# Characterization of Heavy Metal Tolerance and Accumulation in Indian Mustard Overexpressing Bacterial γ-ECS Gene

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**Abstract**   The tolerance and accumulation of multiple metals by Indian mustard (*Brassica juncea*) plants overexpressing the γ-glutamylcystein synthetase (γ-ECS) gene was examined in order to study heavy metal tolerance in plants.  Seedling growth was tested on four different metals, including Mn, Mo, Pb, and Ni.  The ECS seedlings had significantly longer root lengths and higher tolerance than wildtypes for all metals tested.  However, it was likely that the ECS plants did not hyperaccmulate metals as expected since the fresh weights of the transgenic plants did not differ much from the wildtypes.  Nevertheless, the increased root length tolerance in transgenic plants still offers a promising strategy for the production of plants with superior heavy metal phytoremediation capacity.

**Introduction**

   Heavy-metal pollution of soils and waters caused by the mining and burning of fossil fuels is a major environmental problem, and exposure to these metals can be toxic to living cells  (Qian *et al*., 1999).   Unlike organic pollutants, heavy metals cannot be chemically degraded or biodegraded by microorganisms.  One alternative biological approach to deal with this problem is phytoremediation-- the use of trees and plants to detoxify chemical waste sites.  Compared with other technologies, phytoremediation is less expensive (Cunningham and Ow 1996) and is particularly suitable for treatment of large volumes of substrate with low concentrations of heavy metals.

   Heavy metals and metalloids can be removed from polluted sites by phytoextraction, which is a method of phytoremediation and involves the accumulation of pollutants in plant biomass (Zayed *et al*., 1998).  As a result,  hyperaccumulators (plant species that accumulate extremely high concentrations of heavy metals in their shoots) become particularly useful.  In addition, one can genetically engineer these species to improve their metal tolerance and metal-accumulating capacity.  A suitable target species for this strategy is Indian mustard (*Brassica juncea*), which has a large biomass production and a relatively high trace element accumulation capacity.  Most importantly, it can easily be genetically engineered (Zhu et al., 1999b).

   Generally, plants have evolved a number of mechanisms to cope with heavy metal stress.  One example is the production of gluthathione (GSH).  GSH serves as an antioxidant, directly detoxify metals by conjugating them, forming a non-toxic complex through glutathione-S-transferase catalyzed reaction (Coleman *et al*., 1997) (Fig. 1).  In addition, GSH is the precursor to the heavy metal-binding peptides phytochelations (PCs) which are involved in heavy metal tolerance and sequestration (Steffens 1990).  It is proposed that the rate-limiting step for GSH synthesis in the absence of heavy metals is believed to be the reaction catalyzed by the $\gamma$-ECS and GS enzymes (Noctor *et al*., 1996).  Therefore, manipulating the expression of such enzymes involved in GSH and PC synthesis may be a good approach to genetically engineer the plants to enhance their heavy-metal tolerance and accumulation.

Cysteine + Glutamate

γ-ECS

γ–Glutamylcysteine
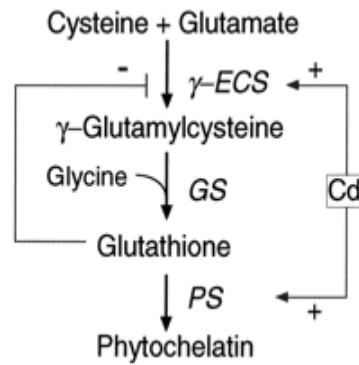
Glycine — GS

Cd

Glutathione

PS

Phytochelatin

Figure 1. Regulation of GSH/PC
Biosynthesis in plants.

The basis for increased production of GSH and PCs during metal stress was addressed through numerous past studies. In one experiment conducted by Zhu et al. (1999a), the overexpression of GS in Indian mustard plants showed an increased concentration of GSH and PCs when the plants were treated with Cadium (Cd). Moreover, the transgenic plants in the experiment exhibited higher Cd tolerance and accumulation. The transgenic seedlings adapted better under metal stress since they had longer root length and higher fresh weight than wildtype plants. As for the adult Indian mustards, they also had a higher relative growth than wildtype plants.

Similar results were obtained for Indian mustards overpressing γ-ECS in the cytosol. Transgenic seedlings tolerated Cd better than wildtype and had significantly higher levels of GSH (Zhu 1999). Mature transgenic γ-ECS plants grew better and accumulated more Cd than wildtype as well.

Thus, overexpression of enzymes GS and γ-ECS are positively correlated to improvements in heavy metal stress response. However, the majority of previous studies with transgenic plants have focused on the change in tolerance to a single metal, primarily Cd. In natural environments where phytoremediation techniques are applied, a variety of different metals are found. Therefore, further examination of these transgenic plants' tolerance to other heavy metals should be conducted.

In this research, I would like to characterize the tolerance and accumulation of seedling transgenic Indian mustard overexpressing bacteria gene γ-ECS to a variety of metals. The metals to be tested include Manganese (Mn), Molybdenum (Mo), Nickle (Ni) and Lead (Pb), which are

all commonly found at heavy metal polluted sites.  Therefore, the objective of this study is to determine if transgenic plants are successful at tolerating other metals besides Cd.  I predict that the transgenic plants will have longer root lengths than wildtypes since they can adapt better; the modified GSH levels help the plants to detoxify metals better.  Likewise, I also hypothesize that the transgenic plants will be heavier than wildtype plants since they will be able to accumulate more metals.

**Methods**

The objective of this study is to test the hypothesis that transgenic γ-ECS seedlings have superior heavy metal tolerance and accmulation as compared to wildtypes .  The experiment can be divided into two main parts.  The focus of the first part is to determine the appropriate level of metal concentration to be used in each treatment solution.  This is essential as metal concentration levels cannot be too high or otherwise all the wildtype plants will be killed, and no data will be available to act as comparison to the results of the transgenic plants.  Similarly, the levels cannot be too low or the wildtype plants will grow just as well as the transgenic ones, and no tolerance difference can be observed between them.  The goal is to find the right metal concentration levels so that the wildtype plants will be significantly affected but not die.  This means that the metal treated wildtype plants should have root lengths and fresh weights that are about 30 to 40 percent lower than those of the control .

The second part of the experiment involves growing both the transgenic and wildtype seeds simultaneously at the concentration levels determined in part I.  The untransformed wildtypes serve as control and their root lengths and fresh weights will be compared to the transgenic γ-ECS seedlings.  Plants with better adaptation will have longer root lengths and heavier fresh weights.

**Part I.  Determining the Appropriate Metal Concentration Levels**  Seedling growth was tested on four different metals, including manganese (Mn), molybdenum (Mo), nickel (Ni), and lead (Pb).  The compound used for Mn is manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) (Fisher, 99%), for Mo is disodium molybdate dehydrate ($Na_2MoO_4 \cdot 2H_2O$; Sigma, 99%), for Ni is nickel chloride hexahydrate ($NiCl_2 \cdot 6H_2O$; Sigma, 99.9%), and for Pb is lead dinitrate ($Pb(No_3)_2$; Fisher, 99%).

For stock solutions, the goal was to make 5 ml of 100,000mg/L for each element. 1.80 g of MnCl$_2$, 1.26g of Na$_2$MoO$_4$, 2.025g of NiCl$_2$, and 0.799g of Pb(No$_3$)$_2$ were added to separate test tubes, each containing 5 ml of sterilized water. Next, a stirring rod was used to dissolve the chemicals.

The growth medium for the seeds was made up of 1.1g strength Murashige and Skoog salt (Sigma), 5g of sucrose (Sigma), 2.5g of phytagar (Sigma), and 0.5L of sterilized water. A stir bar was added to dissolve the contents, and the pH was then adjusted to 5.6, which had been determined as suitable for seedling growth from previous experiments (Zhu et al., 1999a).

Indian mustard (*Brassica juncea*, accession no. 173874) seeds were obtained from the North Central Regional Plant Introduction Station (Ames, IA). Two hundred and twenty five seeds were surface sterilized by rinsing one minute in 95% ethanol, treating for 30 minutes with 20% bleach, and washing five times with sterile water for 10 minutes (Zhu et al., 1999b)

100 ml of growth medium was transferred to each of four autoclaved flasks. Next, the stock solutions were added and then pipetted to sterilized magenta boxes (Sigma). The amount of stock solutions added was determined by the desired metal concentrations (Zhu et al., 1999a). For instance, if the desired concentration level for manganese was 65 ppm, then I would add 0.65 ml of Mn stock solution into the flask, mix it thoroughly, and pipette the medium into magenta box. Likewise, the same procedure was performed for the remaining three metals. Altogether, there were 5 boxes (one for each metal and one for control).

When the medium became hardened, 25 seeds were sown in each box. Afterwards, the boxes were placed in a cold room for two days and then transferred to a growth chamber at 24ºC under constant illumination. Seedlings were harvested after five days of growth, and root lengths and fresh recorded and measured (Zhu et al., 1999b). All statistical analyses carried out in the experiment were performed using the JMP IN statistical package (SAS institute, Cary, NC).

**Part II. Transgenic and Wildtype Seedling Tolerance** The chemicals and stock solutions used were the same as in Part I. Wildtype seeds were also identical to those used previously. The mutated seeds were from transgenic line ECS8, which had been transformed beforehand by other laboratory technicians (Norman Terry Lab, UC Berkeley). The seeds were then surface sterilized by rinsing in 95% ethanol for one minute, treating with 20% bleach for 30 minutes, and washing with sterile water for 10 minutes five times.

100 ml of growth medium was transferred to each of the nine autoclaved flasks (four for wildtype, four for transgenic, and one for control). Next, the stock solutions were added and then pippeted to sterilized magenta boxes (except for control, where no stock solution was added). When the media became solidified, 25 seeds were sown in each box. The boxes were then placed in a cold room for two days and later transferred to a growth chamber at 24ºC under constant illumination. Seedlings were harvested after five days of growth and thoroughly washed under running deionized water to remove any trace elements adhering to the tissue. Total root lengths and fresh weights were measured and recorded.

**Results**

For experiment Part I, the appropriate concentration level for each metal was determined to be: Mn 60ppm, Mo 30ppm, Pb 185ppm, and Ni 0.75ppm. Wildtype and transgenic line ECS8 were sowed on agar medium containing either 60ppm Mo, 30ppm Mn, 185ppm Pb, or 0.75ppm Ni. The root length and fresh weight were measured after each treatment and tolerance index was calculated (Tables I and II). Tolerance index (TI) represents the relative growth rate of the plants and is equal to the growth in metal-containing solution divided by the growth in control solution, the quantity multiplied by 100. TI of root length and fresh weight are commonly used to quantify plant metal tolerance (Tuner, 1994). The higher the TI, the better the tolerance.

For root length tolerance, ECS8 tolerated all metals visibly better than wildtypes (Table I). As for fresh weight tolerance, this transgenic line failed to show any improvement for Mo, but for Mn, Pb, and Ni, the fresh weight TIs were all higher than the wildtype (Table II).

|  | Mn | Mo | Pb | Ni |
|---|---|---|---|---|
| Wildtype | 24.0 | 33.1 | 7.4 | 13.5 |
| ECS8 | 42.8 | 80.8 | 10.4 | 21.5 |

Table I. Root length tolerance index (RLT1) for transgenic and wildtype Indian mustard seedlings treated with 4 metals. RLT1 is equal to the average root length of seedlings when treated with a metal over the root length of control seedlings multiplied by 100

|  | Mn | Mo | Pb | Ni |
|---|---|---|---|---|
| Wildtype | 40.6 | 33.8 | 33.8 | 46.4 |

| | | | | |
|---|---|---|---|---|
| ECS8 | 42.0 | 33.5 | 47.7 | 48.7 |

Table II. Fresh weight tolerance index (FWT1) for transgenic and wildtype Indian mustard seedlings treated with 4 metals. FWT1 is equal to the average fresh weight of seedlings when treated with a metal over the fresh weight of control seedlings multiplied by 100.

In addition to tolerance indexes, two-tailed t-tests and means comparisons were conducted for all metals to find out if the root lengths and fresh weights of transgenic plants were significantly different from those of wildtypes. For root lengths, the values were: Mn, t=1.995, n=20, P=0.0246; Mo, t=2.003, n=23, P=0.031; Pb, t=1.834, n=20, P=0.045, and Ni, t=1.975, n=21, P=0.0293. The statistics revealed that the root lengths for all transgenic plants were significantly longer at a 0.05 level.

On the other hand, the results were not as positive for fresh weights. For Mn, the values were t=1.745, n=24, P=0.082, for Mo, t=1.235, n=21, P=1.203, for Pb, t=1.874, n=20, P=0.0323, and for Ni, t=1.658, n=22, P=0.068. It was only under Pb treatment that transgenic plants demonstrated a significant heavier fresh weight.

**Discussion**

Indian mustard plants overexpressing the bacterial gene for γ-ECS in the cytosol were examined for their tolerance to and accumulation of a number of metals. Previous work had shown an increased tolerance and accumulation of Cd (Zhu *et al.*, 1999a; Zhu, 1999b). However, tolerance to many other metals had not been tested, and was therefore explored in this experiment. Results from the seedling tolerance studies showed that ECS (transgenic) plants have increased tolerance to all metals tested in this study, as determined by root length (Table I). Although transgenic seedlings' fresh weight TI was better than wildtype for Mn, Pb, and Ni, the tolerance for Mo failed to show any improvement.

The results of the experiments highlights the complex nature of plants' tolerance to heavy metals. Root lengths are less substantially impaired by metal stress than fresh weights. The most likely explanation for the increased root tolerance of the ECS plants is that these plants produced more PCs at the roots. High PC levels is expected to lead to a greater capacity to detoxify and sequester metals, and therefore, the roots appear to be the major site of PC synthesis. This finding is consistent with the results obtained from previous studies with cadmium (Cd)

tolerance conducted by Zhu et al. in 1999. The researches found that the PC levels in the roots of ECS plants were about 2-fold higher than in wildtype plants. The translocation of metals from the root to the shoot through the xylem was probably driven by transpiration (Salt et al., 1995).

Another possible explanation for the observed difference between fresh weight and root length tolerance is that gluthathione (GSH) and phytochelatin (PC) are involved in heavy metal tolerance rather than uptake. An increase in metal accumulation will cause the plant to become heavier, but in this case, the transgenic ECS8 line probably did not accumulate more metals than the wildtype. As a result, the weight of the plants remaine unchanged. However, this is subject to verification. An elemental analysis by plasma atomic emission spectroscopy (Fassel, 1978) where the tissues are analyzed for concentrations of trace elements should be carried out to determine the exact amount of metals hyperaccmulated in the transgenic plants.

Another factor to take into account is that the seedlings only have five days of the growth, and the plants did not have time to accumulate a large amount metal (Zhu et al, 1999a). Therefore, one should also examine mature plants for their heavy metal tolerance if time is not a constraint.

Even though the ECS8 transgenic line did improve root length tolerance for all four metals, it should be noted that many other researchers have found that increased tolerance to a particular metal does not necessarily indicate an increase in tolerance of plants to other metals; in fact, a tobacco plasma membrane calmodulin-binding transporter overexpressed in tobacco resulted in an increase in tolerance in tolerance of $Ni^{2+}$ but a decrease in the tolerance of $Pb^{2+}$ (Arazi et al., 1999). Therefore, more metals should be examined to verify that the ECS8 transgenic line would be suitable for usage in the presence of other metals.

In the future, soil experiments where the seeds are germinated directly on contaminated soil should be performed to verify the results of tolerance found in this study and would provide conditions of testing closer to that of the natural environment.

In addition to soil experiments, one should also focus on the tolerance of ECS plants to combinations of metals to study the extent of the ECS transgenic plants' ability to tolerate multiple metals simultaneously.

The work in the paper only characterized the tolerance of seedling transgenic Indian mustard overexpressing bacterial gene ECS, but more studies should be performed to examine other transgenic lines that overexpress GS to investigate if any differences will be obtained

Overexpression of bacterial genes encoding γ-ECS in Indian mustard increased the plant's root length tolerance to a number of metals. The transgenic line did not perform better for fresh weight tolerance. These results extended previous researches' data on transgenic plants' tolerance to heavy metals, which mostly examined only Cd. My results imply that transgenic plants help to increase heavy metal tolerance instead of metal accumulation.

## Acknowledgements

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