Arabidopsis phytochelatin synthase gene overexpression and accumulation of cadmium, arsenic in tobacco

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Abstract- To analyse the partition of heavy metals in various parts of transgenic tobacco plant, accumulation of Cadmium and Arsenics was measured in shoot and root of homozygous *A. thaliana phytochelatin synthase1*- transgenic tobacco lines and wild type controls. Under 50µM, 100 µM, 150µM Cd and As significant difference were observed between the wild type control and transgenic *N.tabacum* lines. Transgenic plants accumulated significantly increased accumulation of Cd (Cadmium) and As (arsenic) in shoots and roots compared to wild type control. The transgenic *N.tabacum* lines shows the noticeable enhancement in tolerance to Cadmium & Arsenic and also revealed enhanced accumulation of these heavy metals both in roots and shoots. The overall results clearly suggest that *Arabidopsis thaliana phytochelatin synthase1*-expressing transgenic tobacco lines exhibit higher-levels of Cadmium and Arsenic accumulation in both shoots and roots when compared to (WT) wild type control.

I. Introduction

Growing advanced technology, the pollution is the major problem due to different heavy-metals, like lead, copper cadmium, mercury, zinc, and others. These heavy metals are reaching the soil in different ways and living in it at a very high altitude, the metals are first absorbed by the plants and become food for animals and humans (Wei *et al.,* 2008). Pollution of soil layers by the heavy-metals is a major concern because lead, cadmium and zinc are often stick on with fertilizers (Vassilev *et al.,* 2002). But advanced conventional tillage technology requires significant investment and may lead to unintended consequences. In addition, these new technologies degrade soil profile and lead to biological degradation [Alkorta *et al.,* (2004); Ghosh and Singh (2005); Van Slycken *et al.* (2009)]. Many of these degradation methods cannot be used for soil cleaning when soil fertility is very important (Chaney *et al.,* 2007). Adequate restoration and protection of the soil environment polluted by heavy metals, requires their specification and repair. Current regulation and legislation body protecting the environment and public health issue at both international and national levels is based on the data revealing the chemical properties of natural events those that reside in our food web (Kabata and Pendias, 2001). Although soil classification may provide insight in the heavy metal specification, the presence of bioavailability of nutrition and other minerals, to correct polluted soil by heavy metals which needs to include information of the source of contamination and basic chemicals, the environmental health issues due to these heavy metals. The possible measurements is an effective tool that enables decision makers to manage highly polluted areas in a cost effective manner, protecting public health and the ecosystem (Zhao and Kaluarachchi, 2002). The present investigation deals with the phyto-remediation technology by using the *Arabidopsis thaliana phytochelatin synthase1*gene and also deals with the accumulation of Cd and As in roots and shoots of transgenic tobacco lines and wild type controls.

II. Materials and Methods

Arabidopsis thaliana phytochelatin synthase1 isolation and cloning

Total RNA, from 15-day-old *Arabidopsis* seedlings (ecotype Columbia) was isolated using the RNeasy plant mini kit (Qiagen) following the manufacturer's instructions. Two µg of total RNA were reverse transcribed using the SuperScript™ III First-Strand Synthesis System (Thermo Fisher Scientific). Two µl of cDNA was used to perform PCR using pfu polymerase employing forward and reverse primers, viz., 5′-GGATCCATGGCTATGGCGAGTTTA-3′ and 5′-GAGCTCCTAATAGGCAGGAGCAGCGAG-3′, specific to *Arabidopsis thaliana phytochelatin synthase1* coding sequence. The underlined regions in the primers depict BamHI and SacI restriction sites introduced for subsequent cloning purpose. Amplified fragments of 1470 bp was cloned at SmaI site of pBluescript KS (+) (Stratagene, USA), then transformed into E. coli Top10 cells and was sequenced using automated DNA sequencer.

Construction of binary vector containing *Arabidopsis thaliana phytochelatin synthase* 1 and GFP expression cassettes

Arabidopsis *phytochelatin synthase1* coding sequence was excised from BamHI and SacI sites of recombinant pBSSK (+) – *Arabidopsis thaliana phytochelatin synthase* 1 vector and cloned between CaMV35S promoter and polyA terminator of *pCambia*1302 hygromycin binary vector constructed earlier in our laboratory. The *pCambia* 1302 binary vector contains expression unit of hyg (CaMV35S-hyg-polyA) which was used as a plant selection marker (www. cambia.org). The recombinant vectors, *viz., pCambia* 1302-hyg- *Arabidopsis thaliana phytochelatin synthase* 1 were maintained in HB101 cells and was mobilized into A. tumefaciens strain LBA4404 by triparental mating (Lichtenstein and Draper, 1985) using the helper vector pRK2013 and the resulting binary vector was designated as *pCambia* 1302-hyg *Arabidopsis thaliana phytochelatin synthase* 1.

Expression of *Arabidopsis thaliana phytochelatin synthase* 1gene in Tobacco plants for their functional validation.

Preparation of tobacco explants:

Seeds of tobacco were treated with 0.1% tween 20 for 5 min and then washed with sterile water later, these seeds were surface sterilized with 0.1% Mercuric chloride and 2% sodium hypochlorite for 5min each followed by sterile distilled water thrice. The seeds were kept for germination in MS basal media at 25º C. After 10-15 days leaf disc of 0.5cm were dissected from mature leaves and inoculated on to Petri dishes containing MS basal media and incubated at 24º C in dark condition for two days before infecting with *Agrobacterium.*

***Agrobacterium*-mediated transformation and regeneration of tobacco transgenic plants**

Single colonies from sub-culture plants *Agrobacterium tumefacians* with *Arabidopsis thaliana phytochelatin synthase* 1gene constructs were inoculated into 50ml of YEP broth and grown for 24hr at 28º C in an orbital sheker set 200rpm. The cells were pelleted at OD_{600} 1.0 and resuspended in MS basal media. Leaf disc collected aseptically from invitro grown tobacco plant were placed in *Agrobacterium* suspension and shaken gently for 15min in orbital shekar set at 200rpm at 28º C. Later, the explants were placed on sterile Whatmann filter paper to blot excess bacteria from the explants and to reduce the overgrowth. The explants were then co-cultivated for two days on co-cultivation medium. After 48hr the explants were washed thrice with a solution of Cephotaxime 250mg/lit for one hr. Later, the explants were transfer to selection media containing hygromycin 50mg/lit.

Analysis of transgenic lines under cadmium and arsenic

Homozygous transgenic tobacco line expressing *Arabidopsis thaliana phytochelatin synthase* 1 of T3 generation along with wild type controls were sown in pots. Plants were grown for 10 days on MS medium and then transferred in fresh MS media containing various concentrations *viz.*, 50 µM, 100µM and 150 µM of CdCl2 and Na2AsO4. Shoots and roots from hydroponic media grown tobacco seedlings were collected and dried in hot air oven at 40ºC to reach constant weight. Dried plant tissues (100 mg) were digested in HNO_3 and H_2O_2 (3:1), through microwave digestion, in Microwave digester (Roychoudhury *et al.*, 2002). This digested solution was diluted from which 10 ml aliquot was quantitatively analysed for As through atomic absorption spectrometry (Perkin–Elmer; AAnalyst 600)fitted with graphite furnace.(Reference standard for calibration was made using 1000 mg/ml (AA03N-5) standard supplied by Accustandard, USA).

III. Results and Discussion

Two types of tobacco plant *viz.*, homozygous transformed tobacco plant and wild type controls were analysed for heavy metals (Cd and As) accumulations in roots and shoots. Transgenic plants accumulated significantly increased accumulation of Cd (Cadmium) and As (arsenic) in shoots and roots compared to wild type control. Under 150µM Cd and As considerable variation were found between the (WT) wild type control and the transgenic (T) *N.tabacum* lines. A mean Cadmium concentrations of 50 µM,100 µM and 150µM is 180 ±2, 300±2 and 450±2 $\mu g.g^{-1}$ dry weight was recorded in root of transgenic *N.tabacum* line respectively(Fig. 37), compared with 100 ±2, 120±2 and 132±2 µg/g dry weight of Cadmium in wild type control respectively Similarly, under 50 µM,100 µM and 150µM is 220 ±2, 500±2 and 650±2 µg/g dry weight in transgenic tobacco line respectively. Whereas, wild type control showed a mean Arsenic concentration of 95 ±2, 110±2 and 117±2 µg/g dry weight respectively (Fig.1&2).

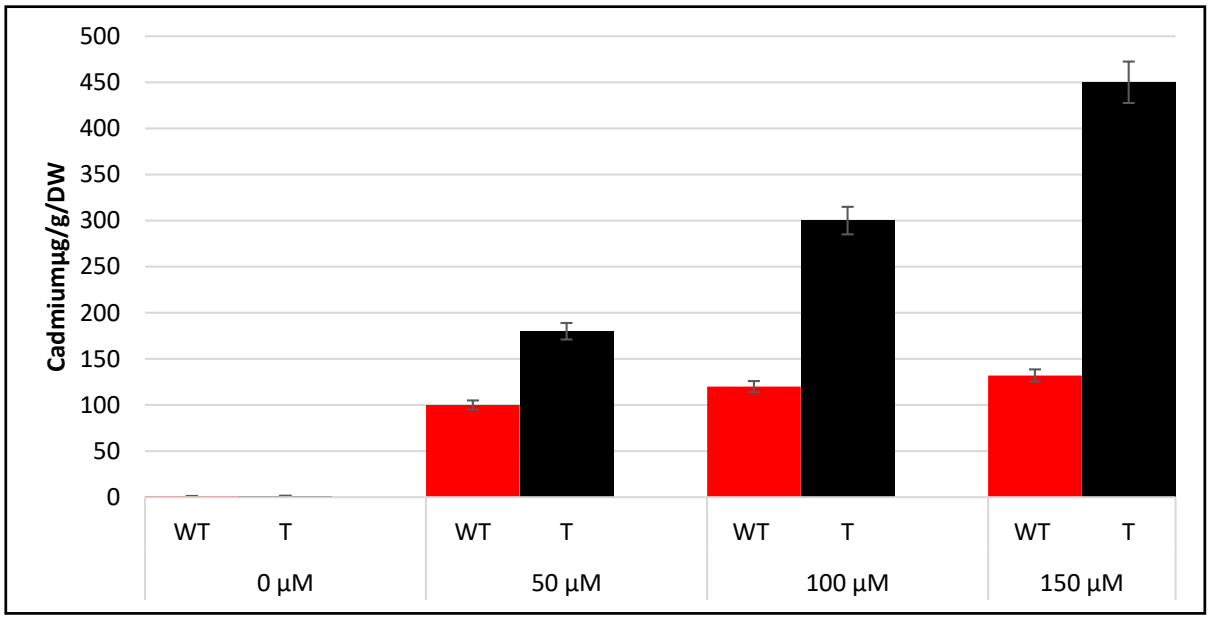

Figure. 1. Cd accumulation in roots of wild type controls (WT) and *AtPCS1* expressing in transgenic tobacco (T).

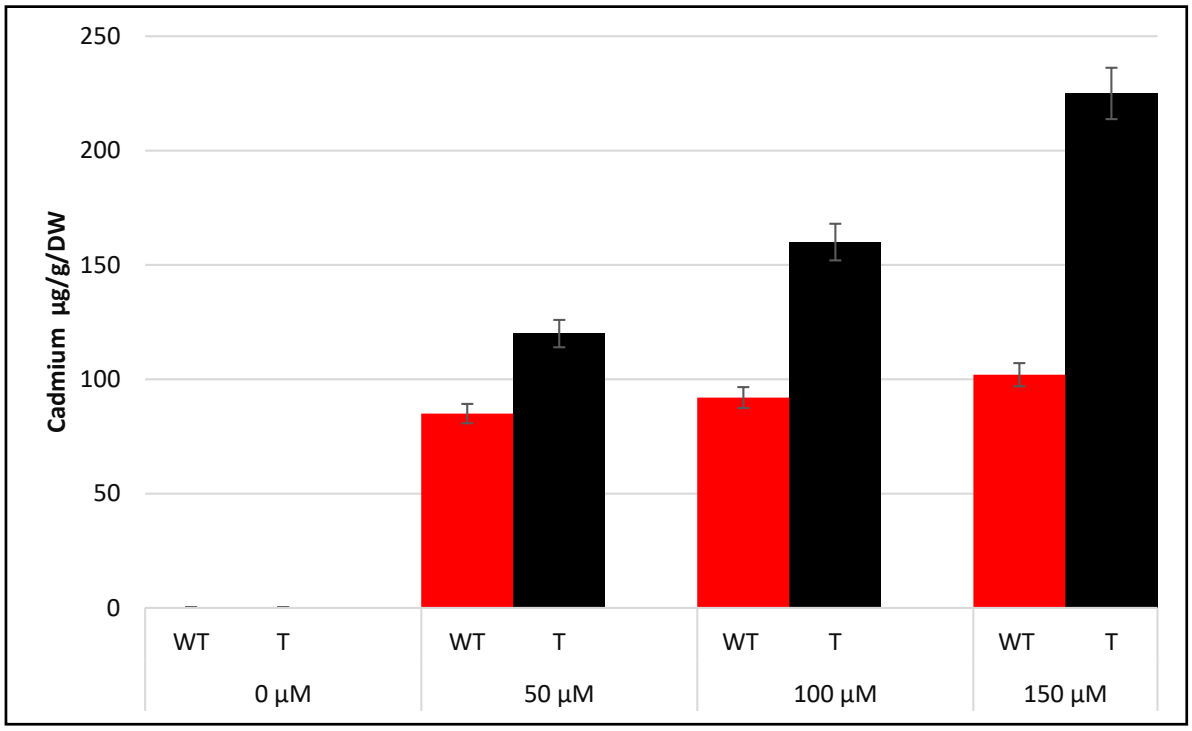

Fig. 2. Cd accumulation in shoots of wild type controls (WT) and *AtPCS1* expressing in transgenic tobacco plant (T).

Moreover, A mean Cadmium concentrations of 50 µM,100 µM and 150µM is 120 ±2, 160±2 and 225±2 µg/g dry weight was recorded in shoots of transgenic *N.tabacum* line respectively, compared with 85 ±2, 92±2 and 102±2 µg/g dry weight of Cadmium in wild type control respectively. Similarly, under 50 µM,100 µM and 150µM As *Arabidopsis thaliana phytochelatin synthase* 1 is 25 ±2, 60±2 and 92±2 µg/g dry weight in transgenic

tobacco line respectively. Whereas, wild type control showed a mean As concentration of 10 ±2, 30±2 and 47±2 µg/g dry weight respectively (Fig.3&4).

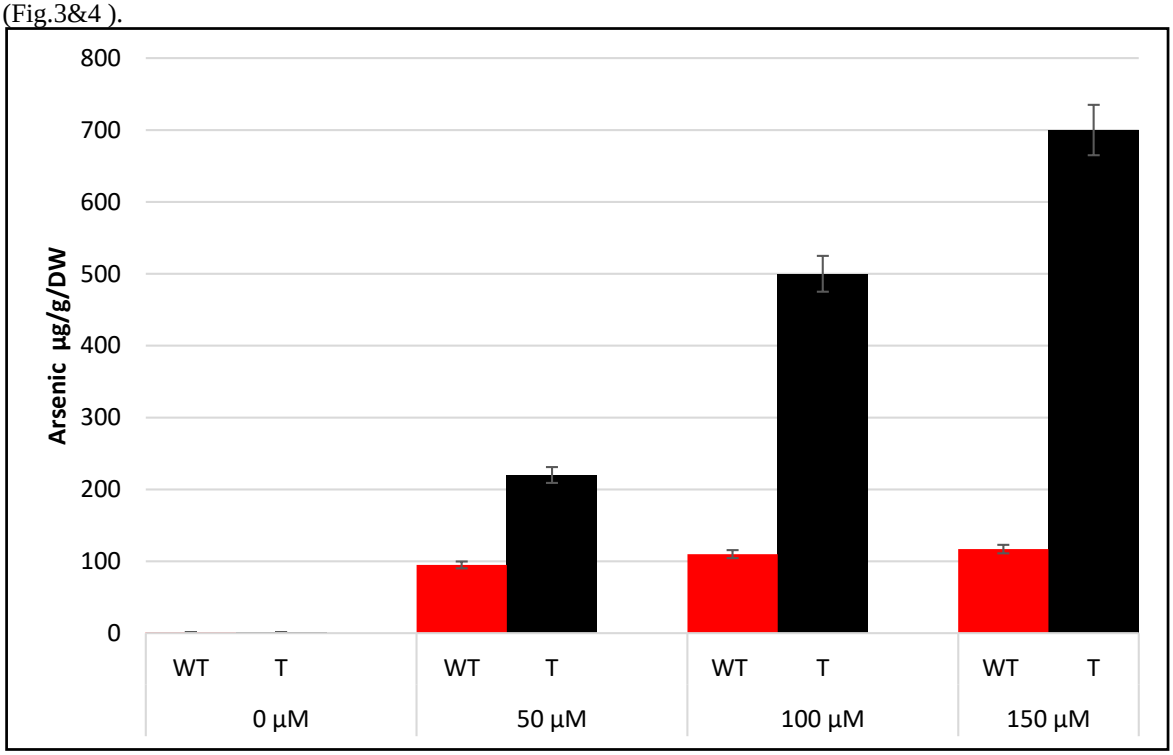

Fig. 3. Arsenic accumulation in roots of wild type controls (WT) and *AtPCS1* expressing in transgenic tobacco plant (T).

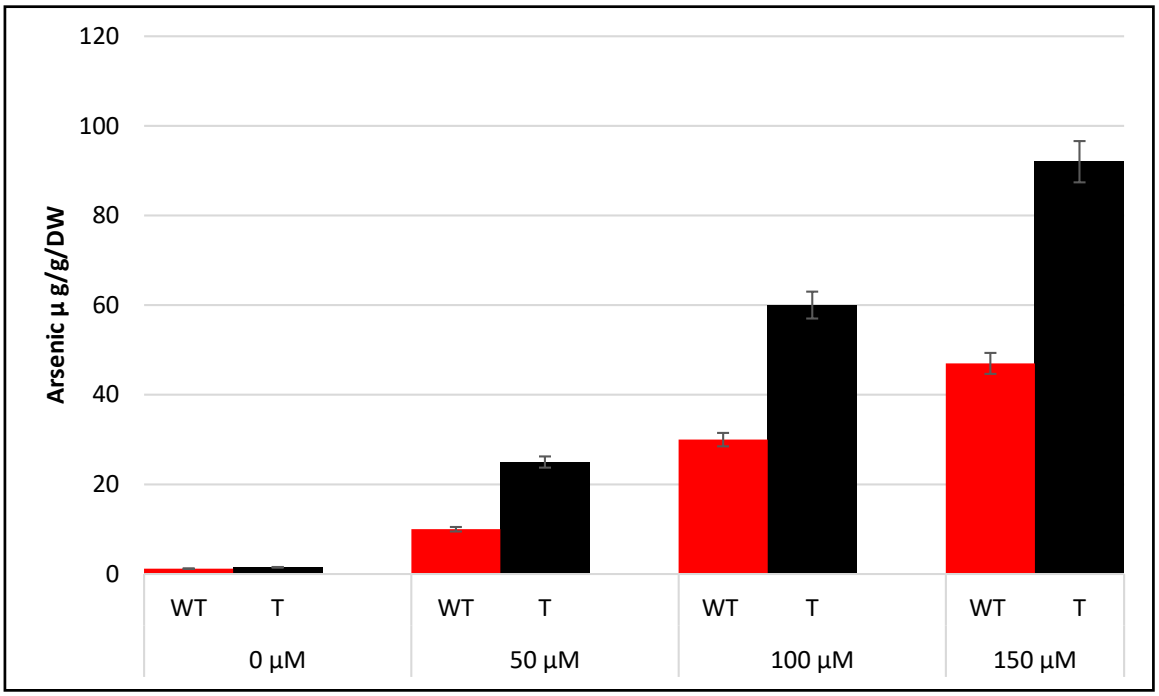

Fig. 4. Arsenic accumulation in shoots of wild type controls (WT) and *AtPCS1* expressing transgenic tobacco plant (T).

The overall results clearly suggest that *Arabidopsis thaliana phytochelatin synthase1*-expressing transgenic tobacco lines exhibit higher-levels of Cadmium and Arsenic accumulation in both shoots and roots when compared to (WT) wild type control. It was reported that heterologous expression of *C. demersum (CdPCS1)* in tobacco and *A.thaliana* led to considerable increased Arsenic or Cadmium accumulation in root accompanied by enhanced Phytochealatins content [Shukkla *et al.* (2012)&(2013)]. Furthermore, ectopic expression of *CdPCS1* complements cad1-3 mutant of *A. thaliyana* to the same level of synthetic Phytochealatins (Shukkla *et al.*, 2013). Earlier, it was reported that expression of *NnPCS1* in *Arabidopsis*, *PtPCS1* and *TaPCS1* in poplar and *TcPCS1* in tobacco disclosed accumulation of various metalloids and heavy- metals, viz., Pb As, Cd,and Zn [Liu *et al.* (2012); Adams *et al.* (2011); Liu *et al.* (2011); Couselo *et al.* (2010)]. Whereas, heterologous expression of *TaPCS1* in wheat, over-expression of *A. thaliana phytochelatin synthase1 (AtPCS1)* in *Arabidopsis thaliana* resulted in Cadmium hypersensitivity [Wang *et al*, (2012); Li *et al.* (2004); Lee *et al.* (2003)]. These disparities in transgenic tobacco plants expressing phytochealatin synthase might have risen due to differential phytochealatin synthase (PCS) activity in source genes and nature of the plant species selected for transformation. There are the enough proofs which suggest role of Phytochealatins in the long distance transport of the heavy-metals through either phloem or xylem [Mendoza *et al.*, (2008); Li *et al.* (2006); Chen *et al.* (2006); Lappartient and Touraine, (1996); Gong *et al.* (2003)]. The similar kind of mechanism might have been involved in *Arabidopsis thaliana phytochelatin synthase1*-expressing tobacco plants which has lead to the higher heavy- metals accumulations in both roots and shoots.

IV. Conclusion

The overall study clearly suggest that *Arabidopsis thaliana phytochelatin synthase1-gene* expressing transgenic tobacco lines exhibit higher-levels of Cadmium and Arsenic accumulation in both shoots and roots when compared to (WT) wild type control.

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