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Accumulation of cadmium (Cd) in T1 transgenic tobacco seedlings expressing metallothionein gene from *Eleusine indica*

Nik Marzuki Sidik[1]*, Roslina Mat Yazid[2], Dhalila Zafirah Mohd Dahlan[2], Babul Airianah Othman[2], Ismanizan Ismail[3].

[1]Faculty of Agro Based Industry, Universiti Malaysia Kelantan, Locked Bag 100, 17600 Jeli, Kelantan, Malaysia
[2]School of Biosciences and Biotechnology, Faculty of Science and Technology Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

[3]Institute of Systems Biology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

***Corresponding author: nikmarzuki@umk.edu.my**

Abstract

Cadmium (Cd) contamination of urban and agricultural soils is toxic to humans, animals and may cause negative effects on plant growth and crop production. The existing conventional methods are found to be not efficient to remove Cd from contaminated soil. The present experiment reports the analysis of nine T1 lines of transgenic tobacco carrying metallothionein gene (*eiMT1*) from *Eleusine indica*, with potential for high efficiency to remediate Cd in contaminated soils. Thirty-days old tobacco plants were treated with different concentrations of $CdNO_3$ (0, 50, 100 and, 150 µmol) for seven days and the accumulation of Cd in the whole seedling was quantitatively determined by using atomic absorption spectrometer (AAS). All transgenic tobacco lines showed greater tolerance and accumulated higher level of Cd than the wild type with lines 18D, 20D1 and, 18C were among the highest (678.7, 623.0 and 611.9 $mgkg^{-1}$ Cd, respectively). Meanwhile, transgenic tobacco lines 18B1 and 20D1 showed higher expression of *eiMT1* gene. These results suggest that the cadmium accumulation in transgenic tobacco did not strictly associate with the expression level of *eiMT1* gene. However, expression of *eiMT1* greatly required for higher accumulation of Cd in transgenic tobacco seedling.

Keywords: Cadmium; gene expression; metal accumulation; metallothionein; phytoremediation
Abbreviations: Cd_cadmium; $CdNO_3$_cadmium nitrate; *eiMT1*_metallothionein gene from *Eleusine indica*; $HClO_4$_perchloric acid; HNO_3_nitric acid; MS_Murashige and Skoog; RT-PCR_Reverse Transcriptase-Polymerase Chain Reaction.

Introduction

Cadmium (Cd) is one of the most harmful pollutant metals which is toxic to organisms amongst all other non-essential heavy metals available in nature. (Jibril et al., 2017). It is widely present in the environment including air, water and most importantly in the soils. Cd in the soils is either naturally present in trace quantities or is derived from human activities such as manure and phosphate fertilizers application, sewage sludge treatment, mining and smelting industries (Shah et al., 2017). Cd enters into the biological cycle through plant roots and then transported to leaves and shoots and finally spread to various parts of the plant. Excessive amount of Cd may cause restriction of photosynthesis, decreases chlorophyll content, alters water status within the plant and disturbs the nutritional content of the plant and its minerals (Ali et al., 2013; Rafiq et al., 2014). Contaminations of this metal element generally not only affect the plant growth but also dangerous to animals as well as to humans when consumed the crop plants with high contaminated Cd.

Existing conventional remediation procedures of Cd-contaminated soils are extremely expensive and environmentally invasive (Li et al., 2012). In recent years, phytoremediation has been considered as one of the important modes of remediation. Phytoremediation is effective as an alternative method to clean up the contaminated soils with potential cost effectiveness, ecofriendly and high efficiency in detoxification. (Hosman et al., 2017). Metallothioneins are cysteine-rich metal-binding proteins which play an important role in mineral homeostasis and also remediating agent against the toxic heavy metals (Hasan et al., 2017). These metallothioneins are found in various types of organisms including bacteria, fungi, plants and animals and they were used in various types of studies as

phytoremediation agents. Genetic modification approaches have been used to examine the ability of various plants after introducing a gene that play an important role in metal homeostasis known as metallothionein to tolerate high concentrations of heavy metals (Vasak, 2005; Benatti et al., 2014). The other effective approaches were attempted to develop transgenic tobacco for Cd phytoremediation (Krystofova et al., 2012; Zhou et al., 2014; Das et al., 2016). Tobacco could be an ideal plant for phytoremediation due to its higher biomass for effective metal accumulation, easy to harvest and also its properties of phytoextraction to remove Cd from the soil through their uptake and transport it to above-ground level through leaves and shoots (Yang et al., 2017).

Plant metallothioneins have been proposed to function in metal homeostasis of essential transition metal ions and detoxification by sequestration of toxic metals (Hossain et al., 2012a). Cysteine molecules have a group of sulfihydriles capable of binding to heavy metals such as zinc (Zn), copper (Cu) and Cd (Sácký et al., 2014). We have previously isolated a metallothionein gene, *eiMT1* from a metal hyperaccumulator plant known as *Eleusine indica* (Sidik et al., 2006). *E. indica* is commonly known as wire grass or goose grass which belongs to Graminae family and is found to be abundant in tropical and subtropical regions such as South Asia and Southeast Asia. A recent study reported by Abdallah et al. (2012) stated that *E. indica* resistant to heavy metals toxicity and capable to accumulate high level of heavy metals without causing any adverse impact on plants.

A better understanding of Cd tolerance and accumulation, specifically in tobacco plant is of great importance. Consequently, the present research was conducted to evaluate the Cd uptake of T_1 seedlings from positive T_0 progenies cell line of transgenic *Nicotiana tabacum* expressing metallothionein gene, *eiMT1* from a hyperaccumulator plant, *Eleusine indica* (Sidik et al., 2010) at various Cd concentrations. Expression of the transgene in the transgenic tobacco plants after exposed to Cd was also evaluated.

Results

Plant morphological observation

Transgenic tobacco seedlings expressing *eiMT1* gene from *E. indica* were selectively grown on kanamycin (75 µg/mL) based on concentration reported by Sidik et al., (2010). Most of the transgenic tobacco seedlings were resistant to the selective agent and none of the non-transgenic seedlings survived. Morphological observations of the shoots showed the clear differences between the transgenic seedlings which were found in fresh green colour and the non-transgenic shoots were observed in yellowish colour. The green colour shows the healthy nature of the transgenic seedlings (Supplementary Fig. 1).

Cd tolerance in transgenic tobacco plants was evaluated through the expression of the *eiMT1* gene, thirty-days old transgenic tobacco and wild type tobacco plants were transferred onto MS medium. The plants were supplemented with different concentrations of $CdNO_3$ (0, 50, 100 and 150 µM) for another seven days of growth. At 0 and 50 µM $CdNO_3$ concentrations, there were no significant difference on development rates and formation of leaves of the wild type tobacco and the *eiMT1*-transgenic tobacco (Supplementary Fig. 2 and Fig. 3). In contrast, at 100 and 150 µM of $CdNO_3$ concentration, *eiMT1*-transgenic tobacco plants exhibited significantly higher growth rate when compared with the wild type tobacco plants. Leaves of wild type plants also exhibited yellowish in (Supplementary Fig. 2) when compared to *eiMT1*-transgenic plants (Supplementary Fig. 3) at 100 and 150 µM of $CdNO_3$ concentration. According to these morphological observations, *eiMT1*-transgenic tobacco plants displayed better Cd tolerance when compared to wild type plants.

Determination of cadmium

After seven days of 0, 50, 100 and 150 µM $CdNO_3$ exposure, an accumulation of Cd by all transgene plants were determined. As shown in Fig. 1, transgenic tobacco plants tolerate to higher concentrations of Cd better than the wild type tobacco which was evidenced with the indication of higher level of Cd presence in the transgenic plants. Transgenic lines 18D, 20D1 and 18C showed the highest accumulation at 150µM $CdNO_3$ with 678.7, 623.0 and 611.925 mg/kg respectively. The lowest Cd concentration accumulation was 281.3 mg/kg by line 20B after exposure with 150µM $CdNO_3$. There were no significant differences in the Cd accumulation between lines 17A, 18B1, 20E, 24C and 24Z for *eiMT1*-transgenic tobacco. In contrast, all wild type tobacco plants showed significantly lower Cd accumulation with 130.8, 194.8 and 220.0 mg/kg at 50, 100 and 150µM $CdNO_3$ respectively.

Expression of eiMT1 gene

The results shown in Fig. 2 demonstrate the different levels of *eiMT1* gene expression. The *eiMT1* transcript was detectable in all transgenic lines and the positive control, whereas it was absent in all wild type tobacco. There was an evident highest expression of *eiMT1* in lines 18B1 and 20D1, which was increased significantly from 0µM to 150µM of Cd. However, the expression of *eiMT1* was very low and decreased slightly in line 20B. Transgenic lines 17A, 18C, 18D, 20E, 24C and 24Z showed no significant level of expression for *eiMT1*. Actin has been used as an internal control or as a housekeeping gene to normalize the expression of target genes between different samples. Actin was one of the most commonly used reference genes because it has more stable expression levels compared with other internal controls (Ruan and Lai, 2007).

Discussion

The morphological differences between the leaves colour of wild type and transgenic tobacco seedlings were clearly observed after growing on MS medium with 75 µg/mL kanamycin. The leaves of wild type seedlings turned yellow and growth of the plants were retarded, while the leaves of transgenic seedlings maintained healthy green and the plants were observed as grown healthy. This is due to the addition of the kanamycin selection agent in the medium culturing cause

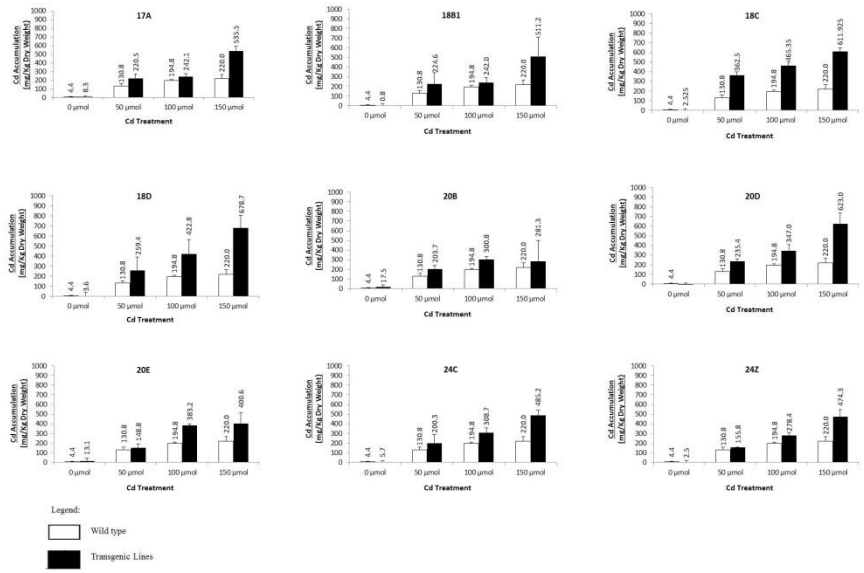

Fig 1. Cd Accumulation in transgenic tobacco plants compared to the wild type tobacco measured by Atomic Absorption Spectrometer

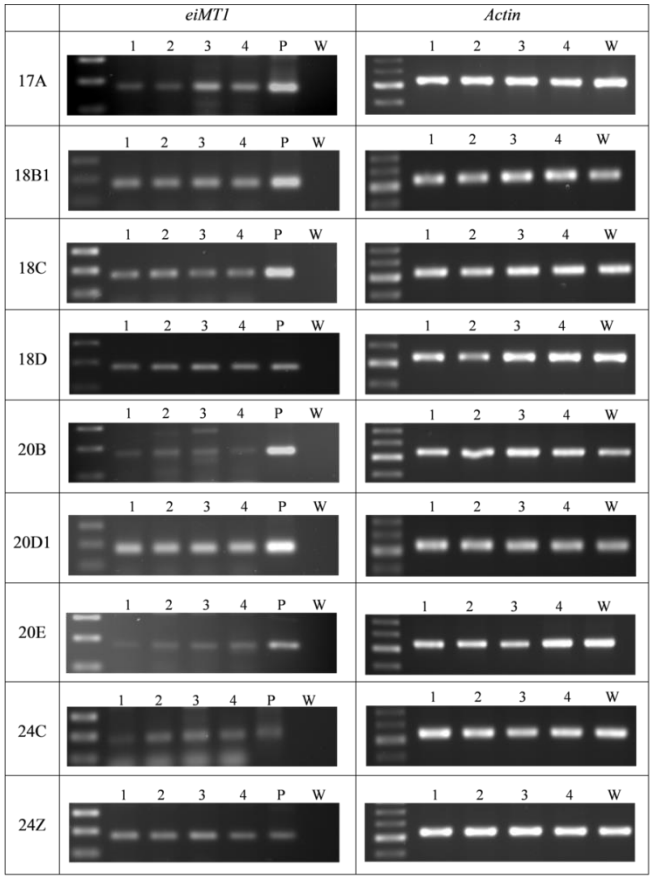

Fig 2. *eiMT1* transgene expression in transgenic plants measured by RT-PCR using gene specific primer. Actin gene serves as a positive control for gene expression to represent a housekeeping gene. Name of the transgenic lines was indicated by the number on the left. Transgenic and control plants were treated with different concentrations of Cd as following; 1, 0 μmol; 2, 50 μmol; 3,100 μmol, 4' 150 μmol. P, *eiMT1* gene as a positive control for RT-PCR; W, wild type non-transgenic tobacco plant.

competition between transformed explants and untransformed cells. Kanamycin serves as a selection agent and promotes the growth of transformant but does not allow wild-type plant to growth. In the genetic transformation system, the resulting transgenic plants usually carry resistance to a selective agent encoding a phenotype in which the screening process can be performed based on physical observations. The results of the present study are in agreement with the previous studies. (Rashid, 2017).

The results clearly showed that Cd accumulation in *eiMT1*-transgenic tobacco was higher when compared to wild type tobacco. The expression level of *eiMT1* genes in both transgenic tobacco and wild type tobacco was determined and as expected, *eiMT1* gene was expressed in the transgenic tobacco and in contrast, no expression was observed in wild type tobacco. As a result, transgenic tobacco plants showed high resistance characteristic toward cadmium as indicated by accumulation of higher cadmium when compared to the wild type.

Besides, the highest cadmium content was determined in transgenic tobacco plants line 18D and 20D1, the expression of *eiMT1* was generally highest in transgenic tobacco plants line 18B1 and 20D1. These findings may be explained by the role of Cauliflower Mosaic Virus 35S (CaMV 35S) promoter simultaneously in transgenic tobacco 18B1. CaMV 35S promoter is the most commonly constitutive, not tissue- and metal-specific promoter in plant biotechnology which has been successfully used to drive high levels of transgene expression (Ferreira et al., 2017).

Plants in general have a range of potential mechanisms which might be involved in the heavy metal detoxification and thus confer a heavy metal tolerance property. Tolerance to heavy metals in plants may be defined as the ability to survive in the environment which is toxic to other plants and is manifested by an interaction between a genotype and its environment (Malik et al., 2017). One of the resistance mechanisms involves is the production of cysteine-rich peptides, such as metallothioneins (MTs) and phytochelatins (PCs) (Cobbett and Goldsbrough, 2002; Sunitha et al., 2013; Hosman et al., 2017; Pandey, 2017).

Many reports have elaborated the structure and function of metallothionein but the specific and precise function of this protein remains not clear. However, many evidences generally support the role of metallothionein to increase plant resistance toward heavy metals toxicity and enhance its accumulation in different parts of the plant. According to Vasak (2005), MTs from animals are involved in homeostasis of essential trace metals and metal sequestration and detoxification. Meanwhile, in plant, MTs play a pivotal role in protecting the cells against the toxic effects of heavy metals by chelating them via cysteine (Cys) thiol groups. The presence of cysteine molecules play a major role in MTs functionality in such a way that, with the presence of thiol side chains, cysteine is classified as hydrophobic amino acids (Vasak and Meloni, 2017). Thiol also known as a mercaptan is a compound which contains the functional group with sulphur and hydrogen atoms (-SH). When the thiol group of two cysteine residues adjacent to each other during protein folding, the oxidation reaction forms cysteine units through the disulfide bond (-S-S-). Cysteine molecules have sulfhydryl group which is capable of binding to heavy metals such as zinc, copper and cadmium (Wang et al., 2017) due to the high affinity between sulphide and metal. Metallothionein synthesis induced by metals through the activation of genes which suggest that MT is a part of early response proteins as well as biological feedback mechanisms.

The introduction and overexpression of MTs genes to plants has shown to be a promising approach to enhance heavy metal accumulation (Fiser et al., 2015). Transgenic plants found to have the higher capability of metal accumulation or a higher resistance to a toxic metal. The accumulation of metals can be further increased using MTs gene (Bulak et al., 2014). A recent study by Yang et al., (2015), reported that *ZjMT*, encoding a type I metallothionein, was cloned from Chinese jujube (*Ziziphus jujuba* Mill) full-length cDNA libraries into *Arabidopsis thaliana*. Transgenic plants accumulate more Cd^{2+} in root when compared to the wild type, suggest that *ZjMT* may have a function in Cd^{2+} retention in roots and, therefore, decrease the toxicity of Cd^{2+}. Consistent with our results, heterologous expression of *ricMT* in transgenic rice (Zhang et al., 2017) and *OsMT2c* in transgenic *Arabidopsis* (Liu et al., 2015) provided increased tolerance against Cd stress.

Materials and methods

Plant sample

Transgenic tobacco plants expressing *eiMT1* gene from *E. indica* was generated as described by Sidik et al., (2010) and the outline strategy used for the *eiMT1* gene cloning was also described. Nine lines of T1 seeds were produced by the positive transgenic tobacco T0 plants, and were named as 17A, 18B1, 18C, 18D, 20B, 20D1, 20E, 24C and 24Z.

Seeds sterilization, germination and seedlings growth

Transgenic T1 *N. tabacum* seeds were disinfected by soaking in 1% sodium hypochlorite for 3 minutes, 70% ethanol for 1 minute and then rinsed thoroughly with sterile distilled water. The seeds were then soaked in sterile distilled water for 30 minutes before transferred onto 1% Murashige and Skoog (MS) agar in a jar bottle and incubated in the dark at room temperature for three to five days. Germinated seedlings in a jar were then transferred into a growth chamber and incubated at 24°C for two weeks.

Seedling selection for the kanamycin resistance characteristic

In order to keep high selective pressure, two weeks old tobacco plants were transferred to fresh MS medium supplemented with 75 µg/mL kanamycin for seven days. Explants that resisted to 75 µg/mL kanamycin in MS medium were transferred into a jar bottle to grow for another 30 days with continuous observation before subjected to cadmium treatment.

Cadmium exposure

Thirty-days old tobacco plants on MS medium were treated with cadmium by pouring 10 mL of $CdNO_3$ in various concentrations (0 µmol, 50 µmol, 100 µmol, and 150 µmol) onto the medium. The plants were left to grow for another seven days in the presence of Cd. Plants were then harvested and divided into two groups for RNA extraction and Cd determination respectively.

Determination of cadmium content in tobacco plants

Thirty-days old transgenic T1 tobacco plants were dried in an oven at 50°C for five to seven days or until a constant weight is measured. A portion of dried plant materials (50 mg) were hydrolysed with 2 mL of concentrated acid mixture of 60% nitric acid (HNO_3) and 70% perchloric acid ($HClO_4$) at 2:1 ratio, v/v, and heated at 160°C for 3 hours or until a clear aqueous solution is obtained. After cooled down to room temperature, the solutions were made up to 20 mL with sterile distilled water. Cd concentration were then determined using Atomic Absorption Spectrometer, Analyst 800 (Perkin Elmer, USA).

Transgene expression analysis by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from 100 mg of fresh tissue using RNeasy Plant Mini Kit according to manufacturer's recommendation (Qiagen, Germany). The quality and quantity of the total RNA was measured by both agarose gel electrophoresis and spectrophotometry. Following DNase digestion, 3 µg of total RNA was reverse transcribed into first strand cDNA using SuperScript™ cDNA Synthesis System (Invitrogen, USA) following the manufacturer's recommendation. The resulted first strand cDNA was used to analyse the *eiMT1* transgene expression by PCR using gene specific primers (Sidik et al., 2010). PCR was also done for the tobacco housekeeping actin gene, *NtActin* as internal control. The *NtActin* gene specific forward primer used was 5'-CGCGAAAAGATGACTCAAATC-3' (NtActinF) and the reverse primer was 5'-AGATCCTTTCTGATATCCACG-3' (NtActinR). PCR cycles for *eiMT1* amplification were as described by Sidik et al., (2010). The same PCR conditions were repeated for *NtActin* gene amplification except that the annealing temperature was changed to 56.7°C. PCR products were analysed on 1.5% agarose gel for DNA band intensity quantification.

Conclusion

The results clearly conclude that the *eiMT1* transgene expression greatly enhances the accumulation of Cd in transgenic tobacco. Transgenic plants that expressed the *eiMT1* transgene accumulated higher level of Cd when compared to the wild types. However, the degree of Cd accumulation does not directly correlate with the quantitative level of *eiMT1* expression. This may suggest that, *eiMT1* gene function was not just to facilitate the metal accumulation but possibly play more other significant roles subjected to further study.

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