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MERCURY DETOXIFICATION USING GENETIC ENGINEERED *NICOTIANA TABACUM*

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ABSTRACT
Phytoremediation is a low cost alternative solution to soil contamination compared with traditional removal and/or disposal techniques. One of the phytoremediation technologies is the phytovolatilization, whereby the contaminant is not primarily accumulated in above-ground tissues, but is instead transformed by the plant into the atmosphere. The detoxification of highly toxic organmercurial compounds and subsequent volatilization of elemental mercury is a unique example for the successful phytoremediation based on genetic engineering approach. For mercury removal techniques we constructed a dicistronic construct containing the bacterial mercury detoxification genes *merA* and *merB* under the control of the *Arabidopsis* Actin2 promoter and terminator. The resulted construct was introduced to *Agrobacterium* competent cells using heat shock transformation method. In the mean time, we germinated tobacco (*Nicotiana tabacum* var. Gold leaf) seeds on MS media and infected leaf discs of 6-8 weeks tobacco seedlings with *Agrobacterium* containing Ti plasmid harboring *merA/merB* dicistronic construct. The results showed that about 90% of tobacco seedlings were carrying the *mer* genes. Tobacco seeds were collected from wild type and transgenic lines and tested for mercury resistance. The results showed that transgenic plants are resistant to both Phenyl Mercuric Acetate (PMA) and $HgCl_2$. The root length and dry weight of wild and transgenic seedlings growing on both media amended with mercury compounds and media without mercury (control) were scored. The results showed that the root lengths and dry weight of the transgenic lines are significantly higher by 60 and 17-folds, respectively, compared to wild type. The results showed clear evidence that the transgenic plants are resistant to both organic and inorganic mercury compounds and can be used to clean up mercury contaminated sites.

KEYWORDS: *Phytoremediation, Phytovolatilization, Transgenic plants, Phenyl mercuric acetate, mercuric chloride, merA, merB.*

1. INTRODUCTION

Phytoremediation is an environmentally friendly, potentially very effective, and less expensive method than the physical and chemical remediation techniques (Chaney *et al.*, 2000; McGrath *et al.*, 2002). Mercury and mercurial compounds are non nutritive heavy metals, which are hazardous to all biological organisms. Although, mercury is typically found at trace levels in the Earth's crust, anthropogenic activities have created areas with elevated and potentially dangerous concentrations (Nriagu, 1979). In our discussion of phytoremediation, three forms of mercury need to be considered. Ionic mercury Hg (II) forms that are water soluble and are found associated with different anions, such as chloride, sulfides, and hydroxides (Anderson, 1979). Organic mercury compounds such as methylmercury (CH_3Hg^+) and Phenylmercuric

acetate (PMA), that are fat soluble and finally metallic mercury Hg (0) that is volatile at room temperature (Langford and Ferner, 1999).

Mercury resistant bacteria detoxify organic and inorganic mercury compounds by producing two enzymes. First, Organ-mercurial lyase (MerB) enzyme that encoded by *merB* gene catalyzes the protonolytic cleavage of the carbon-mercury bond of several forms of organic mercury. The second enzyme mercuric reductase (*merA*) encoded by *merA* gene catalyzes the reduction of Hg (II) to a volatile and less toxic elemental form Hg (0). The transfer of the bacterial *merB* and/or *merA* genes into plants under control of plant regulation sequences resulted in high level resistance to organic and inorganic mercury compounds (Rugh *et al.*, 1996; 1998; Bizily *et al.,* 2000; He *et al.*, 2001). Such plants should direct the management of mercury cycling at contaminated sites and protecting the endangered ecosystems. The translation of eukaryotic mRNAs or transgene RNAs such as these encoding *merA* and *merB* is dependent upon 5`- cap mediated ribosome binding. Ribosomes associate with the 5` cap structure before scanning along the mRNA molecule to the site of transitional initiation, which in most instances the first AUG codon (Mountford and Smith, 1995). While the scanning model of translation initiation accommodates most eukaryotic mRNAs, an alternative model of initiation was discovered that is used by certain viral and cellular mRNAs. For this subset of mRNAs the 40S ribosome subunit associates directly with an internal site in the mRNA leader called an internal ribosome entry site (IRES) (Lewin, 2000).

IRESs are generally 150 - 500nt RNA sequences located in the 5` untranslated region of certain viral and cellular mRNAs. IRESs have been identified in animal and plant viral transcripts, and may be present in a 3% of mammalian genes. Many have exceptionally long 5` untranslated region (UTRs) and are cellular oncogenes. IRESs have been used successfully to express two or polygenes from di or polycistronic transcripts in animal cells (Li *et al.*, 1997; Stoneley *et al.*, 1998; Hennecke *et al.*, 2001), in plant (Urwin *et al.*, 2000) and finally in Yeast (Zhou *et al.*, 2001).

The present study aimed at the co-expression of the bacterial mercury detoxification genes *merB* and *merA* from dicistronic transcripts using the IRES sequence of the human eukaryotic Initiation Factor 4G (*eIF4G*) in Tobacco for mercury detoxification.

2. MATERIALS AND METHODS

2.1. *merB/merA* construct preparation

We design a construct containing the bacterial mercury detoxification genes *merB*, and *merA* in dicistronic system. The *merB/merA* dicistronic construct is made typically with *merB* sequence in the first position, *merA* sequence in the second position and the Human eukaryotic initiation factor 4G (*eIF4G*) IRES sequence in the intercistronic region. The *merA* sequence (*merA* 77) used in the present study is a 1.67 kb sequence reconstructed from the original bacterial *merA* gene by modifying a region covering 77% of the coding sequence with nucleotide combination and codons rich in A and T nucleotides and more common to highly expressed plant genes. Modification of merA77 gene was done by Dr: Rebecca. S. Balish, Genetics department, University of Georgia, USA. The *eIF4G* IRES sequence used in this study is 196 bp synthetic sequences prepared by template independent overlap extension PCR using 4 sense and antisense primers. The *eIF4G* IRES sequence was prepared by Dr: Yujing Li, Genetics department, University of Georgia, USA.

The reconstruction of *merB* gene for dicistronic expression in plant: The 214 codon *merB* gene was amplified by polymerase chain reaction (PCR) using the *merB* sequence in plasmid *pBSmerBpe* as a template (Bizily *et al.*, 2000). Two long synthetic primers were used to generate 36 nt multicloning sites down stream *merB* stop codon for subsequent *eIF4G* IRES and *merA* cloning steps (Figure1). The sense primer, *merBRcaS1*, consisted of the 35 nt sequence 5`– ATG ATG GGT ACC TCA TGA AGC TCG CCC CAT ATA TT– 3` and contained *Kpn1*, *BspH1* (*Rca1*) cloning sites and the first 21nt of the *merB* coding sequence to prime the forward PCR reaction (Rugh *et al.*, 1996).

The antisense primer *merB207A* had the 63 nt sequence 5` – TAG ATC GGA TCC GAT ATC CCA TGG AAG CTT GTC GAC CTC GAG TCA CGG TGT CCT AGA TGA CAT – 3` with *BamH1*, *EcoRV*, *Nco*1, *HindII*I, *Sal1*, *Xho1* cloning sites and anti-codons to the last seven *merB* codons to prime the reverse PCR reaction. PCR was carried out for 35 cycles using the

following conditions: denaturation at 95°C for 1min, annealing at 45°C for 1min, and extending at 72°C for 1min.

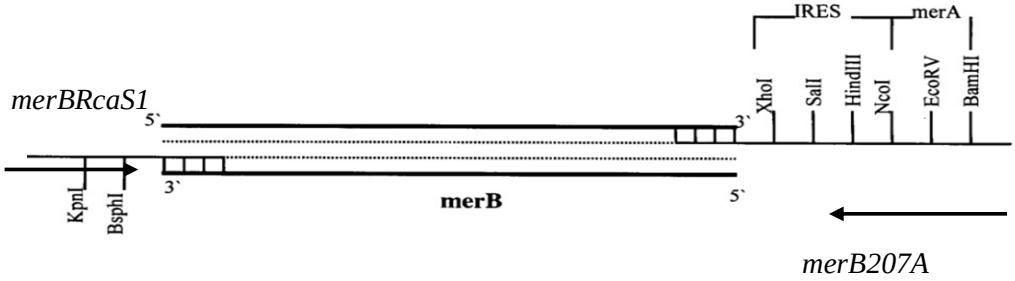

*Figure 1***.** PCR strategy for *merB* amplification**.** During the PCR amplification of the *merB* gene and its cloning, sites were added that were to be used in the subsequent cloning of *eIF4G* IRES and *merA* sequences. A map of the reconstructed *merB* sequence is shown. The sense primer *merBRcaS1* (left arrow) contained *Kpn1* and *Bsph1* restriction endonuclease cleavage sites. The antisense primer *merB207A* (right arrow) contained *BamH1, EcoRV, Nco1, HindIII, Sal1* and *Xho1* cloning sites and the last seven codons of the *merB* gene. The *Xho1* and *Nco1* sites were used to clone *eIF4G* IRES sequence. *Nco1* and *BamH1* sites were used to clone *merA* gene sequence.

2.2. The cloning strategy

The purified *merB* fragment ~750 bp was cloned into the plasmid Bluescript IIKS (Stratagene,USA) containing Act2 promoter and Act 2 terminator to produce *ACT2pt:merB* construct (Figure 2). The cloning strategy included digestion of the *merB* and the plasmid DNA with *Kpn1* and *BamH1* restriction enzymes for 2 hours at 37°C, resolved in agarose gel and purified, then ligated and introduced into *E.coli* competent cells. The *eIf4G* and *merA* sequences are subsequent cloned to *ACT2pt: merB* plasmid using *Xho1*, *Nco1* and *Nco1*, *BamH1* cloning sites respectively to create the *Act2*prom/*merB*/*eIF4G*/*merA*/*Act2*ter construct. The constitutive *Arabidopsis Actin2* promoter (*Act2* prom.) is used to control the *merB* expression, while the expression of *merA* gene is controled by the *eIf4G* IRES sequence. The *Arabidopsis Actin2* terminator sequence (*Act2* ter.) is used to terminate the expression. In every cloning step the new construct is confirmed by restriction analysis of small scale preparation of plasmid DNA. The prepared dicistronic construct was transferred to the plant expression vector pBIN19 and introduce into an LBA4404 *Agrobacterium tumefacience* strain (GIBCO/BRL) using the heat shock transformation method (Sambrook *et al.*, 1989). Transformants were grown in YEP medium amended with streptomycin and kanamycin.

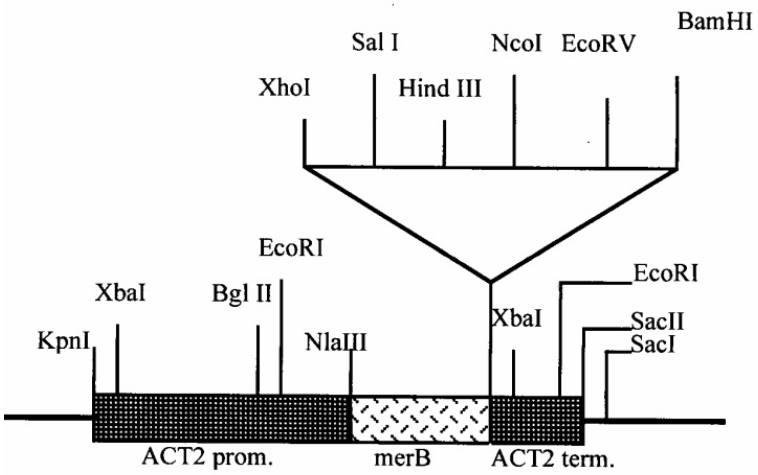

*Figure 2***.** Physical map of the *ACT2pt: merB* gene constructs. The PCR modified *merB* gene was cloned under control of the *Arabidopsis* ACT2 promoter (*ACT2 prom*.) and ACT2 terminator.

2.3. Construction of Tobacco transgenic plants

Tobacco (*Nicotiana tabacum* var. Gold leaf) seeds were kindly provided by Tobacco Research Dep., Agricultural Research Center, Cairo, Egypt. Seeds were surface sterilized and germinated in Murashige and Skoog (MS) medium (4.3 g l^{-1}, GIBCO/BRL) supplemented with 30 g l^{-1} sucrose and 8 g l^{-1} agar. Leaves of 6-8 weeks old tobacco seedlings cut into squares (discs) of about 0.5 cm across and infected with *Agrobacterium tumefacience* carrying the *merB/merA* dicistronic construct. Discs were co-cultivated for 3 days in the dark at room temperature, then transferred to selective MS plates containing 100 mg l^{-1} kanamycin, 500 mg l^{-1} carbincillin, 0.1 mg l^{-1} naphthalene acetic acid (NAA) and 1.0 mg l^{-1} benzyl adenine (BA) and incubated for 3-4 weeks at 24^{o}C in a growth chamber. Shoots and callus were transferred to fresh MS selection plates containing the same antibiotics as above with BA to continue shoot and root induction. The rooted plantlets are transferred to green house to complete growth and produce seeds. Seeds are collected when they completely dried and stored at 4^{o}C. Transformation was confirmed using PCR.

2.4. Mercury resistance assays

Seeds of Tobacco wild type (WT) and *merB/merA* transgenic lines (2 and 5) were sterilized and germinated on MS control or mercury containing plates made with 1% phytagel. 5 µM phenyl mercuric acetate (PMA) or 100 µM $HgCl_2$ final concentration was added to media after autoclaving. Seedlings were grown at 24 ^{o}C with a 16 h light/ 8 h dark regime and photographed at 4-6 weeks.

2.5. Quantitative analysis of seedlings root lengths and dry weighs

Random 21 days old seedlings were chosen and the root lengths and dry weighs were measured for at least 40-60 seedlings for each treatment, data were recorded for statistical analysis.

3. RESULTS

Many attempts were done in the last few years to test the possibility of using different IRES sequences to express mono or dicistrones in animal, plant and yeast cells. Most of this attempts relied on using reporter genes (Li *et al.*, 1997; Urwin *et al.*, 2000; Hennecke *et al*., 2001; Owens *et al.*, 2001). In this manuscript we tested the co-expression of the bacterial mercury detoxification genes *merB* and *merA* from dicistronic construct containing the human eukaryotic initiation factor 4G (*eIF4G*) IRES sequence in the intercistronic region. We prepared the dicistronic construct with *merB* in the first cistron and *merA* in the second cistron and introduced it to tobacco using *Agrobacterium tumefacience* mediated transformation method. Tobacco shoots carrying *merB/eIF4G/ merA* sequences were regenerated, selected for kanamycin resistance encoded on the plasmid vector, and allowed to set seeds. Seeds of several independent transgenic lines were resistant to 5µM PMA and 100µM $HgCl_2$ on mercury containing agar plates (Figure 3). No differences between WT and transgenic lines are observed in control media. The transgenic seedlings grew vigorously in the presence of 5µM PMA or 100µM $HgCl_2$ producing strong shoots, green and healthy leaves and an excellent root system. By contrast, the WT control seeds either failed to germinate or germinated and died during the first 5 days on mercury containing medium.

Table 1. Average numbers of root length (cm) and standard errors of WT and transgenic plants grew on control and mercury containing plates

Plant type	Treatment		
	Control	5 µM PMA	100 µM $HgCl_2$
WT	2.90±0.190	0.05	0.05
merB/merA line # 2	3.07±0.220	3.15±0.185	2.88±0.278
merB/merA line # 5	3.10±0.173	3.21±0.258	3.01±0.191

As seen in table 1 and 2, no significant differences between the root lengths and dry weights of WT and transgenic lines 2 and 5 were observed when they grew on media without mercury (control). By contrast, when the same lines grew side by side on media containing either PMA

or $HgCl_2$, the results showed that the root lengths of the transgenic lines were significantly higher by 60 folds and they gained 17 fold higher dry weights compared to WT. Because of the root system is the system responsible for absorption of metal from media, the results showed clear evidence that the transgenic plants are resistant to both organic and inorganic mercury compounds.

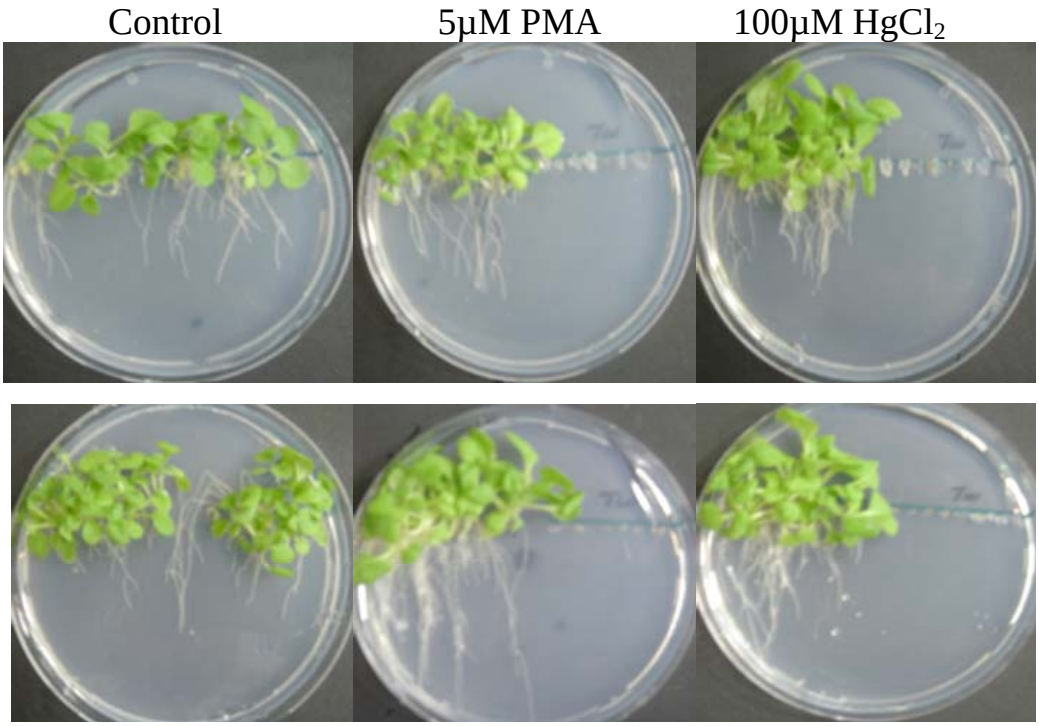

Figure 3. Mercury resistance test of *merB/eIF4G/merA* tobacco transgenic lines. 8-10 seeds of WT and independent transgenic lines were germinated and grew in control media lacking mercury (left hand plates) , 5µM PMA (middle plates) or 100µM $HgCl_2$ (right hand plates). Each plate contains WT seeds (right half) and transgenic seeds (left half). Plates are photographed after 3 weeks of germination. Root lengths and dry weight of 21 day old seedlings grew on either control or mercury containing plates were scored and statistically analyzed. 40-60 seedlings were randomly chosen for each treatment. The results are summarized in tables 1 and 2

Table 2. Average numbers of dry weight (gm) and standard errors of WT and transgenic plants grew on control and mercury containing plates

Plant type	Treatment		
	Control	5 µM PMA	100 µM $HgCl_2$
WT	0.219±0.020	0.012±0.003	0.010±0.006
merB/merA line # 2	0.221±0.040	0.196±0.034	0.228±0.010
merB/merA line # 5	0.233±0.046	0.201±0.033	0.198±0..013

From the quantitative analysis of transgenic lines dry weights and root lengths, it can be concluded that *merB* and *merA* expression in tobacco plants confers resistance to organic mercury compounds such as PMA and inorganic mercury such as $HgCl_2$. The MerB protein that produced by transgenic plants enabled them to germinate and grew well on 5µM PMA, a concentration that 50 fold higher than the concentration that kill WT plants. The data also showed that transgenic plants are resistant to $HgCl_2$ and these plants can be used to clean up mercury contaminated sites and reduce mercury concentrations in the environment.

DISCUSSION

Many genes are involved in metal uptake, translocation and sequestration and transfer of any of these genes into candidate plants is a possible strategy for genetic engineering of plants

for improved phytoremediation traits. Depending on the strategy, transgenic plants can be developed which will be engineered to accumulate high concentrations of metals in harvestable parts. Transfer or overexpression of genes will lead to enhanced metal uptake, translocation, sequestration or intracellular targeting (Eapen and D'Souza, 2006). The enormous costs and environmental undesirability of traditional waste disposal methods has led to a tremendous upsurge in research activity over the last decade to develop alternative, cost-effective technologies to deal with problems of wastes in the environment. In the past few years, decontamination has begun to be carried out by emerging phytoremediation technology. This represents a cost-effective, plant-based approach built on the remarkable ability of plants to grow on toxic soils and eventually concentrate elements and compounds from the environment. In case of mercury, several studies showed that expression of bacterial *merB* and *merA* genes in plant under the control of strong plant promoter conferred resistance to organic and inorganic mercury compounds. (Bizily *et al*., 2000; 2003) demonstrated that both genes are required for plants to detoxify organic mercury by converting it to volatile and much less toxic elemental mercury Hg (0). They also showed that *merB* concentration positively correlated with Hg (0) evolution rates while *merA* expression concentrations do not show a relationship to Hg (0) evolution rates that's why we put *merB* gene in the first cistron under control of a strong plant promoter. Introduction of more than one gene in plant require the use of an equal number of different plant promoters that is not much available and the cloning and transformation strategies require time, effort, and money.

Genetically engineered plants with mer A and mer B genes were produced in three plant species *A. thaliana* [8], *N. tabacum* and *Liriodendron tulipifera* L. (Rugh *et al.*, 1998) and have demonstrated that transgenic plants could grow in the presence of toxic levels of mercury (Rugh *et al.*, 2000). Transgenic Populus deltoids overexpressing mer A9 and mer A18 gene when exposed to Hg (II) evolved 2- to 4-fold Hg (0) relative to wild plant (Che *et al.*, 2003). These transgenic trees when grown in soil with 40 ppm of Hg (II) developed higher biomass.

The present study showed an effective method to introduce multiple genes to plants using IRES sequence which cut promoter use in half and save time. In the phytoremediation field using IRES sequences will help to remediate metals that required many proteins to be expressed. Using IRES sequences and the construct of polycistronic transcripts in plant is a new area of research that needs more investigation.

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