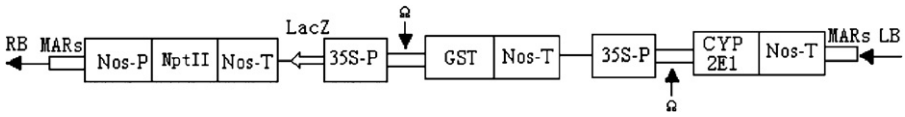

Fig. 1. T-DNA region of the expression plasmid pKHCG for human CYP2E1 and GST. RB, right border; LB, left border; Nos-P, nopaline synthase promoter; Nos-T, nopaline synthase terminator; NPTII, neomycin phosphotransferase II; 35S-P, cauliflower mosaic virus (CaMV) 35S promoter; MARs: Matrix Attachment Regions of tobacco; Ω, Ω enhancer from tobacco mosaic virus (TMV); Lac Z, reporter gene.

medium containing 0.3 mg/L NAA, 50 mg/L Km and 50 mg/L Amp for shoot development. Null vector control plants were derived from transformations using pKH200 instead of pKHCG.

2.3. Selection of transgenic alfalfa plants

Regenerated shoots from alfalfa callus infected with *A. tumefaciens* strains LBA4404 having the recombinant plasmid pKHCG were selected as kanamycin resistant and then subjected to PCR analysis. Regenerated shoots tolerant to Km were transplanted and self-crossed.

2.4. PCR analysis of transgenic alfalfa plants

A genomic DNA prepared from the kanamycin-resistant alfalfa plants transformed with pKHCG was used for PCR analysis for selection of transgenic alfalfa plants with the transgene, and PCR-positive shoots integrating the GST and human CYP2E1cDNAs were transplanted to soil and cultured in a closed greenhouse.

The PCR amplification protocol was: 95 °C for 5 min; followed by 30 cycles of 94 °C for 30 s, 60 °C for 40 s, and 72 °C for 1 min; and finally at 72 °C for 10 min for the final extension. Amplified PCR products were separated by electrophoresis on agarose gels (1%) and bands of ethidium bromide-stained fragments were visualized with an UV light.

PCR primers specific for GST, 5′- GCGGGGATCCTATGTCGCCG-GTGTTGGGTTATT-3′ and 5′- GCGCGAGCTCTTGAAGGAGGAGCAT-CACCACCA-3′; for CYP2E1, 5′- CCATGCGAGTCTACATTGTAC-3′ and 5′- TTCATTCTGTCTTCTAACTGG-3′, were used to confirm the integration of the T-DNA region of pKHCG.

2.5. Western blot analysis of transgenic alfalfa

To confirm the expression of human CYP2E1 and GST from the corresponding integrated genes, Western blot analysis of transgenic alfalfa plants was carried out. Total protein was extracted from a homogenate of leaf tissues taken from each of the kanamycin-resistant alfalfa transformed with pKHCG using 50 mmol/L Tris–HCl buffer (pH 7.4, 0 °C) containing 1 mmol/L EDTA, 1% PVP, 0.001% PMSF (pH8.0), and 5% mercaptoethanol, and then the homogenate was centrifuged at 15,000 rpm for 30 min at 4 °C. The protein fraction was subjected to electrophoresis on 10% SDS–PAGE. After SDS–PAGE, separated proteins were transferred to a PVDF-Plus transfer membrane (Millipore, Bedford, MA, USA). Thereafter, membranes were blocked with 2% (w/v) non-fat dry milk in 0.05 mol/L Tris buffer saline (TBS pH = 7.6), blotted proteins were then detected immunologically using rat anti-human CYP2E1 antibody (Abcam Inc., CA,USA) and rabbit anti-rat GST antibody(GenScript USA Inc., NJ, USA), respectively. Peroxidase-conjugated anti-rabbit IgG for CYP2E1 and GST (Sigma Chemical Co., St. Louis, MO), were used as a secondary antibody.

2.6. Heavy metal-organic pollutants tolerance in transgenic alfalfa plants

Cd and trichloroethylene (TCE) were used for the heavy metal-organic pollutants tolerance tests. Sterilized seeds from transgenic alfalfa plants and non-transgenic control alfalfa plants were embedded in MS medium containing different concentrations of mixtures of $CdSO_4$/TCE (0/0, 0.1/0.15, 0.15/0.20 and 0.2/0.25 mmol/L). The seeds were cultured for 20 days to evaluate the tolerance of transgenic plants. The concentrations of these toxic pollutants were confirmed to damage the control alfalfa plants in preliminary experiments.

2.7. Assay for GST and CYP2E1 activity

Leaves from each of the plants transformed with pKHCG were homogenized in 50 mmol/L Tris–HCl buffer (pH 7.4, 0 °C) containing 1 mmol/L EDTA, 1% PVP, 0.001% PMSF (pH 8.0), and 5% mercaptoethanol and then centrifuged at 15,000 rpm for 30 min at 4 °C. The resultant supernatant were diluted appropriately and then analyzed for GST activity. GST activity was determined by measuring the increase in absorbance at 340 nm, using 2 mmol/L reduced glutathione (GSH) and 1-chloro-2,4-dinithrobenzene (CDNB) according to Khatuna et al. [45].

Aniline hydroxylation has been used extensively to probe the activity of P450 2E1. The aniline hydroxylase activity was measured by measuring *p*-aminophenol formation as reported by Youssef et al. [46]. Activity was determined in a reaction solution containing 5 mmol/L aniline, 5 mmol/L $MgCl_2$, 5 mmol/L isocitrate, 100 mmol/L Tris–HCl buffer and 0.5 mmol/L NADPH. After 30 min at 37 °C, reaction was terminated by addition of 10% ice trichloroacetic acid. Precipitated proteins were removed by centrifugation at 12,000 rpm for 10 min at 4 °C and 1 ml of resultant supernatant was mixed with 1 ml of Na_2CO_3. Absorbance at 630 nm was immediately measured.

Fig. 2. The selection of kanamycin-resistant alfalfa shoots regenerated from callus. (A) Positive control: alfalfa shoots of nontransformational hypocotyl segments on medium without Km, (B) negative control: alfalfa shoots of nontransformational hypocotyl segments on Km medium, and (C) kanamycin-resistant alfalfa shoots of transformational hypocotyl segments on selective medium with Km.

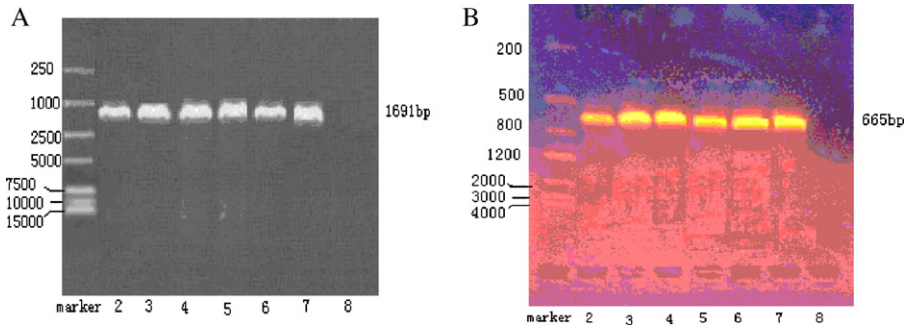

Fig. 3. PCR analysis of the transgenic alfalfa: (A) showing the presence of human CYP2E1: lanes 2–7 represent the transgenic alfalfa plants expressing human CYP2E1 transformed with the pKHCG, lane 8 represents alfalfa plants untransformed with the pKHCG, (B) showing the presence of GST: lanes 2–7 represent the transgenic alfalfa plants expressing GST transformed with the pKHCG, lane 8 represents alfalfa plants untransformed with the pKHCG.

3. Results

3.1. Selection of transgenic alfalfa plants

Alfalfa hypocotyls segments were infected with *A. tumefaciens* strain LBA4404 harboring the constructed recombinant plasmid pKHCG (Fig. 1). Then, kanamycin-resistant shoots regenerated from callus were selected. These plants were subjected to further selection by PCR analysis to confirm the presence of human CYP2E1 and GST cDNAs in the corresponding plant cell genome. As a result, from 30 kanamycin-resistant alfalfa shoots (Fig. 2) transformed with pKHCG, 26 PCR-positive shoots (Fig. 3) were selected.

No amplification was found in non-transformed alfalfa (Fig. 3). In all putative transformants, an expected band was found at 1691 bp for the human CYP2E1 cDNA (Fig. 3 A) and at 665 bp for the GST cDNA (Fig. 3B).

The GST and CYP2E1 activities of the leaf samples of the selected 26 shoots were assayed. 3 transgenic alfalfa plants, C0907, C0909 and C0920 (C represents the co-expression of CYP2E1 and GST) transformed with pKHCG were selected on the basis of both GST and CYP2E1 activities of their leaves. In addition, two transgenic alfalfa plants S0911 (S represents the expression of single CYP or GST gene) transformed with pIGST on the basis of the GST activity and S0917 transformed with pKE1 on the basis of CYP activity, were selected from 30 kanamycin-resistant shoots respectively.

3.2. Western blot analysis

To confirm the expression of human CYP2E1 and GST from the corresponding integrated genes, the Western blotting of transgenic alfalfa plants was carried out. The protein band reacted with anti-CYP2E1 was found in the seedlings extracts prepared from C0907, C0909 and S0917, as shown in Fig. 4A. No protein band corresponding to CYP2E1 was found in the seedlings extracts of C0920, S0911 and the transgenic control plant transformed with the vector pKH200. It was also found that the GST protein band was present in S0911, C0907, C0909 and C0920 (Fig. 4B). No protein band corresponding to GST was found in S0917 and the transgenic control plant.

Consequently, it was found that the CYP2E1 and GST mRNAs were translated into the corresponding CYP2E1 and GST proteins, respectively, in both C0907 and C0909 plants.

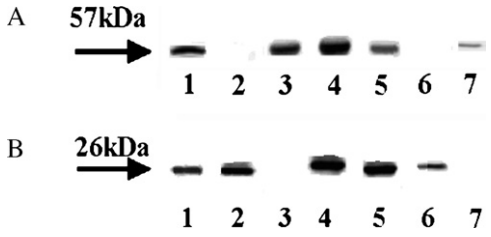

Fig. 4. Western blot analysis of the transgenic alfalfa plants using anti-human CYP2E1 (A), anti-rat GST, (B) antibodies. Lane 1 shows the positive control derived from protein extracts of alfalfa leaves expressing human CYP2E1 (A), GST (B). Lanes 2–7 show S0911, S0917, C0907, C0909, C0920 and the untransformed control, respectively.

3.3. Cd-TCE tolerance in the transgenic alfalfa plants

Tolerance to the Cd-TCE was assayed for S0917, S0911 and C0907 planted in glass tubes for 20 days after treatment with combination of Cd and TCE as shown in Fig. 5. As we expected, C0907 co-expressing CYP2E1 and GST exhibited strong tolerance towards the complex contaminants of Cd and TCE among the transgenic alfalfa plants examined. These plants showed healthy growth in the coexistence of $CdSO_4$ (0.15 mmol/L) and TCE (0.2 mmol/L), whereas the control plants were susceptible and almost could not grow under this condition (Fig. 5). The growth rate of the transgenic seedlings C0907 in the coexistence of $CdSO_4$ (0.15 mmol/L) and TCE (0.2 mmol/L) was nearly the same as that of the control on MS medium without $CdSO_4$ and TCE. In contrast, the transgenic alfalfa plants expressing single gene (S0917 for CYP2E1, S0911 for GST) showed weakly tolerance to the complex contaminants and grew slowly.

As can be seen from Fig. 6, no visual evidence of damage was observed and the pKHCG plants showed healthy growth at 0.15 mmol/L $CdSO_4$ and TCE 0.2 mmol/L. At 0.2 mmol/L $CdSO_4$ and 0.25 mmol/L TCE the plants showed slow growth. Plants did

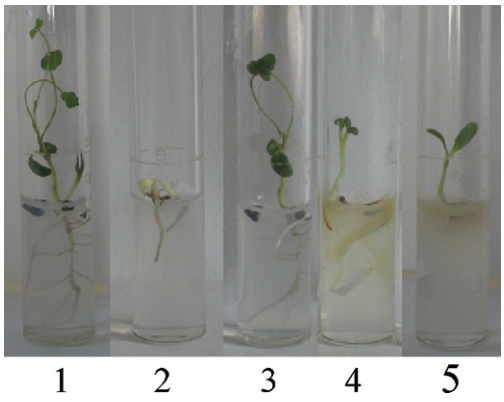

Fig. 5. Tolerance of the transgenic alfalfa plants towards Cd-TCE. 1–5 show the untransformed control plants without Cd-TCE, untransformed control plants, C0907, S0917 and S0911 with Cd-TCE, respectively. The transgenic plants were incubated with mixed contaminants at 0.15 mmol/L of $CdSO_4$ and 0.2 mmol/L of TCE observed after 20 days.

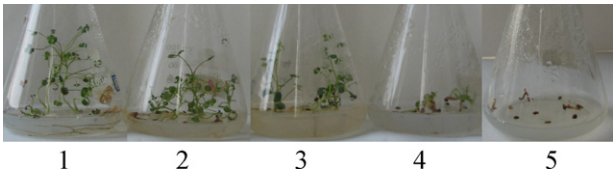

Fig. 6. Tolerance of the transgenic alfalfa plants co-expressing CYP2E1 and GST towards different concentrations of Cd/TCE. 1–5 show the transgenic alfalfa plant with Cd/TCE at 0/0, 0.1/0.15, 0.15/0.20,0.2/0.25 and 0.25/0.30 mmol/L, respectively.

not grow at all at higher concentrations (0.25 mmol/L $CdSO_4$ and 0.3 mmol/L TCE). The complex contaminants concentration damaging transgenic plants C0907 was at 0.25 mmol/L Cd and 0.3 mmol/L TCE which was significantly higher than that of the control.

Based on the above results, the transgenic alfalfa plants co-expressing CYP2E1 and GST were found to show cross-tolerance to Cd and TCE both in germination and vegetative growth stages.

4. Discussion

To our knowledge, this work represents the first development of transgenic alfalfa plants for increased tolerance of a broad range of mixed environmental pollutants from soil. The transgenic alfalfa plants co-expressing human CYP2E1 and GST were generated by *Agrobacterium*-mediated transformation of alfalfa hypocotyl segments for the phytoremediation of the mixed contaminated soil with heavy metals and organic pollutants. In the present study, GST and CYP2E1 species were co-expressed in alfalfa plants under the control of CaMV35S promoter and Nos terminator. The transgenic alfalfa plant C0907 and C0909 simultaneously expressing GST and CYP2E1 clearly showed cross-tolerance towards the complex contaminants of Cd and TCE.

The capacity of mixed contaminants resistance of pKHCG transgenic alfalfa plants became higher compared with the transgenic alfalfa plants expressing single gene (GST or CYP2E1). The present study revealed that the pKHCG alfalfa plants simultaneously expressing human CYP2E1 and GST clearly exhibited strong resistance towards the mixtures of Cd and TCE that were metabolized by the introduced GST and CYP2E1 in combination. However, transgenic alfalfa plants expressing single gene (GST or CYP2E1) did not grow well in the culture medium containing the mixtures of Cd and TCE because each transgenic plant was sensitive to one of the mixed contaminants. Therefore, it was concluded that the two introduced genes worked additively in the pKHCG alfalfa plants.

Phytochelatins(PCs) may play a important role in plant Cd tolerance. GSH is the direct precursor of phytochelatins. PCs are heavy metal-binding peptides involved in heavy metal tolerance and sequestration. They were shown to be induced by heavy metals such as Cd in all plants tested [47]. TCE is known to be taken up by plants and are volatilized [48] to the atmosphere. Suspension cultures of hybrid poplar are known to convert it into metabolites, which finally get bound to unextractable portions of the plant cells [49]. Expression of the CYP2E1 gene was sufficient to confer greatly increased removal of TCE, vinyl chloride, carbon tetrachloride, chloroform, and benzene, all of which are substrates of cytochrome P450 2E1 [28]. In plants, cytochrome 450 form the largest family of plant proteins and they play an important role in deciding the plant's tolerance to xenobiotics [50]. Transgenic tobacco plants having human cytochrome P450 2E1 also showed increased uptake and debromination of ethylene dibromide and a 640 fold enhanced metabolism of TCE [27]. Several cytochrome P450 genes such as CYP1A1, CYP2B6 and CYP2C19, when introduced into rice plants, showed tolerance to herbicide atrazine, metolachlor and norfluazon and could decrease the amount of herbicides [32]. Our data clearly indicate that overexpression of CYP2E1 and GST together leads to enhanced removal of pollutants compared with the control plants. We expected that the pKHCG transgenic alfalfa plants would show stronger metabolic activity by co-operation of the GST and CYP2E1 because some organic compounds were metabolized by not only the introduced P450 species but also the GST in the recombinant alfalfa plants system. It was reported that herbicide safeners reduced injury in corn from treatment with chloroacetanilide erbicides because of the induction of GST and P450 in corn treated with the safeners [51].

For successful phytoremediation, plants need: (a) to have tolerance towards the chemicals or pollutants to be cleaned, (b) to have the ability to metabolize and immobilize them, and (c) to have a large biomass so that they can remediate widespread chemicals in the field. Our results show that the pKHCG transgenic alfalfa plants have good potential for phytoremediation purposes because they have cross-tolerance towards the complex contaminants of heavy metals and organic pollutants. Therefore, these transgenic alfalfa plants co-expressing GST and human P450 species may have a great potential for phytoremediation of environmental contaminants and complex contaminants in particular.

Field studies and risk assessment are important parameters to be taken into consideration while developing transgenic plants. Further investigations for safety assessment of transgenic alfalfa plants and experiments on the reduction of heavy metals and organic pollutants are needed to determine whether the improvements seen in the laboratory studies will ultimately lead to enhanced phytoremediation before such transgenic plants can be put to practical use. In future, pKHCG alfalfa plants could be utilized for engineering of heavy metal-organic pollutants tolerance and for phytoremediation of soils and of ground and surface waters.

5. Conclusions

Transgenic alfalfa plants simultaneously expressing human CYP2E1 and glutathione S-transferase were generated from hypocotyl segments by the use of an *Agrobacterium* transformation system. The capabilities of mixed contaminants resistance of pKHCG transgenic alfalfa plants became markedly increased compared with the transgenic alfalfa plants expressing single gene and the non-transgenic control plants. The pKHCG transgenic alfalfa plants have good potential for phytoremediation because they have cross-tolerance towards the complex contaminants of heavy metals and organic pollutants.

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